A Study On 4.1 Proteins In Brain

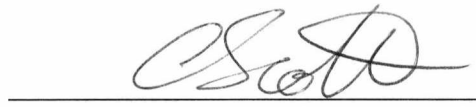
Catherine Scott

Spring 2000

A thesis submitted to the University of Kent for the degree of Doctor of Philosophy in the Faculty of Science, Technology and Medical Studies.

DECLARATION

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or Institute of learning.



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May 2000

ACKNOWLEDGEMENTS

I would like to acknowledge my supervisor, Dr Anthony Baines, for his guidance throughout this research. My thanks go to members of the Research School of Biosciences who have assisted me with this work and to members of the AJB lab for their help and support. In particular I thank Dr. Nandini Hayes for her practical advice and wisdom.

Personal thanks goes to P. Bignone and A. Clow.

This thesis is dedicated to my parents
who have struggled with me to get this far.

ABSTRACT

The 4.1 family consists of a multi-functional group of proteins expressed from four distinct genes. 4.1R, 4.1N, 4.1G and 4.1B have distinct mammalian tissue and developmental expression patterns and each protein exists as multiple isoforms produced by alternative splicing of their respective genes. Functions as diverse as signalling, regulation of pre-mRNA splicing, mitotic spindle rearrangements and anchoring of membrane proteins have been suggested for these proteins based on their tissue and subcellular distribution and the protein binding partners identified. 4.1R was the first family member to be discovered and was identified as a component of the erythrocyte membrane skeleton important for the integrity of these cells.

The role of 4.1 proteins in the brain is of particular interest. All four proteins are expressed but each has a distinct regional localisation. Limited information is available on the subcellular distribution of 4.1 proteins in brain and other tissues. To begin to address this issue, the complement of 4.1 protein isotypes was investigated in postsynaptic density (PSD) fractions of mammalian forebrain. 4.1R was shown to be expressed predominantly in cell bodies and dendrites of primary rat midbrain cultures supporting a postsynaptic distribution for this 4.1 gene product. Antibodies specific to 4.1R, 4.1N and 4.1G and an antibody recognising 4.1R, G and B localised all four 4.1 proteins in PSD fractions. Of these, two 4.1B isoforms ($4.1B^{150kDa}$ and $4.1B^{126kDa}$) were most abundant while the single 4.1R isoform identified ($4.1R^{80kDa}$) was most highly enriched.

To investigate the postsynaptic binding activities of 4.1R, recombinant 4.1R C-Terminal Region (4.1R-CTR) was produced and shown to mimic characteristic spectrin-actin binding and digestion properties of native 4.1R. One and two dimensional gel overlay analysis indicated binding of 4.1R-CTR with dynamin, actin and the intermediate filament proteins α-internexin and NF-L. The identity of actin, α-internexin and NF-L was confirmed using protein specific antibodies. These interactions were supported by data from 4.1R-CTR affinity chromatography but the insolubility of PSDs made it difficult to study 4.1R-CTR-PSD protein interactions under native conditions.

Localisation of 4.1 at PSDs provides evidence supporting a role for these proteins at specialised membrane domains. This is consistent with the localisation of the D*rosophila* homologue of 4.1 (coracle) to septate junctions and the recently identified interaction of mammalian 4.1R with the tight junction protein ZO-2. The interaction of 4.1R-CTR with α-internexin and NF-L indicates a structural role for 4.1R at PSDs. The interaction with actin could in turn suggest a function for 4.1R in the dynamic morphological changes that accompany neuronal plasticity and is consistent with the role 4.1R plays in erythrocyte membrane deformation. The data presented gives further insights into the workings and architecture of PSDs and proposes novel interactions for the C-terminal region of 4.1R.

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ABBREVIATIONS

4.1B	Protein 4.1B
4.1G	Protein 4.1G
4.1N	Protein 4.1N
4.1R	Protein 4.1R
$4.1R^{x}$	Protein 4.1R protein x kDa in size
$4.1G^{x}$	Protein 4.1G protein x kDa in size
$4.1N^{x}$	Protein 4.1N protein x kDa in size
$4.1R^{x}$	Protein 4.1R protein x kDa in size
$4.1X^{xkDa}$	A protein 4.1X species x kDa in size identified in this study
4.1R-CTR	Protein 4.1R-C-terminal region
ABP	AMPA receptor binding protein
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid
AJ	Adherens junctions
APS	Ammonium persulphate
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
C-terminal	Carboxyl terminal
CaM	Calmodulin
CaMKII	Ca^{2+}/calmodulin dependant kinase II
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
CTD	C-terminal domain
DPG	2,3-diphosphoglycerate
dNTPs	Deoxynucleotide triphosphates
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis (β-aminoethylether_ N, N, N', N'-tetraacetic acid
ELF1α	Elongation factor 1α
eIF3	Eukaryotic initiation factor 3
eIF3-p44	Eukaryotic initiation factor 3 – p44 subunit
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
FITC	Fluorescein isothiocyanate
g	Acceleration due to gravity
GABA	γ-aminobutyric acid
GFAP	Glial fibrillary acid protein
GK	Guanylate kinase domain
GPC	Glycophorin C
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
IF	intermediate filament
IPTG	β-D-Isopropylthiogalactopyranoside
IEF	Isoelectric focusing
Kb	Kilobases

K_D	Equilibrium dissociation constant
KDa	Kilodaltons
MAGUK	Membrane associated guanylate kinase
MAP	Microtubule associated protein
MBD	Membrane binding domain
M_r	Relative molecular mass
mGluR	metabotropic glutamate receptor
mRNA	messenger ribonucleic acid
MS	Membrane skeleton
N-terminal	Amino terminal
NEPHGE	Non-equilibrium pH gel electrophoresis
NGF	Nerve growth factor
NLS	Nuclear localisation signal
NMDA	N-methyl-D-aspartate
NMJs	Neuromuscular junctions
NRX VI	*Drosophilia* neurexin VI
nt	Nucleotides
NuMA	Nuclear mitotic apparatus protein
OD_x	Optical density at wavelength x
OPD	o-phenylene diamine dihydrochloride
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pI	Isoelectric point
PIP_2	Phosphatidylinositol (4,5) bisphosphate
PIPES	Piperazine-N, N'-bis-2-ethanesulfonic acid
PKA	Protein kinase A
PKC	Protein kinase C
PP1	Protein phosphatase-1
PSDs	Postsynaptic densities
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
SAB	Spectrin-actin binding domain
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH3	Src homology 3 domain
TEMED	N, N, N', N'-tetramethylethylenediamine
TJ	Tight junctions
Tris	Tris (hydroxmethyl) aminomethane
TRITC	Tetramethylrhodamine isothiocyanate
Tween20	Polyoxyethylenesorbitan monolaureate
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight

Chapter 1

Introduction

Introduction

Complex organisms can be viewed as highly ordered arrangements of cells. Within an organism a hierarchy of organisation exists. For mammals the top of this hierarchy sees the relative positioning of tissues, organs and bone to form the structure we recognise as the animal itself. On the next level we find that tissues, organs and bone are themselves formed by ordered groups of cells organised in an array of like-cell-type with like-cell-type. At an even lower level, this time within the cell, organelles and structural components are organised to promote the functions placed upon the cell. Cell plasma membranes play a pivotal role in the second and third levels of organisation. They delineate cell boundaries marking the area in which organelles can be positioned and aid the association of 'like' cells to form tissues. Inter-communication between each level of organisation is essential. Plasma membranes are fundamental to this communication. They govern the exchange of materials with the extracellular environment and define the complement of extracellular signals that a cell can recognise. They also participate in the transmission of extracellular signals to the intracellular environment enabling a cell to respond as required. Without these features of organisation and communication cells, tissues and organisms would not survive. The integrity of plasma membranes is essential to organism viability.

Erythrocytes have the best studied membrane to date. These cells lack organelles and therefore provide a purifiable system composed solely of plasma membrane. The erythrocyte plasma membrane is a 95% cholesterol/phospholipid bilayer interspersed with transmembrane proteins involved in signalling and transport (McMullin, 1999). On the underside of erythrocyte membranes lies the membrane skeleton (MS), a regular lattice-like meshwork of protein originally identified by its retention of erythrocyte shape after solubilisation of the plasma membrane with non-ionic detergent (Bennett & Gilligan, 1993; Yu *et al.*, 1973). The MS lends strength and durability to the plasma membrane.

1. The Erythrocyte Membrane Skeleton

The MS was first defined when erythrocyte ghosts were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fairbanks *et al.*, 1971). Ghosts are prepared by hypotonic lysis of red blood cells and exist as intact biconcave plasma membranes complete with underlying MS (Dodge *et al.*, 1963). Ghosts give a distinct pattern on electrophoresis and the protein components observed are mainly a mixture of transmembrane and MS proteins that were initially named according to their relative electrophoretic mobility (Figure 1.1). Erythrocyte ghosts have proved a useful tool in the analysis of the MS and its components.

Studies on ghosts have shown that the various protein components associate with the plasma membrane to different extents. Ghosts treated with hypotonic buffer (e.g. 0.3mM phosphate, pH8; 0.1mM EDTA) release MS proteins including spectrin, actin and protein 4.1 with concomitant formation of inside-out-vesicles (Fairbanks *et al.*, 1971; Gratzer, 1982; Steck *et al.*, 1970). Membrane skeletons prepared by triton extraction of ghosts followed by treatment with high ionic strength buffer (e.g. 5mM phosphate, pH7; 1.5M NaCl) selectively elute ankyrin, band 3, band 4.2 and band 4.9 leaving spectrin, actin and 4.1 behind (Shen *et al.*, 1986).

1.1. Gross Structure and Assembly of the Erythrocyte Membrane Skeleton

Electron microscopy of extended membrane skeletons has provided evidence of MS structure, showing a regular lattice of six sided polygons with the occasional pentagon (3%) and heptagon (8%) (Figure 1.2) (Liu *et al.*, 1987; Shen *et al.*, 1986). It has become well accepted that spectrin, actin and 4.1 are the major MS components that interact to form this lattice. Other proteins identified in erythrocyte ghosts act as MS accessory or linker elements.

In the MS structure, rod-like spectrin molecules link to vertices composed of short actin filaments by a binding interaction that is promoted and stabilised by protein 4.1. Adducin promotes a similar interaction between erythrocyte spectrin and actin but does not stabilise complexes the way 4.1 does. The MS is attached to the plasma membrane

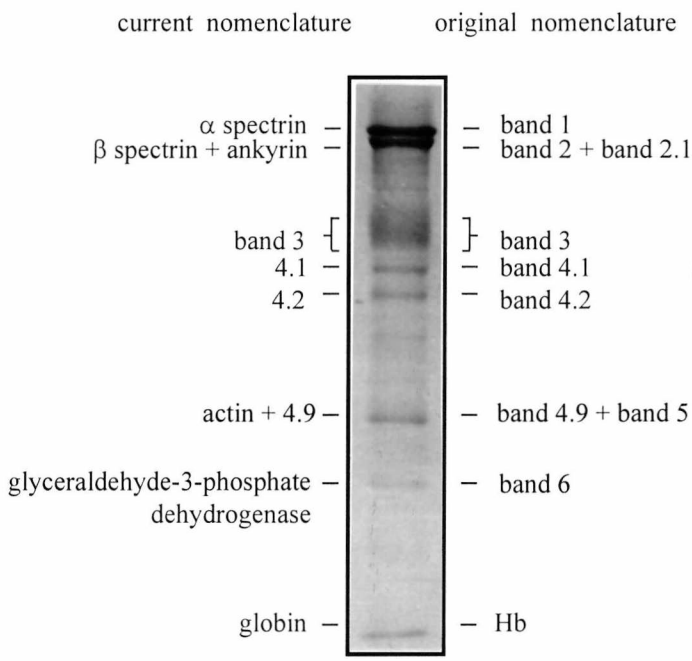
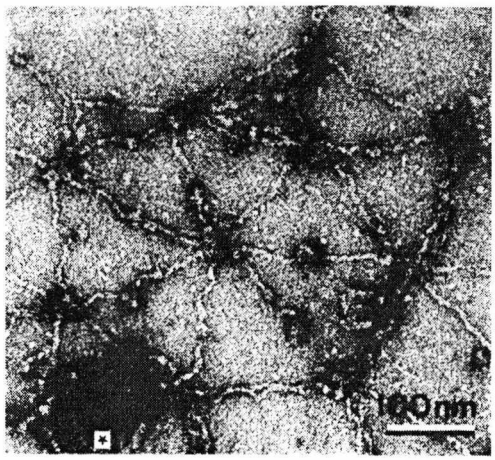


Figure 1.1 Coomassie blue stain of erythrocyte ghosts. Ghosts were prepared from packed human red cells using the method of Gratzer (1982), run on 9% SDS-PAGE and stained with Coomassie blue. The major ghost components are labeled.

(a)



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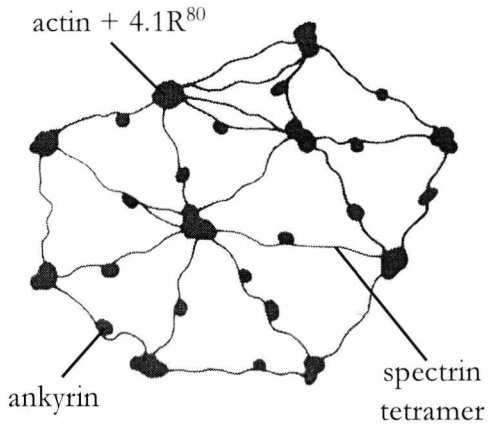

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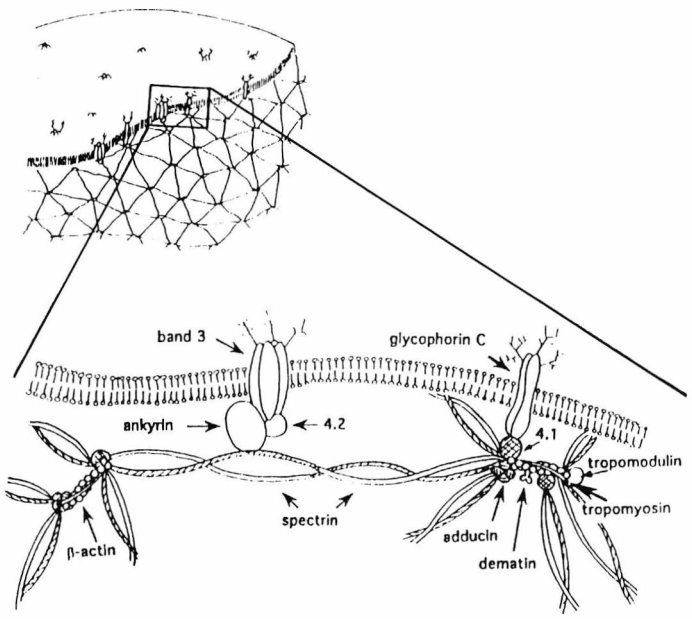


Figure 1.2 Ultrastructure of the erythrocyte membrane skeleton. The membrane skeleton has a regular lattice-like appearance when visualised under the electron microscope (a). The schematic figure in (b) tentatively identifies some of the network structures observed in (a) (both reproduced from Liu *et al.* (1987)). A diagramatic representation of the erythrocyte membrane skeleton is shown in (c), reproduced from Bennett & Gilligan (1993).

via 4.1-band 3 and 4.1-glycophorin C interactions as well as bridges formed between spectrin and band 3 by ankyrin (for reviews on the erythrocyte MS see Marchesi (1985), Bennett & Lambert (1991) and Bennett & Gilligan (1993)).

Assembly of the erythrocyte membrane skeleton occurs at the plasma membrane (for a review see Woods & Lazarides (1988)). Recruitment of skeletal proteins to the membrane is controlled by developmental splicing switches (e.g. spectrin (Chu *et al.*, 1994) and 4.1 (Winardi *et al.*, 1995)) and variations in the rate of protein synthesis, assembly and turnover (spectrin, ankyrin, 4.1 and band 3 (Hanspal *et al.*, 1992)). An unstable MS initially pre-assembles underneath the plasma membrane but does not attach to it. Only 12% of total synthesised spectrin and 20% of synthesised ankyrin associates to form a MS at this point and very little of the expressed 4.1 assembles to the structure (Hanspal *et al.*, 1992). The majority of spectrin, ankyrin and 4.1 remains in the cytosol where it is turned over rapidly. Production of a stable, plasma membrane associated MS does not occur until the late erythroblast stage of erythroid maturation when band 3 is synthesised and inserted into the membrane and 4.1 is expressed with a high affinity spectrin-actin binding site (this Chapter, section 3.1.3.4). It is not clear why this mechanism of assembly is favoured over recruitment of proteins to the membrane skeleton co-translationally.

1.2. Components of the Erythrocyte Membrane Skeleton

Once assembled, the MS becomes a multifunctional complex. It stabilises the lipid bilayer, providing strength and durability (Devarajan & Morrow, 1996; Hansen *et al.*, 1997; Luna & Hitt, 1992), organises the distribution of integral membrane proteins ((Kusumi & Sako, 1996; Tomishige & Kusumi, 1999) and permits rapid cell shape changes in response to intracellular and extracellular signals (Bennett & Gilligan, 1993). It is the nature of the MS protein components that imparts these functions allowing the structure to be dynamic, yet mechanically strong.

1.2.1. The Role of Spectrin

Spectrin structure and functions are reviewed by Hartwig (1995), Bennett & Gilligan (1993), Bennett & Lambert (1991), Viel & Branton (1996) and Devarajan & Morrow (1996).

1.2.1.1. Spectrin structure

The flexibility of the membrane skeleton is largely due to the extended rod shape and elastic properties of spectrin. Erythrocyte spectrin is a heterodimer of αI (240kDa) and βIΣI (225kDa) polypeptides arranged in an anti-parallel helical fashion (Figure 1.3a and b). Each polypeptide is a long flexible rod made up of three domains: the rod domain, the self-association domain and a subunit specific domain. The rod domain encompasses the elongated backbone of the spectrin molecule. It is composed of 22 (αI) or 17 (βIΣ1) protease resistant triple helical repeat segments separated by short protease sensitive linear regions (Speicher & Marchesi, 1984). Each repeat has 106-114 amino acids which arrange into three α helices (A, B and C) folded back on one another. Repeats are thought to be structurally identical regardless of amino acid sequence differences between them (Yan *et al.*, 1993). The spectrin self-association domain is found at the N-terminal of the α chain and the C-terminal of the β chain. Head-to-head assembly of two (αβ) heterodimers at this domain produces spectrin $(\alpha\beta)_2$ tetramers, the functional unit found *in vivo*. Self-association requires that a complete helical repeat unit be formed between each set of 'head on' spectrin chains. The N-terminal of αI spectrin donates a lone C helix to this repeat and the C-terminal of βIΣ1 spectrin donates the A and B helices (Lecomte *et al.*, 1999; Viel & Branton, 1996) (Figure 1.3c). The final domain is subunit specific and differs between the two spectrin chains. The α chain boasts two EF hand motifs at its C-terminal (for a review see Valenta *et al.*, (1998)). These segments undergo conformational changes in response to the binding of Ca^{2+} and may regulate the interaction between spectrin and actin (Viel & Branton, 1996). The β subunit has an N-terminal actin binding domain.

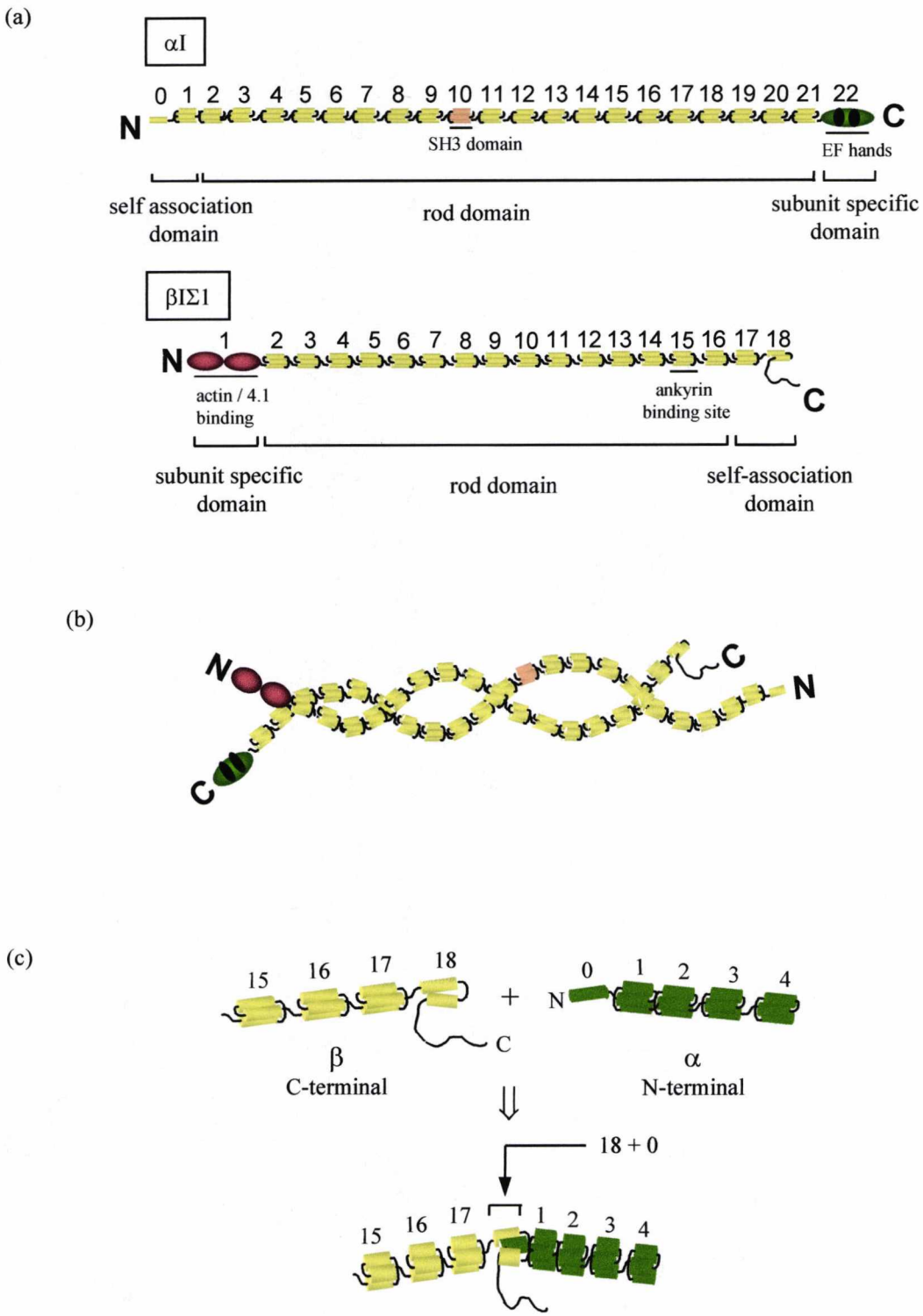


Figure 1.3 Spectrin architecture. Spectrin is a dimer of α and β polypeptides. In erythrocytes it is formed from αI and βIΣ1 polypeptides (a). Each polypeptide chain has a rod domain of triple helical repeats, a self-association domain and a subunit specific domain. Rod domains of the separate α and β polypeptides coil around each other to form spectrin dimers as shown schematically in (b). Self association of spectrin into tetramers occurs by a head-to-head association of dimers where incomplete repeats on opposing polypeptides interact to form a complete triple helical repeat structure (c). Courtesy of P. Bignone (University of Kent).

1.2.1.2. Spectrin binding interactions

Almost all spectrin-ligand interactions take place via the β subunit. The ankyrin binding site has been localised to the 15th repeat of βIΣI spectrin whilst actin filaments, protein 4.1 and adducin bind at the N-terminal of the β subunit. A membrane association domain (MAD1) exists in repeat 1 of the β subunit allowing ankyrin/4.1 independent association of spectrin with membranes (Lombardo *et al.*, 1994) and weak associations of the β subunit have been reported with calmodulin (CaM) (Berglund *et al.*, 1986). In non-erythroid cells, the β subunit of erythrocyte spectrin has also been shown to interact with neurofilaments (Frappier *et al.*, 1991) and is probably responsible for interactions observed with other intermediate filament proteins (Langley & Cohen, 1986; Langley & Cohen, 1987). Binding sites within the α subunit of erythrocyte spectrin are limited to an SH3 domain in repeat 10 and the EF hands in the C-terminal. Despite SH3 domains being common protein binding motifs, very few binding partners have been identified for the SH3 domain of α spectrin (Bialkowska *et al.*, 1997; Cianci *et al.*, 1995; Ziemnicka-Kotula *et al.*, 1998). Similarly the EF hands of spectin have been shown to interact with Ca^{2+} *in vitro* but not *in vivo* (Trave *et al.*, 1995). Spectrin interactions in erythrocytes are regulated mainly by PKA phosphorylation of the β subunit (for a review see Boivin (1988)).

1.2.1.3. Diversity amongst spectrins

Erythrocyte spectrin (αI-βIΣ1) belongs to a larger family of spectrin proteins. Variety is produced from two α spectrin genes (αI and αII) and four[1] β spectrin genes (βI, βII, βIII and *elf*) as well as alternative splicing of some gene products. Products of the βI and βII genes are spliced into either Σ1 or Σ2 subtypes. No splice variants have been identified for αI but αII spectrin can be spliced into subtypes Σ1, Σ2, Σ3 or Σ4 (Cianci *et al.*, 1999). In general, αI pairs with βI and is limited in its tissue distribution while αII pairs with βII and is widely expressed. The α subunit partners for βIII and ELF

[1] The *elf* gene (Spnb-3) resides on mouse chromosme 11 close to the βII spectrin gene (Spnb-2). Although evidence suggests they are two distinct genes conformation is necessary.

spectrins are not clearly defined but αII gene products seem the most likely candidates. Most structural properties and binding activities of spectrin are conserved within the different α and β gene groups. However two major differences exist: (1) αII contains a strong calmodulin binding site in repeat 12 absent from αI, (2) βIΣ2, βIIΣ1 and βIII have a ~ 20kDa C-terminal extension that incorporates a pleckstrin homology domain. Neither βIΣ1, βIIΣ2 (βII short) or ELF3 have this region.

As well as having specific tissue distributions, different isoforms of spectrin can be co-expressed in the same cell where they often localise to distinct regions. The diversity among spectrins and their differential tissue and subcellular distribution leads to the conclusion that there must be cell specific roles for spectrin proteins. Table 1 summarises the different spectrin subunit types and the tissues they are found in.

GENE	MOLECULAR MASS	TISSUE DISTRIBUTION	COMMENTS	REFERENCES
αI	240 kDa SDS-PAGE 281 kDa theoretical	erythrocytes, brain		(Bennett and Gilligan, 1993; Clark *et al.*, 1994; Hartwig, 1995; Marchesi, 1985)
βIΣ1	225 kDa SDS-PAGE 246 kDa theoretical	erythrocytes, brain, heart		(Clark, et al., 1994; Devarajan and Morrow, 1996; Hartwig, 1995)
βIΣ2	235 kDa SDS-PAGE 240 kDa theoretical	brain, skeletal and cardiac muscle		(Bennett and Lambert, 1991; Devarajan and Morrow, 1996; Hartwig, 1995)
αIIΣ1	285 kDa theoretical	brain and muscle	tissue distribution only determined by RT-PCR and awaits conformation with isoform specific antibodies	(Cianci, et al., 1999)
αIIΣ2	284 kDa theoretical	most tissues		
αIIΣ3	282 kDa theoretical	muscle and embryo		
αIIΣ4	282 kDa theoretical	gonads and embryo		
βIIΣ1 (fodrin)	235 kDa SDS-PAGE 274 kDa theoretical	all cell types except erythrocytes and muscle		(Burns *et al.*, 1983; Hartwig, 1995; Levine & Willard, 1981)
βII short	240 kDa SDS-PAGE 253 kDa theoretical	skeletal and cardiac muscle		(Hayes *et al.*, 2000)
βIII	230 kDa SDS-PAGE 271 kDa theoretical	brain, liver, kidney	enriched in synaptic vesicle and plasma membrane fractions	(Ohara *et al.*, 1998; Sakaguchi *et al.*, 1998; Stankewich *et al.*, 1998)
βIII golgi (formerly βIΣ*)	220 kDa SDS-PAGE	golgi and intracellular COPI transport vesicles of MDCK, HeLa and NRK cells. Also brain, liver, kidney, pancreas.	no α subunit detected at the golgi.	(De Matteis & Morrow, 1998; Holleran & Holzbaur, 1998; Stankewich, et al., 1998)
Elf3[2]	200 kDa SDS-PAGE	brain, kidney, liver, heart	expression developmentally regulated in liver.	(Mishra *et al.*, 1999)
βNM	220 kDa SDS-PAGE	NMJ	no α spectrin detected	(Bloch & Morrow, 1989)
TW260/240	α - 260 kDa β - 240 kDa both SDS-PAGE	terminal web of intestinal epithelial cells	β subunit has no ankyrin or 4.1 binding site, specific to birds	(Coleman *et al.*, 1989)
βH	430 kDa SDS-PAGE	*Drosophila* and *C. elegans*	corresponding α subunit similar to mammalian αII	(Hartwig, 1995; McKeown *et al.*, 1998)

[2] ELF1 and ELF2 proteins exist as alternatively spliced isoforms of ELF3. They show less homology to β spectrin than ELF3 and are less well characterised. As a result they have been omitted from the table.

Table 1. Known gene products and isoforms of spectrin. Known products of the spectrin genes are listed along with the tissues each has been recorded in. Spectrin relative molecular masses determined by SDS-PAGE differ to the theoretical molecular masses calculated from the amino acid sequences.

1.2.2. The Role of Actin

Actin and actin binding proteins are generally reviewed in McGough (1998), Yin & Stull (1999) and Steinmetz *et al.*, (1997) and are discussed in the context of the erythrocyte membrane skeleton in Bennett & Gilligan (1993), Marchesi (1985), Shen, Josephs & Steck (1986) and Pinder & Gratzer (1983).

Actin is a bi-lobed 42kDa monomer (g-actin) which can self assemble into long polar helical filaments (f-actin). Within cells the actin cytoskeleton is a dynamic structure that can rearrange and respond to extracellular signals via members of the Rho family of small GTPases (for reviews see Redowicz (1999), Yin & Stull (1999), Aspenstrom (1999), Hall (1994) and Symons (1996)). It is structurally important throughout the cytoplasm of most cells and in particular at specialised domains e.g. those involved in cellular movements such as muscle sarcomeres, laminapodia, filapodia and growth cones.

Erythrocyte actin differs from non-erythroid f-actin in three ways. First, and most significantly, it exists as short proto-filaments 13-15 monomers in length. Second, it is more stable than non-erythrocyte f-actin, presumably due to associations with actin binding or capping proteins. Thirdly, it is composed entirely of β actin rather than a mixture of β and γ isoforms present in non-erythroid cell types.

It is estimated that 25,000-30,000 short actin filaments exist per erythrocyte. The length of these filaments is controlled by the opposing effects of two groups of proteins, tropomyosin and spectin/4.1. Tropomyosin is a rod like α helical dimer that binds along the length of actin filaments ((Ursitti & Fowler, 1994) and reviewed by Bennett & Gilligan (1993)). By doing so it stabilises short actin filaments in erythrocytes and act as a molecular ruler to help maintain their length. Each tropomyosin dimer is 33-34nm in length while short erythrocyte actin filaments are 33 ± 5nm long. There is sufficient tropomyosin in erythrocytes for one tropomyosin molecule to coat each side of an actin filament and delimit its length. Tropomyosin may also regulate the number of spectrin molecules attached to each short actin filament. This may be important

given that in contrast to tropomyosin, spectrin/p4.1 complexes sever actin. Each actin filament is associated with 5-7 spectrin oligomers in a weak binding interaction that is stabilised by 4.1. These complexes limit actin polymerisation and prevent the filaments becoming too long.

The enhanced stability of erythrocyte verses non-erythroid actin filaments is mainly due to filament capping in erythrocytes. Tropomodulin is a tropomyosin binding protein found in erythrocytes that localises to the pointed (slow growing) end of actin filaments (Bennett & Gilligan, 1993). It has been shown to prevent elongation and depolymerisation of actin filaments *in vitro* and may play a similar stabilising role by capping actin filaments in erythrocytes (Figure 1.4).

Immunolabelling has also localised actin bundling proteins 4.9 (dematin) and adducin to spectrin-actin-p4.1 junctional complexes in erythrocytes. Adducin is thought to play a role in regulating spectrin-actin interactions and may help regulate actin filament length and stability in a similar way to spectrin-4.1 complexes. Protein 4.9 may cap the pointed end of actin filaments, stabilising them in a similar way to that proposed for tropomodulin.

1.2.3. The Role of Ankyrin

Ankyrin structure and functions are reviewed by Bennett & Lambert (1991), Bennett & Gilligan (1993), Devarajan & Morrow (1996) and Peters & Lux (1993).

In erythrocytes ankyrin functions to link the plasma membrane to the MS through interactions with spectrin and band 3. Although the adapter role it plays in erythrocytes is important for the function of these cells, studies on non-erythroid ankyrins have described more specialised functions involving the formation and maintenance of polarised membrane domains. More accurately therefore ankyrin is an organiser of cytoskeletal compartments.

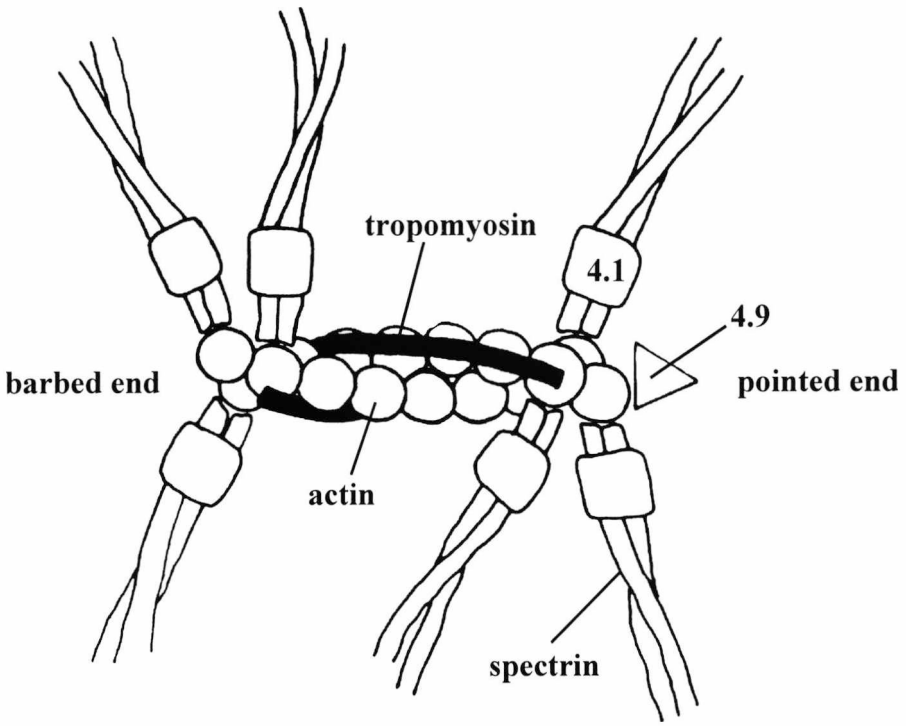


Figure 1.4 Erythrocyte actin and associated proteins. In erythrocytes actin exists as short proto-filaments 13-15 monomers in length. The length of these filaments may be controlled by length delimiting effect of tropmyosin and the actin filament severing action of 4.1-spectrin complexes. Erythrocyte actin may be stabilised by filament capping with tropomoduin, 4.9 or adducin. Reproduced from Marchesi (1985).

1.2.3.1. Ankyrin structure

Most ankyrins share the three domain structure of erythrocyte ankyrin (ANK_R) (Figure 1.5). The globular N-terminal domain (~89kDa) is highly conserved and interacts with the anion exchanger, band 3 (AE1). This domain is made up of 24 repeat units each composed of 33 amino acids and folds into four sub-domains of six repeats each. All four sets of six repeats are required for correct domain folding however the 12 most N-terminal repeats are most important for binding to band 3 in erythrocytes. ANK repeats are found in a diverse range of proteins where they are generally thought to mediate protein-protein interactions (Sedgwick & Smerdon, 1999). The middle domain of ankyrin (62-67kDa) forms the well conserved spectrin binding domain. It is involved in binding to and discriminating between the various β spectrin isoforms. Interactions with spectrin are modulated by ankyrin phosphorylation. Un-phosphorylated ankyrin binds strongly to spectrin tetramers but only weakly to spectrin dimers. Phosphorylated ankyrin shows no preference. Spectrin-ankyrin interactions are also modulated by band 3. Binding of band 3 to the ankyrin repeat domain confers co-operative binding of spectrin by increasing the affinity of ankyrin for spectrin oligomers. The 55kDa C-terminal domain of ankyrin is a regulatory domain that modulates the activities of the other two (Hall & Bennett, 1987)). It is the most variable domain and is subject to alternative splicing. Two important regulatory regions have been identified at opposite ends of the regulatory domain. The C-terminal end can be cleaved by calpain resulting in reduced binding of ankyrin to band 3. Splicing out of a 161 amino acid segment towards the N-terminal of the regulatory domain produces an activated ankyrin isoform referred to as 2.2. $Ankyrin_{2.2}$ has a three-fold greater affinity for spectrin and associates with twice as many band 3 molecules in the erythrocyte membrane. This isoform has binding activities not evident in unprocessed ankyrin, thus the regulatory domain controls binding specificity as well as affinity.

1.2.3.2. Diversity amongst ankyrins

Three ankyrin genes have been identified (*ANK1*, *ANK2* and *ANK3*). $Ankyrin_R$ isoforms ($Ank_{2.1}$ (206kDa), $Ank_{2.2}$ (195kDa)) are derived from the *ANK1* gene and are mainly confined to erythrocytes, muscle and the cell bodies and dendrites of a subset of

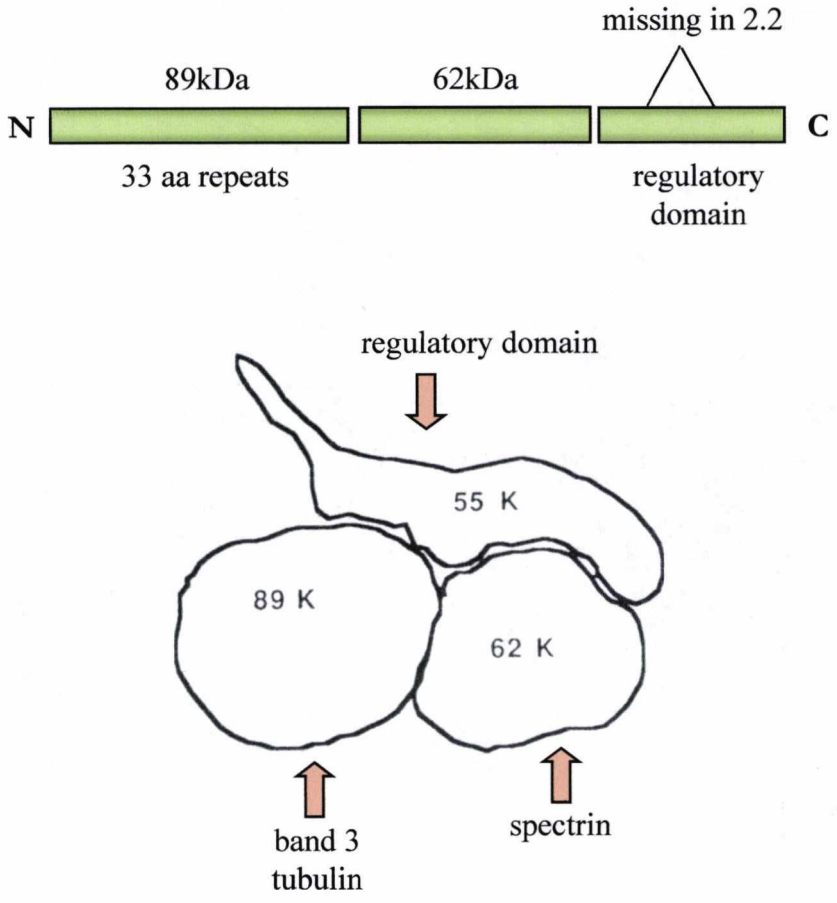


Figure 1.5 Domain structure of ankyrins. Schematic diagram showing the domain structure of erythrocyte ankyrin. This ankyrin shares the three domain structure of most ankyrin proteins where the globular 89kDa domain contains the ANK repeats responsible for binding to the anion exchanger band 3, the middle domain binds spectrin and the C-terminal domain regulates the activities of the other two. Deletion of part of the regulatory domain leads to an activated ankyrin in erythrocytes with an increased affinity for spectrin and band 3. aa, amino acids. Redrawn from Bennett & Lambert (1991).

central nervous system (CNS) neurons. $Ankyrin_B$ species (Ank_{B440} and Ank_{B220}) are produced from the *ANK2* gene and are the major isoforms expressed in brain. A variety of $ankyrin_G$ proteins exist as products of the *ANK3* gene (Ank_{G480} Ank_{G270} Ank_{G190} Ank_{G119}). These are expressed widely throughout the body but are often localised in a tissue or sub-cellular specific manner e.g. $AnkG_{119}$ at the golgi (Devarajan *et al.*, 1996). Variety amongst ankyrins diversifies integral membrane protein interactions to include a range of ion channels and cell adhesion molecules (e.g. the anion exchanger AE3 and voltage dependant sodium channel from brain, the cardiac Na^+/Ca^{2+} exchanger, the Na^+/K^+-ATPase and amiloride-sensitive sodium channel in kidney, the ryanodine and inositol 1,4,5-triphospahte receptors in lymphocytes, the neurofascin/LI/NrCAM family and the hyaluronic acid receptor CD44 (Kordeli *et al.*, 1998). This extended binding repertoire suggests that ankyrins are functionally important to a number of cellular processes.

1.2.4. The Role of Protein 4.1

Protein 4.1 and its role in the erythrocyte membrane skeleton is reviewed by Bennett & Gilligan (1993), Devarajan & Morrow (1996) and Marchesi (1985). A more detailed analysis of protein 4.1 is presented later in this thesis (this Chapter, section 3).

Erythrocyte protein 4.1 is expressed from the *EPB41* gene and is important in the formation and maintenance of a stable MS. It interacts with spectrin and actin to form junctional complexes and manages links between the membrane skeleton and the plasma membrane through interactions with p55, GPC and band 3. Erythrocyte protein 4.1 is monomeric and exists in two electrophoretic forms when resolved on SDS polyacrylamide gels using the Laemmli buffer system (Laemmli, 1970). Protein 4.1a (80kDa) and 4.1b (78kDa) differ only at their C terminus due to post-translational deamidation of asn 502 (Inaba *et al.*, 1992). Erythrocytes have a life span of 120 days. The *in vivo* half life of asn 502 is approximately 41 days and an increasing amount of 4.1a is seen as an erythrocyte ages. The associations of erythrocyte 4.1 are regulated by phosphorylation and Ca^{2+}/CaM. These reduce the affinity of 4.1-membrane interactions and the capacity of 4.1 to promote spectrin-actin binding (Danilov *et al.*, 1990; Ling *et*

al., 1988; Lombardo & Low, 1994; Pinder *et al.*, 1995; Tanaka *et al.*, 1991). Weakening of 4.1 interactions loosens the membrane skeleton allowing the membrane to deform. It is essential if erythrocytes are to squeeze through the small capillaries of the vascular system.

1.2.4.1. The band 4.1 superfamily

4.1R is a member of the band 4.1 superfamily (for reviews see Tsukita & Yonemura (1999), Mangeat, Roy & Martin (1999) and Girault *et al.*, (1999)). All members of this superfamily share some homology to the 30kDa membrane binding domain of 4.1R, also named the FERM domain after the first four members of the superfamily identified, 4.1/Ezrin/Radixin/Moesin (Chishti *et al.*, 1998). Current members of the band 4.1 superfamily include the band 4.1 proteins: 4.1R; 4.1N; 4.1G; 4.1B, coracle, the ERM proteins: ezrin; radixin; moesin; merlin, talin (Schmidt *et al.*, 1999), myosin VIIA and myosin X (Girault *et al.*, 1999; Koyano *et al.*, 1997), CDEP (Chrondocte-Derived-Ezrin-Like protein) (Koyano *et al.*, 1997), the tyrosine phosphatases: PTPH1; PTP-MEG; PTP-BAS; PTPD1 (Chishti *et al.*, 1998; Gu & Majerus, 1996), BZF1 (Shi & Mullersman, 1997), DAL-1 (Tran *et al.*, 1999), NBL1-7 (Novel-Band 4.1-Like protein) (Takeuchi *et al.*, 1994) and various members of the FAK and JAK family (Girault *et al.*, 1999) (Figure 1.6). Ezrin, radixin, moesin, merlin and talin show particular functional similarity to 4.1. These proteins are all actin binding proteins associated at the cortical membrane skeleton. They are regulated in a cell type specific manner and are important in the formation and maintenance of cell shape.

1.2.5. The Role of p55

p55 is an erythrocyte MS accessory protein. It helps link the plasma membrane to the MS through interactions with protein 4.1 and the integral membrane protein GPC. In normal erythrocytes p55 is heavily palmitylated. The reason for this is unknown however patients with chronic myeloid leukaemia show a significant decrease in palmitylation of p55 which may modulate p55 function in these cells, for example by weakening the p55-GPC interaction (Marfatia *et al.*, 1997; Ruff *et al.*, 1999; Ruff *et al.*, 1991).

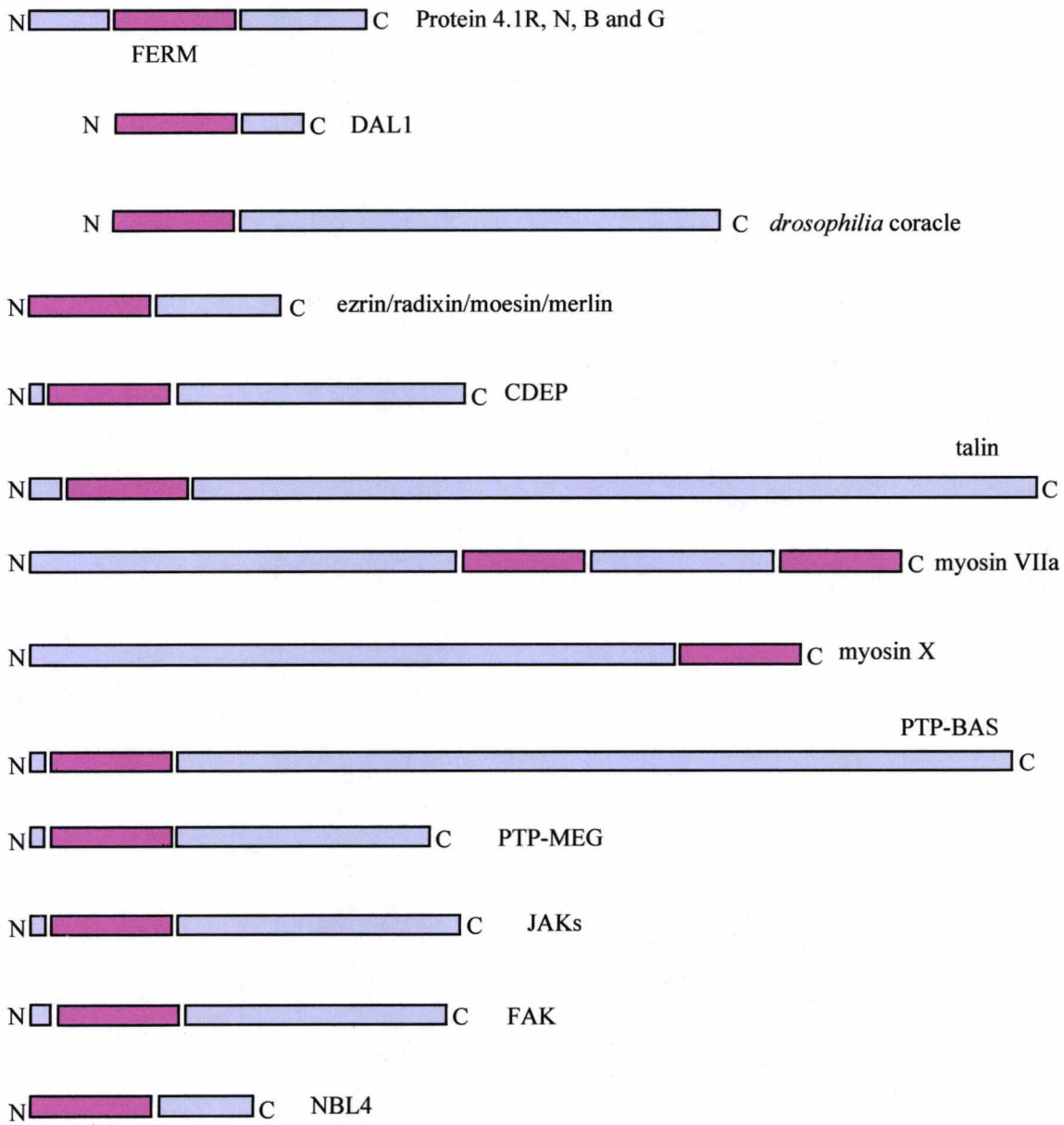


Figure 1.6 Schematic representation of various 4.1 superfamily members showing the position of the FERM domain. 4.1 superfamily proteins are defined by the presence of a FERM domain. The homology of this domain varies between superfamily proteins and while there is ~71% amino acid sequence similarity between the FERM domains of 4.1R, N, G and B, significantly lower sequence similarity exists between the prototypical FERM domain of 4.1R and less well related superfamily members e.g. myosin VIIa (15%), talin (20%), ezrin/radixin/moesin and merlin (~28)%. Members of the 4.1 superfamily have diverse cellular functions but appear to be broadly associated with the structural and regulatory requirements of the actin cytoskeleton. The FERM domain corresponds to the MBD of protein 4.1. Not all superfamily members are shown. Adapted from Girault *et al.* (1999).

1.2.5.1. p55 structure

Sequence studies indicate that p55 has five domains, a PDZ domain, an SH3 domain, a protein 4.1 binding domain, a tyrosine phosphorylation domain and a guanylate kinase (GK) domain (Elder *et al.*, 1996; Marfatia *et al.*, 1997). The organisation of these domains makes p55 a member of the MAGUK (membrane associated guanylate kinase) family of proteins (Figure 1.7) (for reviews see Dimitratos *et al.*, (1999), Hata *et al.*, (1998) and Anderson (1996)). This family includes the *drosophilia* tumour supressor protein dlg, the human homologue of dlg – hDlg (or SAP97), the products of the DLG2 and DLG3, the synapse associated protein PSD95, the tight junction proteins ZO-1 and ZO-2, the *C. elegans* signalling protein LIN2 and the neuronal protein CASK. Two types of MAGUK exist according to the number of PDZ domains present. MAGUKS related to p55 have just one PDZ domain whilst those related to dlg have three.

The GK domain of p55 shares homology with the yeast enzyme guanylate kinase but is not enzymatically active. A yeast-2-hybrid screen using the GK domain of p55 has identified a binding interaction with a novel human homologue of *drosophilia* dlg called P-dlg (Nakamura *et al.*, 1998). The function of this domain in erythrocyte p55 is essentially unknown. The tyrosine phosphorylation domain contains the phosphorylation motif YEXV. This motif is conserved in other MAGUK family proteins and may be a general mechanism for regulation of MAGUK protein binding interactions. Phosphorylation of this domain has not been demonstrated *in vivo*. The SH3 domain has the potential to mediate protein-protein interactions with target substrates containing a specific poly-proline motif (for reviews see Buday (1999) and Mayer & Gupta (1998)). SH3 domains in cytoskeletal proteins are usually involved in the recruitment of binding substrates to specific sub-membranous sites and target substrates are often signal transduction molecules. Binding partners for the SH3 domain of p55 have not yet been identified.

The protein 4.1 binding site exists in the 39 amino acids between the SH3 and GK domain of human erythroid p55. A cluster of lysine residues forms a basic motif within this region that may contribute to binding (Marfatia *et al.*, 1995). A similar motif is

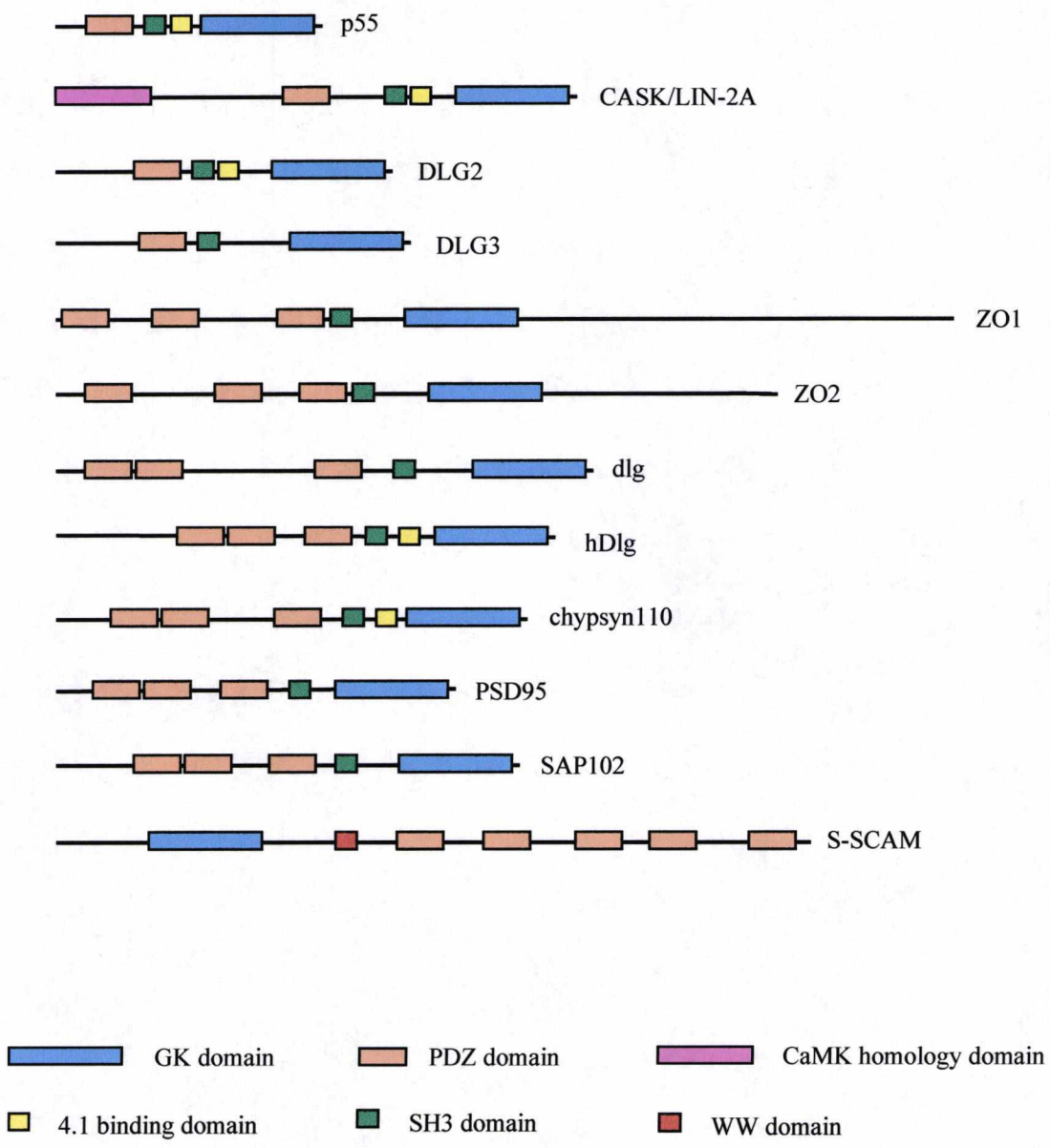


Figure 1.7 Domain organisation of various members of the MAGUK family of proteins. MAGUK proteins are characterised by the presence of an enzymatically inactive guanylate kinase (GK) domain, a variable number of PDZ protein binding domains and an SH3/WW binding motif that interacts with proline rich sequences. Some MAGUK proteins contain a 4.1 binding domain that can interact with the MBD of 4.1R. This domain can be alternatively spliced in both hDlg and chapsyn 110. Diagram adapted from Sheng (1996) and Lue *et al.* (1996).

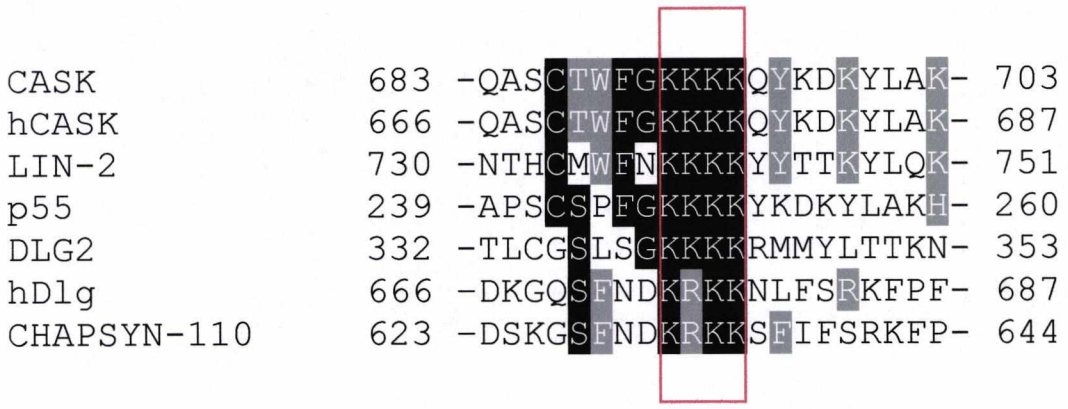


Figure 1.8 4.1 binding motif found in MAGUK proteins. The basic motif (enclosed in red box) in p55, hDlg and CASK is thought to interact with the MBD of 4.1R. This motif is also conserved in chapsyn 110, dlg and DLG2 and may be a general mechanism for 4.1-MAGUK binding.

conserved in other MAGUK proteins, including dlg, hDlg, DLG2, LIN2 (Figure 1.8) and may be a general mechanism for 4.1-MAGUK binding.

The PDZ domain of p55 binds to the C-terminal of the erythrocyte membrane protein GPC. The three amino acid YFI motif at the very C-terminal of GPC is essential for this interaction and defines the PDZ unit of p55 as a class II PDZ domain (this Chapter, section 2.3.3.3.1). The p55-GPC-4.1 ternary complex that forms in erythrocytes is important in the stabilisation of MS interactions and the maintenance of cell shape. Although little is known about the distribution and role of p55 in non-erythroid cells, MAGUK family proteins are well represented and have increasing functional significance.

1.3. Erythrocyte Disorders Linked to the Membrane Skeleton

The functional importance of membrane skeletal components is highlighted by the molecular basis of disorders of the erythrocyte membrane (for reviews see McMullin (1999) and Delaunay (1995)). Hereditary spherocytosis, elliptocytosis and pyropoikilocytosis all occur as a consequence of MS disruption and result in varying degrees of haemolytic anaemia. These disorders are either caused by deficiency of spectrin, ankyrin, GPC or protein 4.1, or by abnormalities in these proteins leading to a reduced binding capacity. Some of the more common mutations are summarised in Table 2. All of the mutations lead to a loss of membrane skeletal integrity with a concomitant reduction in plasma membrane stability required for normal erythrocyte function.

PROTEIN	MUTATIONS LEADING TO ERYTHROCYTE DISORDERS
Spectrin	Reduced α subunit expression. Point mutations in α or β subunit self-association domain leading to inefficient tetramerisation. (Nicolas *et al.*, 1998)
Ankyrin	Decrease in or deficiency of. Mutations leading to ankyrin truncation. Point mutations.
Band 3	Decrease in or deficiency of – due to insertion of stop codons. Mutations in the transmembrane regions that prevent translocation or insertion into the membrane.
Protein 4.1	Decrease in or deficiency of - due to mutation of the erythrocyte protein start codon. Leads to decrease in or absence of p55 at the membrane. (Conboy *et al.*, 1993) Deletion of Lys 447 or Lys 448 in spectrin–actin binding domain – prevents binding of 4.1 to spectrin and spectrin-actin complexes. (Lorenzo *et al.*, 1994)
Glycophorin C	Deficiency – leads to reduced erythrocyte 4.1and p55. (Hemming *et al.*, 1995)

Table 2. Summary of membrane skeleton protein abnormalities that result in membrane disorders in erythrocytes.

2. The Membrane Skeleton in Non-erythroid Cell Types and the Formation of Polarity and Specialised Membrane Domains

Non-erythrocyte membrane skeletons are discussed in Bennett & Gilligan (1993), Devarajan & Morrow (1996), Bennett (1990) and Plumplin & Bloch (1993).

The membrane skeleton paradigm is limited by the simplicity of the erythrocyte and can not be directly extended to all cell types. Cellular processes such as tissue formation, motility, secretion, mitosis, polarisation and the formation of specialised domains are not accounted for by the erythrocyte model. Polarity and specialised membrane domains are formed when the distribution of membrane and cytoskeletal proteins is restricted to pre-determined regions. This is seen with the apical and basolateral membrane domains in epithelial cells, the axonal and dendritic compartments of neurons, neuromuscular junctions (NMJs), lymphocyte caps (Bennett, 1990), nodes of Ranvier and postsynaptic densities (PSDs). Evidence that a membrane skeleton structure is important in the formation and maintenance of these domains comes from the identification of distinct isoforms of cytoskeletal proteins such as spectrin and ankyrin at these regions. These isoforms may help target proteins to their specific polarised locations, stabilise the polarised distribution of membrane proteins and maintain this distribution by restricting membrane protein lateral diffusion (Beck & Nelsom, 1996; Devarajan & Morrow, 1996).

2.1. Epithelial Cell Polarity

Epithelial cell polarity is one of the best characterised examples (for reviews see Morrow *et al.*, (1991) and Devarajan & Morrow (1996)). Non-erythroid (αII-βII) spectrin initially localises at E-cadherin points of cell–cell contact where it stabilises these junctions in order for polarity to be established. Cell junction formation divides epithelial cells into apical and basolateral domains. Polarity is achieved by targeting proteins to one or other of these domains. For example, ankyrin isoforms targeted to basolateral domains interact with membrane proteins such as the Na^+/K^+ ATPase which are then restricted to this domain also. Different isoforms of membrane skeletal

proteins are often localised to different regions of the same cell type. This promotes a polarised distribution of associated proteins through isoform specific interactions.

2.2. Neuromuscular Junctions

NMJs are specialised postsynaptic domains in skeletal muscle cells. They align opposite pre-synaptic motor neuron nerve terminals and cluster receptors, ion channels and signalling molecules to ensure rapid and efficient transmission of nervous signals to the effector muscle (for reviews see Colledge & Froehner (1998) and Sanes & Lichtman (1999)). Various isoforms of β spectrin and ankyrin are found in skeletal muscle. Specific isoforms localise at the NMJ where they are thought to help cluster voltage gated sodium channels (Kordeli *et al.*, 1998; Wood & Slater, 1998) and acetylcholine receptors (spectrin αII-βIΣII). In addition, a NMJ specific protein, rapsyn is essential for acetylcholine receptor clustering, while dystrophin, a member of the spectrin superfamily, concentrates at NMJs and may contribute to receptor and ion channel localisation (Kong & Anderson, 1999; Yoshihara & Hall, 1993).

2.3. Neuronal Polarity

Neurons are morphologically polarised consisting of a rounded cell body with dendritic protrusions (somatodendritic compartment) and a long axonal compartment terminating in a presynaptic nerve terminal. To fully establish polarity axonal and somatodendritic compartments formed by developing neurons segregate distinct isoforms of proteins to one or other location (reviewed by Winckler & Mellman (1999), Foletti, Prekeris & Scheller (1999), Colman (1999) and Craig & Banker (1994)). The structural polarity produced by the asymmetric distribution of proteins confers a functional polarity essential for the efficient propagation and transmission of action potentials. Examples of proteins with a polarised distribution in neurons are the microtubule associated protein MAP2 which is confined to cell bodies and dendrites and MAP3, MAP4 and tau which are considered axonal. Ion channels also have a polarised distribution and α type I and III sodium channels and A-type K4.2 potassium channels are somatodendritic while α type II sodium channels and A-type K1.4 potassium channels are axonal. The MS protein spectrin is also polarised with αI-βIΣII spectrin found in the

somatodendritic compartment and αII-βIIΣI spectrin (fodrin) found predominantly in axons. Other proteins that show a polarised distribution include motor proteins, cell adhesion molecules, integrins and neurotransmitter receptors. Little is known about the targeting mechanisms responsible for creating these polarised distributions however evidence suggests that both direct and indirect targeting may occur. As defined in epithelial cells (and reviewed by Keller & Simons (1997)), direct targeting involves sorting of proteins into distinct vesicle populations in the trans-golgi-network followed by vesicle transport to the correct membrane domain. In contrast to this, indirect targeting involves the initial delivery of proteins to basolateral membranes where they are then endocytosed, sorted and transcytosed to the appropriate membrane domain. Neuronal targeting is less well understood than targeting in epithelial cells and apical and basolateral membranes do not correlate directly with axonal and somatodendritic surfaces as once thought. Research is ongoing to elucidate the mechanisms that generate and maintain neuronal polarity.

2.3.1. Axon Initial Segments

Besides structural and morphological polarity, neurons have distinct regions of specialised membrane domains, namely the axon initial segment, nodes of Ranvier and postsynaptic densities. Axon initial segments define the boundaries of axonal and somatodendritic neuronal compartments (Winckler & Mellman, 1999). It is characterised by a dense, detergent insoluble complex of proteins and functions as a physical barrier to the lateral diffusion of membrane proteins maintaining their polarised distribution. Proteins localised to the axon initial segment include voltage dependant sodium channels, the cell adhesion molecules neurofascin and NrCAM, dynamin and amphiphysin, two molecules associated with clathrin coated vesicle endocytosis, αII-βIIΣI spectrin, f-actin and two specific isoforms of ankyrin$_G$ (ank$_{G480}$ and ank$_{G270}$). Disruption of f-actin with DMSO has been shown to abolish the diffusion barrier formed by the axon initial segment and leads to the redistribution of previously polarised proteins (Winckler & Mellman, 1999). Studies in which the ankyrin$_G$ gene has been knocked out also show that this gene product helps maintain the distribution

of axon initial segment proteins by anchoring them to the cytoskeleton (Zhou *et al.*, 1998). It may also help target proteins to this region during neuronal development.

2.3.2. Nodes of Ranvier

A similar structural morphology occurs at nodes of Ranvier in myelinated axons where specific isoforms of the Na^+/K^+-ATPase, voltage dependant sodium channels, neurofascin, NrCAM, spectrin (αII-βIIΣI) and ankyrin$_G$ (ank$_{G480}$ and ank$_{G270}$) congregate to form a specialised membrane domain. Ankyrin$_G$ is responsible for the heterotypic co-clustering and anchoring of the Na^+/K^+-ATPase, voltage dependent sodium channel, NrCAM and neurofascin, presumably limiting their lateral diffusion by tethering them to a spectrin based MS structure. Ankyrin$_G$ is also suggested to target proteins to nodes of Ranvier. Prior to myelination two types of axonal ankyrin exist (ankyrin$_B$ and ankyrin$_G$) and are expressed along the entire length of the axon. During myelination and node formation ankyrin$_B$ expression is down regulated while ankyrin$_G$ redistributes to sites containing NrCAM/neurofascin clusters. It was initially believed that ankyrin$_G$ was recruited to these sites via interactions with NrCAM and neurofascin however a more recent study indicates that ankyrin$_G$ may instead be responsible for their recruitment (Lambert *et al.*, 1997; Zhou *et al.*, 1998). Much remains to be discovered about the formation of specialised domains and the protein interactions that maintain them. Cytoskeletal proteins such as spectrin, actin and ankyrin clearly have a pivotal role and are essential for the efficient functioning of the nervous system.

2.3.3. Postsynaptic Densities

Postsynaptic densities are specialised membrane domains that have important roles in synaptic transmission (for recent reviews see Van Rossum & Hanisch (1999), Kim & Huganir (1999), Ziff (1997) and Kennedy (1993)). They lie underneath the postsynaptic membrane opposed to presynatptic active zones and are concentrated on dendritic spines (Figure 1.9a). Two broad categories of synapse exist, as noted by Gray (Gray, 1959) (Figure 1.9b). Type I, asymmetric synapses are mainly excitatory (glutamatergic) and have thicker and more dense postsynaptic specialisations than type II, symmetric synapses, which are generally inhibitory (GABAergic). Some variation

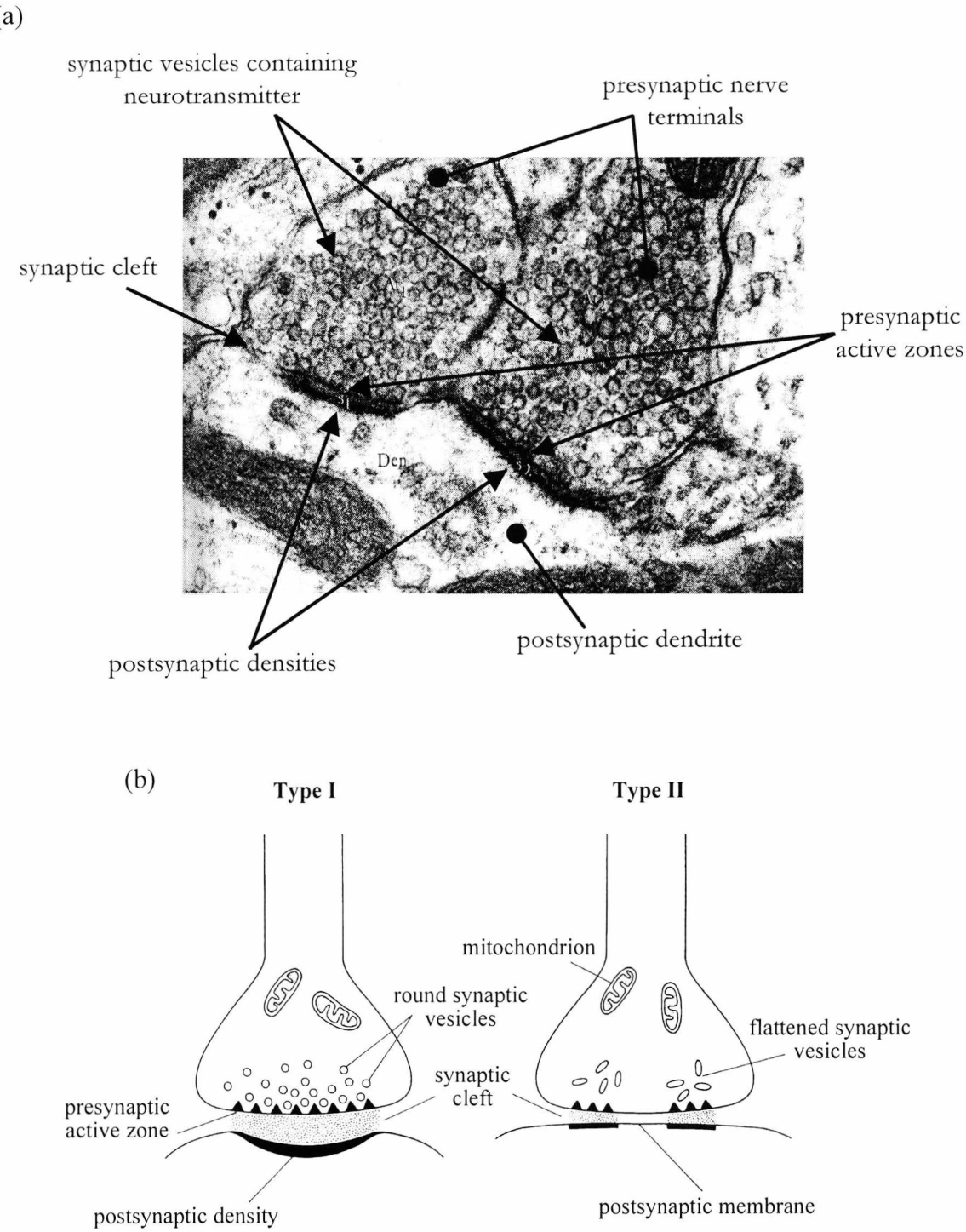


Figure 1.9 Synaptic structure. (a) Electron micrograph of rat auditory cortex showing asymmetric type I synapses. Two presynaptc nerve terminals are seen synapsing with a single postsynaptic dendrite. PSDs appear as dense regions underneath the postsynaptic membrane (reproduced from Peters *et al.* (1991)). (b) Schematic representation of synaptic structure. Gray type I synapses are generally excitatory and have thicker PSDs than Gray type II, inhibitory synapses (reproduced from Strange (1995)).

probably exists within these classes. For example, 'perforated' PSDs have been found in hippocampal neurons (Jones *et al.*, 1991) and there are reports that PSDs in different brain regions contain different classes of proteins (Carlin *et al.*, 1980; Zhang *et al.*, 1999). Glutamatergic synapses are the better characterised of the two main classes. For this reason, the information on PSDs outlined below and in the rest of this thesis refers to studies carried out on glutamatergic synapses unless specifically stated otherwise.

2.3.3.1. Functions of postsynaptic densities

Several functions have been proposed for PSDs based upon the classes of protein identified at the structure so far. None of these functions are mutually exclusive and it seems likely that PSDs are involved in them all to a certain extent. Immunocytochemical detection of cell adhesion molecules at PSDs and synapses suggests that these structures are important in the formation and maintenance of synaptic contacts (Apperson *et al.*, 1996; Hsueh & Sheng, 1999; Persohn & Schachner, 1990; Song *et al.*, 1999). The precise positioning of PSDs opposite pre-synaptic terminal active zones suggests that regulated cell-cell adhesion mechanisms establish the correct contacts between pre- and postsynaptic sites, delimit the boundaries of these zones and maintain the contacts between them once they are formed.

A high concentration of neurotransmitter receptors (e.g. NMDA and AMPA receptors) and ion channels (e.g. shaker type potassium channels and voltage-dependant anion channel 1) at postsynaptic sites indicates that PSDs function to recruit, cluster and anchor these proteins to enhance synaptic efficacy (for reviews see Ziff (1997), Sheng & Kim (1996) and Gomperts (1996) and also Moon *et al.*, (1999)). Postsynaptic cytoskeletal components (e.g. actin (Ratner & Mahler, 1983) and spectrin (Goodman *et al.*, 1995)) previously implicated in anchoring membrane proteins may effect these functions. Similarly, MAGUK proteins with receptor binding and clustering capacity (e.g. PSD95, chapsyn 110, SAP102 see above reviews and Niethammer, Kim & Sheng (1996) and Kim *et al.*, (1996)) may be involved. Recent reports suggest that our understanding of the protein clustering and anchoring mechanisms at PSDs is far from complete (Migaud *et al.*, 1998; Passafaro *et al.*, 1999). PSDs also function to recruit

and localise signalling molecules and proteins important in the regulation of receptor function. The major protein in excitatory synapse PSD fractions is the serine/threonine kinase, calmodulin dependant kinase II (CaMKII). CaMKII is recruited to PSDs in an autophosphorylated state where it phosphorylates a number of PSD proteins including the NR2 subunit of NMDA receptors and the AMPA receptor GluR1 (Omkumar *et al.*, 1996; Strack *et al.*, 1997b). Molecules involved in Ras signalling pathways have also been identified at PSDs (e.g. SynGap (Kim *et al.*, 1998) and MAPK (Suzuki *et al.*, 1999)).

The final major function associated with PSDs is synaptic plasticity. Key proteins involved in the establishment of long term potentiation (LTP) and long term depression (LTD) (such as CaMKII (Tompa & Friedrich, 1998), NMDA receptors and PSD95 (Migaud *et al.*, 1998)) are localised at PSDs or recruited to the structure in response to strong NMDA receptor mediated depolarisation of the postsynaptic membrane (e.g. AMPA receptors (Kim & Huganir, 1999; Leonard *et al.*, 1998)). These proteins and others are thought to induce poorly understood synaptic changes that underlie learning and memory. Neurons stimulated to produce NMDA-receptor-dependant LTP show increases in the number of dendritic spines, the number of perforated PSDs and the area of non-perforated PSDs. Regulation of a PSD associated cytoskeleton could provide a mechanism for such changes (Geinisman *et al.*, 2000; Kennedy, 1993; Matus, 1999). The multiple functions of PSDs infer a complex structure. PSDs are required to provide a fixed structural support for the anchoring and localisation of molecules but must also be capable of rapid activity dependant morphological change. The structure and organisation of these domains is clearly paramount to the execution of PSD function.

2.3.3.2. PSD morphology and ultastructure

Under the electron microscope (EM) type I PSDs are curved structures approximately 400nm long and 40nm wide, containing granular particles ~20nm in diameter (Figure 1.10a and b). Filaments 3-5nm and 10-20nm in diameter appear to form a lattice or web around the granular particles. Diffuse material extends out from the dense band

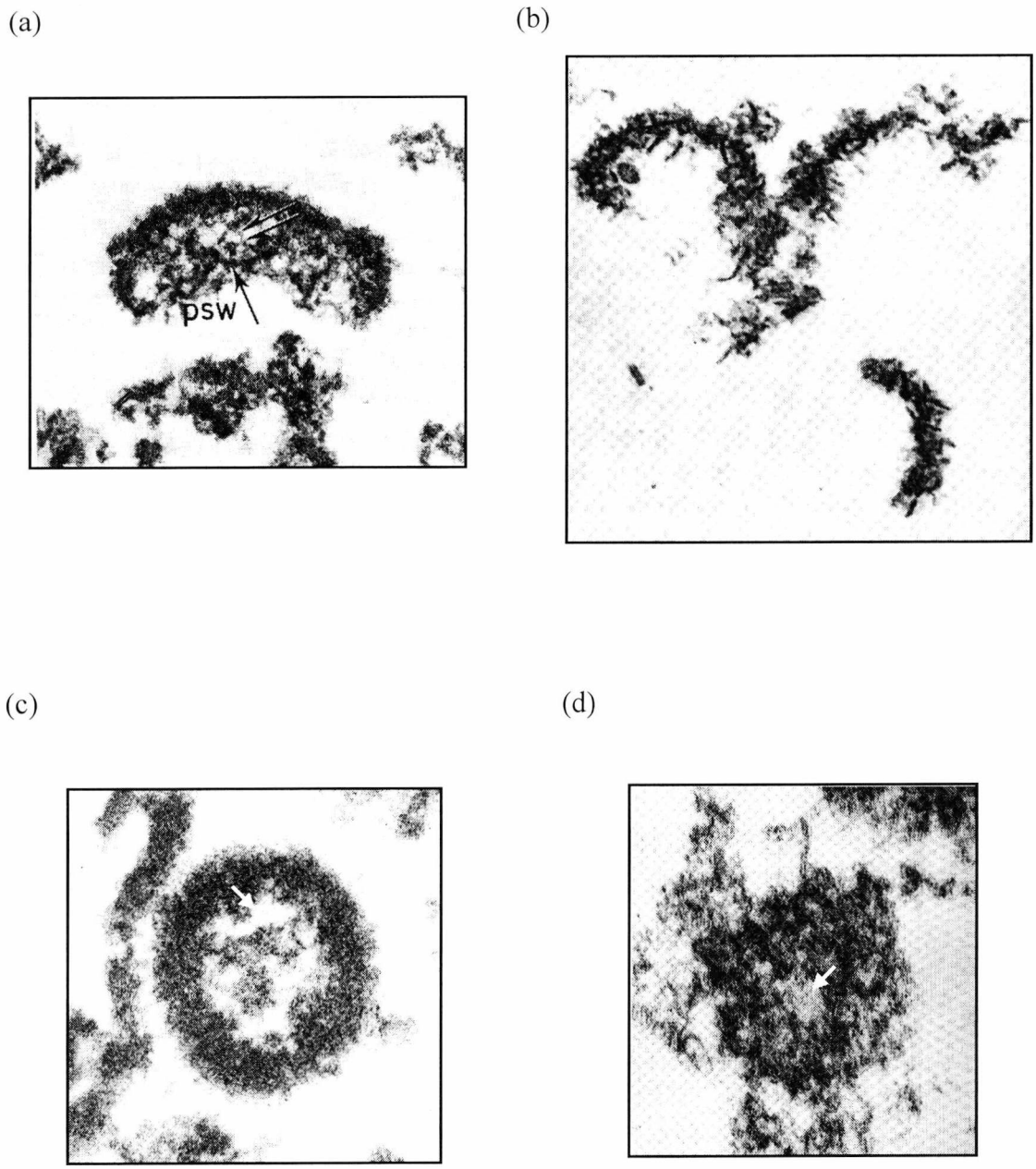


Figure 1.10 Electron micrographs showing the morphology of PSDs purified from mammalian forebrain. From the side PSDs appears as curved granular structures ~ 400nm in length (a) and (b). In (a), particles ~ 13nm in diameter (arrow) are linked by filamentous material (double arrow) to form a postsynaptic web (psw). *En face*, PSDs are disc shaped (c) and (d) with less dense regions forming pores or 'islands' within the PSD structure (arrows). (a) and (c) reproduced from Cohen *et al.* (1977), (b) and (d) reproduced from Gurd *et al.* (1982).

~100nm into the cytoplasm and makes the exact boundary of the PSD difficult to define. *En face* type I PSDs appear to have one or more regions of less dense material that form 'pores' or 'islands' within the PSD structure. (Figure 1.10c and d) (Blomberg *et al.*, 1977; Cohen *et al.*, 1977; Lai *et al.*, 1998; Matus, 1981). How this structure is formed from the individual protein components of PSDs is unclear.

PSD components have mainly been identified through the preparation of PSD fractions from brain. This can be done by isolating synaptosomes or synaptic plasma membranes and extracting the membrane lipids with detergent. Detergent insoluble material is isolated by sucrose gradient density centrifugation and corresponds to a fraction enriched in PSDs (Blomberg *et al.*, 1977; Cotman *et al.*, 1974). The ultrastructure and composition of PSDs is dependant on the nature of the detergent used to purify them (Matus & Taff-Jones, 1978; Somerville *et al.*, 1984). Harsh ionic detergents such as *n*-lauryl sarcosinate and sodium deoxycholate produce PSD fractions with a much simpler composition than non-ionic detergents such as Triton X-100. This is evident in the complement of proteins visualised by SDS-PAGE; at least 30 proteins are resolved when Triton X-100 is used for purification but only ~15 of these remain when the extraction is carried out with sarcosinate. PSD ultrastructure observed by EM also differs according to the detergent used. Triton extraction yields PSDs with granular and filamentous components whilst those visualised after sarcosinate/deoxycholate extraction consist mainly of a polygonal filamentous lattice with no associated granular bodies (Adam & Matus, 1996; Blomberg *et al.*, 1977; Cotman *et al.*, 1974). Proteins therefore associate with PSDs to different extents leading to the idea of a PSD core. The core may consist of a functional framework around which the PSD is assembled.

2.3.3.3. Components of postsynaptic densities

Along with NMDA receptors, AMPA receptors, PSD95, CaMKII, spectrin and actin, a whole host of proteins have been identified as PSD components (Table 3). They fall loosely into four classes: receptors and ion channels, signalling molecules, cell adhesion molecules and structural/anchoring proteins. Some proteins fall into more than one category. In particular many anchoring proteins (e.g. PSD95 family proteins and S-

SCAM) can affect postsynaptic signalling because of the role they have in localising receptors and signalling molecules to their site of activity. PSDs therefore not only appear as macromolecular structures but function as one too. Most PSD components have been identified within the last five years. Like spectrin and actin, some are previously characterised proteins that exist in multiple cell types, or as multiple isoforms within the same cell type (e.g. α actinin, tubulin, MAPs, NF-L and the IP3 receptor (De Smedt *et al.*, 1994; Muller *et al.*, 1995)). Others are novel proteins and are often neuron specific (e.g. densin-180, synamon, S-SCAM and BEGAIN (Apperson *et al.*, 1996; Deguchi *et al.*, 1998; Hirao *et al.*, 1998; Yao *et al.*, 1999a)). Proteins containing PDZ domains are particularly abundant at PSDs. Some of these belong to the MAGUK family of proteins (this Chapter, section 1.2.5.1) (e.g. PSD95 family, S-SCAM and CASK) whilst others contain single or multiple PDZ domains but do not have the SH3 and GK domains characteristic of MAGUKs (homer, nNOS and GRIP).

PROTEIN	REF
RECEPTORS & CHANNELS	
NMDA	(Petralia *et al.*, 1998)
AMPA	(Petralia *et al.*, 1998)
Kainate	(Petralia *et al.*, 1998)
Metabotrophic Glutamate	(Petralia *et al.*, 1998)
TrKB	(Wu *et al.*, 1996)
IP3	(Tu *et al.*, 1999)
Potassium Channels	(Kim *et al.*, 1995)
SIGNALLING	
CaMKII	(Kennedy *et al.*, 1983)
SynGAP	(Kim *et al.*, 1998)
nNOS	(Brenman *et al.*, 1996)
nRAP GEP	(Ohtsuka *et al.*, 1999)
PKA	(Westphal *et al.*, 1999)
PKC	(Suen *et al.*, 1998)
MAPK	(Suzuki *et al.*, 1995)
Fyn kinase	(Grant *et al.*, 1998)
Calmodulin	(Carlin *et al.*, 1980)
PPI	(Strack *et al.*, 1997)
CITRON	(Madaule *et al.*, 1995)
CELL ADHESION	
Densin-180	(Apperson *et al.*, 1996)
Neuroligin	(Song *et al.*, 1999)
N-CAM	(Persohn & Schachner, 1990)
Neurexins?	(Missler & Sudhof, 1998)

PROTEIN	REF
STRUCTURAL/ANCHORIN	
PSD95 family (PSD95, chapsyn 110 SAP102, Hdlg/SAP97)	(Sheng, 1996)
Spectrin	(Malchiodi-Albedi *et al.*, 1993)
Actin	(Cohen *et al.*, 1977)
α-actinin	(Wyszynski *et al.*, 1997)
Tubulin	(Blomberg *et al.*, 1977)
Neurofilaments	(Walsh & Kuruc, 1992)
MAPs	(Walsh & Kuruc, 1992)
CASK	(Hsueh *et al.*, 1998)
CRIPT	(Niethammer *et al.*, 1998)
GKAP/SAPAP	(Kim *et al.*, 1997)
SHANK	(Naisbitt *et al.*, 1999)
Homer	(Tu *et al.*, 1999)
Syndecan	(Hsueh *et al.*, 1998)
Yotiao	(Lin *et al.*, 1998)
BEGAIN	(Deguchi *et al.*, 1998)
S-SCAM	(Hirao *et al.*, 1998)
MAGUIN	(Yao *et al.*, 1999)
CIPP	(Kurschner *et al.*, 1998)
Cortactin	(Naisbitt *et al.*, 1999)
CortBP1	(Boeckers *et al.*, 1999)
GRIP	(Wyszynski *et al.*, 1998)
PICK1	(Xia *et al.*, 1999)
ABP	(Srivastava & Ziff, 1999)

Table 3. Components identified at postsynaptic densities. Proteins identified at PSDs can be loosely divided into four categories; receptors/ion channels, signalling molecules; cell adhesion molecules and structural/anchoring proteins. The list is not exhaustive and novel proteins and new functions for already identified proteins are continually being discovered.

2.3.3.3.1. PDZ domains

PDZ domains are reviewed by Hata, Nakanishi & Takai (1998), Sheng (1996) and Gomperts (1996).

The extent of PDZ domain interactions are only just being recognised. Like the SH3 domain, PDZ domains are protein binding units that are often responsible for recruiting cytoplasmic proteins to the inner surface of the plasma membrane. They are common motifs found in over 50 unrelated proteins however they were first identified in the MAGUKs and are named to reflect this – PSD95/Dlg/ZO-1. Different protein PDZ domains have different substrate specificity's (Songyang *et al.*, 1997). Class I PDZ domains bind the amino acid motif X(S/T)X(V/I) (where X is any amino acid) at the C-terminal of a protein. Examples of class I PDZ domains are PDZ1 and PDZ2 of PSD95 family proteins that bind NMDA receptor NR2 subunits and shaker type potassium channels in this way (Kim *et al.*, 1995; Kornau *et al.*, 1995; Niethammer *et al.*, 1996).

Class II PDZ domains bind the C-terminal motif (Y/F)X(F/V/A/I). The erythrocyte protein p55 contains a class II PDZ domain and binds to GPC via such a sequence (Marfatia *et al.*, 1997). Other less related PDZ domain binding sequences also exist. For example, the sequence SSSTL in mGluR binds the PDZ domain of homer and KEYYV in neurexin binds the PDZ domain of CASK. Internal PDZ domain binding sites have also been reported (e.g. the interaction of InaD PDZ with the TRP Ca^{2+} channel (Shieh & Zhu, 1996)) as well as a PDZ-PDZ interaction between PDZ2 of PSD-95 and the PDZ domain of nitric oxide synthase (Jaffrey *et al.*, 1998). Not all proteins containing a 'binding motif' are able to interact with PDZ domains indicating that additional amino acids must contribute to PDZ domain-substrate binding. The huge diversity of PDZ domain binding proteins and the variety of sequence motifs involved makes it virtually impossible to predict PDZ domain binding partners at present. The tertiary structure of PSD95 PDZ3 elucidated by X-ray crystallography (Doyle *et al.*, 1996) should aid future studies in this direction.

The fact that different PDZ domains act independently means that proteins containing multiple PDZ domains can cluster multiple related or non-related proteins. This may

contribute significantly to the structure of the PSD scaffold; NMDA receptors and potassium channels could be clustered by PSD95 family members and AMPA receptors could be clustered by GRIP and AMPA receptor binding protein (ABP). The formation of PSD95 tetramers through intermolecular disulphide bonds is essential for PSD95 protein clustering (Hsueh *et al.*, 1997). Similarly, ABP and GRIP are reported to homo- and hetero-multimerise (Srivastava & Ziff, 1999). The presence of a PDZ domain in a protein does not guarantee that a binding interaction will take place. Other poorly understood factors may control the *in vivo* associations that actually occur.

3. Protein 4.1

Protein 4.1 may play a general role in the organisation, stability and morphological flexibility of polarised membranes and specialised membrane domains. This is suggested by Leto, Pratt & Madri (1986) and Mattagajasingh *et al.*, (1999a) in endothelial cells, by Rousseaux-Prevost *et al.*, (1994) in testis and by Fehon, Dawson & Artavanistsakonas (1994) in *Drosophila* epithelia. 4.1 in mammals exists as a small gene family consisting of four 4.1 genes: *EPB41* (4.1R), *EPB41L1* (4.1N), *EPB41L2* (4.1G), and *EPB41L3* (4.1B) (Peters *et al.*, 1998). Each gene has its own distinct pattern of expression where 4.1R is found mainly in hematopoietic cells and a specific subset of neurons (Parra *et al.*, 1998; Walensky *et al.*, 1998b), 4.1N is expressed most abundantly in the central and peripheral nervous system (Walensky *et al.*, 1999), 4.1B is expressed in brain and broadly throughout a number of other tissues (Parra *et al.*, 2000) and 4.1G is expressed generally across most mammalian tissues (Parra *et al.*, 1998). As with spectin and ankyrin, 4.1 gene products may be co-expressed in certain cell types and different gene products may be targeted to distinct cellular domains. Chromosomal mapping of the 4.1 genes in human and mouse showed that each resides on a separate chromosome (Peters *et al.*, 1998). The 4.1 family of proteins are becoming ever more intriguing due to diversity amongst the gene products, their distinct patterns of expression and the variety of 4.1 protein functions suggested by recently identified binding activities. The best characterised member of the 4.1 gene family is 4.1R. In order demonstrate the full capabilities of these proteins the 4.1R

gene and its products are discussed in detail before outlining what is known about 4.1N, G and B. For a recent review on the 4.1 family see Hoover & Bryant (2000).

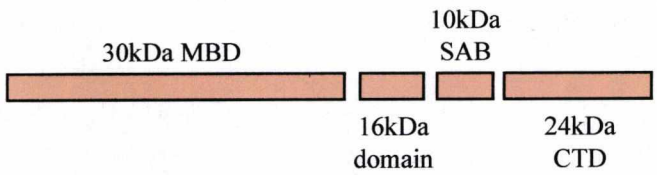
3.1. An Analysis of Protein 4.1R

4.1R was originally identified in erythrocytes as an 80kDa MS protein important for erythrocyte integrity (Yu *et al.*, 1973). Later it was discovered that 4.1R immunoreactive products were present in a variety of non-erythroid cells where they localised to a number of subcellular domains. These non-erythroid products showed great heterogeneity in size which at the time, was attributed to alternative splicing of the 4.1R gene. Discovery of the new 4.1 genes revealed that some of the 4.1R gene products identified previously may in fact be 4.1B, G and N proteins detected as a result of antibody cross reactivity. The products of the 4.1R gene and their distribution are therefore being reassessed in light of the new gene products. Despite this it is still accepted that (1) the 4.1R gene is extensively alternatively spliced into isoforms of heterogeneous size and (2) these isoforms are not confined to erythrocytes but are found in distinct populations of non-erythroid cells (Huang *et al.*, 1993; Walensky *et al.*, 1998b).

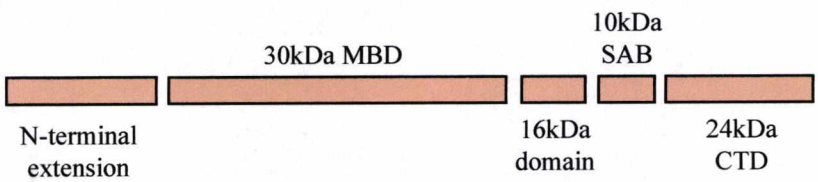
3.1.1. Domain Structure of Protein 4.1R

Limited chymotrypsin digestion of the 80kDa erythrocyte 4.1R protein ($4.1R^{80}$) yields four domains (Leto & Marchesi, 1984): the membrane binding domain (MBD), 16kDa domain, spectrin-actin binding (SAB) domain and C-terminal domain (CTD) (Figure 1.11a). Cloning of 4.1R cDNAs from other cell types revealed a fifth, N-terminal extension domain virtually absent from erythrocytes but expressed in non-erythroid tissues (Figure 1.11b). More recently, sequence comparisons of the four 4.1 gene products demonstrated three distinct regions of homology along with three variable regions. The domain structure of 4.1R has been redefined according to this sequence homology (Figure 1.11c). It now has a total of six domains in which unique region 1 (U1) corresponds to the N-terminal extension domain, U2 is the 16kDa domain plus a small part of the SAB domain and U3 corresponds to a bit from the end of the SAB domain and sequences expressed specifically in skeletal muscle and epithelial cells.

(a) Domain structure of 4.1R according to chymotrypsin digestion of 4.1R[80]



(b) Chymotryptic domain structure of 4.1R plus the N-terminal extension region



(c) Domain structure of 4.1R according to sequence alignment of 4.1R, N, G and B

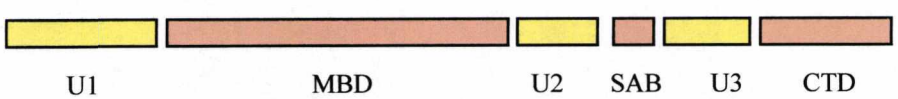


Figure 1.11 Domain structure of 4.1R. The domain structure of erythrocyte 4.1R[80] was originally defined by proteolytic fragments observed on chymotrypsin digestion of the protein (a). The N-terminal extension was later identified in 4.1R species from non-erythroid cell types and was designated a domain on its own (b). More recently cloning of 4.1N, B and G demonstrated amino acid sequence similarities between 4.1 family members in three distinct regions: the membrane binding domain (MBD), part of the spectrin-actin binding domain (SAB) and the C-terminal domain (CTD). Three regions of unique sequence were also identified (U1-U3) and the domain structure of 4.1R is now based on its sequence homology with other 4.1 protein family members.

The MBD of 4.1R is basic and contains a cluster of cysteine residues. This domain is responsible for the membrane associations of 4.1R and in erythrocytes binds GPC, p55, band 3, calmodulin and phosphatidyl serine (Cohen *et al.*, 1988; Hemming *et al.*, 1995). It has also been called the FERM domain because of shared sequence similarity with the ERM proteins and other members of the band 4.1 superfamily (this Chapter, section 1.2.4.1). The 10kDa SAB domain binds spectrin and actin forming junctional complexes that are central to the MS lattice structure. The 10kDa fragment alone can promote binding of spectrin to actin almost as efficiently as intact 4.1 (Correas *et al.*, 1986). The interaction between these three proteins is essential for MS stability. The acidic 22kD-24kDa CTD interacts with NuMA (Mattagajasingh *et al.*, 1999b), elongation factor 1α (Mattagajasingh *et al.*, 1996), eIF3 (Hou *et al.*, 1999) and ZO-2 (Mattagajasingh *et al.*, 1999a) in non-erythroid cells but has no definite binding partners in erythrocytes although an interaction with the immunophilin FKBP13 has been implied (Walensky *et al.*, 1998a). Its functions are unclear.

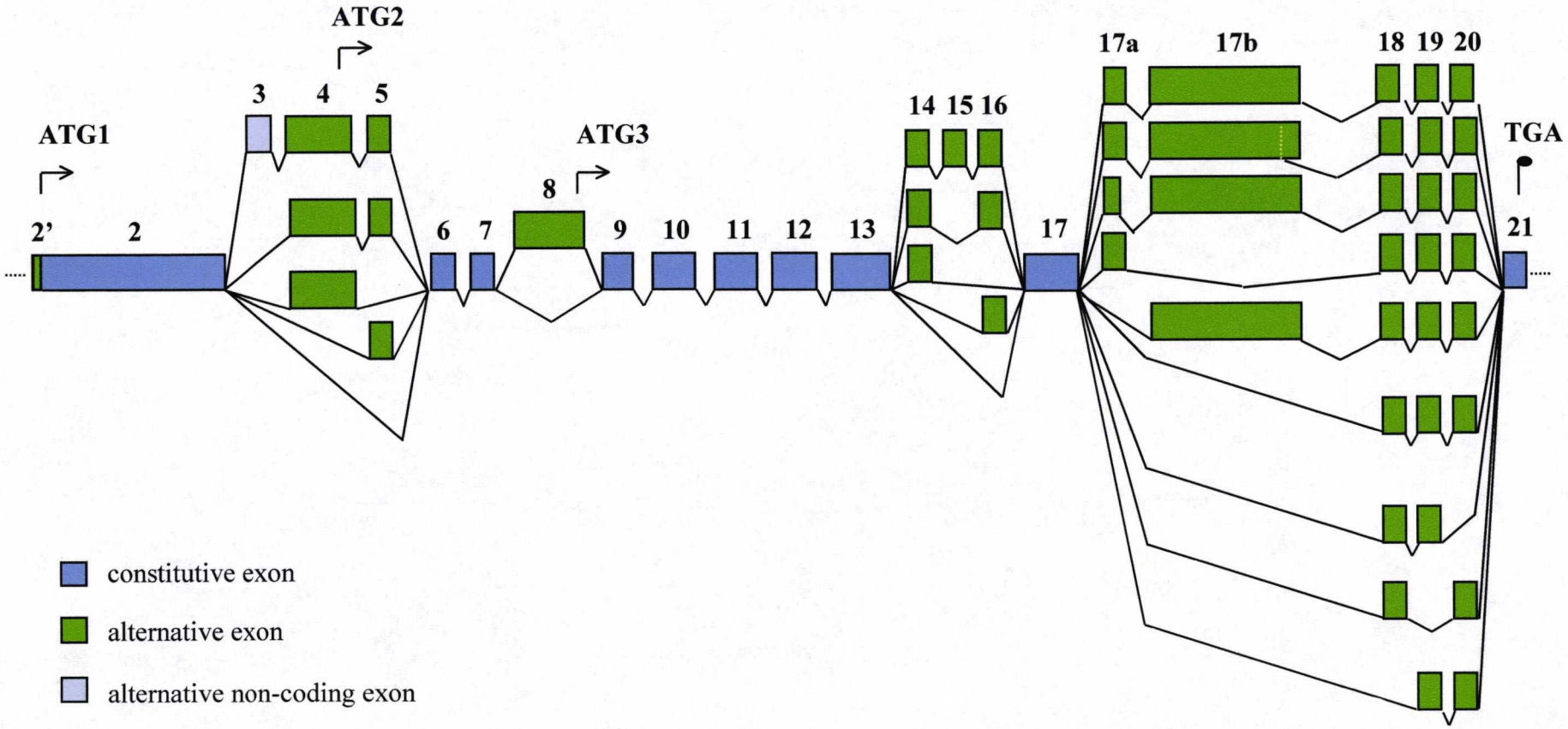
3.1.2. Protein 4.1R Sequence Homology

4.1R proteins from different species show a high level of sequence homology. Human and mouse, and mouse and *Xenopus* 4.1R sequences share 91% and 71% overall amino acid homology respectively (Huang *et al.*, 1993). A high level of conservation between these sequences suggests that the functions of 4.1R are well established through evolution. Coracle, a *Drosophila* 4.1R homologue, shares amino acid sequence homology with human and *Xenopus* 4.1R sequences at the N- (~54%) and very C-terminal but shows little similarity in the SAB domain. As a consequence, coracle does not co-localise with actin in epithelial cells and may have distinct functions that do not include maintenance of cytoskeletal integrity e.g. the formation and organisation of septate junctions (Fehon *et al.*, 1994; Lamb *et al.*, 1998).

3.1.3. Genomic Structure of Protein 4.1R

The 4.1R gene sequence is >200kb in length and contains 12 constitutive and 13 alternative exons (Baklouti *et al.*, 1997; Conboy, 1999; Huang *et al.*, 1993; Schischmanoff *et al.*, 1997) (Figure 1.12). Pre-mRNA splicing of the 4.1R gene

Figure 1.12 Genomic structure of 4.1R. The 4.1R gene consists of 12 constitutive (blue) and 13 alternative exons (green). Alternative splicing of the 4.1R gene is regulated in a tissue and developmental specific manner and produces multiple 4.1R isoforms heterogeneous in size. The diversity of 4.1R isoforms is increased by the presence of three translation initiation sites in exons 2', 4 and 8 respectively. Redrawn from Schischmanoff *et al.* (1997).


ATG1
ATG2
ATG3
TGA
2'
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
17a
17b
18
19
20
21
constitutive exon
alternative exon
alternative non-coding exon

produces multiple 4.1R isoforms (Huang *et al.*, 1993) which are expressed in a tissue and developmental specific manner. Alternative splicing is a feature of many other structural proteins e.g. the cytoskeletal proteins actin, tubulin, keratin, troponin, tropomyosin and myosin, the membrane skeletal proteins α and β spectrin, band 3 and ankyrin, extracellular matrix fibronectins, collagen and laminins, and nuclear matrix lamins (Conboy *et al.*, 1991). Alternative exons often contain specific protein binding sites or targeting motifs. By regulating exon splicing a cell therefore controls the interactions and subcellular location of particular protein isoforms.

Alternative exons in the 4.1R gene are clustered into particular functional domains (Huang *et al.*, 1993). For example, alternative exon 4 and 5 are important in the formation of a functional MBD in erythrocytes and exons 14 – 16 dictate the properties of the SAB domain. Not all of the exons have a known function but some are expressed in a tissue specific way (e.g. exon 17a found in muscle and 17b found in epithelial cells) and presumably confer functions uniquely related to these cells.

3.1.3.1. Exon structure of the protein 4.1R gene

The MBD of 4.1R is formed from exons 4-8. Alternative exon 5 contains the LEEDY sequence motif required for GPC and band 3 interaction (this Chapter, section 3.1.5.1). Isoforms lacking this exon are rare but have been identified in erythroblasts, endothelial cells and mouse embryos where they presumably have a distinct function (Table 4). The SAB domain was originally formed form exons 14-17. Exon 16 is particularly important in 4.1R-spectrin binding and nuclear import of non-erythroid 4.1R isoforms (Luque *et al.*, 1998; Schischmanoff *et al.*, 1995) (this Chapter sections 3.1.5.2 and 3.1.4.1. Whilst exon 16 is expressed in many tissues, exons 14 and 15 are less common and their expression is limited to particular tissues types (Table 4). Isoforms containing all three exons are expressed almost exclusively in brain (Baklouti *et al.*, 1997; Huang *et al.*, 1993). The functions of exons 14 and 15 are unknown and their amino acid sequence does not share high homology with 4.1N, G and B proteins. On redefining the domain structure of 4.1R exons 14 and 15 were regrouped as part of the U2 region of 4.1R (Conboy, 1999). The CTD of 4.1R is formed by exons 18-21. Relatively little

is known about their differential expression (Table 4) but alternative splicing of exons 18, 19 and 20 will disrupt most 4.1R CTD binding activities identified to date. Exons 17a and 17b are tissue specific exons identified in skeletal and heart muscle and epithelial cells respectively (Baklouti *et al.*, 1997; Schischmanoff *et al.*, 1997). Splicing of these exons is complicated by the fact that each has two 5' splice sites and can produce two related peptides, 17a (17 amino acids) or 17a' (12 amino acids) and 17b (150 amino acids) or 17b' (137 amino acids). Functions for these exons have yet to be established.

EXON GROUP	SPLICE VARIANT	TISSUE SPLICE VARIANT WAS DETECTED IN
2'/3	+2'/-3	Erythroblasts, T-lymphocytes, erythrocytes
4 /5	+4/+5	Liver, brain, skeletal muscle, testis, endothelial cells, reticulocytes, endothelial cells, erythroblasts, mammary epithelial cells
	+4/-5	Endothelial cells, erythroblasts, mammary epithelial cells, mouse embryo
	-4/+5	Brain, erythroblasts, mammary epithelial cells
	-4/-5	Endothelial cells, erythroblasts
14/15/16	+14/+15/+16	Brain, mouse embryo, endothelial cells
	+14/-15/+16	Spleen, liver, erythroblasts
	+14/-15/-16	Brain, liver, intestine, erythroblasts
	-14/-15/+16	Erythrocytes, erythroblasts, spleen, bone marrow, skeletal muscle, testis, brain, kidney, liver, intestine, mammary epithelial cells, salivary gland, mouse embryo
	-14/-15/-16	Brain, spleen, kidney, liver, pancreas, testis, erythroblasts, bone marrow, T-lymphocytes, B-lymphocytes, liver, intestine, salivary gland, mammary epithelial cells, endothelial cells, mouse embryo
17	+17a	Skeletal and heart muscle
	+17b	Mammary epithelial cells
18/19/20	+18/+19/+20	Brain, reticulocytes, endothelial cells, erythroblasts, mouse embryo
	+18/-19/+20	Erythroblasts, reticulocytes, MOLT-4 lymphocytes
	+18/-19/-20	Erythroblasts
	-18/+19/+20	Mammary epithelial cells, reticulocytes, erythroblasts, MOLT-4 lymphocytes
	-18/-19/+20	MOLT-4 lymphocytes

Table 4. Summary of the splice variants of protein 4.1R. The splicing combinations listed have all been observed in human, mouse or rat tissues (Baklouti et al., 1997; Conboy et al., 1988; Conboy et al., 1991; Gascard et al., 1998; Huang et al., 1993; Luque et al., 1999; Schischmanoff et al., 1997; Tang et al., 1990). Variants were identified by a combination of cDNA cloning and rtPCR.

3.1.3.2. Alternative translation initiation sites in the protein 4.1R gene

At present, protein 4.1R is unique among gene family members in having three functional translation initiation sites (AUG1-3) (Gascard *et al.*, 1998; Tang *et al.*, 1990). These reside in alternative exons 2', 4 and 8 respectively and further add to the size heterogeneity of 4.1R isoforms. Erythrocyte $4.1R^{80}$ is expressed from AUG2 while isoforms produced from AUG1 occur predominantly in non-erythroid cells and contain the 209 amino acid U1, N-terminal extension domain. For AUG1 to be functional, exon 2' must be spliced in to the 4.1R mRNA and constitutive-non coding exon 3 must be spliced out. This inserts the upstream AUG and causes a frame shift necessary for 'sense' protein to be made. $4.1R^{135}$ is a common isoform expressed from AUG1 and has been described in erythroblasts and a variety of nucleated non-erythroid cells (Baklouti *et al.*, 1996; Chasis *et al.*, 1993; Correas, 1991; DeCarcer *et al.*, 1995; Tang *et al.*, 1990). When 4.1R mRNA contains both AUG1 and AUG2 expression occurs preferentially from the AUG1 site (Tang *et al.*, 1990). The third start site was suggested by Conboy *et al.* in 1991 (Conboy *et al.*, 1991) but has only recently been confirmed (Gascard *et al.*, 1998). Expression from AUG3 produces N-terminal truncated isoforms, which presumably have unique functions that do not require interactions of the MBD. Exon 8 is rarely spliced out of 4.1R isoforms (Conboy *et al.*, 1991) but whether AUG3 elicits translation from transcripts containing AUG1 and/or AUG2 is not known. Low molecular weight 4.1R immunoreactive species identified previously may be a result of expression from AUG3 (Anderson *et al.*, 1988; Rousseaux-Prevost *et al.*, 1994).

The complexity of 4.1R splicing is epitomised in erythroblasts where a total of 18 different splice forms have been identified (Gascard *et al.*, 1998). Seven of these are major 4.1R isoforms while the other 11 appear as minor products. Nine of the 18 isoforms are produced form AUG1, seven from AUG2 and two form AUG3. Why so many isoforms are required during erythroid differentiation is not known. This is

especially intriguing given that mature erythrocytes only require a single 4.1R product ($4.1R^{80}$) to be viable.

3.1.3.3. Mechanism of protein 4.1R alternative splicing

Protein 4.1R exon splicing may be controlled by a combination of *cis* and *trans* acting elements. The *cis* acting elements are particular nucleotide sequences and reside in the vicinity of the splicing event. Because of this splicing can occur in distinct units and does not require the presence of the whole gene. For example, sequences important in exon 16 splicing reside within exon 16 and at the adjacent 5' and 3' splice sites (Gee *et al.*, 1995; Huang *et al.*, 1996). Only the two nearest constitutive exons (i.e. exons 13 and 17) plus the intervening intron sequences are required to reproduce exon 16 splicing *in vitro* (Conboy, 1999). In addition, N-terminal truncated 4.1R mRNA species from certain HE patients show normal exon splicing in the remainder of the message despite the fact that they only begin at exon 13 (Venezia *et al.*, 1998). Presumably these species were not expressed as protein due to the lack of a translation initiation site.

Splicing of certain 4.1 exons may be controlled by the selective presence or absence of pyrimidine and purine rich sequences. Pyrimidine rich sequences are commonly found in the intron prior to a splice site and a purine rich region in the intron prior to exon 15 may signal tissue specific or developmentally regulated splicing (Huang *et al.*, 1993). Pyrimidine rich and pyrimidine poor sites are thought to control splicing at exon 2' and exon 3, regulating synthesis of the $4.1R^{135}$ (Conboy *et al.*, 1991) (Figure 1.13). Splicing at the pyrimidine rich site selects for AUG1 and allows the synthesis of high molecular weight 4.1R isoforms. Splicing at the pyrimidine poor site 17nt downstream, excises the upstream AUG1 and results in translation from the $4.1R^{80}$ start site (AUG2) in exon 4. Two heptad repeats situated 5' to the pyrimidine rich sequence may act as binding sites for *trans* acting factors involved in this selection. *In vitro*, a 40kDa SR protein (for a review see Blencowe *et al.*, (1999)) enhances selection of exon 16 in a concentration dependant manner and may participate in the upstream events related to the splicing of this exon (Huang *et al.*, 1995).

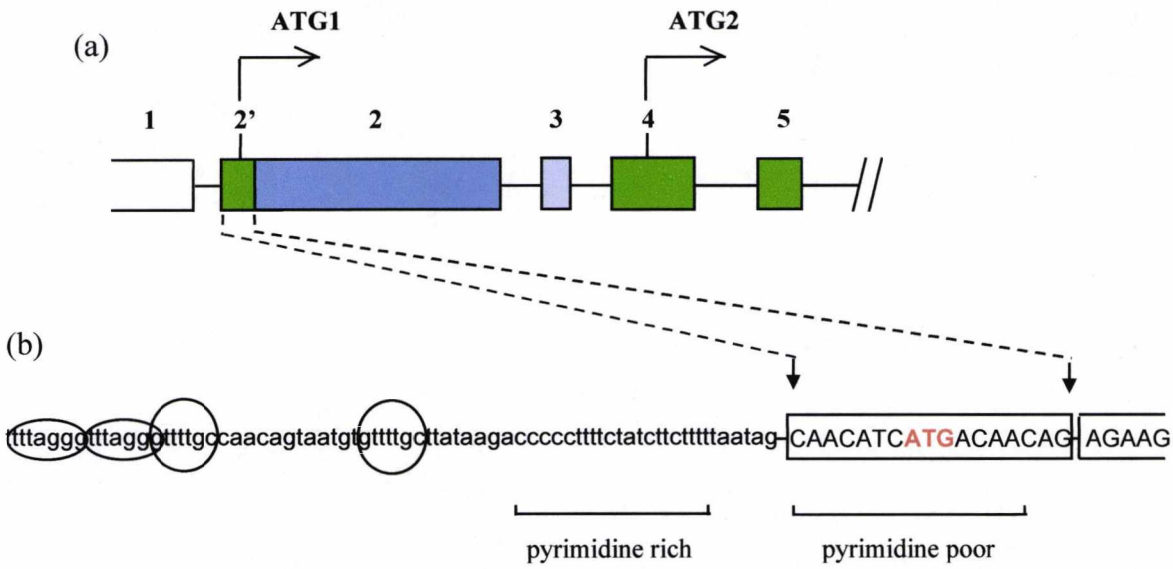


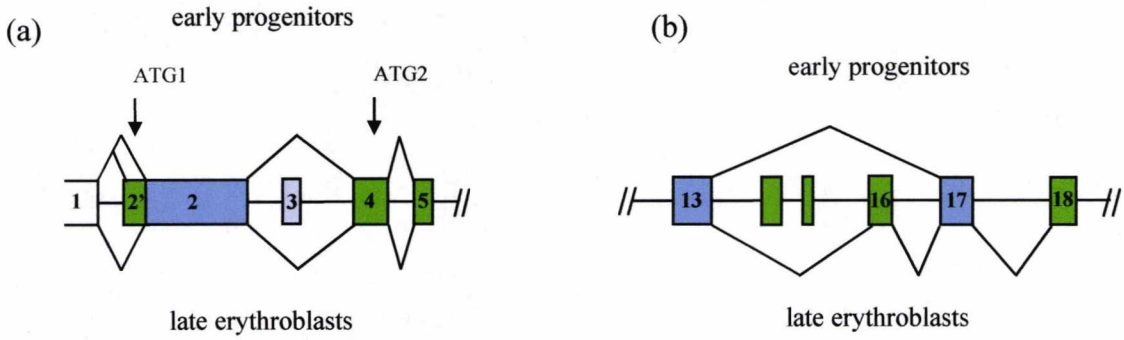
Figure 1.13 Possible mechanism regulating 4.1R splicing at exon 2'. The exon structure of the 5' region of the 4.1R gene is represented schematically in (a) where alternative coding exons are green, constitutive coding exons are blue and alternative non-coding exons are lilac. A more detailed analysis of the sequence in and around exon 2' (b) shows heptad repeats (circles and ovals) that may act as sites for binding splicing factors and pyrimidine rich and pyrimidine poor sites involved in the regulation of exon 2' splicing. Splicing at the pyrimidine rich splice acceptor site (upstream arrow) inserts exon 2' and translation can take place from the upstream AUG1 site. Splicing at the pyrimidine poor splice acceptor site 17nt downstream (downstream arrow) deletes exon 2' and the resulting mRNA is unable to synthesise high molecular weight 4.1R products. Redrawn from Conboy *et al.* (1991).

RNA secondary structures can control mRNA splicing. Pre-mRNA secondary structure has been identified at the intron/exon boundary of exon 2', 14, 15 and 16 (Huang *et al.*, 1993). Tissue specific splicing is controlled by similar secondary structural elements in the tropomyosin gene (Libri *et al.*, 1991) and a similar mechanism may apply to protein 4.1. The spatial arrangement of regulatory elements and the splicing order is also important in the production of viable 4.1R isoforms. For exon 16 to be spliced effectively the downstream intron joining exons 16 and 17 must be excised first (Gee *et al.*, 2000). Excision of the upstream intron first leads to less efficient and less accurate splicing. Cells unable to perform this initial downstream excision step follow a route that leads to exon 16 skipping.

3.1.3.4. Regulation of protein 4.1R alternative splicing

Regulation of 4.1R splicing allows a cell to express specific 4.1R functions at different time points during development or in particular cell types. Alternative splicing switches during erythrpoiesis lead to a redistribution of 4.1 isoforms critical for the proper development of erythrocyte morphology and function. During the early stages of erythroid differentiation (prior to erythroid burst-forming unit day 5) 4.1R is expressed as high molecular weight isoforms from the upstream translation initiation site in exon 2' (AUG1). Activation of an upstream splice acceptor site in exon 2 leads to splicing in of exon 2' for these purposes (Figure 1.14a). Soon after, a splicing switch occurs that abrogates expression of these high molecular weight 4.1R isoforms (Baklouti *et al.*, 1996; Chasis *et al.*, 1993). The upstream splice acceptor site in exon 2 is rejected in favour of a downstream splice acceptor site causing exon 2' to be skipped (Figure 1.14b). Translation initiation starts from AUG2 in exon 4 instead leading to an increase in the amount of $4.1R^{80}$ expressed. The significance of this splicing switch is unclear although it may be important in committing cells to their erythrocytic fate given that mature erythrocytes are one of the few cell types that do not express high molecular weight 4.1R isoforms.

A second regulated splicing switch involves exon 16. It occurs later in erythroid differentiation and is important in the formation of morphologically stable erythrocytes.



Cell stage	*4.1R mRNA structure*	*4.1R protein produced*
early progenitors	E2' partially included	AUG1 → $4.1R^{135}$ AUG2 → $4.1R^{80}$
late erythroblasts	E2' skipped	no AUG1 AUG2 → $4.1R^{80}$

Cell stage	*4.1R mRNA structure*	*4.1R protein activity*
early progenitors	E16 skipped	low affinity spectrin-actin binding unstable membrane skeleton
late erythroblasts	E16 included	high affinity spectrin-actin binding stable membrane skeleton

Figure 1.14 Developmental splicing switches of the 4.1R gene that occur during erythropoesis. An early splicing switch leads to the exclusion of exon 2' and results in the synthesis of low molecular weight isoforms of 4.1R only (a). The splicing switch to include exon 16 (b) occurs later in erythroid development and results in redistribution of 4.1R to the plasma membrane and the production of a stable membrane skeleton. Both redrawn from Conboy (1999).

4.1R isoforms expressed in early erythroid progenitors generally lack exon 16 and localise to the cytosol (Baklouti *et al.*, 1996; Gascard *et al.*, 1998). Activation of a splicing switch to include exon 16 at the erythroblast stage of differentiation leads to redistribution of 4.1R to the plasma membrane and coincides with the production of a stable membrane skeleton (Winardi *et al.*, 1995). 4.1R proteins lacking exon 16 bind spectrin and actin only weakly whilst isoforms incorporating exon 16 form strong ternary complexes with these proteins (Schischmanoff *et al.*, 1995). Developmentally regulated expression of exon 16 therefore controls the extent of spectin-actin-4.1R interactions, which in turn control the formation of a functionally stable membrane skeleton. Alternative splicing of β spectrin and band 3 also affects the subcellular distribution and/or binding interactions of these proteins (Cox *et al.*, 1995; Malchiodi-Albedi *et al.*, 1993). This may be a common phenomenon amongst MS associated proteins.

In nucleated cells control of exon 16 splicing influences 4.1R translocation to the nucleus (Luque *et al.*, 1998). Splicing of exon 16 next to exon 13 produces a cluster of basic amino acids (KKKRER) important in 4.1R nuclear targeting (this Chapter, section 3.1.4.1). A similar splicing event has been shown to target CaMKII isoforms to the nucleus (Srinivasan *et al.*, 1994). 4.1R isoforms including exon 16 localise to the plasma membrane as well as the nucleus in transfected COS cells (Gascard *et al.*, 1998). Additional factors must therefore be involved in the subcellular localisation of 4.1R. Regulated splicing of exon 16 is thought to occur in humans, mouse and dog (Baklouti *et al.*, 1996; Conboy *et al.*, 1991). It has also been demonstrated in the amphibian *Xenopus laevis* (Winardi *et al.*, 1995) implying that the nuclear machinery required to induce this phenomenon is conserved in evolution.

Exon 17b is also expressed in a differentiation specific manner (Schischmanoff *et al.*, 1997). When human mammary epithelial cells are grown adherent to culture dishes they show a flattened morphology and a relatively undifferentiated phenotype. When grown in suspension the same cells differentiate to a state where they stop dividing, assume a rounded morphology, cluster together and demonstrate cytoskeletal and nuclear reorganisation. Differentiation is accompanied by a 4.1R splicing switch that

induces expression of exon 17b. Transfer of suspended cells back to adherent conditions down regulates this splicing event and cells revert to exon 17b exclusion. The reversible nature of this splicing event suggests that the signals required to initiate it are also required to maintain it and highlights how cells are able to adapt to changes in their physical environment. The functions of exon 17b are unknown.

In summary, the diversity among 4.1R isoforms is attributed to the presence of 13 alternative exons and three translation initiation sites. Although a large variety of 4.1R isoforms are theoretically possible only a certain proportion of permissible combinations are probably expressed *in vivo* (Baklouti *et al.*, 1996; Gascard *et al.*, 1998). The complement of 4.1R isoforms expressed at any one time will relate to the specific functional requirements of the cell. Because these functional requirements change during development and cellular differentiation regulated splicing switches exist to ensure that the correct isoforms are expressed at the correct time. Defects in splicing are likely to be detrimental to cell viability. This is evident in sperm cell differentiation where failure of the $4.1R^{135} \rightarrow 4.1R^{80}$ splicing switch leads to mislocalisation of 4.1R and infertility (Rousseaux-Prevost *et al.*, 1994).

3.1.4. Tissue and Subcellular Distribution of Protein 4.1R

4.1R proteins are distributed in both a tissue and subcellular specific manner. In new born mice 4.1R mRNA expression is mainly restricted to heamatopoietic and nervous tissues such as bone marrow, spleen, liver, thymus and neuronal populations of the cerebellum, dentate gyrus, olfactory system, adrenal medulla and retina (Walensky *et al.*, 1998b). RNA blot analysis shows a similar but broader distribution, extending to tissues such as the testis, kidney, pancreas and salivary gland (Parra *et al.*, 1998). Although the functions of 4.1 in these tissues is not known, mice in which the 4.1R gene has been knocked out show defects in movement, co-ordination, balance and spatial learning and memory in addition to haemolytic anaemia and altered erythrocyte morphology (Shi *et al.*, 1999; Walensky *et al.*, 1998b). This is consistent with 4.1R localisation in the cerebellum and dentate gyrus and suggests an important neuronal function for this protein.

In brain 4.1R distribution seems to complement the distribution of 4.1N and B (this Chapter, section 4.3) (Parra *et al.*, 2000). In other tissues there appears to be an overlap of protein 4.1 expression. To avoid functional redundancy the different 4.1 proteins may be targeted to different subcellular compartments where they could perform distinct functions specified by the unique regions in their sequence (Parra *et al.*, 1998). High molecular weight isoforms of 4.1R (encoded from AUG1) are expressed predominantly in nucleated, non-erythroid cells and exist only as early developmental intermediates in erythroid cell counterparts (Luque *et al.*, 1999; Tang *et al.*, 1990). In nucleated cells 4.1R immunoreactive products have been localised to the plasma membrane, cytoplasm (Chasis *et al.*, 1993; Gascard *et al.*, 1998), nucleus (Correas, 1991; DeCarcer *et al.*, 1995; Lallena & Correas, 1997), endoplasmic recticulum (Luque *et al.*, 1999), centrosome (Krauss *et al.*, 1997a), stress fibres (Cohen *et al.*, 1982) and sub-membranous locations at intercellular junctions and membrane ruffles (Gascard *et al.*, 1998; Tang *et al.*, 1990). The functions of 4.1 at these subcellular locations remain speculation. Nuclear 4.1R species are the best explored and isoform exon structures and possible nuclear targeting mechanisms have recently been revealed (Gascard *et al.*, 1998; Gascard *et al.*, 1999; Luque *et al.*, 1999).

3.1.4.1. Protein 4.1R in the nucleus

4.1R immunoreactive species have been localised to the nucleus in cell lines developed from a variety of mammalian species (Correas, 1991; DeCarcer *et al.*, 1995). Immunofluorescence studies indicate that they do not localise predominantly with nuclear membrane pores or the underlying lamin network (Krauss *et al.*, 1997b). Instead, 4.1 is distributed throughout the nucleus where it exists in two distinct populations, one associated with the nucleoplasm and one associated with nuclear speckles (DeCarcer *et al.*, 1995; Krauss *et al.*, 1997b). The nucleolas remains free from 4.1 antibody staining in all cell types examined (Correas, 1991; DeCarcer *et al.*, 1995; Krauss *et al.*, 1997b; Lallena & Correas, 1997). The population of 4.1 found diffusely in the nucleoplasm can be extracted by treatment of cells with 0.5M NaCl (DeCarcer *et al.*, 1995). In contrast, nuclear speckle associated 4.1 remains after this treatment and after removal of chromatin with DNAse I indicating that the two distinct populations of

4.1 are associated with nuclear structures to different extents. The insolubility of speckle associated 4.1 suggests that it is a component of the nuclear matrix, a framework of polymorphic filaments enmeshing large dense bodies involved in maintaining spatial order within the nucleus (Capco *et al.*, 1982). The matrix may organise nuclear machinery into distinct functional compartments and anchor splicing factors, although its precise function is unclear (Capco *et al.*, 1982; Krauss *et al.*, 1997b; Lallena *et al.*, 1998).

Nuclear speckles are enriched in pre-mRNA splicing factors and are retained in nuclear matrix preparations (DeCarcer *et al.*, 1995). Protein 4.1 is localised to speckles containing the non-snRNP splicing factor SC35. Both proteins are dynamic within the nucleus and reversibly redistribute in response to levels of transcriptional activity and heat shock (Lallena & Correas, 1997). 4.1 also interacts with the splicing factor $U2AF^{35}$ (Lallena *et al.*, 1998). Although not thought to be involved in the actual splicing process, 4.1 may play a role in targeting splicing factors to specific domains within the nucleus. The importance of nuclear 4.1 isoforms is demonstrated by the virtual loss of splicing activity in 4.1 depleted nuclear extracts (Lallena *et al.*, 1998). Other known 4.1R binding proteins have been localised to cell nuclei including p55, hDlg (Lutchman *et al.*, 1997), spectrin (Bachs *et al.*, 1990; McMahon *et al.*, 1999), actin (Rando *et al.*, 2000) and NuMA (Cleveland, 1995).

Nuclear 4.1R immunoreactive species are also shown to redistribute during the cell cycle (Krauss *et al.*, 1997b). They often reflect the distribution of NuMA (Mattagajasingh *et al.*, 1999b) implying that 4.1R-NuMA interactions are important in the cell division process. During mitosis the nucleoskeleton dismantles and a mitotic spindle assembles requiring massive reorganisation of nuclear proteins. Chromatin condenses to chromosomes (prophase) which then duplicate, align at the spindle equator (metaphase) and partition into separate daughter nuclei (anaphase and telophase). The nuclear structure reforms and cytokinesis divides the nuclei into two separate daughter cells. Nuclear speckle associated and nucleoplasmic 4.1 observed in interphase cells dramatically redistributes to the spindle fibres and spindle poles during metaphase. The presence of 4.1 at the spindle poles is consistent with reports that it

localises to centrosomes during interphase (Krauss *et al.*, 1997a) as these structures migrate to opposite ends of the cell during mitosis to form the poles of the spindle. During anaphase/telophase 4.1 accumulates around the separating chromosomes and during cytokinesis it retains this localisation and appears at the midbody region connecting the two daughter cells. The function of 4.1 in the cell cycle is unclear but the SAB domain (in a non-spectrin-actin binding capacity) and the CTD are essential for proper nuclear, centrosomal and spindle assembly (Krauss *et al.*, 1999).

Proteins are generally too large to freely diffuse into the nucleus. Instead they are transported by shuttle proteins to nuclear pore complexes where they are then actively translocated across the nuclear membrane. Only a subset of proteins get transported to the nucleus in this way and in order to be recognised as a nuclear protein, 4.1 must express a nuclear localisation signal (NLS). Splicing of exon 16 next to exon 13 in 4.1R produces a cluster of basic amino acids (KKKRER) that fit with the nuclear localisation consensus K(K/R)X(K/R) proposed by Chelsky *et al.*, (Chelsky *et al.*, 1989). Immunofluorescence and transfection studies have shown this sequence to be essential, but not sufficient for nuclear targeting of 4.1R and a second charged sequence in exon 5 (EED) is necessary for efficient nuclear import (Gascard *et al.*, 1999; Luque *et al.*, 1998). To enter the nucleus 4.1R binds the α subunit of the importin complex (Figure 1.15). αβ importin dimers are nuclear shuttle proteins that act as accessory proteins in nuclear import. While the α subunit interacts with protein NLS, the β subunit mediates docking to the nuclear pore. It is suggested that both the basic KKKRER sequence and the acidic EED sequence in 4.1R are important in the 4.1R-α-importin interaction, hence the necessity for both sequences in efficient nuclear transport. The EED sequence is a known protein binding motif of 4.1R and is part of the LEEDY sequence responsible for interactions with GPC and band 3 (this Chapter, section 3.1.5.1).

Identification of 4.1R isoforms lacking exon 5 and exon 16 in the nucleus of MOLT-4 lymphoid cells (Luque *et al.*, 1999) suggests that other factors influence 4.1R nuclear localisation. The 209 amino acid N-terminal extension sequence of 4.1R may be such a factor. Fusion of this sequence to polypeptides normally distributed in the nucleus

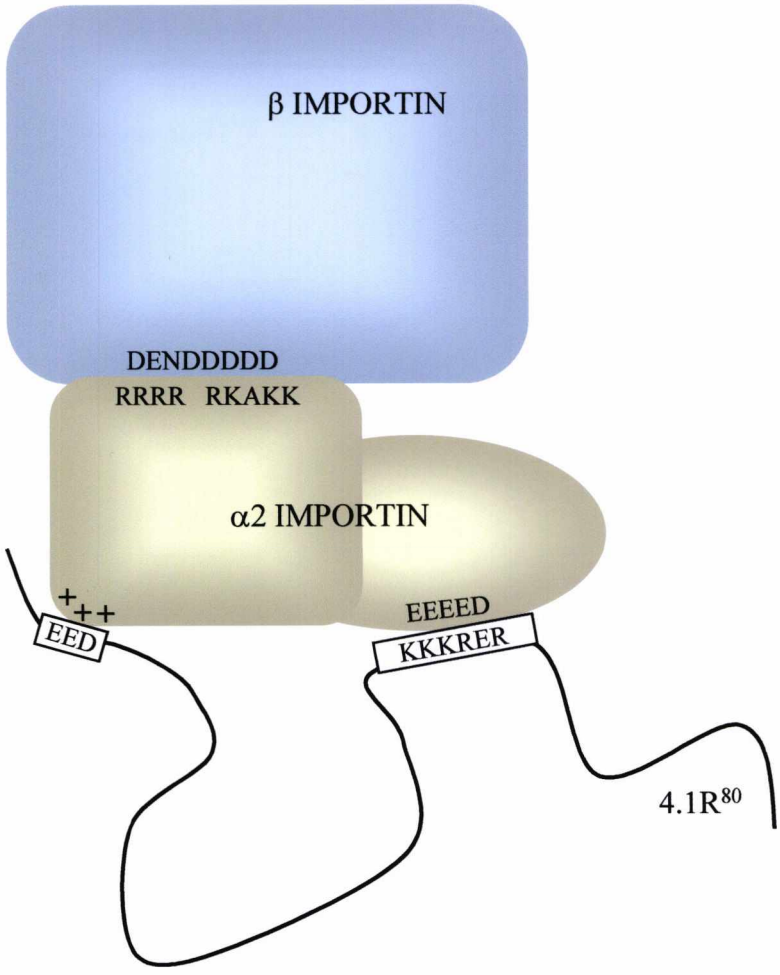


Figure 1.15 Proposed model for the interaction of 4.1R with the nuclear shuttle protein importin. 4.1R isoforms containing exon 16 have a nuclear localisation signal (KKKRER) that interacts with the α subunit of the importin complex. The EED sequence located in the MBD of 4.1R is also necessary for efficient nuclear import and may interact with α-importin at a second site. Redrawn from Gascard *et al.* (1999).

(e.g. $4.1R^{80}$ or the NLS of the SV40 T antigen) suppresses their nuclear localisation and enhances their cytoplasmic distribution (Luque *et al.*, 1999) suggesting a role for this domain in the differential cellular localisation and targeting of 4.1R isoforms. The nuclear distribution observed is consistent with reports that 4.1R isoforms expressed from AUG1 are less concentrated in the nucleus than isoforms expressed from AUG2 (Gascard *et al.*, 1998). Recently however Mattagajasingh *et al.*, (Mattagajasingh *et al.*, 1999b) co-localised $4.1R^{135}$ with NuMA in the nucleus of interphase cells and suggested that the isoforms involved in this interaction were predominantly of the high molecular weight class.

Unlike 4.1R, 4.1G and 4.1N are excluded from the nucleus in transfected COS cells (Gascard *et al.*, 1999). Consistent with this data, neither 4.1G nor 4.1N conserves the 4.1R nuclear localisation signal: KKKRER becomes KKNSLR in 4.1G, and KIKELK in 4.1N. In 4.1B the sequence becomes SLIKRN and although no nuclear localisation data is available on this gene product it seem unlikely that this sequence would act as a NLS. 4.1G, N and B and their presence in the nucleus can not be completely ruled out until more data is accumulated regarding their subcellular localisation. Although not targeted to the nucleus through its own amino acid sequence, 4.1N does translocate to the nucleus in response to nerve growth factor (NGF) signalling (Ye *et al.*, 1999). Nuclear functions for 4.1B and 4.1G can not therefore be ruled out.

3.1.5. Binding Interactions of 4.1R

To elicit its functions protein 4.1 must bind to other proteins. Identification and characterisation of these interactions is essential in understanding 4.1 functions in different cell types and at different subcellular locations. Binding interactions have now been identified for the U1 region, MBD, SAB domain and CTD of 4.1R. These interactions will be discussed below.

3.1.5.1. Interactions of the membrane binding domain of protein 4.1R

The MBD of 4.1R interacts with a number of transmembrane proteins (i.e. GPC (Hemming *et al.*, 1995), band 3 (An *et al.*, 1996), CD44 (Nunomura *et al.*, 1997b), pICln (Tang & Tang, 1998), MAGUK proteins (i.e. hDlg (Lue *et al.*, 1994), p55 (Marfatia *et al.*, 1994), CASK (Cohen *et al.*, 1998) and calmodulin (Tanaka *et al.*, 1991). Interactions with band 3 and GPC are the best characterised of these.

3.1.5.1.1. Protein 4.1R-glycophorin C interactions

GPC is a major sialoglycoprotein found in a wide variety of cell types. In erythrocytes it carries blood group antigens (Reid & Spring, 1994) and links the plasma membrane to the MS. 4.1R interacts with the cytoplasmic C-terminal domain of GPC directly via its MBD (K_D 0.125μM) (Anderson & Marchesi, 1985; Hemming *et al.*, 1995) or through ternary interactions with both GPC and p55 (4.1R-GPC; K_D 2.5nM, GPC-p55; K_D 4.5nM, p55-4.1R; K_D 2.5nM) (Hemming *et al.*, 1995; Marfatia *et al.*, 1994). Ternary interactions are the stronger of the two as p55 is thought to enhance the affinity of 4.1R for GPC (Hemming *et al.*, 1995). The 4.1R binding site on GPC localises to the 12 amino acid peptide RYMYRHKGTYHT (amino acids 82-93) in the C-terminal of the GPC molecule (Marfatia *et al.*, 1995). Site directed mutagenesis has shown that the basic triplet RHK is essential for binding to occur. The GPC binding site on 4.1R is thought to be LEEDY in alternative exon 5 (amino acids 247-251 in mouse 4.1R Acc L00919) (Marfatia *et al.*, 1995), although a direct interaction with this sequence has yet to be demonstrated. Interactions between 4.1R and GPC are important for erythrocyte stability (Nash *et al.*, 1990; Telen *et al.*, 1991) and function as the primary mode of attachment for the 4.1R-anchored membrane skeleton (Workman & Low, 1998).

3.1.5.1.2. Protein 4.1R-band 3 interactions

Band 3 is the major anion channel in the erythrocyte membrane. It mediates HCO^{3-}/Cl^{-} exchange essential to CO_2 transport and blood pH homeostasis (for a review see Cabantchik (1999)) and links the plasma membrane to the membrane skeleton through interactions with $ankyrin_R$. Compared to GPC, band 3 provides only a weak binding site for 4.1R in the erythrocyte membrane (K_D 0.11μM) (Hemming *et al.*, 1995). It also

has a narrower tissue distribution than GPC but is particularly important in maintaining pH homeostasis in the kidney (for a review see Casey & Reithmeier (1998) and also Bruce *et al.*, (1998)). In erythrocytes 4.1R binds predominantly to band 3 tetramers (von Ruckmann *et al.*, 1997) and competition studies have localised the binding site on 4.1R to the basic sequence LEEDY in exon 5 (Jons & Drenckhahn, 1992). A similar sequence exists in the U1 region of 4.1R (LDEEI, amino acids 124-128 in mouse 4.1R Acc: L00919) and may also be able to interact with band 3. Binding of band 3 to this site has not been demonstrated and the relevance of an interaction is questionable given that high molecular weight isoforms of 4.1R are not expressed in erythrocytes. The upstream LDEEI motif may instead initiate 4.1R binding interactions with unidentified ligands in non-erythroid cells instead. Both LEEDY and LDEEI sequences are conserved in *Xenopus* protein 4.1 (VEEDY and LEDDV respectively). This supports a functional role for both sites and suggests that these roles have been conserved through evolution. Band 3 also has two binding sites for 4.1R. Both reside in the N-terminal cytoplasmic domain (LRRRY at amino acids 343-347 and IRRRY at amino acids 386-390) and are functionally active *in vitro* (Jons & Drenckhahn, 1992). The extent to which 4.1R associates with its various erythrocyte membrane attachment sites has been inconsistently reported and between 15 and 70% of 4.1R may be associated with band 3 (Danilov *et al.*, 1990; Hemming *et al.*, 1995; Moriyama *et al.*, 1993). A more recent study trying to clarify the situation (Workman & Low, 1998) concludes that band 3 may contribute up to 60% of the 4.1R binding sites in the erythrocyte membrane but that the interaction between 4.1R and band 3 is not responsible for linking the plasma membrane to the membrane skeleton; this function falls exclusively to 4.1R-GPC (Workman & Low, 1998). Binding of 4.1R to band 3 instead displaces ankyrin from a high affinity band 3 binding site to a low affinity binding site. This disruption relaxes MS structure and increases deformability of the plasma membrane (An *et al.*, 1996). Based on the results of this study and their own, Workman & Low (1998) propose that 4.1R-band 3 interactions modulate membrane flexibility by managing the interactions between band 3 and ankyrin.

In non-erythroid cells, the MBD of 4.1R binds to a 14 amino acid peptide in the cytoplasmic domain of CD44 (NSRRRCGQKKKLVI amino acids 628-641)

(Nunomura *et al.*, 1997b). The sequences SRRRC and QKKKL resemble the IRRRY and LRRRY protein 4.1R binding motifs in band 3 and are essential for high affinity CD44-4.1R binding. The exact binding site on 4.1R has not been identified but the nature of the CD44 binding motif suggests that it may interact with LEEDY and possibly the upstream site LEEDI. No details are known about the interaction of 4.1R with pICIn.

3.1.5.1.3. Protein 4.1R-MAGUK protein interactions

In vitro binding studies have shown that the MBD of 4.1R binds the MAGUK protein p55 (Marfatia *et al.*, 1994) and this Chapter, section 1.2.5, hDlg (Lue *et al.*, 1994) and hCASK (Cohen *et al.*, 1998). The exact region of 4.1R involved in binding is not known, however the site on MAGUKs has been localised to a cluster of four basic residues located between the SH3 and guanylate kinase domain (KKKK in p55 and hCASK, KRKK in hDlg). Various other MAGUK proteins also contain this motif including LIN-2 (a CASK homologue in *C. elegans*), DLG2 and chapsyn 110. These proteins may also interact with 4.1R but there is no direct evidence to confirm this.

In addition to the basic amino acid cluster, Lue *et al.*, 1996 (Lue *et al.*, 1996) suggest that 4.1R binds to a second site in hDlg formed by the boundary between PDZ1 and PDZ2. The eight amino acids that link the two PDZ domains contains a cluster of four basic residues (KRRK) similar to the 4.1R binding motif found between the SH3 and GK domain. Neither PDZ1 nor PDZ2 interacts with 4.1R alone. Instead binding requires the higher order structure produced by the folding of both domains into a functional binding unit. This presumably presents the PDZ1-2 linker region in a conformation 4.1R is able to recognise. Similar basic motifs are present in the PDZ1-2 linker region of dlg, ZO-1, chapsyn 110 and PSD95 (Lue *et al.*, 1996). Marfatia *et al.*, (1996) contest this second 4.1 binding site stating that the interaction with 4.1 is weak and an artifact of the positively charged residues around the PDZ region. Whether this functions as a 4.1R binding site *in vivo* remains to be seen.

3.1.5.1.4. Protein 4.1R-calmodulin interactions

The MBD of 4.1R interacts with calmodulin at Ca^{2+} dependant and Ca^{2+} independent sites. In addition the U1 region of 4.1R contains a second Ca^{2+} dependant binding site that may be important in non-erythroid cells. Ca^{2+}/CaM regulates the membrane binding and spectrin-actin binding interactions of erythrocyte 4.1R. This will be discussed in more detail in section 3.1.6.1.

3.1.5.2. Interactions of the spectrin-actin binding domain of protein 4.1R

The SAB domain of 4.1R is composed of alternative exon 16 (21 amino acids) and constitutive exon 17 (59 amino acids) (this Chapter, section 3.1.3). The NLS in exon 16 has been reported to bind the nuclear shuttle importin, an interaction that is significant in non-erythroid isoforms of 4.1R. Conversely, association of this domain with spectrin and actin in erythrocytes is essential for the formation of morphologically stable cells. Both binary and ternary interactions occur between 4.1R, spectrin and filamentous actin and are the best characterised interactions of this domain.

3.1.5.2.1. Binary interactions with spectrin and actin

4.1R binds spectrin towards the N-terminus of the β subunit, at a site indistinguishable from the actin binding site (Becker *et al.*, 1990; Karinch *et al.*, 1990; Tyler *et al.*, 1979). The 4.1R-spectrin interaction was measured to have a K_D of 0.1-0.2μM and an *in vivo* stoichometry of 1:1 (spectrin:4.1R) dictated by the number of 4.1R molecules present in erythrocytes (Morris & Lux, 1995).

4.1R-actin binary interactions also have a stoichiometry of 1:1 (Morris & Lux, 1995) suggesting that 4.1R binds to the sides of actin filaments. This is consistent with electron micrographs showing spectrin dimers binding end-on to the sides of actin filaments (Cohen & Foley, 1980). Binding of 4.1R to actin filaments increases the stability of filaments as indicated by a lowering of the critical concentration of actin in the presence of 4.1R (Morris & Lux, 1995). Like spectrin, 4.1R binds to filaments in a co-operative manner (Morris & Lux, 1995) but the interaction with spectrin is weaker

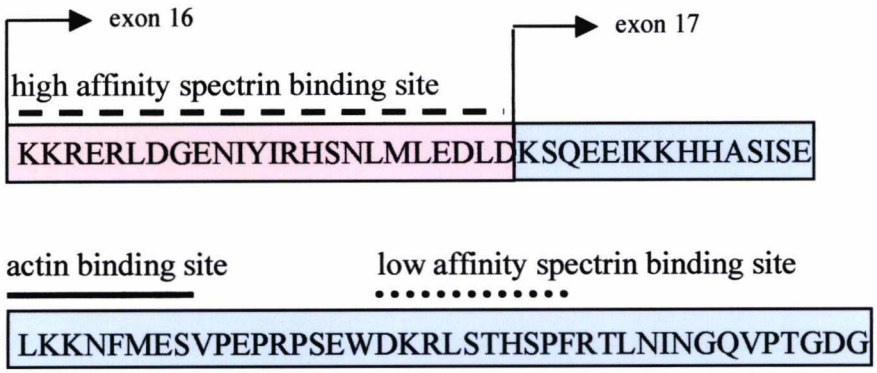
than that involving 4.1R (spectrin K_D 5-200μM, 4.1R K_D 1-20μM depending on the concentration of actin present). Co-operative binding could result from two scenarios: Firstly, binding of 4.1R to an actin monomer could induce a conformational change in adjacent monomers allowing 4.1R to bind more easily. Alternatively binding of 4.1R to actin may alter the quaternary structure of the adjacent actin filament making 4.1R binding sites on adjacent monomers more accessible (Morris & Lux, 1995). So far, neither model has been confirmed. The 4.1R-actin interaction is sensitive to temperature, pH and polyvalent anions (e.g. ATP, 2,3 diphosphoglycerate) (Becker *et al.*, 1990; Morris & Lux, 1995). Although the two former parameters are unlikely to have an effect under physiological conditions, disruption of the interaction by polyvalent anions may contribute to the regulation of 4.1R-actin binding *in vivo*.

3.1.5.2.2. Ternary interactions with spectrin and actin

Spectrin binds to actin weakly in the absence of protein 4.1R (Ohanian *et al.*, 1984). Both α and β spectrin polypeptides are necessary for the interaction but it is the β chain that confers 4.1R sensitivity (Coleman *et al.*, 1987). 4.1R strengthens the interaction between spectrin and actin and forms a highly stable ternary complex essential for the strength and durability of the erythrocyte plasma membrane. Evidence for a ternary complex was first provided by *in vitro* binding studies where spectrin and actin sedimented in a 4.1R dependant manner (Ungewickell *et al.*, 1979).

Within the SAB domain of 4.1R there are two spectrin binding sites (high and low affinity) and one actin binding site. The high affinity spectrin binding site is encoded by exon 16 (amino acids 643-663 in mouse 4.1R Acc: L00919) and the low affinity site by amino acids 36-43 of exon 17 (amino acids 699-706 in the full length sequence of mouse 4.1R Acc: L00919) (Schischmanoff *et al.*, 1995) (Figure 1.16a). The actin binding site has recently been localised to amino acids 19-26 of exon 17 (682-689 in the full length sequence of mouse 4.1R Acc: L00919) and shares similarity to the actin binding motifs of profilin and actobindin (Gimm & Mohandas, 1999), A. J. Baines, personal communication (Figure 1.16a). Deletion studies helped define these regions and have shown that all three sites are required for optimal ternary interactions (Discher *et al.*, 1995; Schischmanoff *et al.*, 1995) (Figure 1.16b).

(a)



(b)

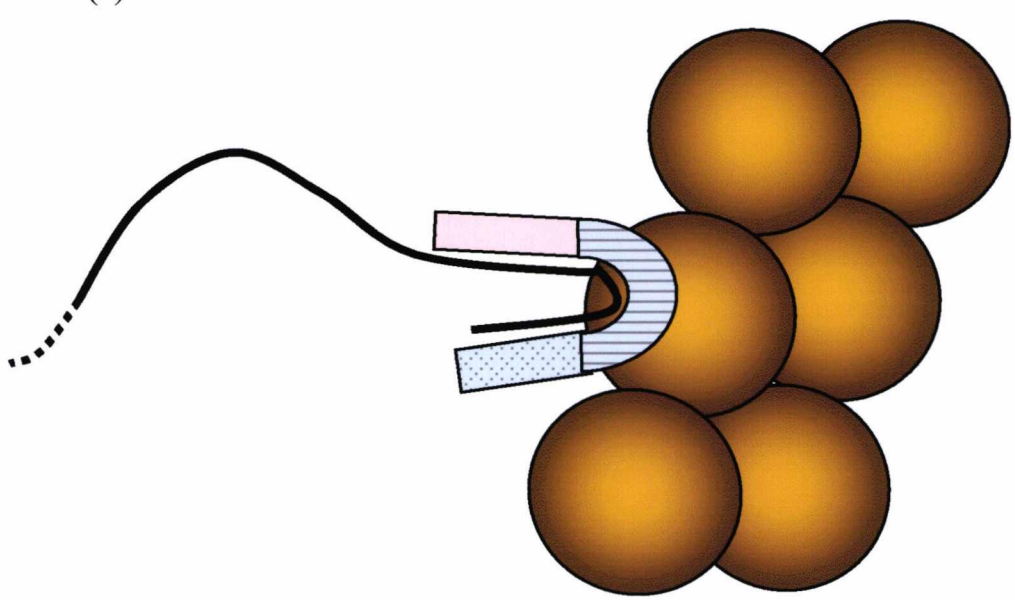


Figure 1.16 Possible model for the binding of 4.1R to spectrin and actin. 4.1R isoforms containing exon 16 are able to form high affinity ternary complexes with spectrin and actin. (a) shows the amino acid sequence of exon 16 (pink) and 17 (blue) from mouse 4.1R showing the location of the spectrin and actin binding sites. All three sites are required for optimal ternary interactions to occur. (b) Schematic model suggesting one way that 4.1R may interact with β-spectrin (black line) and actin (orange) to form a ternary complex. Only the spectrin-actin binding domain of 4.1R is shown: Exon 16; pink, actin binding site in exon 17; blue stripes, spectrin binding site in exon 17; dotted blue. .Panel (b) is redrawn from Discher *et al.* (1995).

In the absence of the high affinity spectrin binding site in exon 16 only weak spectrin-actin-4.1R interactions occur. Very high levels of exon 16 expression are only found in muscle cells and erythrocytes (Baklouti *et al.*, 1997). This implies that muscle 4.1R has a major spectrin-actin binding function that could be important in the morphological stability and organisation of these cells. Other non-erythroid cells show lower expression or splicing out of exon 16. The major functions of 4.1R are unlikely to involve spectrin-actin binding in these cells and exon 16, if present may function more as a nuclear targeting sequence than in spectrin-actin binding. In accordance with this, spectrin-actin binding has not yet been demonstrated with high molecular weight nuclear 4.1R species (Mattagajasingh *et al.*, 1999b).

The SAB domain of 4.1R also binds myosin heavy chain *in vitro* (Pasternack & Racusen, 1989). Whether this interaction co-exists with spectrin and/or actin interactions is not clear however binding of 4.1R reduces the actin-activated ATPase activity of myosin by 50% (Pasternack & Racusen, 1989). This could be one reason why exon 16 is expressed at high levels in muscle cells. Myosin and 4.1R could also participate in Mg^{2+}/ATP dependent erythrocyte shape changes (Pasternack & Racusen, 1989) and the cytoskeletal contractions essential to cytokinesis (Zang & Spudich, 1998). 4.1R-myosin interactions have been overlooked in favour of 4.1R-spectrin-actin interactions.

3.1.5.3. Interactions of the C-terminal domain of protein 4.1R

Interactions of the CTD of 4.1R are only now starting to be recognised and are less well characterised than MBD and SAB domain interactions. The presence of three alternative exons (exons 18, 19 and 20) suggests that there are tissue specific functions for this domain, while a high degree of sequence homology across constitutive exon 21 of all 4.1 gene products suggests that some functions of the domain are conserved. The CTD of 4.1R has been shown to interact with NuMA (Mattagajasingh *et al.*, 1999b), elongation factor 1α (EF1α) (Mattagajasingh *et al.*, 1996), the p44 subunit of eukaryotic initiation factor 3 (eIF3-p44) (Hou *et al.*, 1999) and ZO-2 (Mattagajasingh *et al.*, 1999a). There is also an FKBP13 binding site which was originally identified in the

CTD of 4.1G (Walensky *et al.*, 1998a). Although a direct interaction between 4.1R and FKBP13 has not been demonstrated sequence identity with 4.1G in this region suggests binding can occur.

3.1.5.3.1. Interactions of 4.1R with NuMA

Binding of 4.1R to NuMA reinforces a nuclear function for 4.1R. NuMA localises to the interphase nucleus of proliferating and terminally differentiated cells and has an essential role in spindle formation and nuclear reformation during mitosis (for reviews see Cleveland (1995) and Compton & Cleveland (1994)). NuMA has also been suggested to play a structural role in nuclear architecture due to its identification as a component of the nuclear matrix and its multimerisation properties (Harborth & Osborn, 1999; Merdes & Cleveland, 1998). 4.1R and NuMA co-localise in interphase nuclei and during the cell cycle (Mattagajasingh *et al.*, 1999b). Addition of excess recombinant CTD to interphase and mitotic nuclei leads to mislocalisation of NuMA and phenotypes similar to those with a targeted disruption of NuMA (Krauss *et al.*, 1999; Merdes & Cleveland, 1998; Yang & Snyder, 1992). One function of 4.1R may therefore be to localise and anchor NuMA at various nuclear structures.

Both high and low molecular weight 4.1R species can bind NuMA but it is hypothesised that only high molecular weight isoforms will interact *in vivo* (Mattagajasingh *et al.*, 1999b). This does not correlate with findings that nuclear localisation of 4.1R is abrogated by the presence of the U1 region (Luque *et al.*, 1999) and further work will be needed to account for these data. The NuMA binding site on 4.1R has been localised to exons 20 and 21 and both exons are necessary for an interaction to take place (Mattagajasingh *et al.*, 1999b). The requirement for both exons implies that 4.1R-NuMA binding can be regulated by alternative splicing of exon 20. The 4.1R binding site on NuMA maps to amino acids 1788-1810 and resides in an alternative exon of the C-terminal globular tail domain (Mattagajasingh *et al.*, 1999b). Signals for NuMA nuclear localisation exist within this exon and the 4.1R binding site partly overlaps the region of NuMA involved in association with the mitotic spindle (within amino acids 1750-1800). The precise role of 4.1R-NuMA complexes still remains to be determined.

3.1.5.3.2. Interactions of 4.1R with EF1α and eIF3-p44

The C-terminal domain of 4.1R binds EF1α and eIF3-p44. EF1α is an abundant protein involved in the translation elongation process where it mediates GTP dependant transfer of aminoacyl-tRNAs to the ribosomal A site. It has also been associated with a number of non-translation related functions and may help co-ordinate the regulation of cellular growth, division and transformation (for a review see Negrutskii & El'skaya (1998)). In particular EF1α may be a precursor component of centrosomes a site where 4.1R has been localised previously (Krauss *et al.*, 1997a). The binding site for EF1α has not been mapped to any specific region in the CTD of 4.1R but as with NuMA the interaction has been shown to occur with high molecular weight protein 4.1R species (Mattagajasingh *et al.*, 1996).

eIF3 is a complex of at least 10 subunits each of which plays an essential role in the pathway of translation initiation. In particular it maintains ribosomal subunits in a dissociated state and promotes mRNA binding to the 40S subunit (for a review see Hershey *et al.*, (1996)). Recent characterisation of the p44 subunit indicates that it binds 18 S rRNA and β-globin mRNA species (Block *et al.*, 1998). eIF3-p44 depleted cell free translation systems do not synthesise proteins efficiently (Hou *et al.*, 1999). The binding site for eIF3-p44 on 4.1R begins half way through exon 18 and ends half way through exon 21 (amino acids 735-832 in mouse 4.1R Acc:L0091956) (Hou *et al.*, 1999). This has been defined as the minimal binding domain thus 4.1R-eIF3-p44 interactions require alternative exons 18, 19 and 20 and can easily be regulated by alternative splicing of the 4.1R gene. The binding site for 4.1R on p44 exists towards the middle of the molecule at amino acids 54-185. The significance of 4.1R-EF1α and eIF3-p44 interactions are not known but suggest that 4.1R may play a role in localising translation apparatus.

3.1.5.3.3. Interactions of 4.1R with ZO-2

Binding of 4.1R to ZO-2 requires exons 19, 20 and 21 of the 4.1R sequence and amino acids 1054-1118 in ZO-2 (Mattagajasingh *et al.*, 1999a). The binding site on ZO-2 is in the C-terminal of the protein downstream from the GK domain. This differs to other MAGUK proteins who interact with 4.1R via a basic motif located between the SH3

and GK domain (this Chapter, section 1.2.5.1). Both 4.1R isoforms shown to bind ZO-2 were high molecular weight species (135kDa and 150kDa (Mattagajasingh *et al.*, 1999a)) but an interaction with low molecular weight 4.1R proteins expressing the right exons can not be excluded. The interaction with ZO-2 may also be conserved in 4.1N, B and G due to the high level of amino acid sequence homology that exists in the CTD of 4.1 proteins. ZO-2 localises to epithelial tight junctions (Jesaitis & Goodenough, 1994) and adherens junctions (Itoh *et al.*, 1999) where it may function to organise the cortical cytoskeleton and mediate signalling (for a review see Fanning *et al.*, (1996) and also Wittchen, Haskins & Stevenson (1999)). Actin and spectrin have been identified at these junctions together with the transmembrane cell adhesion molecules occludin (tight junctions) and cadherins (adherens junctions) (Furuse *et al.*, 1993; Hirokawa & Tilney, 1982; Kaiser *et al.*, 1989; Piepenhagen & Nelson, 1998). ZO-2 binds the C-terminal of occludin at tight junctions (Itoh *et al.*, 1999) and a complex may exist between occludin, ZO-2 and 4.1R analogous to the GPC-p55-4.1R complex in erythrocytes. In adherens junctions, ZO-2 binds α-catenin and may crosslink cadherin-catenin complexes with the actin cytoskeleton via 4.1R. Protein 4.1 has previously been immunolocalised to septate junctions, the *Drosophilia* equivalent of tight junctions (Fehon *et al.*, 1994). Tight Junctions are particularly important in maintaining membrane domains (Devarajan & Morrow, 1996). Thus 4.1R may be involved in the establishment and maintenance of epithelial polarity.

3.1.5.4. Interactions of protein 4.1R with tubulin, phospholipids and 2,3-diphosphoglycerate

Other less well characterised binding interactions of 4.1R immunoreactive proteins include interactions with tubulin, phospholipids and glycolytic enzymes. $4.1R^{80}$ binds to the C-terminal of tubulin and distributes along microtubules in a manner similar to the microtubule associated proteins MAP2 and tau (Correas & Avila, 1988). Mitotic spindles are composed of microtubules. An interaction between 4.1R and tubulin may have implications during the cell cycle when 4.1R is known to distribute to the spindle (Krauss *et al.*, 1997b). The tubulin binding site on 4.1 has not been mapped.

Protein 4.1R[80] interacts with various membrane phospholipids *in vitro*. The *in vivo* relevance of these interactions are unclear but in erythrocytes they may contribute to plasma membrane-membrane skeleton binding. Phosphatidyl serine and phosphatidyl ethanolamine are both enriched in the inner leaflet of plasma membranes. Binding of 4.1R to both classes of phospholipid has been reported (Cohen *et al.*, 1988; Pradhan *et al.*, 1989). The interaction between 4.1R and phosphatidyl ethanolamine is relatively weak but the 4.1R-phosphatidyl serine interaction occurs with high affinity (K_D 0.3μM) and involves the MBD of 4.1R (Cohen *et al.*, 1988). Acidic conditions, Ca^{2+} and Mg^{2+} disrupt 4.1R-phosphatidyl serine binding indicating that the interaction may also be under regulatory control (Cohen *et al.*, 1988; Shiffer *et al.*, 1988). Erythrocytes deficient in 4.1R have an altered pattern of membrane lipid distribution (Cohen *et al.*, 1988). 4.1R may be responsible for maintaining the asymmetric distribution of lipids in the erythrocyte plasma membrane as well as the distribution of integral membrane proteins.

2,3 DPG is a glycolytic intermediate in cellular metabolism. It binds directly to 4.1R (Moriyama *et al.*, 1993) and may enhance 4.1R phosphorylation by PKC. Binding of 2,3 DPG to 4.1R in erythrocytes can modulate 4.1R based MS-plasma membrane attachments (this Chapter, section 3.1.6.3).

In summary, the MBD interactions of 4.1R facilitate linkage of transmembrane proteins to the actin cytoskeleton via interactions of the SAB domain. This is a well characterised phenomenon in erythrocytes but is probably also true in non-erythroid cells given that 4.1R has been shown to interact with the transmembrane proteins CD44 and pICIn. MAGUK proteins are important in the organisation of specialised membrane domains. The interaction between the MBD of 4.1R and MAGUK proteins suggests that 4.1R may function at specialised membrane sites in non-erythroid cells. The CTD of 4.1R interacts with a diverse range of ligands and suggests a number of novel functions of 4.1R (e.g. roles in transcription and translation) not recognised from the interactions of the other domains. There are probably a number of 4.1R binding interactions that remain to be identified in non-erythroid cells. In particular the U1 domain has only been shown to bind CaM to date but is likely to interact with other

proteins and may confer a number of 4.1R specific functions upon high molecular weight isoforms.

3.1.6. Regulation of 4.1R Binding Interactions

Regulation of 4.1R binding is complex. The interactions of 4.1R are controlled by Ca^{2+}/CaM, phosphorylation, inositol phospholipids and 2,3 DPG according to cellular needs. Each specific regulatory mechanism can affect multiple interactions of 4.1R. The 4.1R interactions that prevail in a cell at any one time will reflect the overall consensus of the individual regulatory effectors.

3.1.6.1. Regulation of 4.1R binding interactions by Ca^{2+}/CaM

Increases in intracellular Ca^{2+} affects erythrocyte membrane deformability suggesting that Ca^{2+} modulates membrane skeleton interactions. Ca^{2+} mediates its effects through CaM, which binds to the MBD of $4.1R^{80}$ in a Ca^{2+} dependent and Ca^{2+} independent manner (Nunomura *et al.*, 1997a). Ca^{2+}/CaM binding to erythrocyte 4.1 inhibits both 4.1R-membrane and 4.1R-spectrin-actin interactions (Lombardo & Low, 1994; Tanaka *et al.*, 1991). 4.1R-membrane associations are decreased by 43% when CaM binds to the MBD of 4.1R (Lombardo & Low, 1994). This increases to 83% in the presence of Ca^{2+} and both 4.1R-band 3 and 4.1R-GPC interactions are affected (Lombardo & Low, 1994). Ca^{2+} inhibition of 4.1R-band 3 binding requires the presence of both Ca^{2+} dependant and Ca^{2+} independent CaM binding sites (Nunomura *et al.*, 1997a). It is thought that CaM binds constitutively to the Ca^{2+}independent site and that Ca^{2+}/CaM binding to the Ca^{2+} dependant site then stimulates a conformational change leading to inhibition of 4.1R-band 3 binding. The interaction between 4.1R and CD44 is also regulated in a Ca^{2+}/CaM dependent manner (Nunomura *et al.*, 1997b). Whether a similar mechanism applies is yet to be seen.

Binding of CaM to the MBD of $4.1R^{80}$ also confers Ca^{2+} sensitivity to spectrin-actin-4.1 interactions (Tanaka *et al.*, 1991). The mechanism of inhibition is similar to the regulation of 4.1R-band 3 interactions: CaM binds to 4.1R in the absence of Ca^{2+} but this time has no effect on 4.1R-spectrin-actin interactions. Addition of Ca^{2+} causes

Ca^{2+}/CaM to bind 4.1R and induces a conformational change in the CaM-$4.1R^{80}$ complex. This change affects the extent to which spectrin can crosslink actin and consequently weakens the MS structure.

A second functional Ca^{2+} dependant CaM binding site has also been identified in the N-terminal extension of human (amino acids 76-88), mouse (amino acids 78-90; (Leclerc & Vetter, 1998)) and *Xenopus* (amino acids 68-80; (Kelly *et al.*, 1991)) 4.1R isoforms. The interactions of CaM with this site can be modified by serine phosphorylation and may impose another level of regulation on 4.1R binding. This site may regulate interactions of 4.1R in non-erythroid cells.

3.1.6.2. Regulation of 4.1R binding interactions by phosphorylation

With the exception of actin, most erythrocyte membrane skeleton proteins are targets for phosphorylation and a number of different kinase activities have been isolated from erythrocytes including PKC, PKA, casein kinase, tyrosine kinase and Ca/CaM kinase activities (for a review see Boivin (1988)). $4.1R^{80}$ contains multiple phosphorylation sites and all four domains produced by limited chymotryptic digestion are substrates for one or more kinase (Figure 1.17).

Phosphorylation of the MBD by PKC decreases $4.1R^{80}$-plasma membrane interactions by 60-70% (Chao & Tao, 1991). Most of this reduction comes from disruption of $4.1R^{80}$-band 3 interactions (Danilov *et al.*, 1990) and only a minor proportion is attributable to disruption of $4.1R^{80}$-GPC binding sites (Pinder *et al.*, 1995). Phosphorylation of the CTD by casein kinase A also reduces in $4.1R^{80}$-membrane binding (25-30%). As the CTD is not involved in membrane binding in erythrocytes, phosphorylation by casein kinase A may induce a conformational change that directly affects the capacity of the MBD to associate with the membrane (Chao & Tao, 1991).

Spectrin-actin binding associations of $4.1R^{80}$ are also affected by phosphorylation. PKA, PKC, casein kinase and a tyrosine kinase have all been shown to decrease the

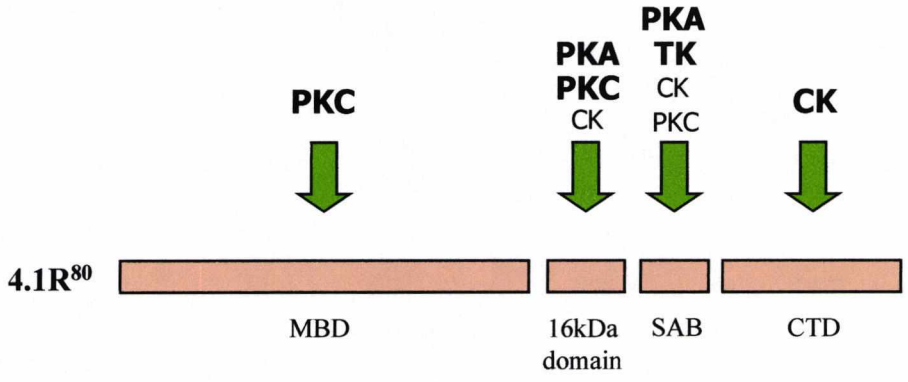


Figure 1.17 Phosphorylation of 4.1R chymotyptic domains. The chymotryptic domains of 4.1R act as substrates for different protein kinases. Kinase names in non-bold text indicate minor phosphorylation events. PKC, protein kinase C; PKA, protein kinase A; CK, casein kinase A; TK, an unspecified tyrosine kinase. Adapted from Chao and Tao (1991).

affinity of $4.1R^{80}$ for spectrin or reduce ternary complex formation (Eder *et al.*, 1986; Horne *et al.*, 1990; Ling *et al.*, 1988). Three specific phosphorylation sites have been identified in 4.1R at serine 541, serine 703 and tyrosine 654 of the mouse sequence Acc: L00919 (Horne *et al.*, 1990; Subrahmanyam *et al.*, 1991). Phosphorylation at all three sites reduce the capacity of 4.1R to promote spectrin-actin interactions. Serine 541 is located in the 16kDa domain produced by chymotrypsin digestion of 4.1R. Phosphorylation at this site probably affects 4.1R-spectrin-actin binding indirectly, maybe via a conformational change. In contrast, tyrosine 654 and serine 703 reside in the high and low affinity spectrin binding sites respectively. Phosphorylation at these sites is more likely to affect 4.1R binding directly through steric hindrance or charge repulsion.

Whether all these phosphorylation events occur *in vivo* as well as *in vitro* is not clear. Generally phosphorylation of 4.1R leads to a loosening of the MS lattice structure and a reduction in its association with the plasma membrane. Similar events would be required for erythrocyte deformation.

3.1.6.3. Regulation of 4.1R binding by inositol phospholipids and 2,3 diphosphoglycerate

Binding of $4.1R^{80}$ to GPC may require phosphatidyl inositol 4,5-bisphosphate (PIP_2) as an obligate cofactor (Anderson & Marchesi (1985) but see Gascard & Cohen (1994)). The capacity of PIP_2 to promote an association between these two proteins can not be replaced by any other phospholipid. Hydrolysis of PIP_2 in membrane vesicles containing GPC decreases $4.1R^{80}$-vesicle binding (Gascard *et al.*, 1993). Phospholipase C (PLC) is responsible for PIP_2 hydrolysis *in vivo* (for a review see Meldrum, Parker & Carozzi (1991) and is activated by G-protein linked plasma membrane receptors in response to extracellular ligands. Agonists of this signalling pathway have the potential to regulate membrane structural rearrangements by activating PLC. It is questionable whether this occurs *in vivo* given that no erythrocyte agonists have been identified and PIP_2 levels do not appear to alter significantly enough to elicit a major effect (Gascard *et al.*, 1993).

In addition to its role in cellular metabolism the glycolytic intermediate 2,3 DPG regulates haemoglobin-oxygen affinity. Erythrocytes treated with 2,3 DPG show decreased membrane deformability and stability suggesting a role for 2,3 DPG in regulating MS interactions (Chasis & Mohandas, 1986). 2,3 DPG binds to 4.1R and disrupts MS-plasma membrane interactions directly and by enhancing PKC phosphorylation of 4.1R (Moriyama *et al.*, 1993). Elevated levels of 2,3 DPG also interfere with 4.1R-spectrin-actin ternary complex formation (Cohen & Foley, 1984). The apparent regulatory effects of 2,3 DPG may not apply to intact cells under physiologic conditions but may instead be a specific phenomenon associated with erythrocyte ghosts (Waugh, 1986).

It is clear from the studies outlined in this section that the combined effects of many cellular modulators precisely control 4.1R-membrane and 4.1R-MS interactions. It should not be forgotten that 4.1R interactions are also controlled by alternative pre-mRNA splicing (this Chapter, section 3.1.3.4). Thus 4.1R is regulated at multiple levels to effect its cellular functions.

4. Protein 4.1N, G and B

Protein 4.1R belongs to a small gene family that currently consists of four members (4.1R (*EPB41*), 4.1N (*EPB41L1*), 4.1G (*EPB41L2*), and 4.1B (*EPB41L3*)) (Peters *et al.*, 1998). Each gene localises to a separate chromosome in mouse and human and unlike members of the 4.1 superfamily, the products of these genes share sequence homology in regions other than the 4.1R MBD (Figure 1.18a). In addition to the 4.1B protein reported by Parra *et al.*, 2000 (Parra *et al.*, 2000) a human ortholog of 4.1B named DAL1 was described in a separate study on primary lung tumours (Tran *et al.*, 1999). DAL1 possesses tumour supressor activity and in this study is grouped as a member of the 4.1 superfamily. Although the predicted DAL1 protein lacks the N and C-terminal domains of mouse 4.1B it is nearly identical in a 600 amino acid overlapping region. DAL1 is therefore more likely to be a 4.1B variant. As discussed in section 3.1.1, comparison of the 4.1R, N, G and B sequences has led to the assignment of a slightly new 4.1 domain structure (Figure 1.18b).

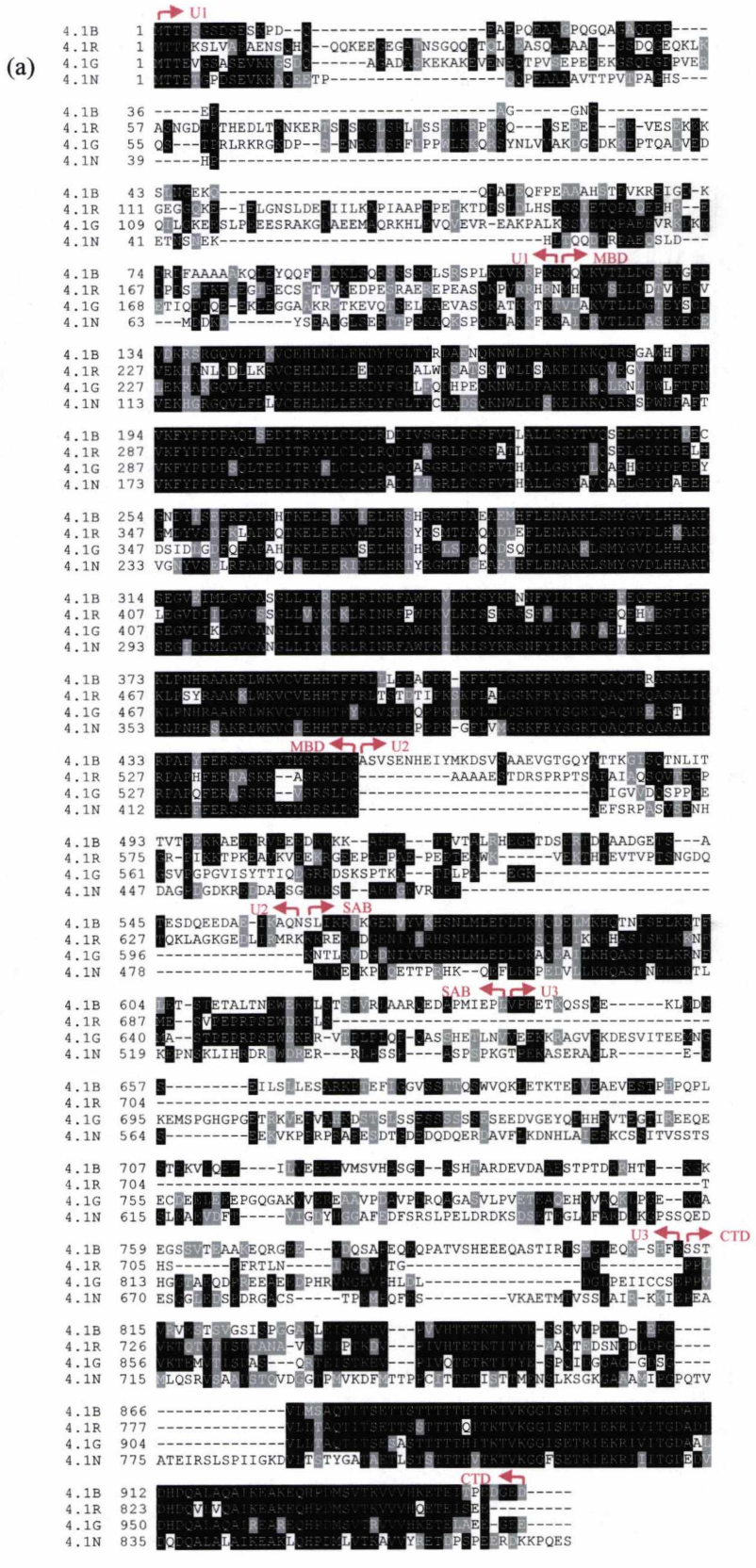


Figure 1.18 Domain organisation and sequence of mouse 4.1 proteins. (a) Amino acid sequence alignment of mouse 4.1B cDNA (accession number AF152247 with addition of amino acids 559-580 expressed only in muscle), mouse 4.1R cDNA (L00919), mouse brain 4.1G (AF044312) and mouse brain 4.1N (AF061283). Three regions of homology and three regions of unique sequence exist and are now used to define the domain structure of the 4.1 proteins. The start of each domain is indicated in the alignment. (b) Schematic representation of 4.1B, R ,G and N domain structure. (MBD) membrane binding domain, (SAB) spectrin-actin binding domain, (CTD) C-terminal domain, (U1-U3), unique regions 1-3. (a) and (b) redrawn from Parra *et al.* (2000).

The known functional domains of 4.1R are essentially maintained (MBD, SAB domain and CTD) in all gene products (Conboy, 1999; Parra *et al.*, 2000) and the high sequence homology suggests that some of the characteristic 4.1R functions could be conserved in 4.1N, G and B. In the same context, the three regions of unique sequence (U1-U3) may confer distinct functions upon each gene product. These may be expressed in a tissue specific manner and could be important in defining each proteins tissue specific roles.

4.1. Amino Acid Sequence Comparison of 4.1R, N, G and B

When used in pairwise comparisons the 4.1 MBD shows 70-74% amino acid sequence identity. Interestingly, the LEEDY sequence responsible for the interaction of 4.1R with GPC, band 3 and CD44 is altered in 4.1N, G and B to LEKDY. The introduction of a basic amino acid into the acidic triplet is likely to alter the binding characteristics of this motif dramatically and suggests that membrane protein targets for 4.1N, G and B are different to those identified for 4.1R. The calcium-dependant and calcium-independent CaM binding sites identified in the membrane binding domain of 4.1R are conserved in 4.1N, G and B. The leucine residue at the end of the calcium dependant binding site in 4.1R is changed to serine in 4.1N, G and B. Rather than disabling 4.1-CaM binding this substitution may lead to phosphorylation dependant modulation of CaM-4.1 interactions as shown for the Ca^{2+} dependant CaM binding sequence identified in U1 of 4.1R (Leclerc & Vetter, 1998). This upstream CaM binding site is only partly conserved in 4.1G and is not present in 4.1B or N. It may therefore be a unique feature of 4.1R high molecular weight isoforms.

The SAB domain shows only 50-70% sequence identity across the four gene products. 4.1N is the least similar to 4.1R and may not be able to promote binding of spectrin to actin *in vivo*. The SAB domain of 4.1B is the most highly conserved and 4.1B isoforms containing this region retain the ability to interact with spectrin and actin to form a ternary complex (Parra *et al.*, 2000). Sequence differences between 4.1R and 4.1B in this region render 4.1B less effective at promoting this interaction. It is unclear whether 4.1G binds spectrin and actin at present although the 73% sequence homology shared with 4.1R in this domain suggests that it probably will.

The CTD is 70-80% similar in all four proteins. The highest region of homology resides in the second half of this domain and implies that functional similarities may exist between the 4.1 proteins. The CTD of 4.1G for example binds the immunophilin FKBP13 (Walensky *et al.*, 1998a). The binding site for this interaction is well conserved in 4.1R, N and B and may reflect a common binding interaction among these proteins. Similarly, the CTD of 4.1R and 4.1N both interact with NuMA (Mattagajasingh *et al.*, 1999b; Ye *et al.*, 1999). Conserved 4.1 binding interactions do not result in conserved 4.1 functions however, as demonstrated by 4.1-NuMA interactions: Stimulation of PC12 cells with nerve growth factor (NGF) mediates translocation of 4.1N to the nucleus where it binds NuMA and arrests mitotic cells in G1 phase of the cell cycle (Ye *et al.*, 1999). In contrast, 4.1R-NuMA interactions are important for mitotic spindle formation and the localisation of NuMA in interphase cells (Mattagajasingh *et al.*, 1999b). The phenomenon of a conserved binding interaction with functional diversity provides a cellular mechanism for fulfilling a number of functions via a single protein type. This could explain why all 4.1 proteins co-exist in certain cells as discussed in the next section.

4.2. Tissue Distribution of 4.1N, G and B

RNA blot analysis and *in situ* hybridisation studies on mice have been used to investigate the tissue distribution of the various 4.1 gene products. 4.1B transcripts appear most abundant in brain with lower expression in testis, adrenal gland and kidney (Parra *et al.*, 2000). Brain isoforms of 4.1B insert a 12 amino acid exon in the U2 region of the transcript that is not found in other tissue types. This is presumably important for the function of 4.1B in brain. Expression of 4.1N is observed in most central and peripheral neurons. The kidney is the major non-neuronal site of 4.1N expression although transcripts have also been detected in heart and pancreas. A variety of different sized transcripts have been detected suggesting that 4.1N is also subject to alternative splicing. As with 4.1B, brain tissue shows a unique splicing bias and a 7.5kb transcript expressed poorly in other tissues is the most abundant 4.1N species is brain (Walensky *et al.*, 1999). Similar results were found in a separate study by Yamakawa *et al.*, (1999). These authors describe a 'brain 4.1' protein that appears to be identical to the 4.1N protein described by Walensky *et al.*, (1999). Unlike 4.1B

and 4.1N, significant levels of 4.1G transcripts are observed in most tissue types including nervous tissue, respiratory and gastrointestinal systems, hematopoietic tissue, skeletal muscle, salivary gland and testis (Parra *et al.*, 1998; Walensky *et al.*, 1998b). 4.1G is again alternatively spliced and the transcripts seen in the brain are distinct from those seen in other tissue types. Alternative splicing of the 4.1 gene products is confirmed at the protein level and a number of differently sized products exist across the various tissues investigated (4.1B ~148kDa and 128kDa (Parra *et al.*, 2000); 4.1N ~ 207kDa, 165kDa, 135kDa, 125kDa, 100kDa and 66kDa (Walensky *et al.*, 1999; Yamakawa *et al.*, 1999); 4.1G ~ 160kDa, 140kDa and 100kDa (Parra *et al.*, 2000; Parra *et al.*, 1998))

4.3. Distribution of 4.1N, G and B in Brain

Regional localisation of 4.1 gene products in brain reveals both overlapping and complementary patterns of 4.1 protein expression. The best examples are the cerebellum and the hippocampus. In cerebellum, 4.1N localises to the granular cell cytoplasm and is found diffusely throughout the molecular layer (Ohara *et al.*, 1999) but is not expressed in purkinje cells (Ohara *et al.*, 1999; Walensky *et al.*, 1999)). 4.1R co-exists with 4.1N in the cerebellar granule layer and is similarly absent from purkinje cells. 4.1B on the other hand is specifically expressed in purkinje cells and complements the expression of 4.1R and 4.1N. In the hippocampus, 4.1N is expressed in pyramidal cells of the CA1-3 region as well as the dentate gyrus (Parra *et al.*, 2000). In contrast, 4.1R is expressed specifically in the dentate gyrus while 4.1B is restricted to the CA1-3 region. Co-expression of certain 4.1 gene products is therefore combined with distinct gene expression in certain cell types. These cell types probably exploit a unique function of the specific 4.1 protein they express.

4.4. Subcellular Distribution of 4.1N, G and B in Brain

Studies with 4.1R have shown that 4.1 proteins can distribute to different subcellular locations within a cell. Initial immunofluorescence investigations with antibodies specific to 4.1N, G and B have indicated that 4.1B accumulates at areas of cell-cell contact (Parra *et al.*, 2000), 4.1N localises to synapses (Ohara *et al.*, 1999; Walensky *et*

al., 1999) and 4.1G has a peri-nuclear and diffuse cytoplasmic distribution (Parra *et al.*, 1998). These studies are by no means complete. Further investigation may reveal that the subcellular distribution of these proteins varies between cell types and according to the exon structure of the particular isoform being expressed as is true for 4.1R.

In summary, the tissue distribution data suggest that multiple 4.1 gene products are co-expressed in a number of (if not all) mammalian tissues. This parallels other MS proteins such as spectin and ankyrin which have for some time been known to express more than one related gene product in the same cell (this Chapter, section 1.2.1.2, section 1.2.3.2 and section 2). Different brain regions are enriched in different 4.1 gene products suggesting that these proteins have region specific functions. These functions could be encoded by the unique sequence domains or by expression of certain brain specific exons as inferred by northern blot analysis. Little is known about the subcellular localisation of the new 4.1 proteins but initial analysis suggests that 4.1N and B may be importance at specialised membrane domains.

5. Aims of this Thesis

The identification of 4.1N, G and B requires a rethinking of the 4.1 story. Most of the studies on 4.1R (pre-Sept 1998) were carried out before these new products were identified and it is not always clear which gene products these studies now refer to. This is particularly true of the tissue distribution and subcellular localisation of 4.1R immunoreactive products as some 4.1R antibodies are expected to cross react with 4.1N, G and B gene products (for examples see Mattagajasingh *et al.*, (1999b)). The definitive tissue distribution of 4.1R has now been investigated using a 4.1R deficient mouse model (Shi *et al.*, 1999), RNA blot analysis (Parra *et al.*, 1998) and *in situ* hybridisation (Walensky *et al.*, 1998b) with 4.1R specific probes. In contrast the subcellular localisation of 4.1R proteins has not been completely redefined and remains vague for all 4.1 gene products described.

The work presented in this thesis begins to address the cellular and subcellular localisation of 4.1 proteins in brain concentrating on the isotypes present in

postsynaptic density fractions. To demonstrate a physiological relevance for postsynaptic 4.1 proteins, binding of 4.1R to PSD components was investigated. There are several reasons why 4.1 products are likely to localise to PSDs: (1) PSD lattice-like structures visualised by EM are reminiscent of the membrane skeleton network of erythrocytes. (2) Functional requirements of PSDs and erythrocytes are similar. PSDs need to be able to target and anchor functionally important proteins in an organised and regulatable manner. They are also dynamic and need to be capable of rapid morphological changes in response to the extracellular environment. (3) Protein 4.1 has previously been localised to synapses (i.e. 4.1N (Ohara *et al.*, 1999; Walensky *et al.*, 1999)) and the specialised membrane domains that form points of cell-cell contact (i.e. 4.1B (Parra *et al.*, 2000), 4.1R (Mattagajasingh *et al.*, 1999a) and coracle (Lue *et al.*, 1994)). (4) Many known 4.1 binding proteins and MS proteins localise to PSDs and other specialised membrane domains.

The complement of 4.1 gene products in PSD fractions was investigated using protein specific antibodies and the binding activities of the C-terminal of 4.1R were determined using a recombinant polypeptide corresponding to the SAB domain and the CTD. Chapter three describes the production of bacterially expressed recombinant 4.1R C-terminal region showing it to exhibit characteristics associated with native erythrocyte 4.1R. The use of recombinant 4.1R C-terminal region to produce a rabbit polyclonal antibody specific to 4.1R is also described. The reactivity of this antibody and that of 4.1N, 4.1G and 4.1R/G/B are analysed in detail.

The localisation of 4.1R in brain was investigated by immunofluorescence with anti-4.1R on embryonic rat midbrain dissociated cultures. These studies are discussed in chapter four and establish the presence of 4.1R gene products in brain, with a mainly neuronal distribution. An analysis of 4.1R RNA and protein products confirms the expression of 4.1R in the cultures used. Chapter four forms a small preliminary study to demonstrate expression of 4.1R and assess its distribution in different cell types of the brain. It was not extended to 4.1N, G and B partly because this work was undertaken at a time when these gene products had not been identified.

Chapter five describes work localising products of the 4.1R, N, G and B genes in PSD fractions purified from pig and rat forebrain. Purified PSDs are characterised in this chapter, showing enrichment of known PSD proteins and depletion of axonal and presynaptic components. The 4.1 specific antibodies characterised in Chapter three are put to use in identifying specific 4.1 isoforms and calculating their relative enrichment over original brain homogenates. A subset of postsynaptic 4.1 proteins are shown to be intrinsic to the PSD 'core' and the question of 4.1 proteins as PSD artifacts is addressed.

Recombinant 4.1R C-terminal region characterised in Chapter three is used in Chapter six to identify binding activities of 4.1R with PSD components. Binding interaction specificity is assessed together with the identity of 4.1R binding proteins. The postsynaptic relevance of these interactions and the possible functions of 4.1R at PSDs are discussed in full in an attempt to extend our knowledge of both the protein and this specialised membrane domain.

Chapter 2

Materials and Methods

Introduction

Unless stated otherwise, all chemicals and reagents were of analytical grade and purchased from Sigma Chemicals (Poole, UK). Restriction enzymes, DNA ligase enzymes, dNTPs and *Taq* polymerase were from Promega (Southampton, UK).

cDNA encoding mouse embryo 4.1R clone pE15 in the plasmid pGEM4 (Huang *et al.*, 1993) was kindly provided by Tang K. Tang (Institute of Biomedical Sciences, Academia Sinica, Taiwan).

An antibody to the NMDA receptor subunit NR1 was a gift from Professor A. Stephenson (School of Pharmacy, University of London). Other antibodies were obtained from the following suppliers: CaMKII; Santa Cruz Biotechnology and Transduction Laboratorie, PSD95; Affinity Bioreagents, synaptophysin and α-internexin; Chemicon, GAP43; Sigma and Chemicon, actin, tubulin and NF-L; Sigma.

Mass spectrometry was carried by Judy Hardy (University of Kent) on an LCQ electrospray mass spectrometer (Thermoquest).

1. Molecular Biology Techniques

1.1. PCR

The polymerase chain reaction (PCR) allows *in vitro* amplification of a specific region of DNA using short oligonucleotide primers. The primers are formed from single stranded DNA and are complementary to the ends of the DNA sequence to be amplified. Heat denaturation of the template DNA under suitable reaction conditions allows primers to anneal and become extended by DNA polymerase to form a new double stranded DNA fragment. The cycle is repeated several times to produce multiple copies of the DNA region of interest.

Primers and PCR cycling conditions are given in Table 5. Primers incorporated either an *NcoI* (5' primers) or *XhoI* (3' primers) restriction site (underlined) for cloning into pET23d.

	4.1R-CTR amplification	4.1R^{80} amplification
Primer sequences		
Forward:	5'-ACTGCCATGG-AGCCCACAGAAGCATGGAAG-3'	5'-CACACCATGG-TGCACTGTAAGGTCTCCTTG-3'
Reverse:	5'-GTCCCTGCTCGAGCTCCTCAGAGA-3'	5'-GTCCCTGCTCGAGCTCCTCAGAGA-3'
Template DNA		
	Mouse 4.1R clone pE15 in pGEM4	Rat midbrain culture cDNA
Cycling conditions		
Denature:	96°C 1 minute	95°C 1 minute
Anneal:	50°C 1 minute	64.5°C 45 seconds
Extension:	72°C 1 minute	72°C 1 minute
Number of cycles:	30	40
Final cycle extension:	72°C 5 minutes	72°C 5 minutes

Table 5. PCR primers and cycling parameters.

Reactions were set up with 1X PCR buffer (sold as 10x from Advanced Biotechnologies, Surrey, UK), 2mM $MgCl_2$ (Advanced Biotechnologies, Surrey, UK), 20 pmole of each primer (Cruachem, Glasgow, UK), 200μM each dNTP and variable amounts of template DNA. Reagents sufficient for a 20μl reaction were added to a 0.5ml thin-walled eppendorf tube and heated at 96°C for 5 minutes. Reactions were snap cooled on ice before adding 0.5 units of *Taq* DNA polymerase (Advanced Biotechnologies, Surrey, UK) and returning the tubes to the Cycler. Cycling took place

in a GeneE thermal cycler (Techne, Cambridge, UK).

1.2. Electrophoretic separation of DNA

For a 1% agarose gel, 0.5g of agarose was added to 50ml of 0.5x TBE buffer (45mM tris borate buffer containing 1mM EDTA (Boehringer Mannheim, Lewes, UK)) and heated until the agarose had dissolved. The agarose was allowed to cool slightly before adding ethidium bromide to 0.3μg/ml concentration. Gels were poured into a Flowgen mini-gel unit (Flowgen, Staffordshire, UK) and left to set for 30-60 minutes before covering with 0.5x TBE. Samples for electrophoresis were prepared by adding sufficient 6x loading dye (40% (w/v) sucrose (BDH Laboratory Supplies, Poole, UK), 0.5x TBE, 0.25% (w/v) bromophenol blue) to give a 1x solution, mixed and loaded directly. Electrophoresis was carried out at 50-60 volts on a Bio-Rad model 250/2.5 powerpack (Bio-Rad, Hemel-Hempstead, UK) and allowed to proceed until the loading dye had run a sufficient distance into the gel (typically $^{2}/_{3}$ of the way down the gel). Separated DNA was visualised under UV light. Gels were calibrated using Boehringer DNA molecular markers YI (range 2176-154 bp) (Boehringer Mannheim, Lewes, UK).

1.3. Agarose Gel cleaning of DNA

Bands were excised from agarose gels using a clean razor blade and the DNA extracted using the Hybaid Recovery™ DNA Purification Kit II (Hybaid Ltd, Middlesex, UK) according to the manufacturers instructions. Briefly, the agarose band was added to 400μl of binding buffer and melted for 5 minutes at 55°C. Melted agarose was removed by spinning in an MSE Microcentaur microfuge (Fisher Scientific, Loughborough, UK) and washed using the wash buffer provided. DNA was eluted with 2 X 10μl of elution buffer and stored at -20°C until needed.

1.4. Restriction digestion and ligations

DNA for restriction digestion was added to 10x restriction enzyme buffer and made up to the desired volume with distilled water. 5-10 units of restriction enzyme were added and the reaction was incubated under the conditions specified by the restriction enzyme

supplier - typically 37°C for 2-3 hours. Digested DNA was ligated at insert:plasmid volume ratios of 2:1, 4:1, 7:1, and 9:1. DNA was added to 10x ligation buffer and made up to the required volume with distilled water. Three units of T4 DNA ligase were added and reactions were left in a beaker of 30°C water, overnight at 4°C.

1.5. Isolation of Plasmid DNA

Plasmid DNA was isolated from bacteria using the method of Sambrook, Fritsch & Maniatis (1989). A single colony forming unit was inoculated into 2ml of Luria-Beritani broth (LB) (1% (w/v) NaCl (BDH Laboratory Supplies, Poole, UK) 1% (w/v) bactotryptone (DIFCO Laboratories, USA) and 0.5% (w/v) yeast extract (Oxoid, Basingstoke, UK) containing 100µg/ml ampicillin and grown for 16 hours at 37°C with shaking. Bacteria were harvested by centrifuging 1.5ml of the overnight culture at 13000 rpm in an Microcentaur microfuge (Fischer Scientific, Loughborough, UK) for 30 seconds at 4°C. The supernatant was removed by aspiration and the bacterial pellet resuspended by vortexing in 100µl of ice cold, sterile solution I (50mM glucose (Fisons, Loughborough, UK), 25mM Tris-Cl, pH 8, 10mM EDTA, pH 8). 200µl of freshly prepared solution II (0.2M NaOH (Fisons, Loughborough, UK), 1% SDS) was added and mixed by inverting the tube 5 times. The tube was incubated on ice for 5 minutes followed by addition of 150µl of ice-cold solution III (5M potassium acetate (Fisons, Loughborough, UK), 2.1M acetic acid. After vortexing in an inverted position for 10 seconds the tube was stored on ice for 3-5 minutes. The tube was centrifuged in an MSE Microcentaur microfuge (Fisher Scientific, Loughborough, UK) at 13000rpm for 5 minutes at 4°C and the supernatant was transferred to a fresh tube, noting the approximate volume of the liquid. An equal volume of phenol chloroform isoamyl was added and the solutions mixed by vigorous vortexing. The tube was centrifuged at 13000rpm for 2 minutes at 4°C and the top aqueous layer was transferred to a clean tube, again noting the volume of liquid removed. Double stranded DNA was precipitated with 2 volumes of ethanol. The solutions were vortexed and allowed to stand at room temperature for 2 minutes after which they were centrifuged at 13000rpm for 5 minutes at 4°C. The supernatant was aspirated carefully and the DNA pellet allowed to dry before washing in 1ml of ice-cold 70% ethanol (BDH Laboratory Supplies, Poole, UK). The sample was centrifuged again and the supernatant removed.

The pellet was air dried for 10 minutes before resuspending it in 50µl sterile dH_2O with 1µg of DNase free RNase (Boehringer Mannheim, Lewes, UK). The solution was left at 4°C for 1 hour before transferring it to -20°C for storage. The concentration of double stranded DNA was calculated from the absorbance at 260nm. At this wavelength 1 OD unit is equal to 50µg of double stranded DNA.

Plasmid to be sent for DNA sequencing was purified using mini- or midi- prep kits from Qiagen (Crawley, UK) according to the instructions provided. The concentration and purity of samples was estimated by reading the absorbance at 260nm and the A260/280 ratio.

1.6. Production of electroporation competent E. coli cells

In order to take up plasmid DNA, bacterial cells need to be made competent. The method used to make electroporation competent *E. coli* cells was based upon that given in the BioRad Gene Pulser manual. One litre of LB (1% (w/v) NaCl (BDH Laboratory Supplies, Poole, UK) 1% (w/v) bactotryptone (DIFCO Laboratories, USA) and 0.5% (w/v) yeast extract (Oxoid, Basingstoke, UK)) was inoculated with $^1/_{100}$ volume of an overnight *E. coli* starter culture. Cells were grown at 37°C with vigorous shaking to an OD_{600} of 0.5-0.8 and chilled on ice for 15-30 minutes before harvesting by centrifugation at 4000g for 15 minutes, 4°C in a Beckman model J2-21 centrifuge (Beckman Instruments Inc., High Wycombe, UK). The pellet was resuspended in 1 litre of ice cold sterile water and centrifuged again. This pellet was resuspended in 0.5 litres of ice cold sterile water and the centrifugation repeated a second time. The pellet resulting from this spin was resuspended in 20ml of ice cold, sterile 10% glycerol (Fisons, Loughborough, UK) before centrifuging again with half the maximum brake. Cells were then resuspended in ice cold, sterile 10% glycerol to a final volume of 2-3ml and stored in 50µl aliquots at -80°C until required for use. Cells stored in this way remained viable for ~ 6 months.

1.7. Transformation by electroporation

Electroporation is an efficient method for introducing plasmid DNA into competent bacterial cells. Aliquots of cells were thawed gently whilst touching, but not embedded in ice. Electroporation cuvettes (Bio-Rad, Hemel-Hempstead, UK) and the electroporation chamber slide were cooled on ice and the BioRad Gene Pulser and pulse controller were set to 25μF, 1.5KV and 1000Ω respectively. 1-2μl of plasmid DNA was added to the cell suspension, mixed gently but thoroughly and placed on ice for 1 minute. The mixture of cells and DNA was transferred to an electroporation cuvette, inserted into the chamber slide and pulsed once at the settings outlined above. The cuvette was removed immediately and the cells resuspended in 1ml of sterile SOC medium (1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 1mM $MgSO_4.7H_2O$ (Fisons, Loughborough, UK), 1mM $MgCl_2.6H_2O$ (Fisons, Loughborough, UK) and 20mM glucose. The cell suspension was transferred to a 1.5ml eppendorf and incubated at 37°C for 1 hour before plating onto selective media (typically LB containing 1.5% (w/v) agar (DIFCO Laboratories, USA) and 100μg/ml ampicillin).

2. Preparation of Recombinant Protein

2.1. Expression of 4.1R-CTR

The 4.1R-CTR*c* was expressed in *E. coli* BL21 (DE3) pLysS. A 5ml overnight culture was inoculated into 500ml of LB broth containing 100μg/ml ampicillin and 30μg/ml chloramphenicol. Cultures were grown at 37°C with vigorous shaking to an $OD_{600} \approx$ 0.3 at which pint they were induced by adding IPTG (isopropyl-ß-D-thiogalactopyranoside) (Melford, Suffolk, UK) to 0.25mM. After induction, cultures were grown at either 37°C for 3 hours or ~24°C for 2 hours. Cultures were harvested by centrifugation at 4000g for 20 minutes, 4°C, then resuspended in PBS (phosphate buffered saline; 20mM $Na_2HPO_4.12H_2O$, 150mM NaCl, pH7.4) and recentrifuged as above. The bacterial pellet was stored frozen at -80°C and kept for no longer than 2 months. During the induction, levels of expressed protein were analysed by SDS-PAGE (this chapter, section 3.1). To ensure equal numbers of bacteria were compared

at each sampling stage the following equation was used to determine how much of the bacterial culture to remove:

$$y \times \frac{42.2}{OD_{600}}$$

(where y is any constant)

The correct volume of bacterial culture was centrifuged at 13000rpm for 1minute in an MSE Microcentaur microfuge (Fischer Scientific, Loughborough, UK) and the bacterial pellet was made into a gel sample by resuspending it in 40µl of 2.5x SDS-PAGE sample buffer (this Chapter, section 3.1).

2.2. Immobilised Metal Chelate Affinity Chromatography Purification of 4.1R-CTR

The presence of a hexa-his tag at the C-terminal of 4.1R-CTR made it a suitable polypeptide to be purified using immobilised metal chelate affinity chromatography. Both Ni^{2+}-IDA (iminodiacetic acid) and Ni^{2+}-NTA (nitrilotriacetic acid) based purification systems were used.

2.2.1. Hi-trap purification of 4.1R-CTR

Pre-packed IDA His-Trap™ columns were obtained from Amersham-Pharmacia Biotech (St. Albans, UK) and 4.1R-CTR was purified based on the manufacturers instructions.

Frozen bacterial pellets produced by induction of *E. coli* BL21(DE3)pLysS (this Chapter, section 2.1) were thawed and resuspended in 10ml Hi Trap™ start buffer containing Complete™ Protease Inhibitor Cocktail solution (Boehringer Mannheim, Lewes, UK) used as per the manufacturers guidelines. Lysis was effected by sonnicating cells on an MSE Soniprep 150 sonnicator at 14µm amplitude for 2 x 10 second bursts followed by sheering with a 0.8 x 40mm needle, keeping the suspension on ice as much as possible. The lysate was spun at 23000g for 20 minutes at 4°C using a Beckman JA20 rotor and the supernatant containing soluble recombinant protein was removed and filtered through a 0.45µm filter (Sartorius) before applying to the Ni^{2+}

activated Hi Trap™ column. Purification was achieved by washing the column with increasing concentrations of imidazole (5ml each of start buffer containing 20mM, 40mM, 60mM, 100mM, 300mM and 500mM imidazole). Fractions were analysed by 9% SDS-PAGE (this Chapter, section 3.1). Those containing 4.1R-CTR (typically 300mM and 500mM imidazole fractions) were dialysed against 10mM HEPES pH7.4, 10mM NaCl, 0.01% Tween 20 and stored in aliquots at -80°C.

2.2.2 Ni-NTA purification of 4.1R-CTR

Ni-NTA resin was obtained from Qiagen (Crawley, UK) and purification of 4.1R-CTR was carried out according to the manufacturers instructions. Bacterial lysates were prepared as with the Hi-Trap™ purification method except that the buffer used to resuspend the starting bacterial pellet was Ni-NTA lysis buffer (50mM NaH_2PO_4, pH 8, 300mM NaCl (BDH Laboratory Supplies, Poole, UK), 10mM imidazole).

The Ni-NTA resin was resuspended and 2ml (equivalent to 1ml beads) was transferred to a 5ml bijou. The resin was equilibrated with 3 x washes with 4ml Ni-NTA lysis buffer, allowing the resin to settle by gravity and removing the supernatant before adding new buffer. After the final wash, buffer was replaced with 4ml bacterial supernatant and the resin was stirred gently with a magnetic flea for 1 hour at 4°C. Beads were poured into a Bio-Rad glass Econo-column (15cm x 0.39cm, Bio-Rad, Hemel-Hempstead, UK) and allowed to settle by gravity. The column was washed sequentially with 5 column volumes each of Ni-NTA wash buffer (50mM NaH_2PO_4, pH8, 300mM NaCl) containing 20mM, 50mM, 100mM and 250mM imidazole, followed by 5ml of Ni-NTA lysis buffer. The column flow rate was 0.34ml/min and 1ml fractions were collected throughout.

Fractions were analysed by 9% SDS-PAGE gels (this Chapter, section 3.1). Those containing 4.1R-CTR of similar concentration and purity were pooled and dialysed overnight against 10mM HEPES, pH 7.4, 10mM NaCl, 0.01% Tween 20 at 4°C. Samples were stored in aliquots at -80°C.

3. Protein Electrophoretic Techniques

Proteins were separated according to their molecular mass by linear sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a discontinuous tris-glycine buffer system as described by Laemmli (1970). Proteins electrophoresed in two-dimensions (IEF or NEPHGE) were separated by charge according to the methods of O'Farrell (1975) and O'Farrell, Goodman & O'Farrell (1977), prior to separation by mass.

3.1. SDS-PAGE

SDS-PAGE was carried out using the Hoefer SE250 Mighty Small II mini gel system (Amersham-Pharmacia Biotech, St Albans, UK). Resolving gels were made by diluting 40% acrylamide solution (acrylamide:bisacrylamide 37:1, Bio-Rad, Hemel-Hempstead, UK) to the desired percentage with 2M Tris, pH 8.8, 10% SDS and distilled water. Final concentrations were 372mM Tris and 0.1% SDS, thus for a 9% resolving gel, reagents were mixed as shown in Table 6.

Reagent	*Volume*
Acrylamide (40%)	4.50ml
Tris (2M)	3.70ml
SDS (10%)	0.20ml
Distilled water	11.40ml
APS (10%)	0.20ml
TEMED	0.02ml

Table 6. Preparation of SDS-polyacrylamide resolving gels for electrophoresis. The volume of each reagent required to make 20 ml of a 9% resolving gel solution are given. This volume is sufficient for two 1.5mm thick gels using the Hoefer SE250 Mighty Small II mini gel system.

Polymerisation was initiated by the addition of ammonium persulphate (APS) to 0.1% and N,N,N',N'-tetramethlyethylenediamine (TEMED) to 0.01%. The mixture was poured into gel plates and overlayed with water-saturated isobutanol. Gels were allowed to polymerise for at least 30 minutes and were either used immediately or stored for up to one week at 4°C with a small volume of solution containing 372mM Tris and 0.1% SDS to prevent drying.

Prior to electrophoresis resolving gels were overlayed with a 4% stacking gel (0.125M Tris pH6.8, 4% acrylamide solution, 0.2% SDS, 0.1% APS and 0.1% TEMED) to increase the resolution of protein separations. The stacking gel was allowed to polymerise fully before adding SDS-PAGE running buffer (25mM Tris, 192mM glycine, pH 8.6, 0.1% SDS) to both upper and lower buffer chambers and loading gel samples. Gel samples were prepared by adding 5x SDS-PAGE sample buffer (62.5mM Tris pH 6.8, 10% SDS, 50% (w/v) sucrose, 10% β-mercaptoethanol and trace bromophenol blue) to give a 1x solution and boiled for 5 minutes before loading. Gels were run at 60 volts until the dye front traversed the stack and at 120 volts thereafter.

3.2. NEPHGE/IEF gel electrophoresis

Non-equilibrium pH gel electrophoresis (NEPHGE) and isoelectric focusing (IEF) were carried out using the Hoefer (Amersham-Pharmacia Biotech, St Albans, UK) SE250 Mighty Small mini gel two-dimensional adapter system. First dimension gels were cast in a Hoefer SE 225 Tube Gel Caster (Amersham-Pharmacia Biotech, St Albans, UK) according to the manufacturers instructions using the solution outlined in Table 7. All solutions were made up with Elgar Maxima ultra pure water (Elgar). The 30% acrylamide solution was made by dissolving acrylamide and bisacrylamide powder (Bio-Rad, Hemel-Hempstead, UK) in water at a acrylamide:bisacrylamide ratio of 17.5:1.

Reagent	*Volume*
Solid Urea	2.75g
Acrylamide (30%)	665µl
Nonidet P40 (10%)	1ml
Water	1ml
Ampholines (pH 3.5 – 10)	150 µl
Ampholines (pH 5 – 7)	100µl
APS (10%)	5µl
TEMED	5µl

Table 7. Preparation of first dimension polyacrylamide gels for NEPHGE/IEF. Volumes given allow the preparation of 6 first dimension stick gels using the Hoefer SE 225 Tube Gel Caster.

A mixture of urea, acrylamide, nonidet P40 and water was warmed gently to 37°C to allow the urea to dissolve. It is important that the temperature of the mixture does not exceed 37°C to minimise hydrolysis of urea. Once the urea had been solubilised ampholines were added, followed by the APS and TEMED just prior to casting. Gels were left to polymerise for 2–4 hours.

First dimension stick gels were run on the Hoefer SE250 Mighty Small II mini gel system using the Hoefer SE 220 tube gel adapter kit (both from Amersham-Pharmacia Biotech, St Albans, UK). For NEPHGE 0.01M phosphoric acid (Fisons, Loughboroguh, UK) was used as the upper electrode buffer (anode) and 0.02M NaOH as the lower electrode buffer (cathode). For IEF, the buffers and hence polarity were reversed and sticks were pre-focused at 250 volts for 30 minutes before loading. Sample dissolved in IEF sample buffer (9.5M urea, 2% (w/v) nonidet P40, 5% β-mercaptoethanol, 20µg/ml leupeptin, 1.2% ampholines pH 3.5-10, 0.08% ampholines pH 5-7) was loaded on the top of each stick and overlayed with 10µl of IEF sample buffer diluted 1:2 with water. Sticks were topped up with upper electrode buffer

ensuring there were no air bubbles to disrupt the flow of current and electrophoresis was carried out at 500 volts for 4 hours.

On completion of the run the sticks were ejected from their casting tubes and either equilibrated for 30 minutes in equilibration buffer (62.5mM Tris, pH 6.8, 10% (w/v) glycerol, 5% (5/v) β-mercaptoethanol, 2.3% SDS and trace bromophenol blue) making a note of stick orientation and frozen at -20°C until required, or cut into 0.5 cm pieces and used to determine the pH gradient. Stick pieces used to determine the gradient were placed into 100µl dH_2O and left overnight at 4°C to equilibrate before reading the pH. Fragment pH was plotted against stick length.

Flat top, second dimension gels were cast as in outlined in section 3.1 of this Chapter. A single stick was placed along the top of the stacking gel and sealed in place with a solution containing 1% agarose and running buffer diluted 1:4 with dH_2O. It is important to make sure no air bubbles exist between the stick and the second dimension gel to allow uniform current flow during electrophoresis. Second dimension gels were run at 60 volts for 3.5 hours or until the dye front reached the bottom of the gel and were either western blotted or silver stained using one of the methods outlined in section 3.3 of this Chapter.

3.3. Protein Staining methods

A number of staining methods with varying sensitivities were used to detect proteins on polyacrylamide gels or nitrocellulose after western blotting.

3.3.1 Coomassie blue

To directly visualise SDS-PAGE separated proteins present at >0.1µg, gels were transferred to a clean plastic container and gently shaken for ~1 hour in a solution of Coomassie blue (50% methanol, 10% glacial acetic acid (both from BDH Laboratory Supplies, Poole, UK) and 0.1% (w/v) Coomassie blue R250). The Coomassie blue was replaced with a solution of destain (50% methanol, 10% acetic acid) which was changed periodically until the desired level of destaining was achieved.

3.3.2. Silver staining

Proteins not abundant enough to be detected by Coomassie blue staining were visualised by one of two silver staining methods.

3.3.2.1. Rapid silver staining method

Rapid silver staining of polyacrylamide gels was carried out according to the method of Shevchenko *et al.*, (1996) adapted from Rabilloud, Carpentier & Tarroux (1988) and Swain & Ross (1995). All steps were performed with continuous shaking. The gel was fixed for 20 minutes in 50% methanol, 5% acetic acid and washed for a further 10 minutes in 50% methanol alone. A second wash with distilled water for 10 minutes was followed by sensitisation in 0.02% sodium thiosulphate (BDH Laboratory Supplies, Poole, UK) for 1 minute. The gel was rinsed in 2 changes of distilled water over 1 minute and submerged in 0.1% silver nitrate (Fisons, Loughborough, UK) for 20 minutes. Following this, the gel was rinsed in 2 changes of distilled water over 1 minute as before and developed in 0.04% formalin, 2% sodium carbonate (BDH Laboratory Supplies, Poole, UK) until the protein bands could be seen clearly. Development was carried out in transparent solution. Developer that had turned yellow was replaced with fresh solution. Development was stopped by submerging the gel in 5% acetic acid and stained gels were stored at 4°C in 1% acetic acid.

3.3.2.2. Enhanced sensitivity method

Silver staining for enhanced sensitivity was carried out according to Morrissey (1981). All steps involve shaking at room temperature. Polyacrylamide gels were fixed in a solution of 50% methanol and 10% acetic acid for 30 minutes then transferred to a solution of 5% methanol, 7% acetic acid for a further 30 minutes. Gels were rinsed in distilled water overnight followed by a fresh 30 minute rinse and soaked in a solution of dithiothretol (DTT; 5μg/ml) for 30 minutes. The DTT solution was poured off and replaced without rinsing, with a solution of silver nitrate (0.1%) for a further 30 minutes. Gels were rinsed rapidly with a small amount of distilled water and twice rapidly with a small amount of freshly prepared developer (0.02% formaldehyde in 100ml 3% sodium carbonate (Fisons, Loughborough, UK)) and submerged until the

desired level of staining was achieved. Development was stopped by the addition of 2.3M citric acid (Fisons, Loughborough, UK) and agitating the solution at 4°C for 10 minutes.

3.3.3. Ponceau S staining

Ponceau S is a reversible stain. It does not affect protein antigenicity and can be used fix proteins to the nitrocellulose membrane prior to immunoblotting. After protein transfer by western blotting, the nitrocellulose membrane was stained with Ponceau S solution (1 vial of Ponceau S concentrate, 20% propan-2-ol and 5% acetic acid made up with distilled water). After shaking in this solution for 1-2 minutes excess Ponceau was removed with distilled water allowing protein bands to be visualised. The stain was completely removed prior to immunoblotting by shaking the nitrocellulose membrane in PBS/0.1% Tween 20.

3.3.4. Staining with colloidal gold

The colloidal gold solution for staining proteins on nitrocellulose membranes was prepared according to Yamaguchi & Asakawa (1988). Membranes were placed in colloidal gold solution (0.25ml/cm^2) and shaken for several hours at room temperature or overnight as required. Once the colour had developed sufficiently the membrane was washed in distilled water and left to air-dry. If staining of the nitrocellulose membrane was to take place after immunoblotting, membranes were blocked during the blotting procedure (this Chapter, section 3.5) by shaking in a solution of PBS, 0.05% Tween 20, 1% PVP-40 for 2 hours at room temperature and the antibodies used were diluted in block. After development the membrane was washed for 10 minutes in PBS/0.1% Tween 20 before adding the colloidal gold solution for staining.

3.4. Western blotting

Western blotting was carried out by transfer of proteins to Protran® nitrocellulose transfer membrane (0.4μm pore size, Schleicher and Schuell GmbH, Germany). SDS-PAGE was carried out initially to separate proteins as outlined in this Chapter, section

3.1. Both gel and nitrocellulose were soaked in transfer buffer (SDS-PAGE running buffer containing 10% methanol and 0.01% SDS) and the 'blotting sandwich' was assembled: A scotchbrite pad soaked in transfer buffer was lined with filter paper (Whatman 3mm, Whatman, Maidston, UK). The soaked nitrocellulose was placed onto the filter paper and the polyacrylamide gel was laid on top of the nitrocellulose ensuring no bubbles were trapped. A second piece of filter paper soaked in transfer buffer was laid over the gel and air bubbles were excluded. The blotting 'sandwich' was completed with another pre-soaked scotchbrite pad and was assembled into the blotting tank (Bio-Rad, Hemel-Hempstead, UK) with the nitrocellulose orientated towards the anode. Blotting was at 0.1 amps overnight or 0.3 amps for 3 hours.

3.5. Immunoblotting

Immunoblotting is a technique that probes proteins transferred to nitrocellulose with specific antibodies as a means of identification. All incubations were performed at room temperature with shaking. Primary and secondary antibodies were spun at top speed in an MSE Microcentaur microfuge (Fischer Scientific, Loughborough, UK) for 5 minutes at 4°C prior to dilution. Protein transferred to nitroellulose were fixed with Ponceau S and the stain removed as outlined in section 3.3.3. Non-specific binding sites on the nitrocellulose were blocked by incubating the blot with milk proteins (5% Marvel) in a solution of phosphate buffered saline (PBS: 20mM $Na_2HPO_4.7H_2O$, 2mM KH_2PO_4, 140mM NaCl, 3mM KCl pH7.4) and 0.1% polyoxyethylenesorbitan monolaureate (Tween 20) for 1 hour. Primary antibody was diluted to the appropriate concentration with a solution of PBS, 0.1% Tween 20 containing 2% milk proteins. This solution was used to replace the blocking solution and blots were incubated for a further 1 hour period. Blots were washed by shaking for 5 minutes in PBS, 0.1% Tween 20, followed by 5 minutes in High Salt Tween (HST: 10mM Tris pH7.4, 1M NaCl, 0.5% Tween 20) and another 5 minutes in PBS, 0.1% Tween 20. The appropriate horseradish peroxidase (HRP) conjugated secondary antibody was diluted with PBS, 0.1% Tween and blots were incubated with this solution for 1 hour before washing five times in PBS, 0.1% Tween and HST (middle wash) for 5 minutes each time. Blots were developed by enhanced chemiluminescence (reagents and method from Amersham-Pharmacia Biotech (St. Albans, UK)).

4. Protein Purification and Subcellular Fractionation Methods

4.1. Purification of spectrin

Human erythrocyte spectrin dimer was purified based on the method of Gratzer (1982). 50ml human blood was distributed between 4 x 50ml falcon tubes and topped up with wash buffer (0.15M NaCl, 20mM Tris pH7.6). Tubes were mixed by inversion and centrifuged for 5 minutes at 2000g in a Denley Model 401 bench top centrifuge. The supernatant and buffy coat were removed by aspiration and the process repeated 3 times. Three millilitres of washed cells were aliquoted into each of 8 Beckman JA20 tubes (Beckman Instruments Inc., High Wycombe, UK) and topped up with cold lysis buffer (5mM Tris pH7.6) containing Complete™ Protease Inhibitor Cocktail solution (Boehringer Mannheim, Lewes, UK) used as per manufacturers guidelines. Tubes were mixed immediately by inversion and centrifuged at 48000g for 10 minutes, 4°C. The dark red supernatant was removed by aspiration and the process repeated until the remaining ghosts were almost white. A small sticky pellet forms at the centre of the ghost pellet during the lysis steps. This pellet contains residual white cells that could be a damaging source of proteases. The pellet is removed by rotating the centrifuge tube and aspirating the exposed pellet from the top. Ghosts were pooled and the tube topped up with cold distilled H_2O before centrifuging once more.

To extract spectrin dimers from ghosts an equal volume of extraction buffer (0.3mM phosphate pH8, 0.1mM EDTA and 0.1mM phenylmethanesulphonylfluoride (PMSF) was added to the ghosts and the suspension periodically swirled in a water bath at 35°C for 15 minutes. Centrifugation at 90,000g for 30 minutes gave a supernatant containing 70-90% of the total spectrin plus actin and protein 4.1. Separation of these proteins was effected by gel filtration using a S500 column (column dimensions: 60cm x 3cm, Amersham-Pharmacia Biotech, St. Albans, UK). The column was equilibrated with 0.1M NaCl, 0.05M Tris pH7.6 and run at a flow rate of 64 ml/hr. Allowing for the void volume to pass through the column (~140ml), 5ml fractions were collected and gel samples were made for SDS-PAGE. Fractions containing pure spectrin were pooled and concentrated using solid polyethelyene glycol. After dialysis overnight against

20mM phosphate, 0.1mM EDTA, 0.1mM DTT and 1mM sodium azide the spectrin was aliquoted into 1ml fractions and stored at -80°C until required.

4.2. Purification of actin

Actin was extracted from rabbit skeletal muscle acetone powder using the method of Pardee & Spudich (1982) adapted by A. Lodge (University of Kent) and powder prepared previously by D. Rainham (University of Kent) Acetone powder (5g) was stirred in 90ml buffer I (2mM borate pH8, 0.2mM $CaCl_2$, 0.05mM ATP and 0.5mM DTT) for 30min at 0°C. The extract was spun at 10000g for 10 minutes at 4°C in a Beckman model J2-21 centrifuge (Beckman Instruments Inc., High Wycombe, UK) and the supernatant filtered through filter paper (Whatman 3mm, Whatman, Maidston, UK). The filtrate was stored on ice while the residual pellet was re-extracted with 60ml of buffer I for 15 minutes at 0°C and centrifuged and filtered as before. Filtrates were combined and solid KCl (BDH Laboratory Supplies, Poole, UK) was added to a concentration of 100mM while 0.2M $MgCl_2$ (Fisons, Loughborough, UK) was added to a concentration of 2mM. The suspension was mixed gently by inversion until the KCl had dissolved and the actin was allowed to polymerise at room temperature for 1.5 hours. Solid KCl was added to a final concentration of 0.6M and after gentle mixing the tube was again allowed to stand for 1 hour in order to remove associated tropomyosin and troponin. Polymerised actin was collected by centrifugation at 100,000g for 1.5 hours at 15°C using a Beckman 45Ti rotor. The pellet was rinsed in buffer II (2mM borate pH7.5 (Fisons, Loughborough, UK), 0.2mM $CaCl_2$ (Fisons, Loughborough, UK), 0.05mM ATP, 0.5mM DTT and 0.02% sodium azide) and homogenised in 1ml of buffer II for each 1g acetone powder extracted with a teflon glass hand held homogeniser. F-actin was dialysed over 3 days against 1 litre of buffer II with 6-8 changes of buffer during this period. The resulting g-actin was stored at 4°C in the presence of 0.05mM ATP for up to 3 months replenshing ATP weekly.

4.3. Postsynaptic density preparation

Postsynaptic densities were purified from pig or rat forebrain according to the method outlined by Carlin *et al.*, (1980). Brain tissue (typically 20g) was thawed and

homogenised with 12 up and down strokes of a Teflon-glass homogeniser Using 10g brain/40ml buffer A (0.32M sucrose (BDH Laboratory Supplies, Poole, UK), 1mM $NaHCO_3$ (Fisons, Loughborough, UK), 1mM $MgCl_2$, 0.5mM $CaCl_2$). Homogenates were combined, diluted to 10% (w/v) with buffer A and centrifuged at 1400g, 4°C for 10 minutes. The supernatant was removed and retained on ice while the remaining soft pellet was homogenised with 3 up and down strokes of the teflon glass homogeniser using the same 10% volume of buffer A used previously. Homogenates were pooled and spun at 710g, 4°C for 10 minutes. The supernatant was removed and after pooling with the first supernatant was centrifuged at 13,800g, 4°C for 10 minutes. The pellet resulting from this spin contained synaptosomes and mitochondria and was resuspended with 6 up and down strokes of the homogeniser using 24ml buffer B/10g starting brain tissue (0.32M sucrose, 1mM $NaHCO_3$, 1mM azide). The suspension was loaded on to sucrose gradients consisting of 10ml each of 0.85M sucrose–1mM $NaHCO_3$, 1M sucrose–1mM $NaHCO_3$, 1.2M sucrose–1mM $NaHCO_3$, at approximately 8ml per gradient. Gradients were run on a Beckman model L8-70m ultracentrifuge using a Beckman SW28 rotor, (Beckman Instruments Inc., High Wycombe, UK) for 2 hours at 82,500g with no brake. Synaptosomes band between 1M and 1.2M sucrose and were removed by aspirating off the solution above the band and removing the band with a plastic pasteur pipette. The synaptosomes were diluted with 60ml buffer B/10g starting tissue and an equal volume of 1% (v/v) Triton X-100 in 0.32M sucrose–12mM Tris-HCl (pH8.1) was added. The suspension was stirred for 15 minutes at 4° and centrifuged at 32,800g, 4°C for 20 minutes in a Beckman 45Ti rotor. The resulting pellets were resuspended in 2.5ml buffer B/10g starting brain ready for loading on a second set of sucrose gradients. Sucrose gradients consisting of 4ml of 2M sucrose, 3ml of 1.5M sucrose–1mM $NaHCO_3$, and 3ml of 1M sucrose–1mM $NaHCO_3$ were each layered with 2ml of the suspension and run at 201,800g in a Beckman SW40 rotor for 2 hours, 4°C, with no brake. Postsynaptic densities were recovered from the 1.5M–2.0M sucrose interface. The PSDs were diluted to a final volume of 6ml with buffer B and an equal volume of 1% Triton-150mM KCl was added. The suspension was spun for 20 minutes at 201,800g in the SW40 rotor at 4°C with half maximum brake and the pellet was resuspended in minimal buffer B by homogenisation in a hand held teflon-glass homogeniser. PSD were either stored at –80°C in aliquots or were prepared for

use in SDS-PAGE, IEF or affinity chromatography. For SDS-PAGE, 5x SDS-PAGE sample buffer was added to PSDs to give a 1x solution and gel samples were boiled and stored at –20°C until required. To prepare PSDs for NEPHGE and IEF gels, the final PSD pellet was resuspended in 50µl buffer B and 50µl IEF sample buffer (9.5M urea, 2% (w/v) nonidet P40, 5% β-mercaptoethanol, 20µg/ml leupeptin, 1.2% ampholines pH 3.5-10, 0.08% ampholines pH 5-7). Solid urea was added to 9M and dissolved thoroughly. PSDs were stored at –20°C until required. To prepare PSDs for affinity chromatography, the final PSD pellet was resuspended by homogenisation in 1ml of 50nM Tris pH7.4, 1% CHAPS and 1mM PMSF. PSDs were stored at -20°C in 250µl aliquots until required.

4.4. Preparation of High Speed Supernatant

Soluble cytosolic proteins were prepared as a high speed supernatant (HSS) using the first part of the method for purifying CaMKII outlined by Bennett, Erondu & Kennedy (1983). The brains of three rats were removed and homogenised with 12 up and down strokes of a teflon glass homogeniser using 10ml HSS buffer/g brain (20mM Tris, pH 7.5, 1mM imidazole, 0.1mM $CaCl_2$, 1mM $MgCl_2$, 1mM DTT and Complete™ Protease Inhibitor Cocktail solution (Boehringer Mannheim, Lewes, UK) used as per the manufacturers guidelines). The homogenate was centrifuged at 10000g for 20 minutes at 4°C and the supernatant removed and spun in a Beckman 70Ti rotor (Beckman Instruments Inc., California) at 170000g, 4°C for 1 hour. The resulting supernatant, designated the high speed supernatant, was aliquoted and stored at -80°C. Protein concentration was estimated by OD_{280} against a standard curve of BSA and was found to be 10.5mg/ml.

5. Analysis of 4.1R-CTR

5.1. Ternary Complex Formation

Human erythrocyte spectrin and 4.1R-CTR were pre-spun in a Beckman TL-100 ultracentrifuge (Beckman Instruments Inc., High Wycombe, UK) at 85000g for 30 minutes at 4°C. Reactions contained 40µg/ml spectrin, 30µg/ml 4.1R-CTR and

0.35mg/ml g-actin in ternary complex buffer (50mM KCl, 5mM PIPES, pH6.8, 1mM $MgCl_2$, 0.25mM DTT and 0.05mM ATP) in a total reaction volume of 200µl. Where spectrin or 4.1R-CTR were omitted from a reaction they were replaced with ternary complex buffer. Where actin was omitted it was replaced with buffer containing 2mM borate pH7.5, 0.2mM $CaCl_2$, 0.05mM ATP, 0.5mM DTT and 0.02% sodium azide. Reaction tubes were left at room temperature for 90 minutes to allow complexes to form and then centrifuged at 85000g for 30 minutes, 4°C. On completion of the spin supernatants were removed and their volume recorded. SDS-PAGE sample buffer was added to the supernatants to give a 1x solution and the total volume of the sample was determined. Pellets were washed carefully in ternary complex buffer and resuspended in a volume of 1x SDS-PAGE sample buffer equal to the total volume of the corresponding reaction supernatant.

5.2. Chymotrypsin Digestion

4.1R-CTR was subjected to limited enzymatic digestion using TLCK treated α-chymotrypsin (Boehringer Mannheim, Lewes, UK). 4.1R-CTR was dialysed into buffer containing 20mM HEPES buffer, pH7.5, 10mM $MgCl_2$ and 0.5mM $CaCl_2$ and reaction tubes were set up at a 4.1R-CTR:α-chymotrypsin weight ratio of 100µg:1µg. For limited digestion tubes were kept on ice at all times and the reaction was stopped at time intervals by the addition of TPCK (Boehringer Mannheim, Lewes, UK) to 100µM. After 2 minutes on ice to allow TPCK to have an effect, 5x SDS-PAGE sample buffer was added to 1X and samples were run on a 10-20% gradient polyacrylamide gel for better resolution of low molecular weight digestion fragments. For complete 4.1R-CTR digestion tubes were incubated at 4°C overnight before analysing by SDS-PAGE.

6. Analysis of Protein-Protein Interactions

6.1. Blot Overlay Technique

Protein-protein interactions were detected on blots using the blot overlay technique. In this technique the polypeptide of interest is incubated with a nitrocellulose membrane containing a mixture of proteins separated by SDS-PAGE. Specific interactions

between the polypeptide and separated proteins are detected with a primary antibody against the polypeptide and an appropriate secondary antibody conjugated to HRP.

Blots were blocked for 1 hour at room temperature with 5% milk proteins in PBS, 0.1% Tween 20 as outlined in section 3.5 and then rinsed for 5 minutes in PBS, 0.1% Tween 20. 4.1R-CTR was diluted to the appropriate concentration with PBS, 0.1% Tween 20 and incubated with the blot for another 1 hour period. The blot was washed for 5 minutes in PBS, 0.1 Tween 20, 5 minutes in HST and 5 minutes in PBS, 0.1% Tween 20 before adding either anti-4.1R/G/B or anti-*ap*4.1R-CTR diluted to the desired concentration with PBS, 0.1 %Tween 20. Blots were incubated in primary antibody for 1 hour at room temperatre with shaking and washed as above prior to incubating with an appropriate HRP-conjugated secondary antibody for 1 hour at room temperature with shaking. Bound 4.1R-CTR was detected by enhanced chemiluminescence using ECL reagents (Amersham-Pharmacia Biotech, St. Albans, UK) as per the manufacturers instructions. Typically 4.1R-CTR was used at 20μg/ml and 1μg/ml with anti-4.1R/G/B and anti-*ap*4.1R-CTR respectively.

6.2. Affinity Chromatography

Affinity columns can be used to study protein binding activities by immobilising the protein of interest and passing various cell extracts over it. Proteins bound to the immobilised ligand are specifically eluted from the column by a chaotrophic agent and are detected by SDS-PAGE. To make affinity columns, the protein to be immobilised was first dialysed into buffer containing 0.1MNaHCO$_3$, pH8, 0.5M NaCl. Cyanogen bromide activated sepharose 4B suffient to immobilse the ligand was swelled gently in 1mM ice cold HCl and then washed with 200ml 1mMcold HCl per g of gel powder through a scintered glass filter. Washed sepharse beads were incubated with the protein to be immobilised for 2 hours at room temperature with gentle rocking. This allowed coupling of the ligand to the beads. Remaining ligand binding sites were blocked with 0.1M Tris pH8 for 2 hours at room temperature with rocking after which the beads were left to sediment under gravity and the Tris supernatant removed. Beads were washed alternately with 0.05M Tris pH8, 0.5M NaCl and 0.05M formate pH4, 0.5M NaCl in a

batchwise manner until each buffer had be used three times. The final formate buffer was replaced with column running buffer (10mM NaH_2PO_4 pH7.5, 2mN EGTA, 0.05% Tween 20, 0.5mM DTT, 1mM azide) and the beads were mixed, allowed to settle and the buffer removed. Beads were resuspended in a second lot of column running buffer, poured into a Bio-Rad glass Econo-column (15cm x 0.39cm, Bio-Rad, Hemel-Hempstead, UK) and allowed to settle under gravity at 4°C. Column running buffer was pumped through at a low flow rate (~0.1ml/minute) to allow the column to pack properly before use. For 4.1R-CTR affinity columns, 0.5g of cyanogen bromide activated sepharose 4B gave a column of ~1.5ml on which 1.7mg of protein was immobilised. For BSA columns, 0.3g of beads gave a final column volume of ~0.75ml on which 1.1mg of protein was immobilised.

Columns were run at 4°C and before use were equilibriated with 5-6 column volumes of column running buffer. Sample was loaded directly on top of the column bead bed and columns were run at a flow rate of ~ 0.25ml/minute. Columns were washed with 30 column volumes of buffer before eluting bound protein in 0.5ml fractions with column running buffer containing 1.2M NaBr. Elution samples were not always concentrated enough to be visualised clearly by SDS-PAGE staining techniques. In these cases elution samples were dialysed into 10mM Tris pH7.4, 0.1%SDS ~24 hours with one change of buffer. Samples were lyophilised using a Modulyo freeze dryer (Edwards, Crawley, UK) and resuspended in 50µl 2.5x SDS-PAGE sample buffer to concentrate the sample 10-fold. Samples were then anaylsed by 9% SDS-PAGE.

PSD samples for affinity chromatography were sonnicated on ice using an MSE Soniprep 150 sonnicator for 4 x 5 second bursts at an amplitude of 6µm. They were then sheered through a 0.15x16 gauge needle and centifuged at top speed in an MSE Microcentaur microfuge (Fischer Scientific, Loughborough, UK) for 10 minutes at 4°C. The supernatant was loaded on to the column.

6.3. Immunoprecipitation

HSS was mixed with anti-*ap*4.1R-CTR under various conditions to examine 4.1R binding activities. HSS, prepared as outlined in section 4.4 was diluted to 2.1mg/ml with IP buffer (HSS buffer + 0.5% Triton X-100) and incubated on ice for 2 hours with 1-3μg antibody in a total reaction volume of 400μl. Protein A beads (1:4 suspension of protein A:buffer) were equilibrated with IP buffer and 35μl of homogenous suspension was added to each reaction. Tubes were incubated at 4°C overnight with shaking after which beads were pelleted by centrifugation for 1 minute at top speed in an MSE Microcentaur microfuge (Fischer Scientific, Loughborough, UK) and the supernatant removed and retained for gel samples. Reactions were kept on ice as much as possible while the beads were washed with 3 x 1ml of IP buffer. Beads were pelleted and the supernatant discarded after each wash. Protein bound to the beads was solubilised in 30μl of 2.5x SDS-PAGE sample buffer and boiled for 2 minutes prior to loading on an SDS-PAGE gel. Gels were silver stained using the enhanced detection method of staining, this Chapter, section 3.3.2.2.

7. Miscellaneous

7.1. ELISA

ELISA reactions were performed in 96 well plates (Greiner, Gloucestershire, UK) using an automatic plate washer (Denley, model well wash 4 mk2). Before starting the ELISA reaction, plates were coated overnight at 4°C with 4mg/ml strepavidin in 0.1M $NaHCO_3$ pH8.2 and 20μg of 4.1R-CTR (10μg/ml) was biotinylated for 1 hour at room temperature at a 2:1 mole ratio of biotin:4.1R-CTR. The biotinylated polypeptide was dialysed overnight at 4°C into 10mM HEPES pH7.4, 10mM NaCl and 0.01% Tween 20. Strepavidin coated plates were stored at 4°C and biotinylated 4.1R-CTR not used directly was stored at -20°C.

A strepavidin coated plate was washed x 3 with PBS, 0.1% Tween. Biotinylated 4.1R-CTR was diluted to 1μg/ml with 0.1M $NaHCO_3$ pH8.2, 4% NaCl, 0.1% Tween 20 and added to the strepavidin coated plate which was then incubated for 1 hour at 37°C. The

plate was washed as before and blocked for 1hour at 37°C with 1% gelatin in PBS. After a further 3 washes the plate was incubated for 1 hour at 37°C with primary antibody. Antibodies were spun at top speed in an MSE Microcentaur microfuge (Fischer Scientific, Loughborough, UK) for 5 minutes at 4°C before diluting them to the appropriate concentration with 0.5% gelatin in PBS. At the end of the incubation the plate was washed again and incubated for another hour at 37°C with anti rabbit-HRP secondary antibody (DAKO, Cambridge, UK). The secondary was spun as described for the primary antibody and diluted to $^{1}/_{5000}$ with 0.5% gelatin in PBS before use.

Binding was detected by incubating the plate with a freshly prepared solution of 2mg/ml OPD (O-phenylenediamine dihydrochloride), 0.03% H_2O_2 (30% (w/w) solution, Sigma), 0.1M citric acid, pH5. Colour development was allowed to continue in the dark for up to 15 minutes at room temperature and was stopped by adding 25% H_2SO_4. The absorbance in each well was read at 492nm on a Jbio plate reader.

7.2. Affinity Purification of Anti-4.1R-CTR

Anti 4.1R-CTR was affinity purified from nitrocellulose strips bound with 4.1R-CTR protein. Hi-Trap purified 4.1R-CTR (this Chapter, section 2.2.1) was electrophoresed on two 9% flat top polyacrylamide gels and western blotted overnight. The band corresponding to 4.1R-CTR was excised and blocked with 5% milk proteins in PBS, 0.1% Tween 20 for 1 hour at room temperature with shaking. Blocking solution was replaced with rabbit serum containing 4.1R-CTR immunoreactivity diluted 1:1 with PBS, 2mM EDTA, 0.1% Triton X-100, 1mM PMSF and 1mM azide and incubated overnight at 4°C with shaking. The serum was removed and retained and the strips were washed for 5 x 5 minutes in wash buffer 1 (0.1M sodium phosphate pH7.4, 0.5M NaCl, 0.2% Triton X-100, 1mM EDTA, 1mM azide), 3 x 5 minutes in wash buffer 2 (2M urea, 0.1M glycine, 0.1% Triton X100) and 3 x 5 minutes in wash buffer 3 (50mM sodium acetate (Fisons, Loughborough, UK), pH5). Antibody was eluted in 2ml of 0.2M glycine hydrochloride, pH2.5 by repeatedly flushing the solution over the strips whilst at a 45° angle. The solution was neutralised by the addition of 300µl of 1M Tris base and dialysed overnight against 0.1M sodium phosphate pH7.1, 1mM azide.

Antibody was stored at -80°C in aliquots and was designated *ap*4.1R-CTR. The retained serum was checked for residual 4.1R-CTR activity before being discarded.

7.3. Immunofluorescence

Immunofluorescence was carried out on rat embryonic day 17 midbrain cultures (supplied by Fiona E. McCann) using a variety of antibodies. All procedures were performed at room temperature unless otherwise stated. The culture media was removed and cultures were washed in Hank's buffered saline solution (Gibco, Paisley, UK) for 5 minutes before fixing the cells with cold methanol (100%) at -20°C for 5 minutes. The methanol was removed and cultures were rinsed in PBS for 5 minutes before permeablising cells for 30 minutes with a solution of PBS, 0.1% Tween 20 and 0.5% Triton X100. Cultures were blocked with PBS, 0.1% Tween 20, 3% BSA for 1 hour then washed for 3 x 5 minutes in PBS, 0.1% Tween 20. Primary antibodies were spun at top speed in an MSE Microcentaur microfuge (Fischer Scientific, Loughborough, UK) for 5 minutes at 4°C before diluting to the desired concentration with block solution and incubating with the cultures.

7.3.1. Single Antibody Staining

For single antibody staining cultures were incubated with primary antibody for 1 hour at room temperature. After 1 hour cultures were washed 3 x 5 minutes with PBS, 0.1% Tween. The appropriate fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) conjugated secondary antibody was spun at top speed in in an MSE Microcentaur microfuge (Fischer Scientific, Loughborough, UK) for 5 minutes at 4°C and diluted with block before incubating with the cultures for 1 hour in the dark. After washing cultures as before cell nuclei were labelled for 2 minutes with DAPI (4, 6,-diamidino–2-phenylindole, 1μg/ml made up in distilled water), and a final 5 minute wash was carried out in PBS, 0.1% Tween. Samples were mounted onto glass microscope slides (NUNC) with Mowiol 40-88 and left to set overnight at room temperature in the dark. For storage slides were kept in the dark at 4°C.

7.3.2. Double Antibody Staining

Double stained cultures were incubated with the first primary antibody overnight at 4°C. Cultures were washed 3 x 5 minutes with PBS, 0.1% Tween and incubated for 1 hour in the dark with the appropriate secondary antibody. The cultures were washed as before and re-blocked with PBS, 0.1% Tween 20, 3% BSA for 1 hour. After washing again, the second primary antibody was added to the cultures and staining proceeded according to the single antibody staining protocol in section 7.3.1 above. All steps of the second round of staining were carried out in the dark to minimise secondary antibody conjugated fluorescent dye bleaching.

Chapter 3

Cloning of 4.1R C-Terminal Region and Production of Anti-C-Terminal Region Antibodies

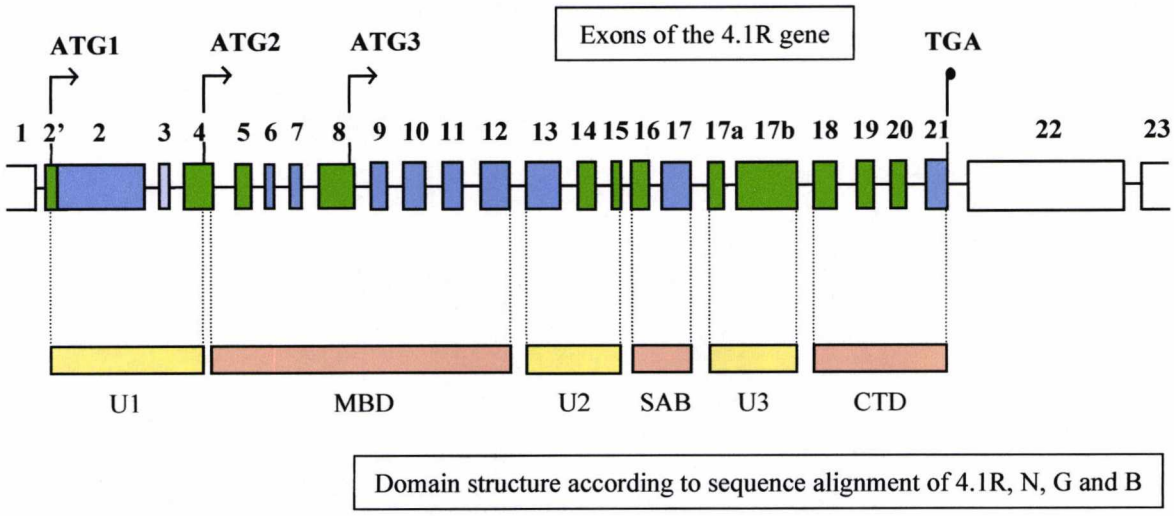
Introduction

In erythrocytes the MBD of 4.1R interacts with Band 3 (An *et al.* 1996 271 p33187) GPC (Hemming *et al.*, 1995) and p55 (Marfatia *et al.*, 1994) in a Ca^{2+} and phosphorylation dependent manner. This domain also interacts with pICIn (Tang & Tang, 1998) and CD44 (Nunomura *et al.*, 1997b) in non-erythroid cells and is relatively well characterised. Far less is known about the function and interactions of the CTD of 4.1R, partly due to its apparent inactivity in erythrocytes. In non-erythroid cells this domain becomes considerably more interesting. It is encoded by exons 18-21 (Figure 3.1) of which exons 18, 19 and 20 are alternatively spliced in a tissue and developmental specific manner (Gascard *et al.*, 1998; Huang *et al.*, 1993). The regulated expression pattern of these exons suggests a significant functional role for the CTD supported by recently identified binding activities of this region.

The CTD of 4.1R interacts with NuMA (Mattagajasingh *et al.*, 1999b), EF1α (Mattagajasingh *et al.*, 1996), eIF3–p44 (Hou *et al.*, 1999), and ZO-2 (Mattagajasingh *et al.*, 1999a) (Chapter 1, section 3.1.5.3). These interactions suggest a role for 4.1R in the nucleus of interphase cells and at the spindle during mitosis, in protein translation mechanisms and at specialised membrane domains such as tight junctions. The CTD of 4.1R therefore appears to support a diverse range of functions distinct from the MS functions of the MBD and SAB domain. The importance of 4.1R CTD interactions is highlighted by *Drosophilia* 4.1 (coracle) mutants. *Drosophila* cor^1 and cor^2 gene mutations lead to C-terminal truncated forms of coracle and result in either defects in or failure of dorsal closure and an embryonic lethal phenotype (Fehon *et al.*, 1994). Consequently it was the C-terminal region 4.1R that was chosen to investigate 4.1R binding activities in the ensuing study.

In addition to binding activities of 4.1R, the distribution of 4.1 proteins was to be examined in various subcellular fractions of brain. Protein specific antibodies were required for this and recombinant 4.1R C-terminal region (4.1R-CTR) was produced and used to generate rabbit polyclonal antibodies specific to 4.1R. Antibodies available to 4.1N, 4.1G and a peptide sequence conserved in 4.1R, G and B were characterised to

(a)



(b)

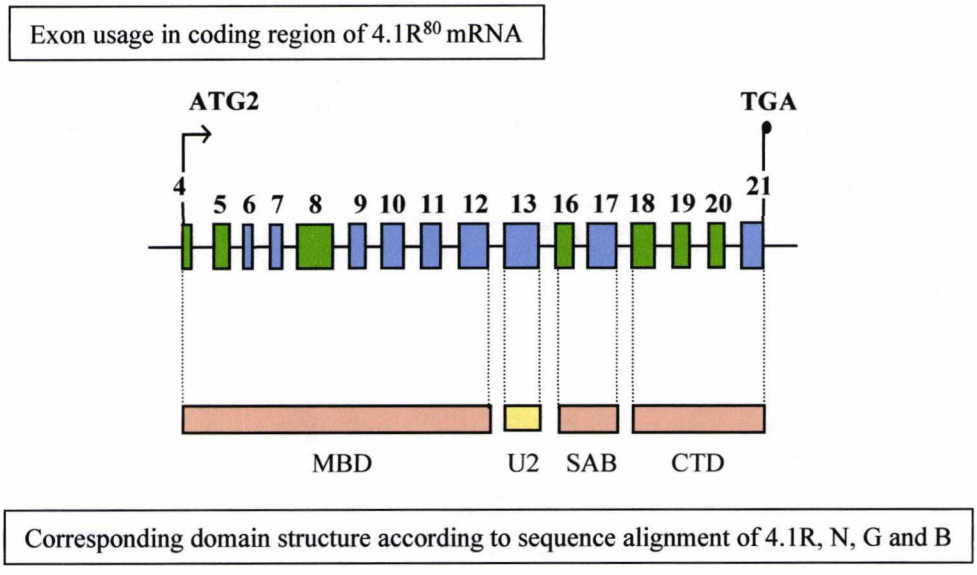


Figure 3.1 Schematic diagram showing the exon and domain structure of full length 4.1R and erythrocyte 4.1R^{80}. The exon structure of the full length 4.1R gene is shown in (a) along with the corresponding protein domain structure. The same is shown for 4.1R^{80} in (b). Constitutive coding exons are blue; alternative coding exons are green; constitutive non-coding exons are white and alternative non-coding exons are lilac. Domain structures are based on sequence alignment of 4.1R, N, G and B. Domains conserved between these gene products are orange, sequences unique to each gene product are yellow. Diagrams are not drawn to scale. MBD: membrane binding domain, SAB: spectrin-actin binding domain, CTD: C-terminal domain, U1, U2 and U3: unique regions 1, 2 and 3.

determine their reactivity and with a view to using recombinant 4.1R-CTR in future binding studies this polypeptide was characterised and assessed for biological activity. The first part of this chapter presents data relating to the cloning, expression and characterisation of 4.1R-CTR. The latter part concentrates on the production and characterisation of the 4.1 antibodies mentioned.

1. Cloning the C-Terminal Region of 4.1R

When designing a cloning strategy for the C-terminal of 4.1R it was necessary to assess the characteristics required of the final protein product. Two properties were considered particularly important; the polypeptide would need to be easily purified and obtained in relatively large amounts; also, for the polypeptide to be used in binding studies it would be necessary to demonstrate shared characteristics with native 4.1R. To overcome the first of these problems the C-terminal of 4.1R was expressed as a C-terminal hexa-his tag fusion protein from the vector pET23d (Novagen) (Figure 3.2). This tag can be used to purify polypeptides by immobilised metal chelate affinity chromatography. To overcome the second problem the SAB domain was expressed in conjunction with the CTD; the resulting recombinant polypeptide was labelled 4.1R-CTR (4.1R-C-Terminal Region). The SAB domain of 4.1R is able to promote the binding of spectrin to actin (Ohanian & Gratzer, 1984) and can be cleaved from the CTD by limited chymotrypsin digestion (Leto & Marchesi, 1984). By incorporating both domains the recombinant polypeptide could be validated by assessing these characteristics.

1.1. 4.1R-CTR Cloning Details

The C-terminal region of 4.1R was amplified by PCR. cDNA encoding resides 604-858 of mouse 4.1R omitting exons 14, 15, 17a and 17b was amplified from the plasmid construct pE15 provided by T. K. Tang (Huang *et al.*, 1993). These residues corresponded to the SAB domain and CTD of the mouse sequence provided. The product obtained by PCR was consistent with the size predicted by the Apple Macintosh program 'Amplify' (Engels, 1993) and was cloned into pET23d at *Nco I* and *Xho I* restriction sites. Four positive clones were obtained (5D, 5G, 7B and 7G) and

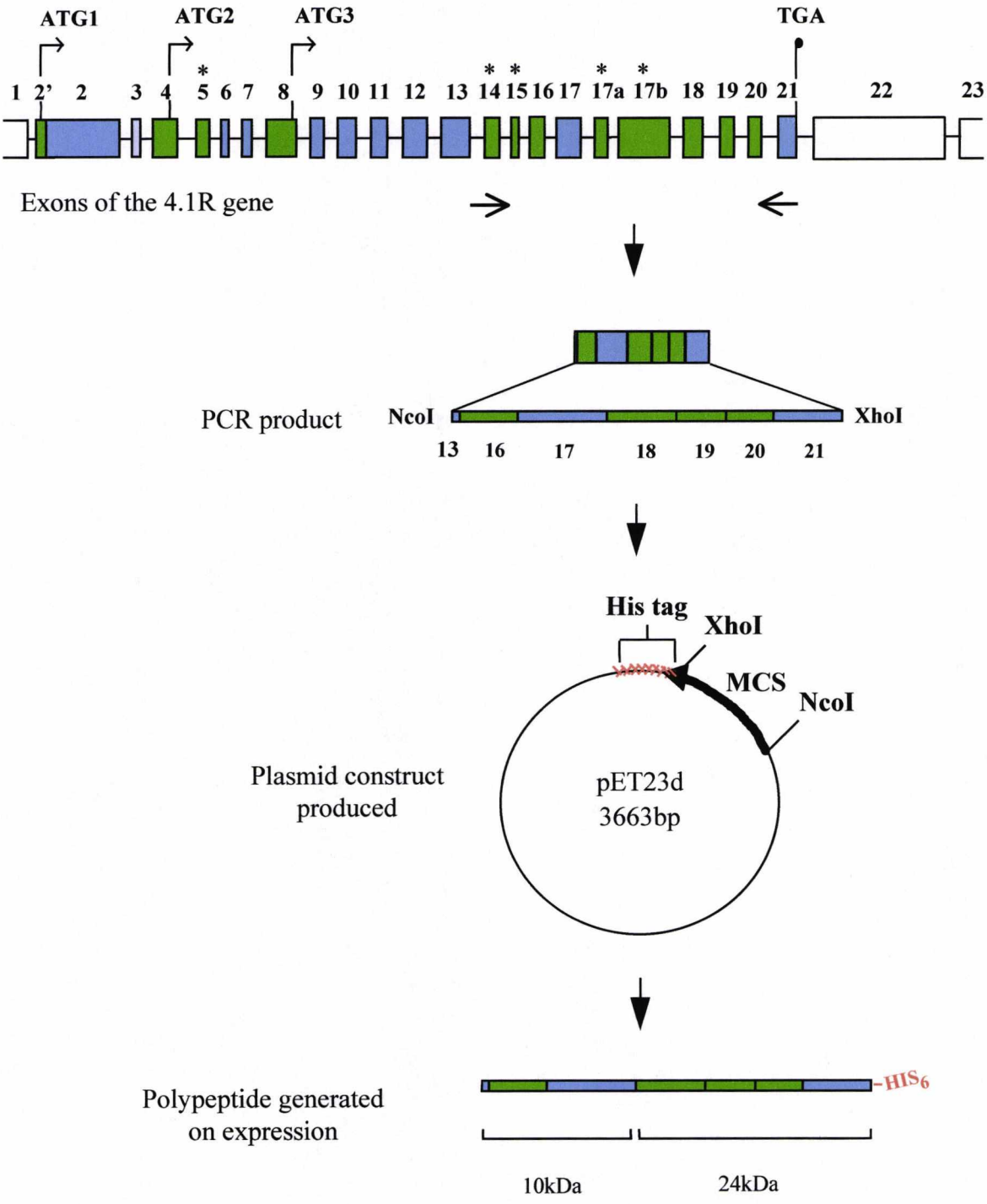


Figure 3.2 Schematic diagram showing the cloning strategy for the production and expression of 4.1R-CTR. The C-terminal (exons 18-21) and spectrin-actin binding domain (exons 16 and 17) of mouse 4.1R cDNA were amplified by PCR and cloned into pET23d at *Xho* I and *Nco* I restriction sites. The position of the primers used for amplification are shown by arrows. The 4.1R C-terminal region (4.1R-CTR) was expressed in *E. coli* BL21 (DE3) pLysS as a hexa-His tagged fusion protein to allow its purification by metal ion chelation. Exons absent from 4.1R-CTR are indicated with *. Constitutive coding exons are blue; alternative coding exons are green; constitutive non-coding exons are white and alternative non-coding exons are lilac.

restriction mapped to confirm the presence of a 4.1R insert. Plasmid constructs were sequenced (ABI) and clone 5D was shown to be free from *Taq* polymerase amplification errors. This clone was designated 4.1R-CTR*c* (C-Terminal Region construct) and was expressed to produce the polypeptide 4.1R-CTR used in all subsequent experiments involving the C-terminal of 4.1R.

2. Expression and Purification of 4.1R-CTR

pET23d is an expression vector that can be induced to synthesise protein when it is introduced into a suitable expression system e.g. *E. coli* BL21 (DE3) pLysS. Addition of IPTG to a growing culture of *E. coli* BL21 (DE3) pLysS leads to production of RNA polymerase II which then expresses genes linked to a suitable promoter. One suitable promoter is the T7 promoter that controls expression of 4.1R-CTR*c* in pET23d. 4.1R-CTR can be produced in this way. Post-induced bacteria are lysed to release 4.1R-CTR so that soluble polypeptide can be purified by virtue of its hexa-his tag.

2.1. Optimisation of 4.1R-CTR Expression Conditions

To produce 4.1R-CTR, 4.1R-CTR*c* was introduced into *E. coli* BL21 (DE3) pLysS by electroporation. Transformed bacteria were grown in culture at 37°C to an OD_{600} between 0.3 and 0.6 and induced to express protein with IPTG. Cultures were grown for a further three hours and samples were taken at hourly intervals to assess 4.1R-CTR synthesis. Harvested bacteria were lysed and the lysate cleared by centrifugation to assess the solubility of the polypeptide. Large amounts of 4.1R-CTR were produced using this method but was mainly found in inclusion bodies as shown by 4.1R-CTR enrichment in the pellet fraction of the cleared bacterial lysate (Figure 3.3). Treatment of the lysate with urea or guanidine-HCl efficiently solubilised the polypeptide, however 4.1R-CTR precipitated on their removal indicating that neither was an effective method for obtaining soluble native protein.

Modification of the expression conditions can sometimes alter recombinant protein solubility. Bacterial cultures grown at 37°C initially were transferred to ~25°C on addition of IPTG and grown at this temperature for a further four hours.

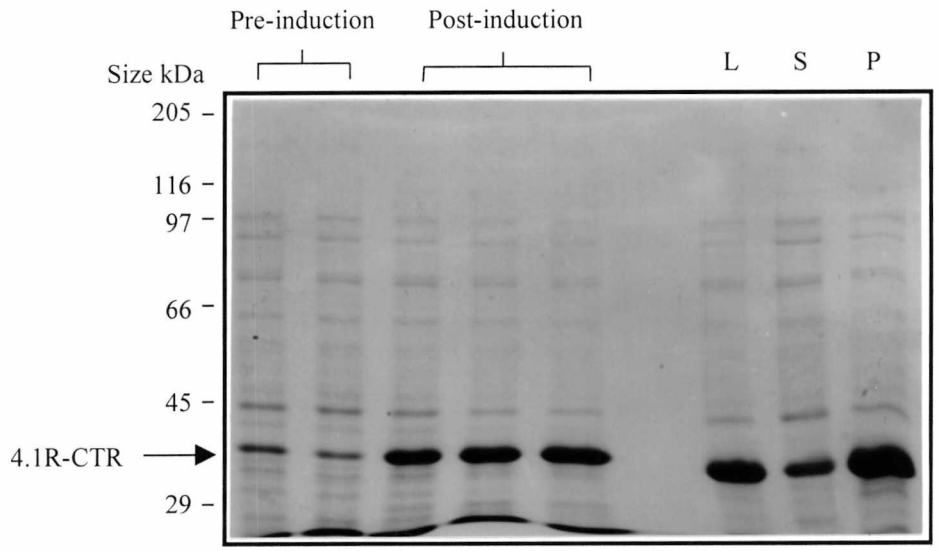


Figure 3.3 Expression of 4.1R-CTR by *E. coli* BL21 (DE3) pLysS at 37°C. Bacterial cultures were grown at 37°C and induced to express 4.1R-CTR by adding IPTG to 0.25mM. Cultures were sampled hourly and the levels of 4.1R-CTR analysed by 9% SDS-PAGE. Post-induced cultures were lysed and the lysates cleared by centrifugation to determine the proportion of soluble 4.1R-CTR. Large amounts of 4.1R-CTR were expressed by *E. coli* BL21 (DE3) pLysS but most of the polypeptide was insoluble in the pellet fraction (P) of the cleared lysate. (L) bacterial lysate, (S) cleared bacterial lysate supernatant fraction, (P) cleared bacterial lysate pellet fraction.

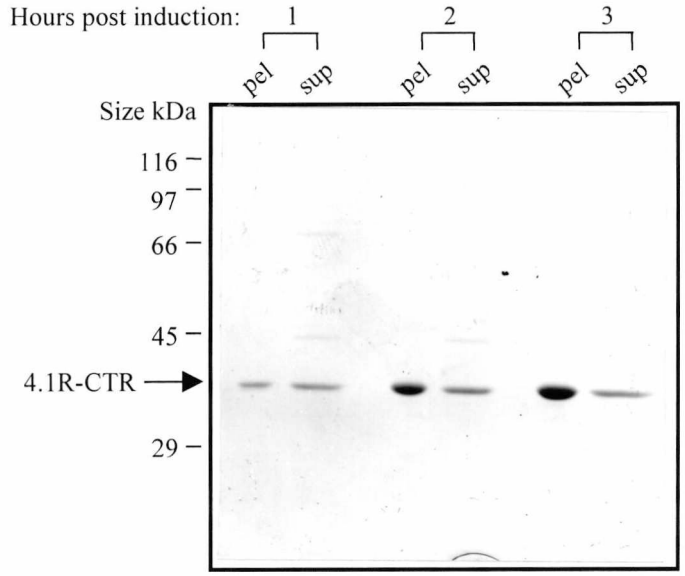
Supernatant and pellet samples were produced from lysed bacteria each hour post-induction and analysed by SDS-PAGE (Figure 3.4). Although less 4.1R-CTR was synthesised by bacteria at ~25°C compared to a parallel culture grown at 37°C, slightly higher levels of soluble 4.1R-CTR were obtained in this way. The amount of soluble 4.1R-CTR was optimum at ~25°C, two hours post-induction. These parameters were used to produce 4.1R-CTR for purification.

2.2. Optimisation of His-trap™ and Ni^{2+}-NTA Purification Procedures

Immobilised metal ion affinity chromatography can be used to purify recombinant proteins expressing his-tags. Histidine residues chelate divalent cations such as Ni^{2+}. Proteins containing multiple histidines can therefore be separated from a complex mixture of proteins by passing the mixture over a column on which nickel ions have been immobilised. Ni^{2+} is immobilised by chelation and a matrix conjugated to either IDA or NTA will retain Nickel ions in this way. The principal behind the NTA and IDA systems is the same but IDA chelates Ni^{2+} at three co-ordination sites while NTA chelates it at four. His-tagged protein is bound by the remaining available Ni^{2+} co-ordination sites and is competed off the column with imidazole.

Both of the immobilised metal ion affinity chromatography systems (IDA and NTA) were used during the course of this work, producing 4.1R-CTR of similarity electrophoretic purity (Figures 3.5 and 3.6). With the His-Trap (IDA) column (Amersham-Pharmacia) optimal purification was obtained by washing with 10ml of buffer containing 10mM imidazole, and eluting consecutively with buffers containing 20mM, 40mM, 60mM, 100mM, 300mM and 500mM imidazole. 4.1R-CTR was concentrated mainly in fractions eluted with 300mM and 500mM imidazole (Figure 3.5b and c). Purification using the Ni-NTA system (Quiagen) used buffers containing 20mM, 50mM, 100mM and 250mM of imidazole. In this case, 4.1R-CTR eluted with greatest purity with 100mM and 250mM imidazole buffers (Figure 3.6). An immunoblot on 4.1R-CTR with a penta-His antibody (Sigma) confirmed the expression of an inframe his-tag prior to purification (Figure 3.7).

(a)



(b)

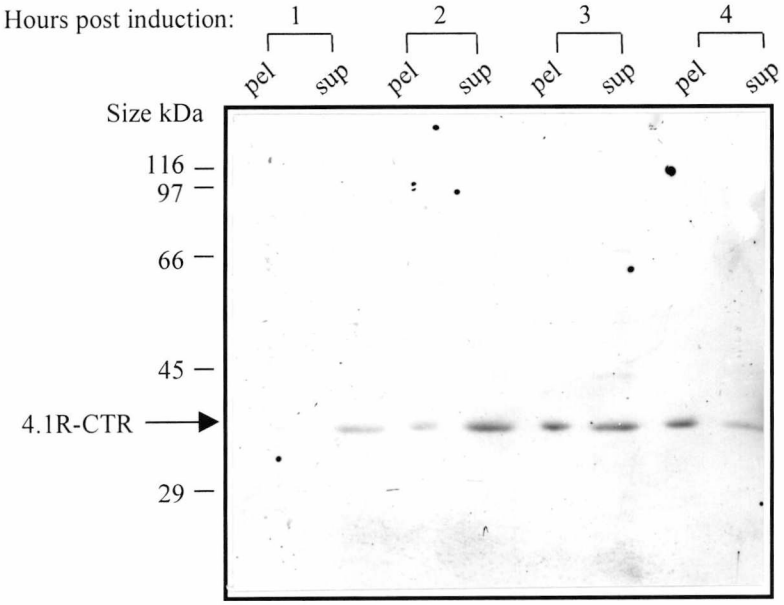


Figure 3.4 Comparison of soluble vs. insoluble 4.1R-CTR expressed by *E. coli* BL21 (DE3) pLysS under different temperature conditions. Samples were taken each hour post-induction, the bacteria lysed and the lysate cleared. Pellet and supernatant fractions were run on 9% SDS-PAGE to compare the relative levels of soluble 4.1R-CTR at each stage of expression. Expression was carried out at (a) 37°C and (b) ~25°C.

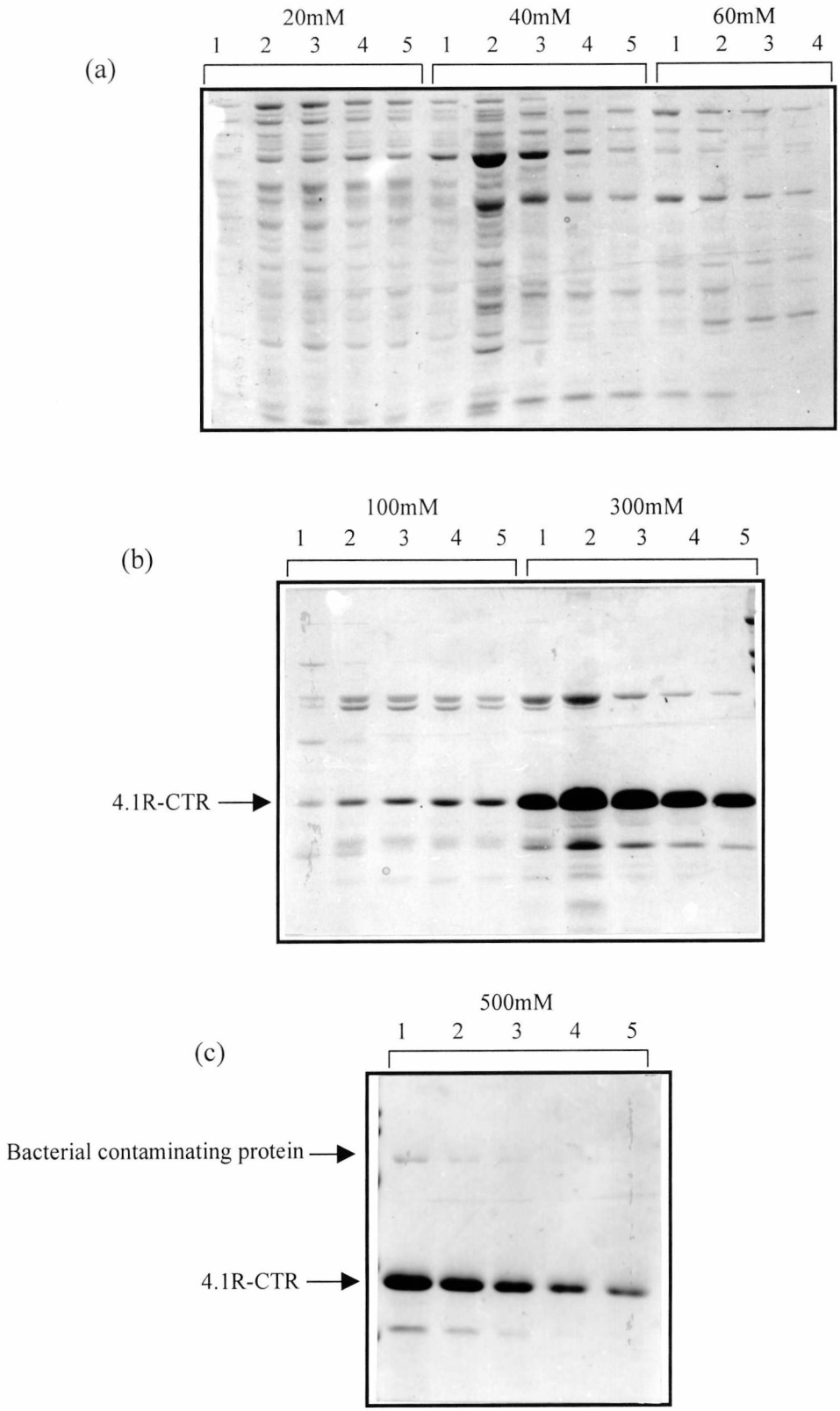


Figure 3.5 His-Trap (IDA) purification of 4.1R-CTR. Cleared bacterial lysate was applied to a nickel activated IDA column and washed successively with buffer containing increasing concentrations of imidazole. Recombinant his-tagged 4.1R-CTR eluted with 300mM and 500mM imidazole. Panel (a) 20mM, 40mM and 60mM imidazole washes, panel (b) 100mM and 300mM washes, panel (c) 500mM wash.

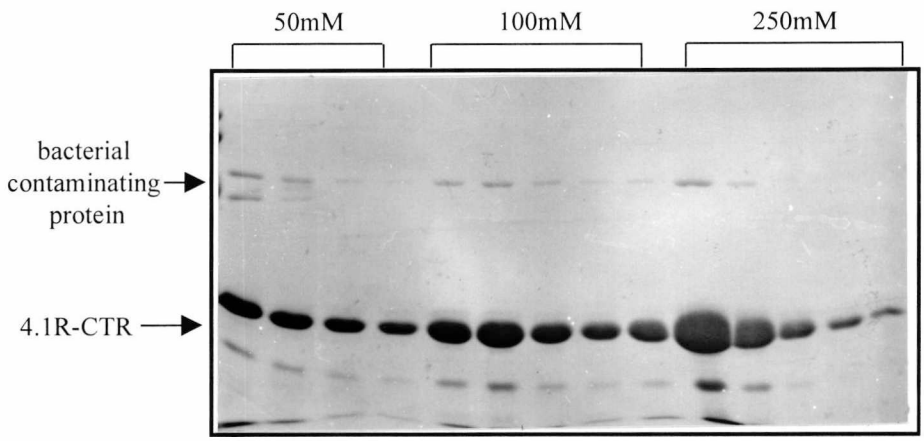


Figure 3.6 Ni^{2+}-NTA purification of 4.1R-CTR. Cleared bacterial lysate was mixed with nickel activated NTA-agarose beads and washed successively with buffer containing increasing concentrations of imidazole. Recombinant His-tagged 4.1R-CTR eluted with 100mM and 250mM imidazole.

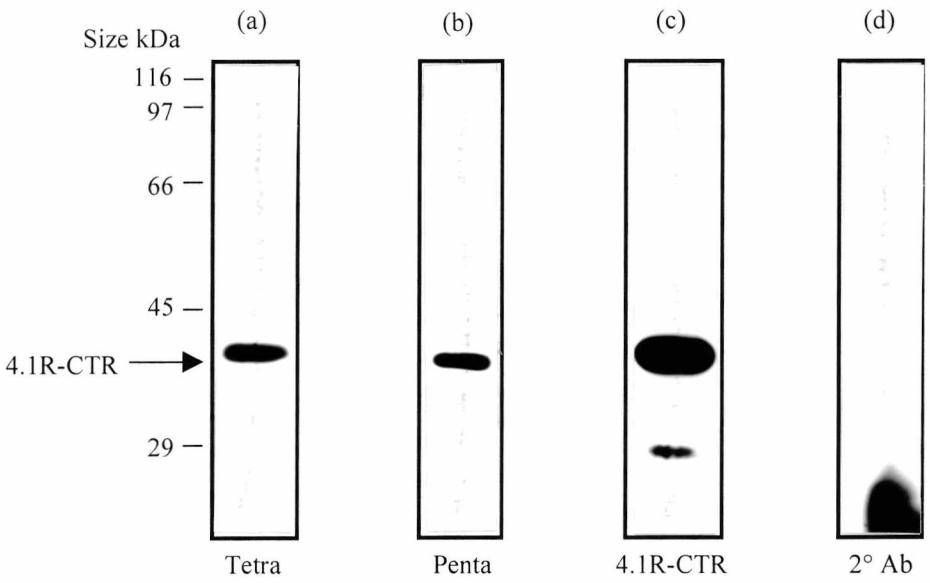


Figure 3.7 Immunoblot on 4.1R-CTR with a His-tag antibody. Recombinant 4.1R-CTR was immunoblotted with (a) tetra- and (b) penta-His antibodies to confirm the expression of an in-frame hexa-His tag. Panel (c) is an equivalent blot with an affinity purified anti-4.1R-CTR antibody to confirm the nature of the band. Panel (d) shows the mouse secondary antibody only control that accompanies panels (a) and (b).

2.3. Analysis of a ~75kDa Contaminating Protein Co-purifying with 4.1R-CTR

A protein of ~75KDa co-purified with 4.1R-CTR regardless of the purification used. Due to its size, it was thought that this contaminating protein might be DnaK (LaRossa & Van Dyk, 1991). Recombinant proteins produced in a bacterial system are at times seen by the bacterium as foreign. In *E. coli* the presence of foreign protein induces expression of the *DnaK* gene. DnaK is a 70kDa protein involved in degradation of foreign proteins to prevent them doing harm. Purification of recombinant protein in the presence of 2mM ATP and 10mM $MgSO_4$ has been shown to facilitate the dissociation of DnaK from the foreign protein allowing uncontaminated recombinant protein to be obtained (Trouble Shooting Guide, GST Gene Fusion System, Pharmacia Biotech, 3rd Edition, Revision 1). An attempt to remove the 75kDa protein co-purifying with 4.1R-CTR using ATP and $MgSO_4$ was unsuccessful (data not shown).

Dimerisation of 4.1R-CTR would also give a complex of ~75kDa by SDS-PAGE. Thus the contaminating protein could be dimer of 4.1R-CTR rather than bacterial in origin. To determine the nature of the 75kDa protein *E. coli* BL21 (DE3) pLysS lacking 4.1R-CTR*c* were immunoblotted with an antibody able to detect both 4.1R-CTR and the contaminating protein (anti-*ap*4.1R-CTR; this chapter, section 4). A ~75kDa immunoreactive protein in untransformed *E. coli* BL21 (DE3) pLysS indicates that the co-purifying protein is bacterial in origin (Figure 3.8). In support of this, tetra- and penta-His antibodies did not react with the 75kDa protein confirming that it does not derive from recombinant 4.1R-CTR (Figure 3.7a and b).

3. Characterisation of Protein 4.1R-CTR

Any recombinant protein used as a tool in scientific investigation must be shown to mimic known activities of its native counterpart. To characterise 4.1R-CTR three properties of the polypeptide were assessed (1) molecular mass; (2) the efficacy at which 4.1R-CTR promotes the binding of spectrin to actin; (3) the domain structure of the polypeptide produced by limited chymotrypsin digestion.

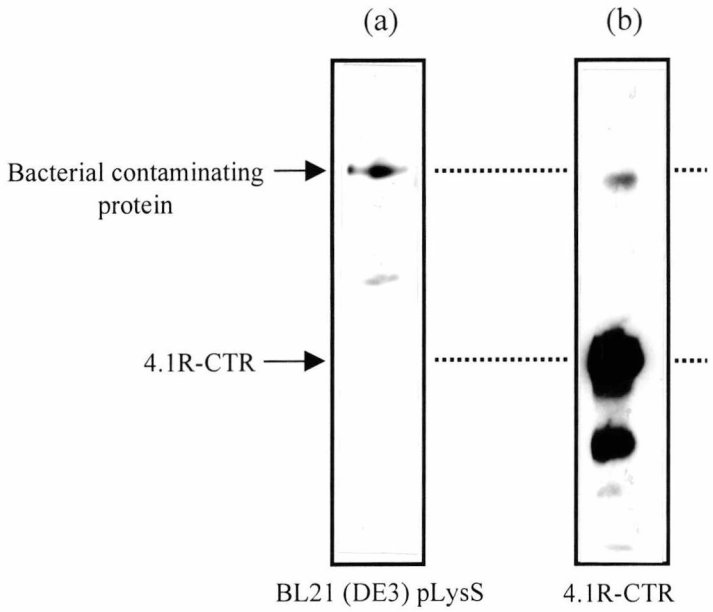


Figure 3.8 Immunoblot *E. coli* BL21 (DE3) pLysS and 4.1R-CTR with an affinity purified anti-4.1R-CTR antibody. (a) Untransformed *E. coli* BL21 (DE3) pLysS, (b) Metal affinity chromatography purified 4.1R-CTR. Reactivity of a ~ 75kDa band in untransformed bacteria suggests that the contaminant co-purifying with 4.1R-CTR is bacterial in origin and not dimeric 4.1R-CTR.

3.1. 4.1R-CTR Mass Determination

The molecular mass and relative molecular mass of 4.1R-CTR were determined and compared with the theoretical mass of this portion of 4.1R. Electrospray mass spectrometry of 4.1R-CTR gave a single peak corresponding to a molecular mass of 26068 daltons (Figure 3.9). This correlates well with the theoretical molecular mass of 4.1R-CTR (26071 daltons) showing a difference of only 3 daltons. 4.1R-CTR had a relative molecular mass of ~37kDa as determined by SDS-PAGE. This is higher than the molecular mass of the polypeptide but correlates well with the sum of the relative molecular masses of the SAB domain (10kDa) and CTD (24kDa) that have previously been determined by SDS-PAGE.

3.2. Ternary Complex Formation

The SAB domain of 4.1R alone is able to promote the binding of spectin to actin as efficiently as full length 4.1R^{80} (Correas *et al.*, 1986). 4.1R-CTR should therefore be able to form a ternary complex with spectrin and actin similar to complexes seen in the MS of erythrocytes. The effectiveness of 4.1R-CTR at promoting complex formation was used as an assay to demonstrate 4.1R-CTR biological activity.

3.2.1. Preparation of human erythrocyte spectrin

Human erythrocyte spectrin dimer was purified from out of date blood bank packed red cells using the method outlined by Gratzer (1982). Following extraction of spectrin dimer from erythrocyte ghosts gel filtration was necessary to separate spectrin from accompanying actin and 4.1R (Figure 3.10a). Spectrin-actin-4.1R^{80} complexes eluted in the void volume of the column (~140-175ml, Figure 3.10b fractions 2-8). Purified spectrin dimer eluted after spectrin-actin-4.1R^{80} complexes and was concentrated across approximately four fractions (Figure 3.10b fractions 13-16) corresponding to 0.42-0.47 column volumes of buffer (~180ml – 200ml). Fractions 13-16 in Figure 3.10b were pooled and concentrated to ~120μg/ml.

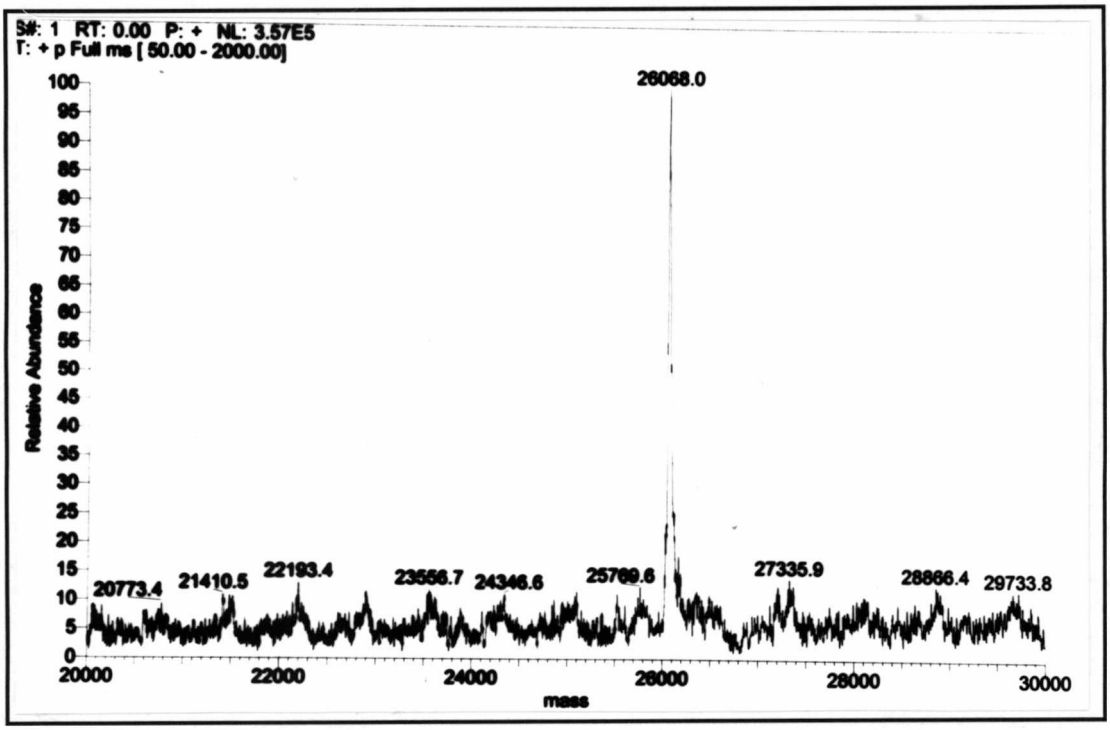
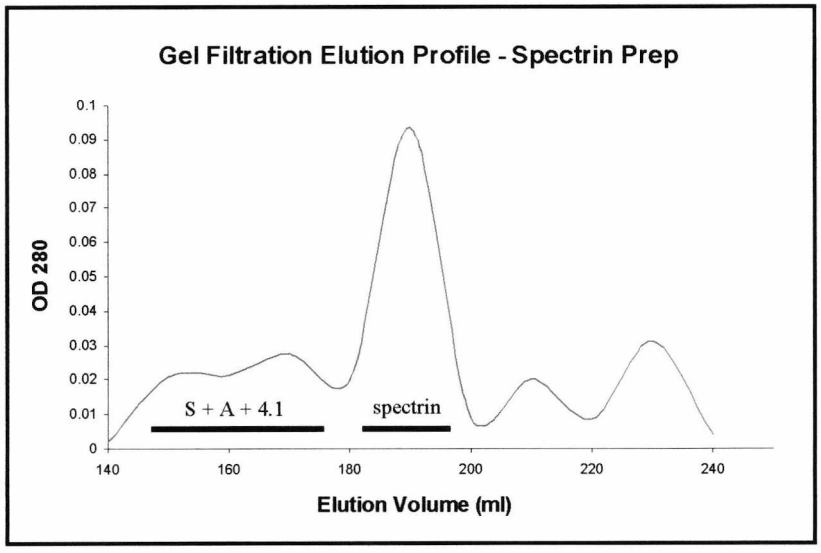


Figure 3.9 Mass spectrum of purified 4.1R-CTR. The single peak corresponds to 4.1R-CTR. The molecular mass of the recombinant polypeptide was determined as 26068 daltons.

(a)



(b)

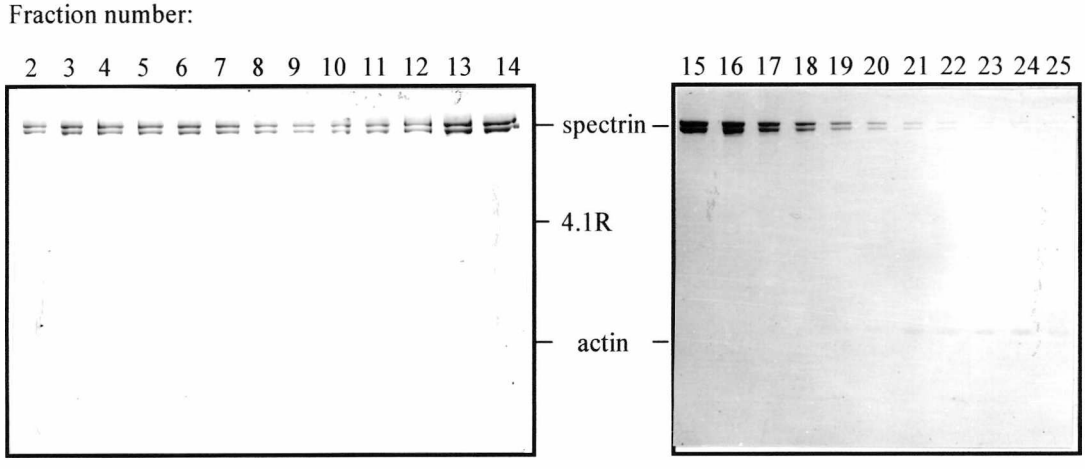


Figure 3.10 Preparation of spectrin dimer from human erythrocytes. After extraction from human ghosts spectrin, actin and protein 4.1R were separated by gel filtration chromatography. (a) S500 gel filtration profile for the elution of spectrin-actin-4.1R complexes (S + A + 4.1R) and spectrin dimer. 5ml fractions were collected. Collection commenced after 135ml of buffer had passed through the column. Fractions collected from gel filtration were analysed by 7.5% SDS-PAGE to assess spectrin purity (b). Fraction 13-16 and 17-18 were pooled and concentrated as described in Chapter 2, section 4.1.

3.2.2. Preparation of rabbit skeletal muscle actin

Rabbit skeletal muscle contains a high concentration of actin making it an ideal tissue for the preparation of this protein. Tropomyosin was dissociated from actin filaments with 0.6M KCl and G-actin was produced by dialysis into low ionic strength buffer. Actin produced in this way was obtained at a concentration of 1.1mg/ml and was electrophoretically pure (Figure 3.11). Removal of trace impurities by gel filtration, such as myosin and α-actinin, was not considered necessary for the purposes of ternary complex formation.

3.2.3. Ternary complex formation

Human erythrocyte spectrin, rabbit skeletal muscle G-actin and recombinant 4.1R-CTR were used to form ternary complexes. Seven reactions were set up containing 4.1R-CTR only, spectrin only, G-actin only, 4.1R-CTR with G-actin, 4.1R-CTR with spectrin, spectrin with G-actin and 4.1R-CTR with spectrin and G-actin. Reactions were incubated for 90 minutes at room temperature to allow protein interactions to occur. At the end of this period actin and associated proteins were pelleted by high speed centrifugation. Protein not bound to actin remained in the supernatant. Supernatant and pellet fractions were analysed by SDS-PAGE (Figure 3.12)

Actin pelleted in all reactions indicating that the incubation time was sufficient to allow polymerisation. The weak interaction between spectrin and actin alone was significantly enhanced by the addition of 4.1R-CTR as indicated by the relative amounts of spectrin pelleted in these reactions. Some 4.1R-CTR was pelleted in the absence of any other protein, possibly due to non-specific aggregation of the polypeptide as has been experienced previously with native 4.1R[80] (Ohanian & Gratzer, 1984; Ohanian *et al.*, 1984). The amount of non-specifically pelleted 4.1R-CTR was less in the presence of spectrin suggesting that specific 4.1R-CTR-spectrin interactions are favoured over the aggregated state.

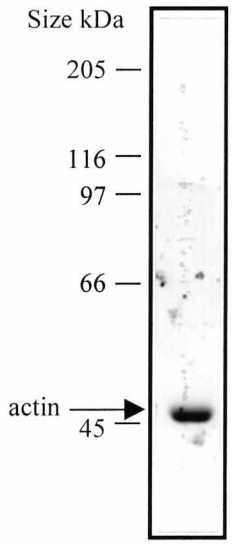


Figure 3.11 Preparation of rabbit skeletal muscle actin. Actin was purified using a method adapted by A. Lodge (University of Kent) (Chapter 2, section 4.2). Tropomyosin depleted G-actin was run analysed by 9% SDS-PAGE to assess electrophoretic purity.

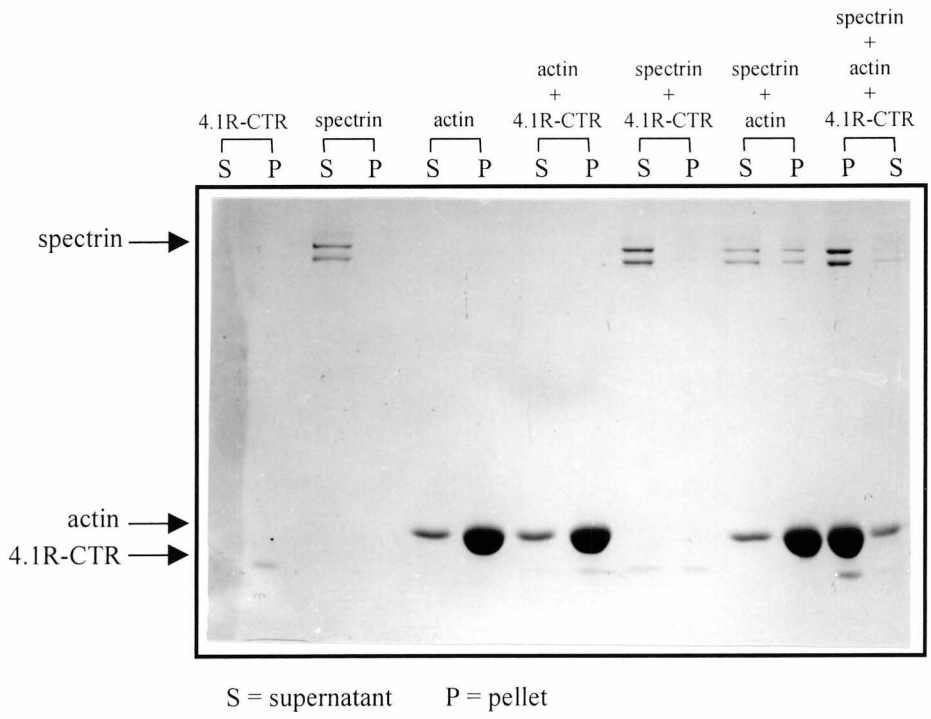


Figure 3.12 Ternary complex formation by spectrin, actin and 4.1R-CTR. Human erythrocyte spectrin dimer (40μg/ml), rabbit skeletal muscle G-actin (0.35mg/ml) and recombinant 4.1R-CTR (30μg/ml) were incubated individually, in pairs and together under conditions that promote actin polymerisation and ternary complex formation. The increased amount of spectrin pelleted in the spectrin + actin + 4.1R-CTR reaction compared to the reaction with spectrin + actin only clearly demonstrates the ternary complex promoting activity of 4.1R-CTR.

3.3. Chymotrypsin Digestion of 4.1R-CTR

Limited α-chymotrypsin digestion of $4.1R^{80}$ cleaves the protein into four domains: MBD, 16kDa domain, SAB domain and CTD (Leto & Marchesi, 1984). In the same way, treatment of 4.1R-CTR with α-chymotrypsin should cleave the SAB domain from the CTD if both recombinant and native proteins share similar structural characteristics. 4.1R-CTR was incubated with TLCK treated α-chymotrypsin for 90 minutes at 4°C and the reaction was quenched with the inhibitor TPCK at 30 minute time intervals. Digestion products were analysed by SDS-PAGE (Figure 3.13a) and immunoblotted with anti-4.1R-CTR (Figure 3.13b) and anti-4.1R/G/B (Figure 3.13c) antibodies. Anti-4.1R/G/B was raised against a 14 amino acid peptide at the very C-terminal of 4.1R while anti-4.1R-CTR was generated using the recombinant polypeptide (this Chapter, section 4 and 5). These antibodies were used to help distinguish the various 4.1R-CTR digestion products.

A 24kDa digestion product observed after 30 minutes was derived from the C-terminal of 4.1R-CTR as inferred from its immunoreactivity with anti-4.1R/G/B. The size of this product suggests that it corresponds to the whole $4.1R^{80}$ CTD. A 15kDa digestion product was also detected by anit-4.1R/G/B implying that the CTD undergoes further cleavage. This product appears to be protease resistant. A ~6kDa band was found to react with anti-4.1R-CTR but not anti-4.1R/G/B suggesting that it derives from the N-terminal of 4.1R-CTR and corresponds to part of the SAB domain. It is interesting to note that the 15kDa product does not react with anti-4.1R-CTR suggesting that the epitopes for this antibody lie N-terminal to the cleavage site for this product (i.e. towards the SAB domain). Detection of 24kDa and ~6kDa products in the 4.1R-CTR digestion mixture indicates that the recombinant polypeptide is folded correctly and behaves like native $4.1R^{80}$.

Additional studies in collaboration with A. J. Baines and J. Hardy (University of Kent) were carried out to determine the nature of the 15kDa α-chymotryptic polypeptide. The results of these studies demonstrated that the 15kDa fragment persists after digestion with α-chymotrypsin overnight at 4°C and runs as a globular monomer in gel filtration.

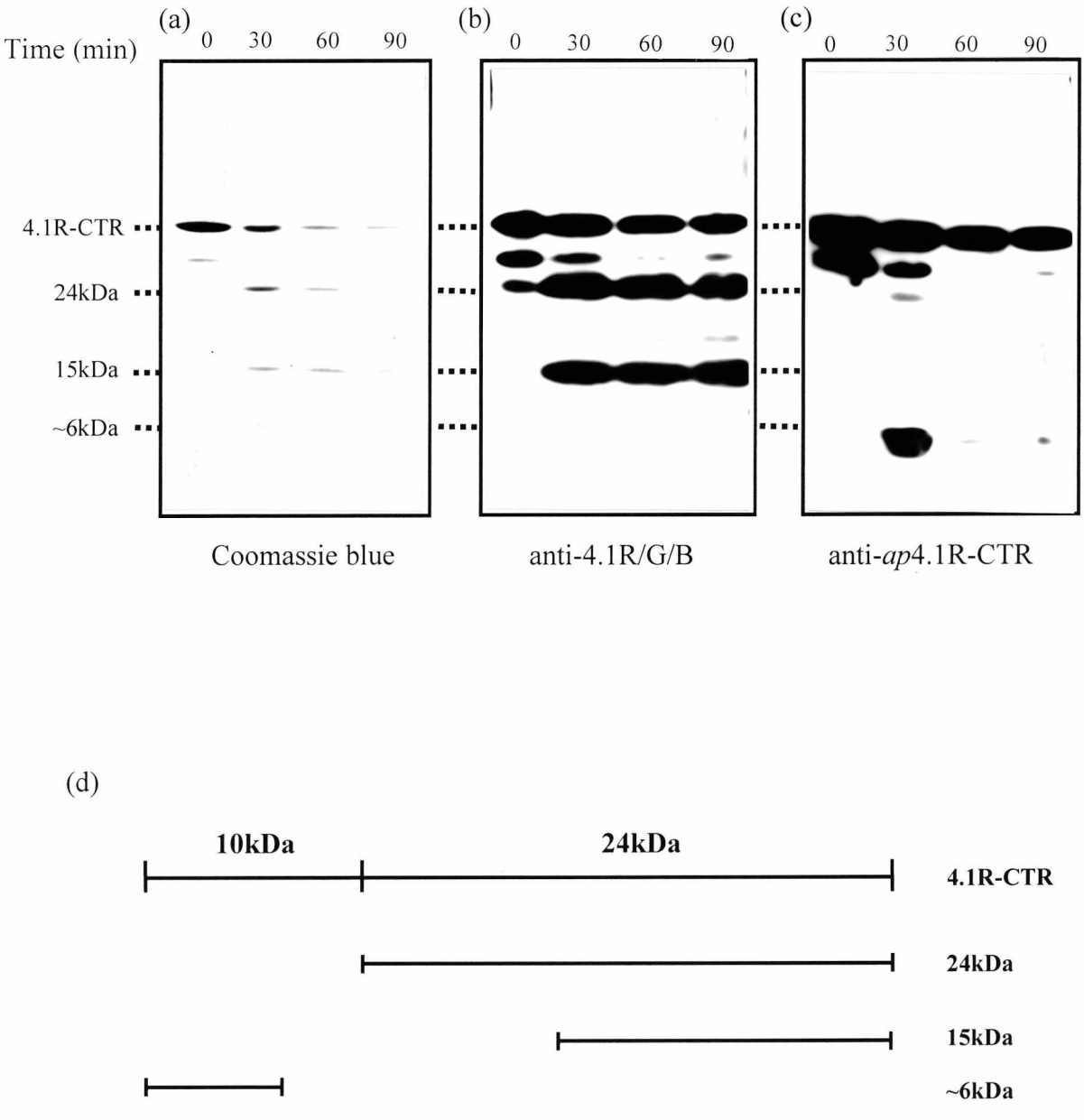


Figure 3.13 Limited α chymotrypsin digestion of 4.1R-CTR. Recombinant 4.1R-CTR was digested with α chymotrypin for 0, 30, 60 and 90 minutes at 4°C before stopping each reaction with TPCK. Digestion products were analysed by10-20% gradient SDS-PAGE and Coomassie blue staining (a) or immunoblotting with anti-4.1R/G/B (b) or anti-*ap*4.1R-CTR (c). The schematic representation in (d) indicates the possible origin of various digestion products.

Figure 3.14 Origin of the 15kDa fragment produced by chymotrypsin cleavage of 4.1R-CTR. The molecular mass of the17199.3 dalton fragment produced by chymotyrpsin digestion of 4.1R-CTR correlates well with a 17199 dalton polypeptide (underlined) that would be produced by cleavage at the chymotryptic site indicated by red arrows.

Amino acid sequence of 4.1R-CTR

TMEPTEAWKKKRERLDGENIYIRHSNLMLEDLDKSQEEIKKHHASISELKKNF MESVPEPRPSEW

17199 dalton fragment

DKRLSTHSPF RTLNINGQVPTGDGPPLVKTQTVTISDTANAVKSEIPTKDVPIVHTETKTITYEAAQ

TEDSNGDLDPGVLLTAQTITSETTSSTTTTQITKTVKGGISETRIEKRIVITGDADIDHDQVLVQAIK

EAKEQHPDMSVTKVVVHQETEISEELEHHHHHH

The molecular mass of the polypeptide (17199.3 daltons) was determined by electrospray mass spectrometry and correlates well with a fragment in the 4.1R-CTR sequence (Figure 3.14). This fragment has a theoretical mass of 17199 daltons and begins at a potential α-chymotrypsin cleavage site (PF-RTLN). The results of this analysis suggest that the 17kDa 4.1R-CTR digestion fragment forms a compact globular domain. In support of this, in the full length 4.1R sequence, the start of the 17kDa digestion fragment is located just ahead of the region defined as the CTD by 4.1 protein sequence homology (Parra *et al.*, 2000).

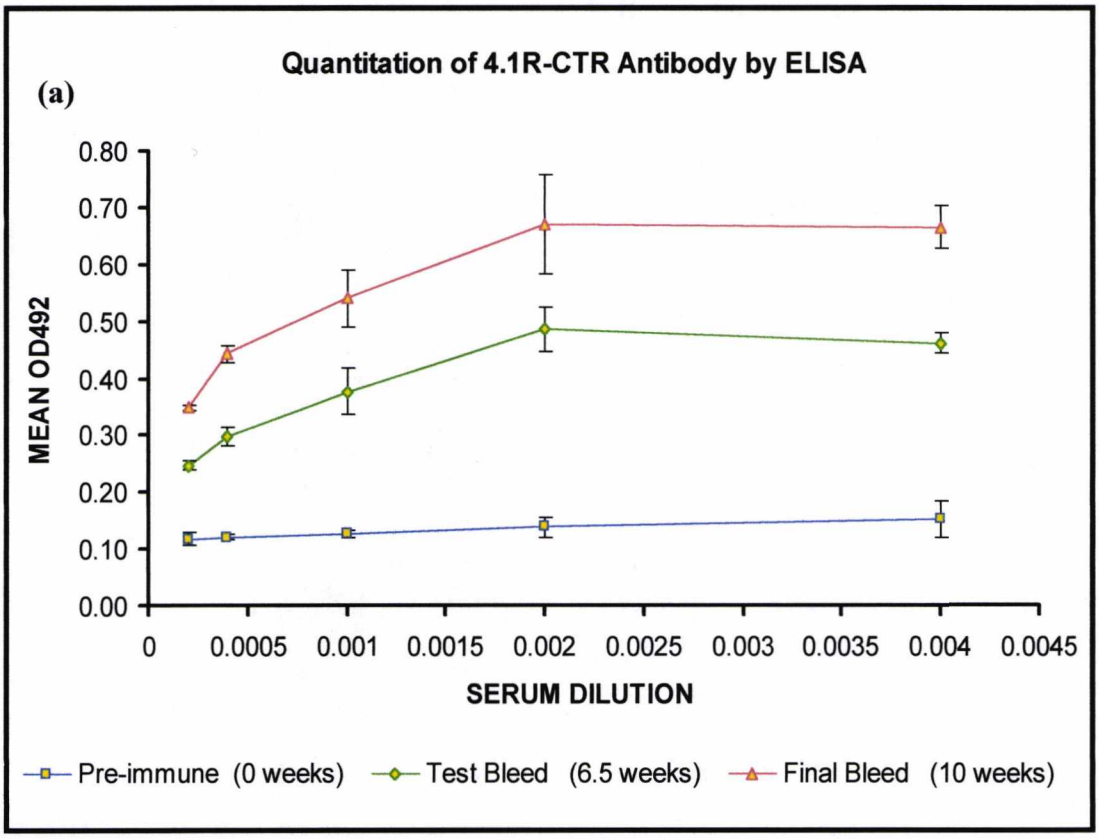
4. Production and Characterisation of an Antibody to Recombinant 4.1R C-Terminal Region

Antibodies are an important tool in cell biology and if characterised carefully can be used to detect specific protein species by a variety of techniques. To aid the detection of 4.1 proteins in brain a polyclonal antibody was raised against recombinant 4.1R-CTR. This antibody was affinity purified and its reactivity assessed against various 4.1 species.

4.1. Immunisation Regime

Polyclonal antibodies are produced by introducing an antigen such as 4.1R-CTR into animal models. Purified 4.1R-CTR was dialysed into buffer containing 10mM HEPES pH7.4 and 10mM NaCl and injected into two rabbits and two rats to induce an immune response. Pre-immune bleeds were taken from the rabbits prior to injection and 0.1mg and 0.01mg of antigen were injected in to rabbits and rats respectively. Injections were repeated approximately every two weeks and continued for a total of 9 weeks. A test bleed was taken 2½ weeks after the third injection and animals were bled out 10 days after the fifth injection. The sera from injected rabbits and rats was tested for 4.1R immunoreactivity by ELISA and immunoblotting (Figures 3.15 and 3.16).

Rabbits were not significantly immune to 4.1R prior to the injection of antigen but contained a high titre of antibody at both the test bleed and final bleed stages. Rat and



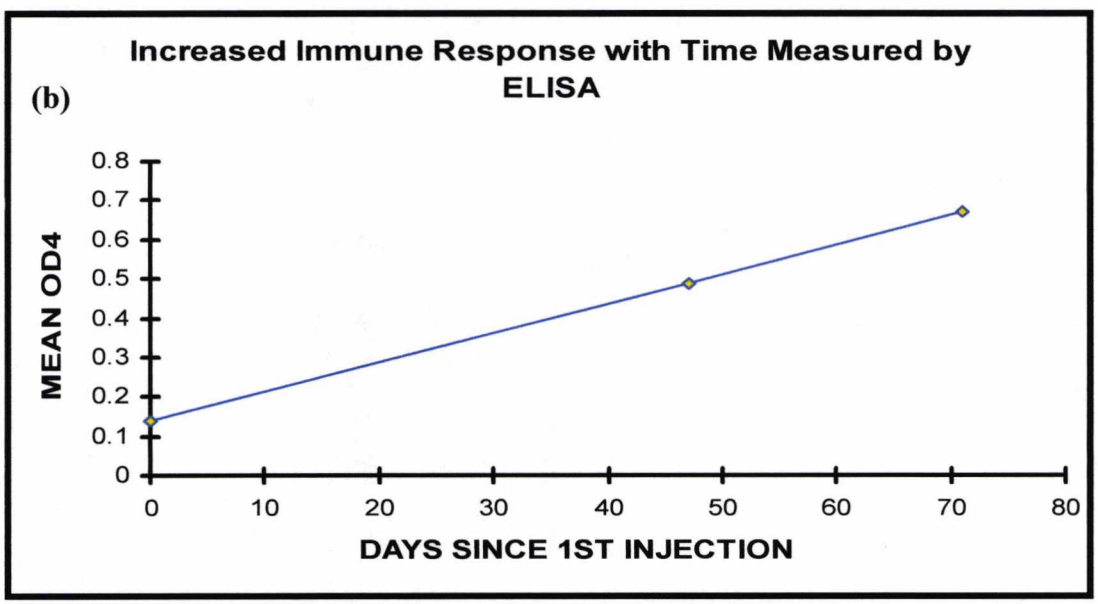


Figure 3.15 ELISA reaction demonstrating rabbit immunity during the production of anti-4.1R-CTR antibody. (a) The levels of 4.1R-CTR antibody in pre-immune, first test bleed and final bleed rabbit serum samples were quantified by ELISA. Antibody-antigen interactions were measured at 1/250 (0.004), 1/500 (0.022), 1/1000 (0.001), 1/2500 (0.0004), 1/5000 (0.0002) dilutions of the serum samples. The OD492 was measured for each sample as an estimate of the extent of antibody-antigen binding and hence amount of antibody present. Binding was shown to be saturable with both test bleed and final bleed serum. (b) A plot of mean OD492 data against time for the 1/1000 dilution of pre-immune, test bleed and final bleed serum to show how the rabbit immune response increases over time.

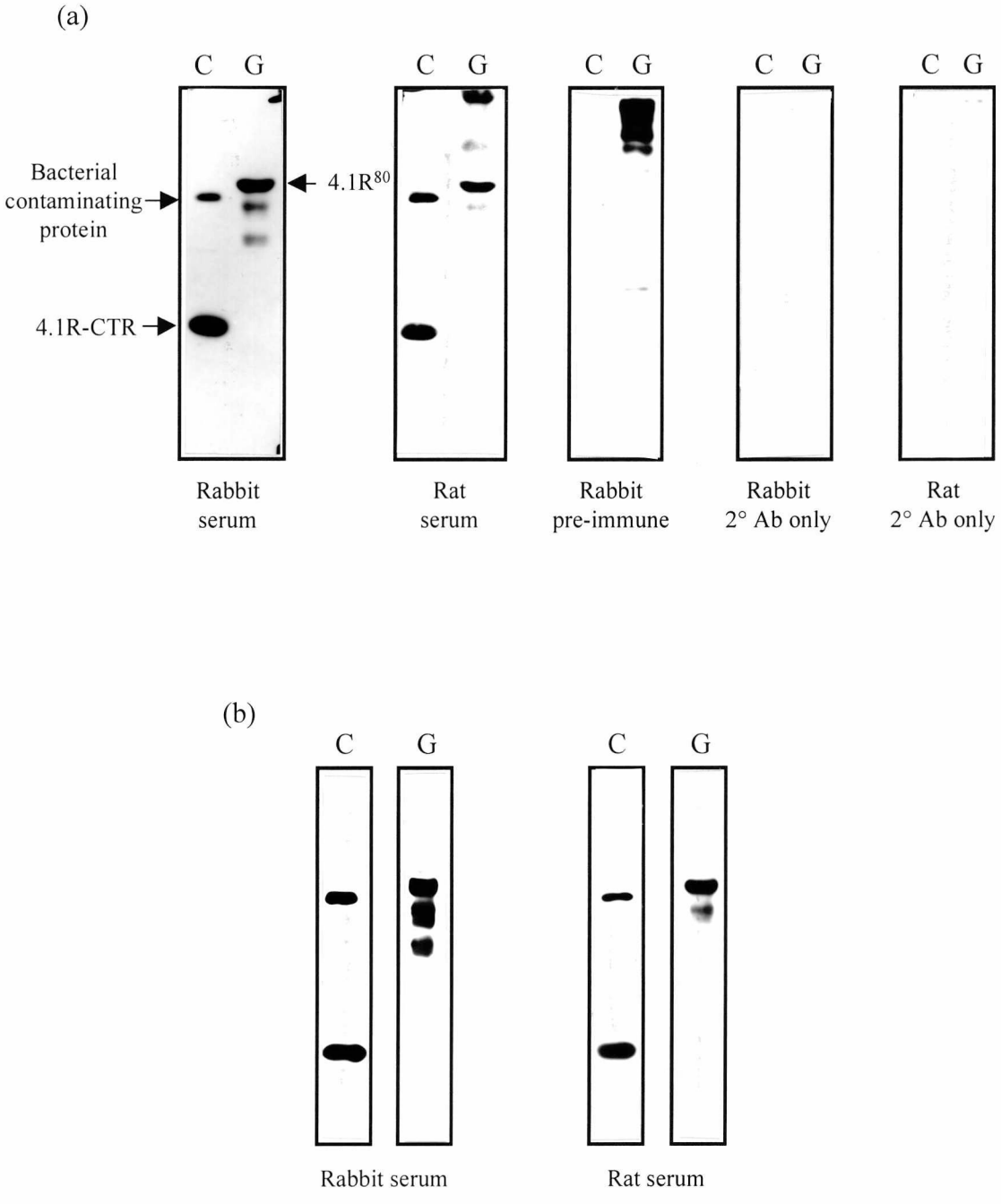


Figure 3.16 Production of anti 4.1R-CTR antibody. Purified 4.1R-CTR was used as an antigen in the production 4.1R-CTR antibodies and along with erythrocyte ghosts in immunoblotting to test serum reactivity. (a) Test bleed serum taken after $2\frac{1}{2}$ weeks, (b) final bleed serum obtained after 10 weeks. (G) erythrocyte ghosts, (C) 4.1R-CTR.

rabbit sera were both immunoreactive against 4.1R-CTR and erythrocyte $4.1R^{80}$ on immunoblots but cross-reactivity was also observed with a ~75kDa protein in 4.1R-CTR samples. This protein corresponds to the *E. coli* contaminating protein that co-purifies with 4.1R-CTR (this Chapter, section 2.3).

4.2. Affinity Purification and Characterisation of Anti-4.1R-CTR

Because rabbit and rat sera were contaminated with antibodies to the bacterial protein, antibodies to 4.1R-CTR needed to be affinity purified out of this mixture. This was achieved by incubating final bleed serum with antigen bound to nitrocellulose strips. Bound antibody was eluted with a buffer of low pH and was designated anti-*ap*4.1R-CTR. To characterise the immunoreactivity of this antibody immunoblots were carried out on 4.1R-CTR, erythrocyte ghosts and antigens used to produce anti-4.1G and anti-4.1N antibodies (Figure 3.17b). Anti-*ap*4.1R-CTR reactivity was directed specifically against 4.1R-CTR and $4.1R^{80}$ and no cross-reactivity was observed with recombinant 4.1G or 4.1N. Over exposed blots indicated that anti-*ap*4.1R-CTR retained reactivity against the ~75kDa bacterial protein (not shown). In contrast, antibodies purified against the bacterial protein reacted specifically against a ~75kDa product and showed no cross-reactivity with 4.1R-CTR (Figure 3.17c). This antibody was used as a control in forthcoming experiments to affirm results obtained with anti -*ap*4.1R-CTR.

5. Characterisation of Other 4.1 Antibodies

Anti-*ap*4.1R-CTR was specific for products of the 4.1R gene. To assess the full complement of 4.1 proteins in subcellular fractions of brain anti-*ap*4.1R-CTR was to be used in conjunction with antibodies raised against 4.1N, 4.1G and a 14 amino acid peptide conserved in 4.1R, 4.1B and 4.1G. It was necessary to characterise each of these antibodies in a similar way to anti-*ap*4.1R-CTR to confirm their immunoreactivity.

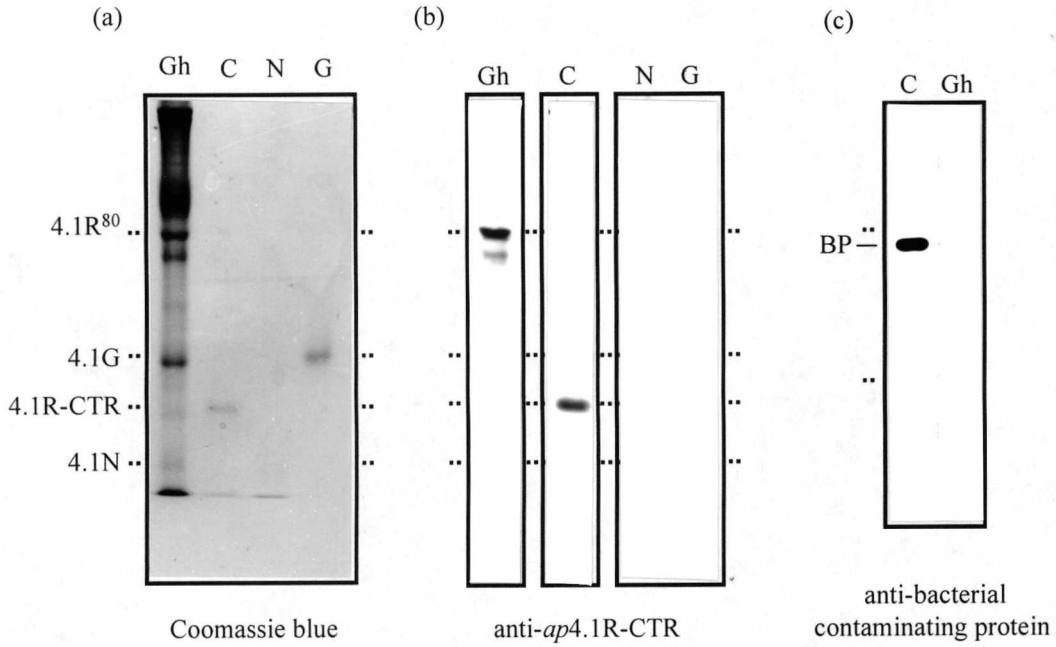


Figure 3.17 Characterisation of anti-*ap*4.1R-CTR. The reactivity of anti-*ap*4.1R-CTR was tested on erythrocyte ghosts, 4.1R-CTR and 4.1N and 4.1G recombinant antigens. Only 4.1R species were detected by the antibody and no cross reactivity occurred with 4.1N or G polypeptides. (a) Coomassie blue stain equivalent of immunoblot in panel b, (b) immunoblot with anti-*ap*4.1R-CTR, (c) immunoblot with affinity purified anti-bacterial contaminating protein antibody. Note that the affinity purified bacterial contaminating protein antibody was specific to the bacterial protein (BP) in 4.1R-CTR preparations and did not react with 4.1R-CTR or $4.1R^{80}$. This antibody was used as a control in future experiments to confirm anti-*ap*4.1R-CTR specificity. See text for full details. (Gh) erythrocyte ghosts, (C) 4.1R-CTR, (N) recombinant 4.1N polypeptide, (G) recombinant 4.1G polypeptide.

5.1 Characterisation of Anti-4.1R/G/B

Anti-4.1R/G/B was made by Dr. M. L. Bellamy in the laboratory of Dr. A. J. Baines (University of Kent) and was raised against a 14 amino acid peptide from the C-terminal of 4.1R (amino acids 830-843 in mouse 4.1R). The peptide was conjugated to Keyhole Limpet Haemocyanin (KLH) and used as antigen for rabbit polyclonal antibody production. Immunoblots on 4.1R-CTR, human erythrocyte ghosts and 4.1N and G polypeptides demonstrated strong anti-4.1R/G/B reactivity against 4.1R-CTR and $4.1R^{80}$ (Figure 3.18b). The lack of reactivity against recombinant 4.1N and G polypeptides confirmed that neither fragment contained the sequence antigen for this antibody.

Similarity between the 4.1R peptide antigen and equivalent sequences in 4.1N, G and B (Figure 3.19a) demonstrate that the antibody will recognise 4.1B proteins and will possibly also detect 4.1N and 4.1G. To clarify the specificity of this antibody, equivalent peptide sequences in 4.1N and G were synthesised (J. Hardy, University of Kent) and used in competition immunoblots (Figure 3.19b). The antibody was incubated with 1000M excess of the relevant peptide before using it as the primary antibody in standard immunoblotting procedures (Chapter 2, section 3.5). Antibody reactivity was competed out by 4.1R/B and 4.1G peptides but not 4.1N. To reflect the specificity of this antibody it is designated anti-4.1R/G/B.

5.2. Characterisation of Anti-4.1G and Anti-4.1N

Rabbit polyclonal antibodies to 4.1G and 4.1N were produced by Dr. L. A. Keating in the laboratory of Dr. A. J. Baines (University of Kent). Both antibodies were raised against recombinant polypeptides from the U3 region of human 4.1 (amino acids 675-837 in 4.1G and 570-768 in 4.1N). Characterisation of these antibodies by immunoblotting recombinant 4.1G and N antigens, 4.1R-CTR and human erythrocyte ghosts confirmed their specificity and demonstrated that cross-reactivity with other 4.1 proteins was negligible (Figure 3.18c and d).

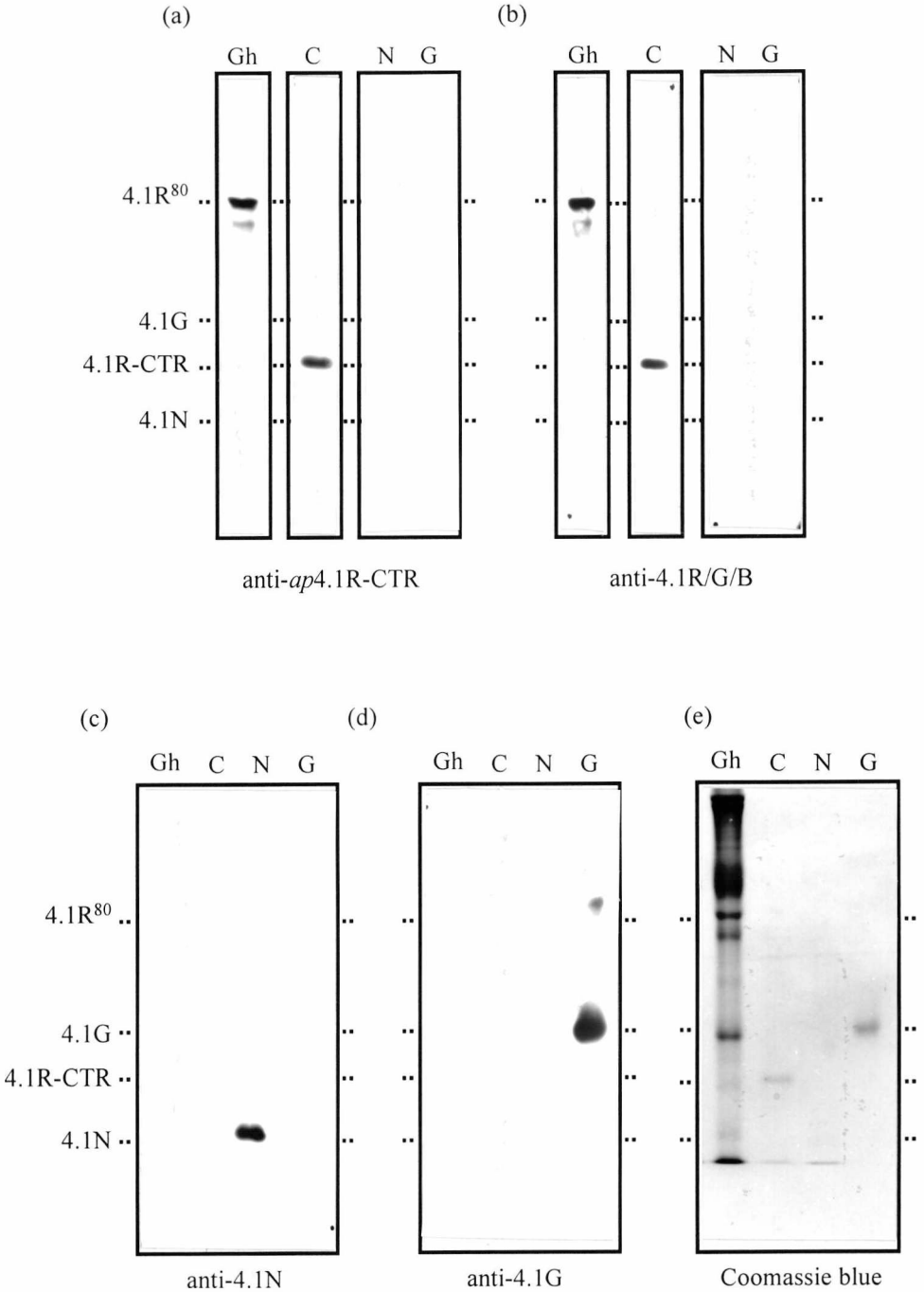
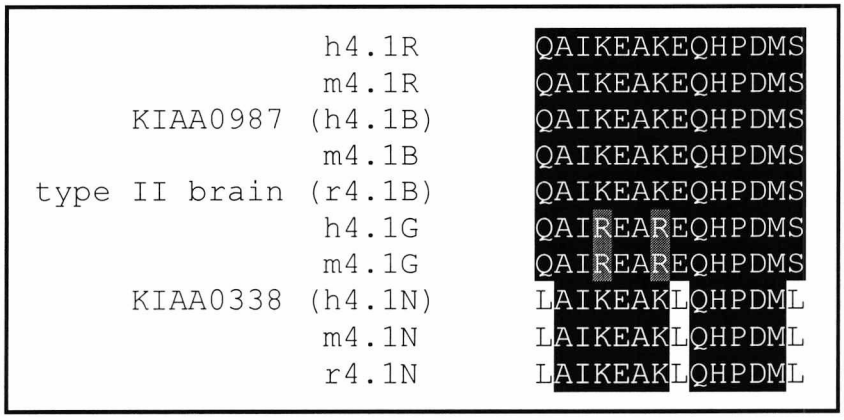


Figure 3.18 Characterisation of anti-*ap*4.1R-CTR, anti-4.1R/G/B, anti-4.1G and anti-4.1N. The reactivity of each 4.1 antibody was tested on erythrocyte ghosts and on 4.1R-CTR, 4.1N and 4.1G recombinant antigens. (a) Anti-*ap*4.1R-CTR, (b) anti-4.1R/G/B, (c) anti-4.1N, (d) anti-4.1G, (e) Coomassie blue stain. As expected, all antibodies were specific for their respective antigens and only anti-*ap*4.1R-CTR and anti-4.1R/G/B detected erythrocyte $4.1R^{80}$ and 4.1R-CTR. See text for full details of antibodies. (Gh) erythrocyte ghosts, (C) 4.1R-CTR, (N) recombinant 4.1N polypeptide, (G) recombinant 4.1G polypeptide.

(a)



(b)

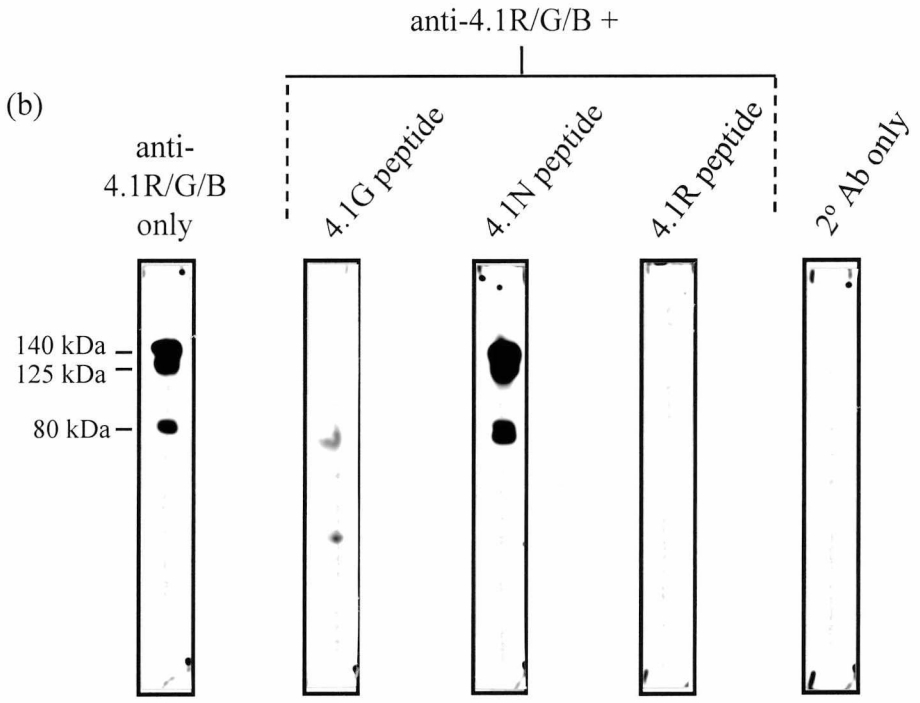


Figure 3.19 Competition blot to demonstrate 4.1R/G/B specificity. (a) 4.1G, 4.1N and 4.1B peptides were made, corresponding to the 14aa antigenic region of 4.1R used to raise the 4.1R/G/N antibody. The sequence alignment shows that all known 4.1 R and B sequences are identical in this region while 4.1G sequences contain two conserved amino acid changes (lys → arg). 4.1N sequences are more variable in this region. (b) Competition blots using the 4.1 peptide antibody with the peptides shown demonstrated that 4.1G and 4.1B could successfully compete out antibody immunoreactivity, whereas 4.1N could not. This indicates that the antibody cross reacts with both 4.1B and 4.1G species and is designated anti 4.1R/G/B as a result. 4.1N isoforms are not detected by this antibody.

Discussion

The C-terminal region (SAB domain and CTD) of 4.1R[80] was successfully cloned into *E. coli* and expressed as a his-tag fusion protein. The his-tag allowed easy purification of the recombinant polypeptide by immobilised metal chelate affinity chromatography. Sufficient quantities of protein were obtained for structure and activity analysis and 4.1R-CTR was shown to exhibit characteristics similar to erythrocyte 4.1R[80]. 4.1R-CTR was used to generate polyclonal antibodies and these were found to be specific for 4.1R proteins. Antibodies to 4.1N and 4.1G were also specific for their relevant gene products and a peptide antibody to the C-terminal of 4.1R detected 4.1R, G and B but not 4.1N.

Expression of 4.1R-CTR – the his-tag system

The his-tag purification system used to purify 4.1R-CTR was an attractive prospect because the his-tag is small and unobtrusive, is uncharged at physiological pH and can be used to purify polypeptides under denaturing conditions (for a review see Nilsson *et al.*, (1997)). Larger fusion partners such as glutathione S-transferase (GST) are in some cases prone to non-specific protein-protein interactions (N. Hayes: unpublished results, P. Bignone: unpublished results, Murthy *et al.*, (1999)). Although his-tags have been shown to affect protein-DNA interactions (Buning *et al.*, 1996; Jessop *et al.*, 2000) there are no similar reports concerning protein-protein interactions and the six histidine residues fused to the C-terminal of 4.1R-CTR are unlikely to influence polypeptide binding.

A common problem when purifying his-tag polypeptides from bacterial expression systems is contamination with proteins originating from the bacterial host (Hengen, 1995). Furthermore, the expression of chloramphenicol resistance by the bacterial host is thought to interfere with his-tag fusion protein purification (Oswald & Rinas, 1996). The latter was not experienced during purification of 4.1R-CTR despite the use of a chloramphenicol resistant bacterial strain. A 75kDa protein of bacterial origin did contaminate 4.1R-CTR preparations however and could not be removed by increasing the stringency of the purification or modifications to remove a previously identified

fusion system contaminant, DnaK (Trouble Shooting Guide, GST Gene Fusion System, Pharmacia Biotech, 3rd Edition, Revision 1).

One possibility is that the bacterial protein contains a high content of histidine and is able to chelate Ni^{2+} as efficiently as 4.1R-CTR. An alternative is that the bacterial protein binds and therefore co-purifies with 4.1R-CTR. This is an intriguing possibility given that *E. coli* contain FKBP related proteins (Hesterkamp & Bukau, 1996; Trandinh *et al.*, 1992; Wulfing *et al.*, 1994) and 4.1G binds FKBP13 via a CTD binding site conserved in 4.1R, N and B (Walensky *et al.*, 1998a). FKBP proteins exhibit chaperone activity (Bharadwaj *et al.*, 1999) and can be induced in response to stress (Kurek *et al.*, 1999; Vucich & Gasser, 1996). This makes them prime candidates to be present in recombinant protein expressing bacteria grown at sub-optimum temperatures. The contaminating bacterial protein could be identified by peptide sequencing and may help ascertain its relationship to 4.1R-CTR. Production of an anti-bacterial protein antibody ensured that results obtained with 4.1R-CTR in prospective studies were a specific function of the recombinant polypeptide.

Characterisation of 4.1R-CTR

When produced in a prokaryotic expression system recombinant proteins can be mis-folded and may lack post-translational modifications that occur in eukaryotes. This may give the recombinant protein different characteristics to its biological counterpart and if not addressed can lead to artifactual results in experimental analysis. Mass determination, ternary complex formation and chymotrypsin digestion were used to demonstrate that 4.1R-CTR behaves in a similar way to $4.1R^{80}$.

Mass determination

The near identity between the molecular mass of 4.1R-CTR (26068 daltons) and the theoretical mass of $4.1R^{80}$ in this region (26071 daltons) confirms that the recombinant protein was not mistranslated or unnaturally modified during expression. The relative molecular mass of 4.1R-CTR determined by SDS-PAGE is somewhat higher than the absolute mass determined for this region but is a common feature among proteins of the

4.1 family (shown for 4.1G in Parra *et al.*, (1998), for 4.1N in Walensky *et al.*, (1999) and for 4.1R in Conboy *et al.*, (1991)).

Ternary complex formation

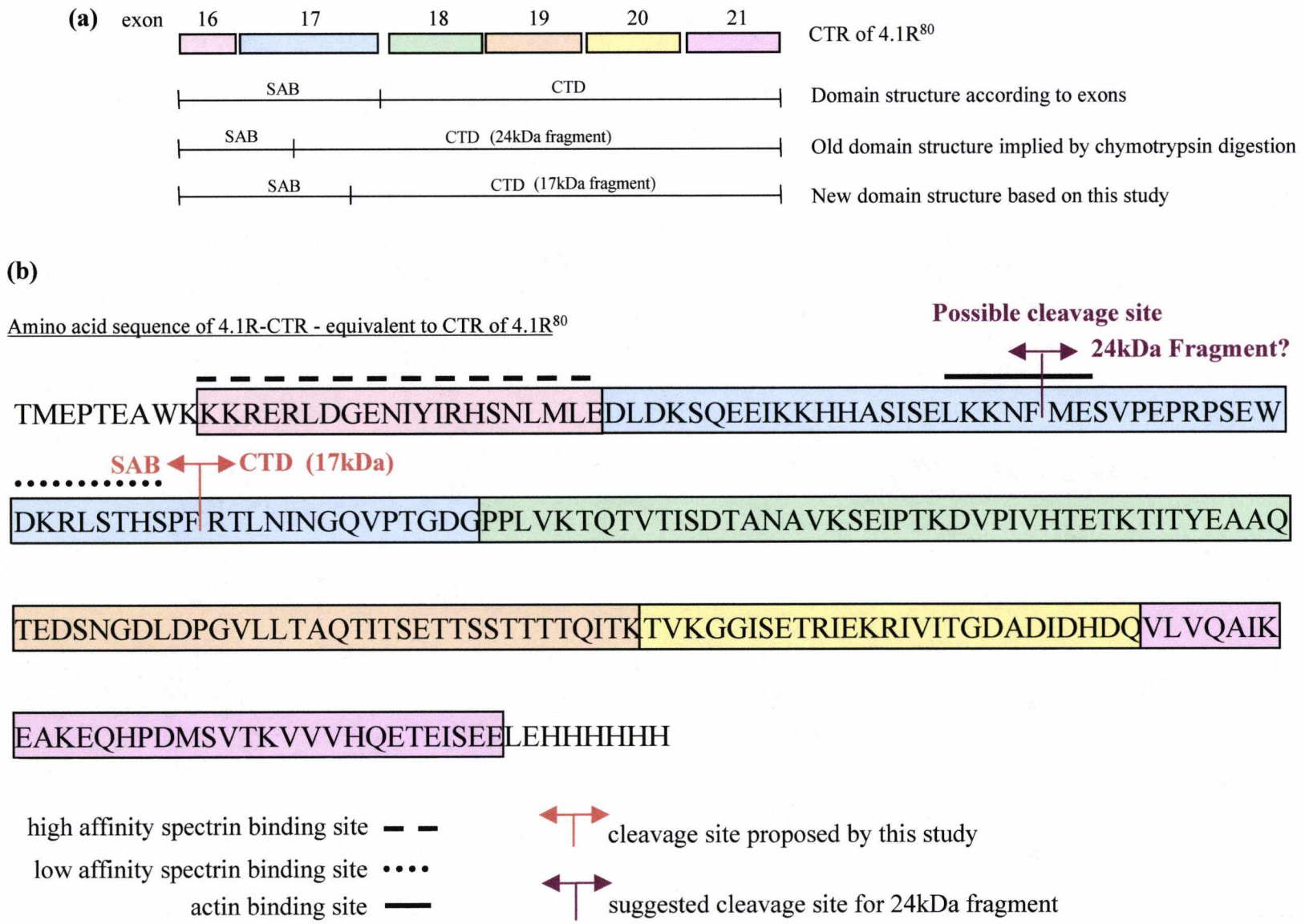
Purified $4.1R^{80}$ from erythrocytes can be unstable and has a tendency to become inactivated (Ohanian & Gratzer, 1984; Ohanian *et al.*, 1984). 4.1R-CTR was able to reproduce the known spectrin-actin binding activity of $4.1R^{80}$ as observed by Ohanian & Gratzer (1984) indicating that the recombinant polypeptide was biologically active. Binding of spectrin to actin is weak in the absence of $4.1R^{80}$ (Ohanian *et al.*, 1984) but has been shown both qualitatively (Ungewickell *et al.*, 1979) and quantitatively (Ohanian et al 1984 biochem 23 4416-4420) to be strongly potentiated when $4.1R^{80}$ is present. A similar phenomenon was demonstrated here.

Spectrin dimer was used in this study yet the association of spectrin, actin and $4.1R^{80}$ has been reported to occur more efficiently in the presence of spectrin tetramer (Ungewickell *et al.*, 1979). Ohanian *et al.*, (1984) and Ohanian & Gratzer (Ohanian & Gratzer, 1984) used spectrin dimer to successfully form ternary complexes and although the cross-linked network that occurs with spectrin tetramer (Ungewickell *et al.*, 1979) is probably not observed, binding of the three proteins can still be detected. The ternary complex data demonstrates functional similarity between recombinant and native 4.1R. The interaction does not appear to be affected by the C-terminal 4.1R-CTR his-tag indicating that polypeptide is competent for use in binding studies.

Chymotrypsin digestion

Chymotryptic digestion of 4.1R-CTR produced a digestion pattern comparable to that produced by Leto & Marchesi (1984) with native $4.1R^{80}$. This implies that 4.1R-CTR has tertiary structure and is folded properly by *E. coli* during expression. In particular, the results of these researchers also indicate the presence of a 15kDa protease resistant species similar to that produced by digestion of 4.1R-CTR.. The work carried out in collaboration with A. J. Baines and J. Hardy showed this polypeptide to be a 17kDa fragment that shares molecular mass identity with a chymotryptic polypeptide present within the sequence of 4.1R-CTR (Figure 3.20). The CTD of 4.1R has previously been

Figure 3.20 Proposed digestion sites and domain structure of 4.1R^{80}. (a) Schematic representation of the exon and domain structure of erythrocyte 4.1R^{80}. The domain structure defined by exons is slightly different to the structure defined by chymotrypsin digestion. (b) A more detailed analysis of the exon and domain structure of 4.1R^{80} where the amino acid sequence for this region is highlighted with the relevant exons and both known and potential chymotrypsin cleavage sites are shown. The 17kDa chymotryptic 4.1R-CTR fragment was identified in this study to correspond to a region beginning just upstream of the low affinity spectrin binding site and it is proposed that this fragment represents the true CTD of 4.1R^{80}. The 24kDa chymotryptic fragment suggested until now to correspond to the CTD of 4.1R^{80} is more likely to be produced by cleavage at an upstream chymotrypsin site located within the actin binding region.


(a)
exon
16
17
18
19
20
21
CTR of 4.1R80
SAB
CTD
Domain structure according to exons
SAB
CTD (24kDa fragment)
Old domain structure implied by chymotrypsin digestion
SAB
CTD (17kDa fragment)
New domain structure based on this study
(b)
Amino acid sequence of 4.1R-CTR - equivalent to CTR of 4.1R80
Possible cleavage site
24kDa Fragment?
TMEPTEAWKKKRERLDGENIYIRHSNLMLEDLDKSQEEIKKHHASISELKKNFMESVPEPRPSEW
SAB
CTD (17kDa)
DKRLSTHSPFRTLNINGQVPTGDGPPLVKTQTVTISDTANAVKSEIPTKDVPIVHTETKTITYEAAQ
TEDSNGDLDPGVLLTAQTITSETTSSTTTTQITKTVKGGISETRIEKRIVITGDADIDHDQVLVQAIK
EAKEQHPDMSVTKVVVHQETEISEELEHHHHHH
high affinity spectrin binding site
low affinity spectrin binding site
actin binding site
cleavage site proposed by this study
suggested cleavage site for 24kDa fragment

referred to as the 24kDa domain and the SAB domain the 10kDa domain. Based on the work carried out here I propose that the CTD of 4.1R is structurally formed by a 17kDa polypeptide that shares high sequence homology with 4.1N, G and B. The SAB domain would therefore be formed from the remaining upstream polypeptide that has a theoretical molecular mass of 7917 daltons. In accordance with this definition, the 17kDa polypeptide cleavage site is located two amino acids after the low affinity spectrin binding site in 4.1R (Figure 3.20) and therefore exists two amino acids after the region defined as the minimal sequence required for spectrin-actin-4.1R^{80} binding (Discher *et al.*, 1995; Schischmanoff *et al.*, 1995). Furthermore, in a study by Correas *et al.*, (1986), 4.1R digested with chymotrypsin produced an 8kDa fragment and an un-sized fragment of ~15kDa. The 8kDa fragment had spectrin-actin binding activity and was shown by sequencing to be generated by 4.1R cleavage at the same site as identified here. The 24kDa region labelled previously as the CTD of 4.1R is probably produced by cleavage at an upstream chymotrypsin digestion site and includes a portion of the region involved in spectrin-actin binding (Figure 3.20). Sequencing of the 24kDa fragment is required to confirm this assertion, however it is the 17kDa fragment that truly defines the CTD of 4.1R^{80}.

Antibody characterisation

Polyclonal rabbit and rat anti-*ap*4.1R-CTR antibodies produced in this study were shown to be specific for 4.1R. In addition to anti-*ap*4.1R-CTR, three other antibodies were characterised in this study, anti-4.1R/G/B, anti-4.1N and anti-4.1G. Anti-4.1N and anti-4.1G were specific for their respective gene products with negligible cross-reactivity with other 4.1 proteins. Together with anti-*ap*4.1R-CTR, anti-4.1N and anti-4.1G allow direct and specific detection of three of the 4.1 gene products. The triple specificity of anti-4.1R/G/B means that it is also possible to identify products of the 4.1B gene if all antibodies are used concurrently. These antibodies therefore provide a valuable set of immunological tools with which to study 4.1 proteins.

In summary, the C-terminal region of 4.1R^{80} was successfully cloned and expressed in a bacterial system. Soluble polypeptide was purified to near homogeneity and demonstrated characteristics reminiscent of native 4.1R^{80}. 4.1R-CTR was used to

generate 4.1R specific polyclonal antibodies, which can be used with antibodies to 4.1N, 4.1G and 4.1R, G and B to assess the distribution of 4.1 proteins in brain. Recombinant 4.1R-CTR can also be used to investigate $4.1R^{80}$-CTR binding interactions in specific subcellular fractions such as PSDs. During the characterisation of 4.1R-CTR it was discovered that the complete CTD of 4.1R corresponds to a 17kDa product produced by chymotrypsin digestion of this species. This product is formed from the 24kDa region referred to as the CTD in previous 4.1 studies and therefore redefines the chymotrypsin domain structure of 4.1R.

Chapter 4

Identification of 4.1R in Cultured Neurons of the Rat Midbrain

Introduction

Initial hypotheses about the distribution and function of protein 4.1 were modified when 4.1 mRNA species were identified in non-erythroid cells (Tang *et al.* 1990; Tang *et al.* 1988). These hypotheses have been altered once again with the realisation that 4.1G, B and N gene products exist. It is now generally accepted that 4.1 proteins are ubiquitous throughout the mammalian body with the different 4.1 gene products differentially distributed across tissues and subcellular locations. RNA blot analysis by Parra *et al.*, (1998) showed 4.1R mRNAs to be most abundant in human hematopoietic tissues such as bone marrow, foetal liver and leukocytes. The amounts are lower in kidney, adrenal and cerebellum but all tissues tested indicate some level of 4.1R expression including whole brain, forebrain and the substantia nigra. *In situ* hybridisation carried out by Walensky *et al.*, (1998b) on new born mice support the data of Parra *et al.* showing 4.1R mRNA levels to be most prominent in hematopoetic and nervous tissues. In brain it is not clear whether 4.1R is predominantly neuronal or is also expressed in glia. Neither is it clear whether 4.1R has a polarised distribution in these cell types. Immuno-localisation of 4.1R should begin to answer these questions.

1. Evidence for Protein 4.1R in Rat Midbrain Cultures

Midbrain refers to the portion of the brain at the top of the brain stem. In rodents it lies beneath the rear portion of the cerebrum, in front of the medulla and cerebellum but distinct from the hippocampus and hypothalamus (Figure 4.1). Mesencephalon tissue present during early developmental stages ≤ embryonic day 14 later becomes replaced by substructures such as the substanita nigra and red nucleus. The substantia nigra contains high concentrations of dopaminegic neurons and is involved in the mesostriatal dopamine pathway that degenerates in Parkinson's disease (Strange, 1995). Dissociated midbrain primary cultures can be established by dissecting the ventral mesencephalon region from rat embryos (Figure 4.2). Dissected cells are treated with trypsin, dissociated by tituration in Dulbecco's Modified Eagle Medium supplemented with fetal calf serum and plated in poly-lysine coated chamber slides (or equivalent) which are then incubated at 37°C/5% CO_2 (Pardo *et al.*, 1997).

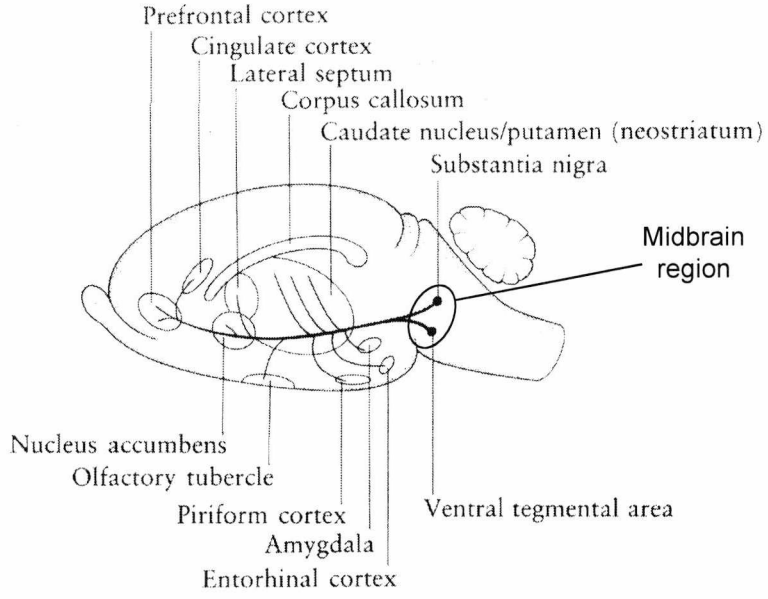


Figure 4.1 Schematic diagram of a rat brain showing the principal long dopamine neuronal pathways in rat brain and thus localising the region referred to as the midbrain. The substantia nigra and ventral tegmental areas both localise to the midbrain region in adults. In early developmental brain this region consists of mesencephalon and tegmental tissue that is dissected to establish dopaminergic primary neuronal cultures. Reproduced from Strange (1995).

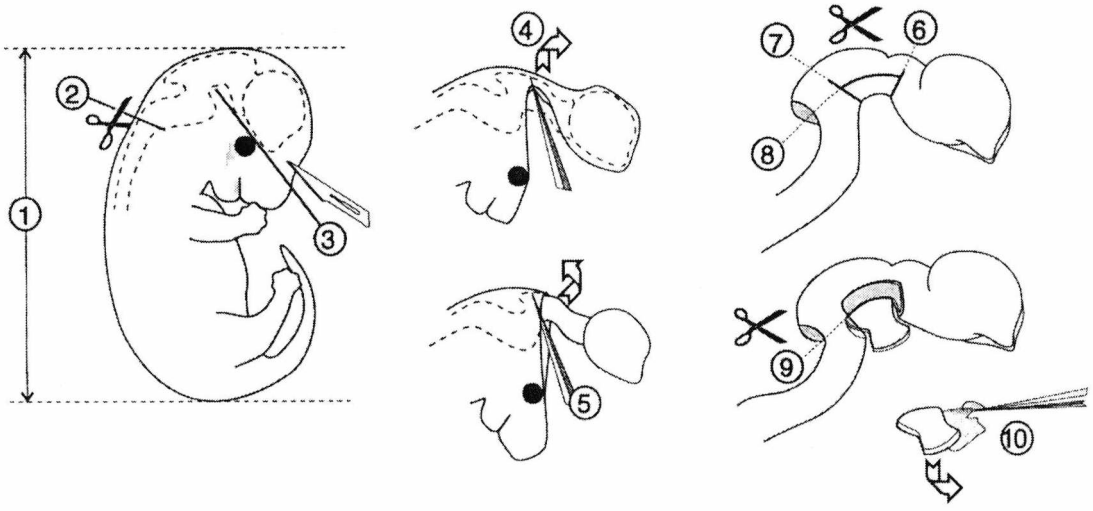


Figure 4.2 Schematic diagram outlining dissection of embryonic day 14 rat ventral mesenchephalon used to establish primary rat midbrain cultures. (1) Measurement of the embryo to confirm its stage of development, (2) Decapitation, (3)-(5) Removal of the brain, (6)-(9) Removal of dopamine cells of the substantia nigra. These are dissected as a wedge from the base of the neural tube at the level of the mesencephalic flexure and results in removal of te ventral mesencephalon, (10) Removal of meninges. Reproduced from Dunnett & Bjorklund (1997).

Immunocytochemical characterisation of such cultures reveals dopaminergic neurons (tyrosine hydroxylase and MAP2 positive), non-dopaminergic neurons (tyrosine hydroxylase negative, MAP2 positive) and to a lesser extent, glia (GFAP positive) (Cheung *et al.*, 1997; Pardo *et al.*, 1997; Shimoda *et al.*, 1992).

A dissociated rat midbrain primary culture system was established in the laboratory of Dr. A. Baines by Fiona McCann. Cultures were established using embryonic day 17 rats and were shown to contain characteristics of normal midbrain neurons: Immunofluorescence with anti-MAP2 revealed neuronal cell bodies and dendrites, staining with anti-GAP43 showed the presence of axons and staining with anti-tyrosine hydroxylase demonstrated that a proportion of neurons were dopaminergic (F. McCann, unpublished results). 4.1R mRNA species are present in midbrain as shown by dot blot analysis on RNA prepared from the substantia nigra (Parra *et al.*, 1998) however the levels are very low. In spite of this, the availability of a characterised rat midbrain culture system provided an opportunity to look at the distribution of 4.1R among different cell types of the brain (i.e. neurons vs glia).

1.1. Immunolocalisation of 4.1R in Rat Midbrain Cultures

To investigate the distribution of 4.1R in rat midbrain cultures antibodies raised to 4.1R, glial fibrillary acidic protein (GFAP) and MAP2 were used in immunofluoresence labelling.

Double labelling with anti 4.1R-CTR rat serum and anti-GFAP revealed no co-localisation of the two proteins (Figure 4.3) indicating that 4.1R was not a component of astrocytes. 4.1R did show very low level, diffuse cytoplasmic staining in glia but staining of neurons was more specific (see below).

Staining with anti 4.1R-CTR rabbit serum and anti-MAP2 (Figure 4.4) demonstrated 4.1R and MAP2 co-localisation in cell bodies and dendrites. Relatively little 4.1R staining was seen in MAP2 negative processes indicating that neuronal 4.1R predominantly localises to somatodendritic compartments. 4.1R and MAP2 staining sometimes segregated to distinct regions within the same neurite. In some processes

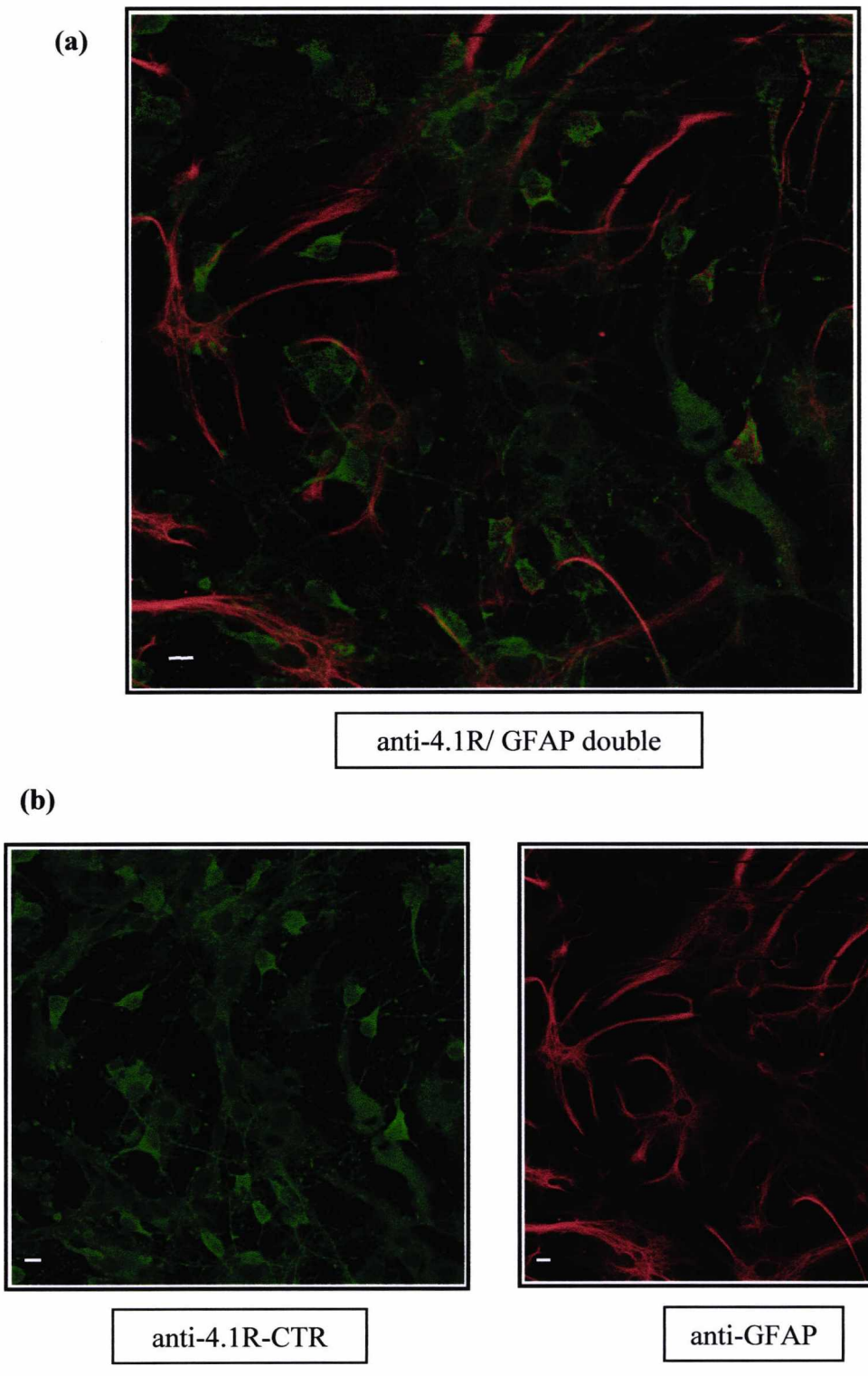


Figure 4.3 Immunofluorescence on rat embryonic midbrain cultures with anti-4.1R-CTR serum and anti glial fibrillary acidic protein. (a) Double stained image with anti 4.1R-CTR serum labeled with FITC (green) and anti-glial acidic fibrillary protein (GFAP) labeled with TRITC (red). (b) and (c) show the corresponding single stained images with anti-4.1R-CTR and anti-glial acidic fibrillary protein respectively. Each bar is equivalent to 20μm.

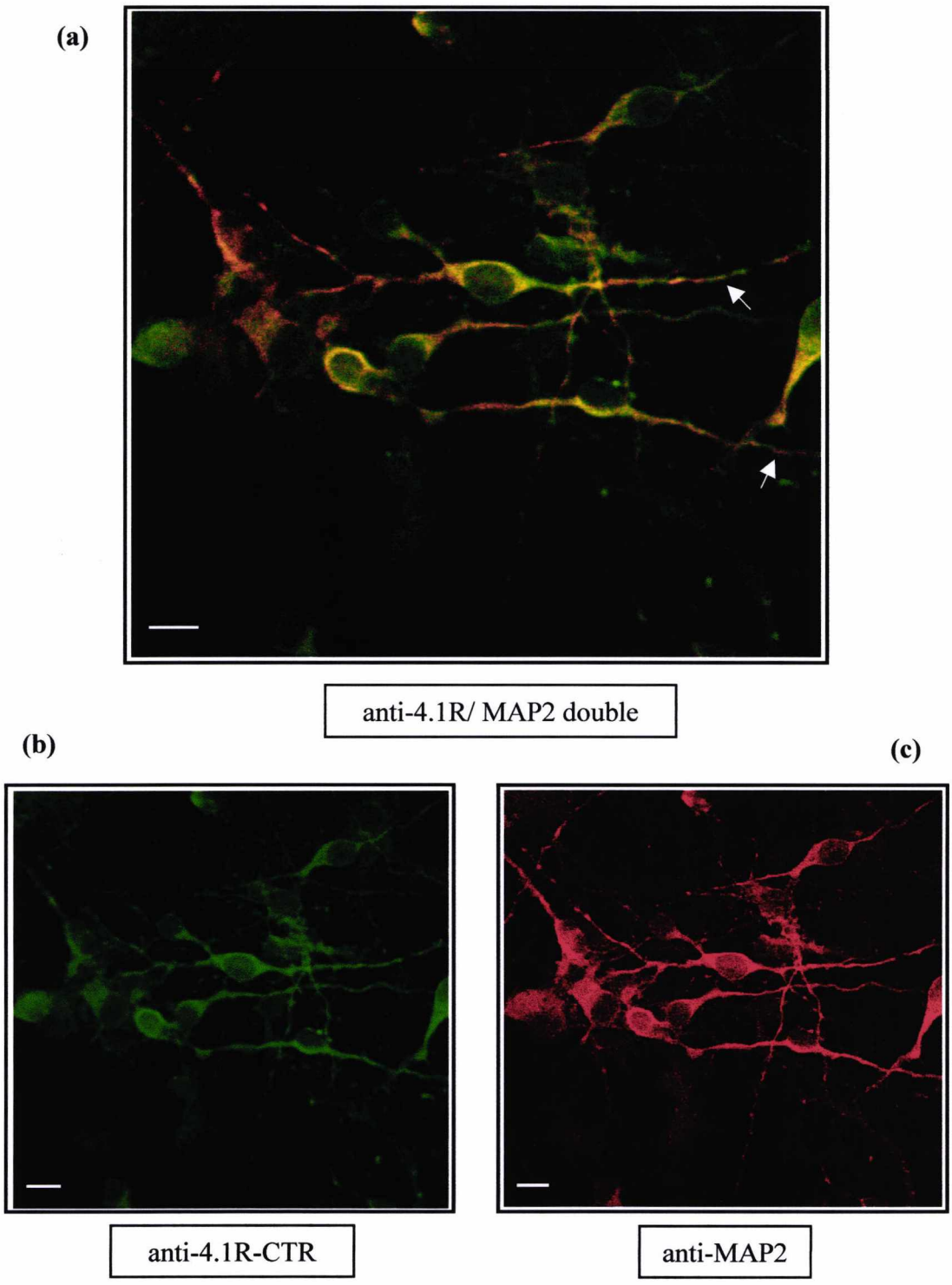


Figure 4.4 Immunofluorescence on rat embryonic midbrain cultures with anti-4.1R-CTR serum and anti-MAP2. (a) Double stained image with anti-4.1R-CTR serum labeled with FITC (green) and anti-MAP2 labeled with TRITC (red). (b) and (c) show the corresponding single stained images with anti-4.1R-CTR and anti-MAP2 respectively. Arrows indicate where MAP2 localises to the centre of a neurite while 4.1R labels the periphery. Each bar is equivalent to 20μm.

anti-MAP2 labelled the centre of the neurite while anti 4.1R-CTR labelled points along the neurite periphery (Figure 4.4, arrows). This would be consistent with a sub-membranous distribution of 4.1R for example, at a MS structure or specialised membrane domains. Very occasionally MAP2 negative neurites stained with anti 4.1R-CTR suggesting that a small proportion of 4.1R exists in axons of these cultures.

1.2. Identification of 4.1R mRNA in Rat Midbrain Cultures

Immuno-staining of rat midbrain cultures infers that 4.1R proteins are present in neuronal cell bodies and dendrites. If this is so, 4.1R mRNA should be detectable amongst RNA isolated from the cultures. Expression of 4.1R within midbrain cultures was demonstrated using 4.1R specific primers and total RNA isolated from cultures by F. McCann. 4.1R primers were designed to DNA sequences corresponding to the N- and C-terminal of mouse erythrocyte $4.1R^{80}$ (5' nucleotides 2186-2206 and 3' nucleotides 4116-4139) and identified mRNA transcripts ~2.0kb, ~1.6kb and ~1.2kb in size (Figure 4.5). Identification of multiple transcripts in isolated rat midbrain culture cDNA suggests 4.1R is alternatively spliced in this brain region. The exon structure of the individual products is not known.

1.3. Identification of 4.1R Proteins in Rat Midbrain Cultures

Rat midbrain culture cells were lysed and made into SDS-PAGE gel samples to investigate the complement of 4.1R protein isoforms present in the cultures. Immunoblots with anti *ap*4.1R-CTR and anti 4.1R/G/B revealed three major 4.1R species at 135kDa, 80kDa and 30kDa respectively (Figure 4.6). The 135kDa and 80kDa species are consistent with previously identified $4.1R^{135}$ and $4.1R^{80}$ isoforms (Tang *et al.*, 1990). $4.1R^{135}$ is produced by expression from AUG-1 of the 4.1R gene and includes the 209 amino acid N-terminal extension found in non-erythroid forms of the protein (Chapter 1, section 3.1.3.2). $4.1R^{80}$ is the erythrocyte protein and is expressed from AUG-2. A minor 66kDa band also evident on the immunoblot may correspond to 4.1R expression from AUG-3 (Gascard *et al.*, 1998).

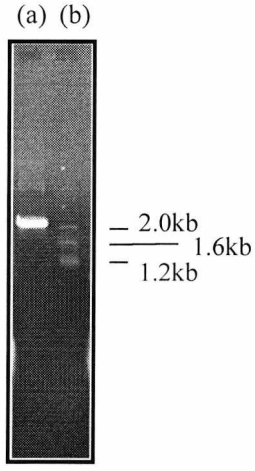


Figure 4.5 RT-PCR on total rat midbrain culture cDNA with 4.1R specific primers. Rat midbrain culture cDNA was amplified with primers corresponding to the N and C-terminal of 4.1R^{80} (b). Three distinct 4.1R transcripts were detected (~2.0kb, 1.6kb and 1.2kb) providing evidence of 4.1R expression in rat midbrain cultures. Identification of multiple transcripts supports the occurrence of 4.1R alternative splicing in this brain region. (a) Amplification with the same primers using mouse 4.1R clone pE15 (Huang *et al.*, 1993) supplied by T. K. Tang.

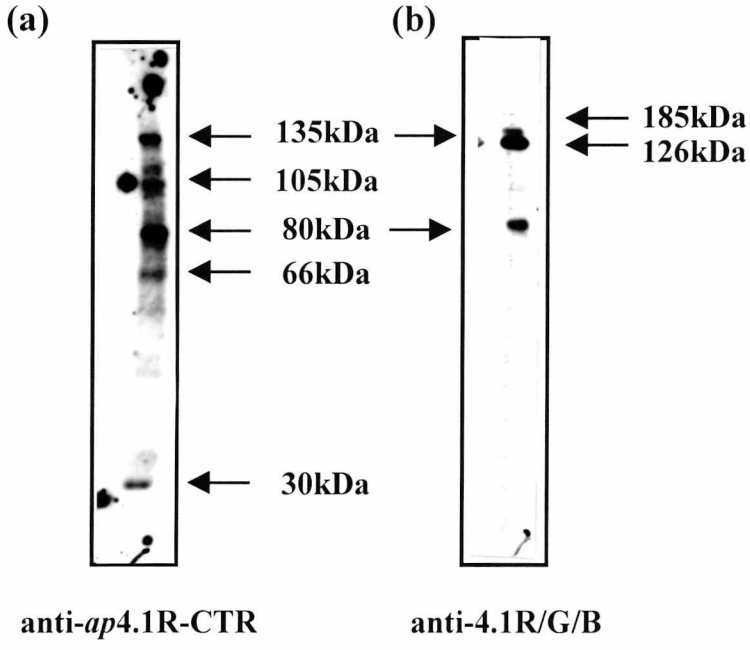


Figure 4.6 Immunoblots on rat midbrain cultures with 4.1R and 4.1R/G/B specific antibodies. (a) An immunoblot on rat midbrain cultures with *ap*4.1R-CTR showed various 4.1R isoforms to be present in rat midbrain cultures. (b) An immunnoblot with anti 4.1R/G/B indicated that in addition to 4.1R, products of the 4.1B and/or 4.1G genes were expressed.

In addition to 4.1R, anti 4.1R/G/B detected a major product at ~126kDa and minor products at ~175kDa and ~100kDa. These must be products of the 4.1B or 4.1G genes according to the antigen specificity of this antibody (Chapter 3, section 5.1).

Discussion

The presence of 4.1R proteins in the somatodendritic component of rat midbrain cultures was demonstrated by immunofluorescent co-localisation of 4.1R with MAP2. Coupled with identification of 4.1R mRNA by RT-PCR and 4.1R protein by immunoblotting, it can be concluded that specific isoforms of 4.1R are expressed in rat midbrain neurons, where they exhibit a polarised distribution. Previous studies demonstrating expression of 4.1R immunologically related species in brain also localised the protein to cell bodies and dendrites, but not axons (Goodman *et al.*, 1984; Krebs *et al.*, 1987; Tsumoto *et al.*, 1988; Yorifuji *et al.*, 1989). In addition, electron microscopy by Yorifuji *et al.*, (1989) showed microtubule bundles along the longitudinal axis of dendrites were immuno-gold labelled on their outside with anti-4.1 antibodies. This is consistent with staining patterns observed in this study where 4.1R lined the edge of dendrites while MAP2 stained the middle section.

Although hippocampal cultures are more common for immunocytochemical studies on 4.1 proteins, rat midbrain dissociated cultures contain machinery for both excitatory (glutamatergic) (Mount *et al.*, 1990; Mount *et al.*, 1989) and inhibitory (GABAergic) (Masuko *et al.*, 1992) neuronal responses, implying the presence of a diverse neuronal population. The distribution of 4.1R was therefore analysed across a variety of neuronal types and there is no reason to suspect that the cellular distribution of this protein will be significantly different in neurons of other brain regions. This is consistent with the results of other researchers who show a similar subcellular localisation of 4.1R related proteins in cerebellum and cultured PC12 cells (Goodman *et al.*, 1984; Krebs *et al.*, 1987; Yorifuji *et al.*, 1989).

Immunoblot analysis indicates that multiple 4.1 proteins are expressed in embryonic day 17 rat midbrain cultures. Recent reports of an 126kDa 4.1B protein and ~160kDa

and ~100kDa 4.1G proteins in mouse brain homogenate (Parra *et al.*, 2000) suggest an identity for the 4.1B/G species detected by anti 4.1R/G/B. These identities were not confirmed with gene product specific antibodies.

The 80kDa 4.1R species is the most abundant 4.1R isoform detected on embryonic rat midbrain culture immunoblots. It is probably produced from the 2.0kb mRNA transcript and is likely to be equivalent to erythrocyte $4.1R^{80}$. Like erythrocytes, neurons require structurally stable yet flexible plasma membranes to permit the morphological changes accompanying synaptic remodelling and plasticity (Matus, 1999; van Rossum & Hanisch, 1999). 4.1R may be part of a membrane skeleton structure that helps generate these properties. It may be particularly significant that the 80kDa 4.1R product is expressed in developing neurons, when morphological transformations are at their highest. Dendritic spines contain high concentrations of PSDs (Trommald & Hulleberg, 1997). A erythrocyte equivalent protein could feasibly localise to these specialised membrane domains to assist the anchoring of transmembrane proteins such as neurotransmiter receptors and ion channels (cf. erythrocyte GPC and band 3).

Less abundant 4.1R proteins were detected on rat midbrain culture immunoblots at 135kDa and 66kDa. The high molecular weight product is assumed to be equivalent to $4.1R^{135}$ expressed from AUG1 in the 4.1R gene (Tang *et al.*, 1990). $4.1R^{135}$ is found predominantly in nucleated, non-erythroid cells (Luque *et al.*, 1999; Tang *et al.*, 1990) but its tissue distribution is not well documented. From the data presented here it can be concluded that $4.1R^{135}$ is a component of embryonic rat midbrain. Whether $4.1R^{135}$ is also expressed in adult rat midbrain can not be ascertained from these results. During erythroid differentiation $4.1R^{135}$ expression is developmentally regulated (Baklouti *et al.*, 1996; Chasis *et al.*, 1993). An early splicing switch removing AUG1 abrogates expression so levels of this isoform in mature erythrocytes become negligible. A similar phenomenon could be true for rat midbrain cultures and adult rat midbrain and developmental series need to be analysed to determine this. It is conceivable that the 66kDa 4.1R protein detected in cultures is encoded by the 1.6kb mRNA transcript identified by RT-PCR. This transcript theoretically produces a protein ~58kDa in size

which correlates well with the 66kDa protein identified on blots. The nature of the 1.2kb RT-PCR product and the origin of the 30kDa 4.1R protein seen on blots is unclear.

In summary, multiple 4.1R isoforms are expressed in embryonic rat midbrain where they localise predominantly to the somatodendritic compartment of neurons. 4.1R proteins may be important in producing a flexible, yet stable plasma membrane and may organise transmembrane proteins to particular specialised plasma membrane domains.

Chapter 5

Identification of 4.1 Proteins in Postsynaptic Density Preparations

Introduction

Little is known about the subcellular distribution of 4.1 proteins. 4.1B has been shown to accumulate at regions of cell-cell contact (Parra *et al.*, 2000), 4.1N at synapses (Ohara *et al.*, 1999; Walensky *et al.*, 1999) and 4.1G at peri-nuclear regions and diffuse throughout the cytoplasm (Parra *et al.*, 1998). In non-erythroid cells, immunocytochemical methods have localised 4.1R-related species to the membrane skeleton, intracytoplasmic fibrillary networks, peri-nuclear regions (Tang *et al.*, 1990), the cytosol (Baklouti *et al.*, 1996) and nucleus (Krauss *et al.*, 1997b). Some 4.1R related species may in fact be 4.1B, G or N proteins detected as a result of antibody cross reactivity.

In brain, 4.1 proteins are differentially expressed in different brain regions (Ohara *et al.*, 1999; Walensky *et al.*, 1999). Expression of 4.1N is detected in all neuronal populations of the brain while 4.1G is expressed in glia and selected neuronal populations, 4.1B in the hippocampus, cerebellum and olfactory bulb and 4.1R in the cerebellum and dentate gyrus (Parra *et al.*, 2000; Walensky *et al.*, 1998b). Overlapping distributions in some brain regions suggests that multiple 4.1 proteins may co-exist in the same cell. Very little is known about the subcellular distribution and function of 4.1 proteins in brain. 4.1N has been suggested to localise at pre-synaptic nerve terminals of cerebellar mossy fibres (Ohara *et al.*, 1999) while 4.1R-immunologically related species localise to purkinje cell bodies of rat cerebellum, exist as cytoskeletal components in the cytoplasm of dendrites and underlie dendritic plasma membranes (Yorifuji *et al.*, 1989). A mouse with the 4.1R gene knocked out shows defects in movement, co-ordination, balance and learning (Walensky *et al.*, 1998b) suggesting a significant role for 4.1R at least, in the brain.

1. Postsynaptic Densities *In Vitro*

In Chapter 4, 4.1R isoforms were immunofluorescently localised to neuronal dendrites. Among the most significant structures in dendrites are the PSDs that concentrate on dendritic spines (for reviews see Van Rossum & Hanisch (1999), Kim & Huganir

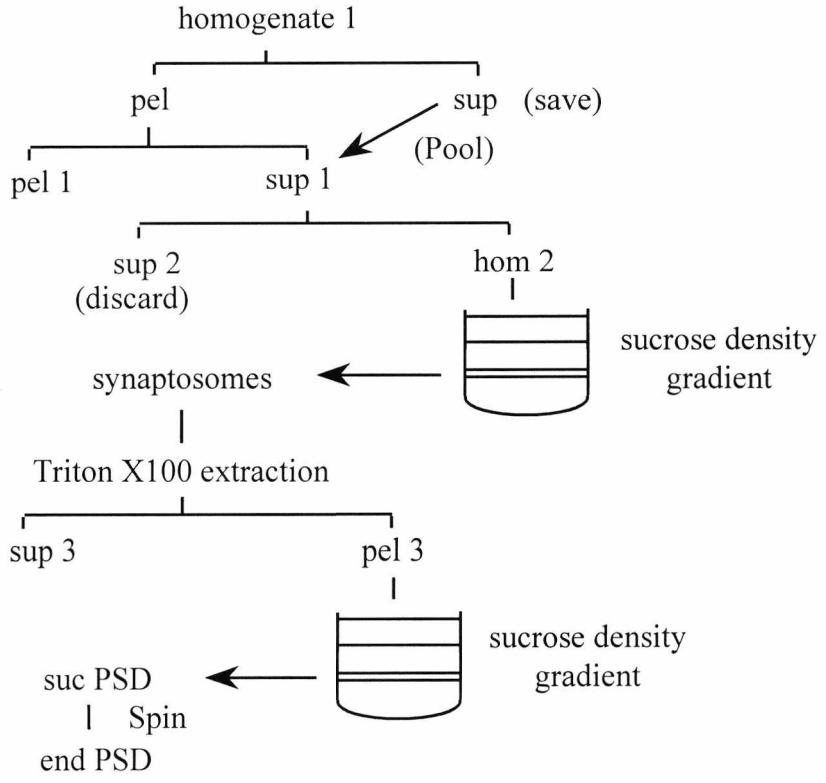
(1999), Ziff (1997) and Kennedy (1993). The importance of these structures in synaptic transmission as well as the mechanisms underlying learning and memory meant that PSDs would be an interesting subcellular fraction in which to look for protein 4.1. The hypothesised functions of PSDs and the elucidated structure to date (Chapter 1, section 2.3.3) imply that they are macromolecular complexes with a lattice-like form similar to the erythrocyte membrane skeleton. 4.1 proteins at PSDs could help stabilise membrane skeletal structures and assist PSD function, for example by minimising the lateral diffusion of receptor proteins and/or recruiting and localising signalling molecules to their site of action.

PSDs can be isolated from brain using various different strength detergents. In general, non-ionic detergents are used to sediment PSDs from synaptosomes or synpatosomal membranes. Ionic detergents lead to wide dispersal of PSDs on continuous density gradients and are avoided as a result (Somerville *et al.*, 1984). PSDs produced from synaptosomes and synaptic plasma membrane were originally shown to have similar compositions (Cohen *et al.*, 1977). Since then it has been suggested that synaptosomal PSDs have increased amounts of certain proteins, including contaminants such as GFAP and neurofilaments (Matus *et al.*, 1980). In spite of this, both methods of purification give a product that is highly reproducible.

1.1. Purification of Postsynaptic Densities

The purification method used here is that set out by Carlin *et al.*, (1980) and is outlined in Figure 5.1a. Purification was via synaptosomes with pig forebrain as the initial source of material. Triton X-100 was used to solubilise the membrane component of synaptosomes as it has been shown to give best preservation of PSD morphology (Cohen *et al.*, 1977; Matus *et al.*, 1980). Figure 5.1b shows the Coomassie blue profile of the purification procedure. Samples were taken from each stage of the purification and run on a 9% SDS-PAGE gel. The final PSD fraction is comparable with that obtained by both Carlin *et al.*, (1980) and Matus *et al.*, (1980).

(a)



(b)

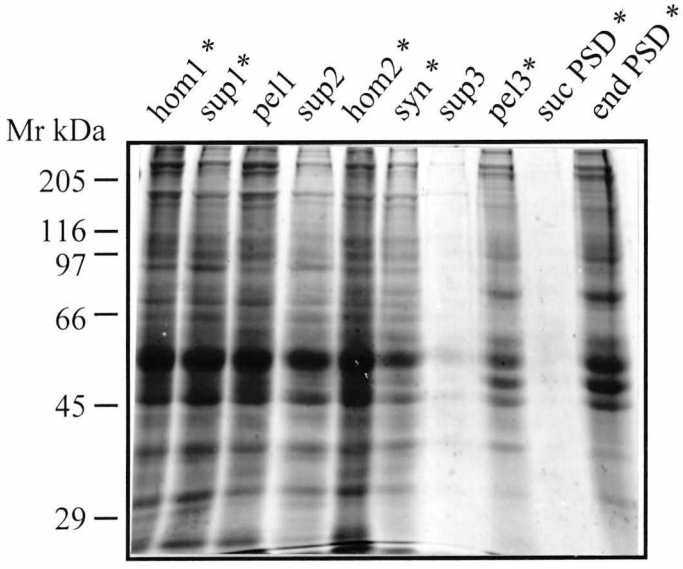


Figure 5.1 Postsynaptic density preparation. (a) Schematic showing the various steps of PSD purification according to Carlin *et al.* (1980). (b) Pig or rat forbrain were used to prepare PSD fractions as outlined in (a). Samples were taken at each step of the preparation and analysed by SDS-PAGE in conjunction with Coomassie blue staining. Samples marked with * were taken on the the next step of the preparation at each stage.

1.2. Characterisation of Postsynaptic Densities

The purified PSD fraction was validated by assessing enrichment of certain known PSD proteins and de-enrichment of non-PSD proteins. Protein specific antibodies were used in immunoblots on equal protein loadings of pig brain homogenate and PSDs. The relative levels of immunoreactive proteins were quantified and expressed in terms of 'fold enrichment'.

Equal protein loadings of brain homogenate and PSDs were determined by densitometry with BioRad GelDoc 2000 Multi-Analyst software (version 1.1). A Coomassie blue stain of the two samples showed enrichment of the major 50kDa PSD protein and an immunoblot with anti-αCaMKII (Santa Cruz Biotechnology) confirmed the identity of this band (Figure 5.2a and b). Subsequent densitometry of the immunoblot showed αCaMKII was enriched in PSDs 2.5 fold over the original brain homogenate. Similar immunoblots with different protein specific antibodies indicated that PSD preparations were enriched in isoforms of the NMDA receptor subunit NR1 (46 and 31 fold over the original brain homogenate) and the PSD specific protein PSD-95 (enriched 14 fold over the brain homogenate) (Figure 5.2c and d). These data confirm that PSD structures were successfully purified using the method of Carlin *et al.* Depletion of GAP43 and the synaptic vesicle associated protein synaptophysin also indicated that PSD preparations were not significantly contaminated with axonal or presynaptic proteins (Figure 5.2e and f)

2. 4.1 Proteins in Postsynaptic Density Preparations

To broadly determine if 4.1 proteins were present in PSD fractions immunoblots were carried out with anti-4.1R/G/B. More exact identification of 4.1 proteins used antibodies specific to 4.1R (*ap*4.1R-CTR), 4.1N and 4.1G. 4.1B isoforms could be identified through a process of elimination.

An immunoblot with anti-4.1R/G/B on samples taken from each step of the PSD preparation revealed as many as six different 4.1 species present across the whole preparation. Three major 4.1 species were found in the final postsynaptic density

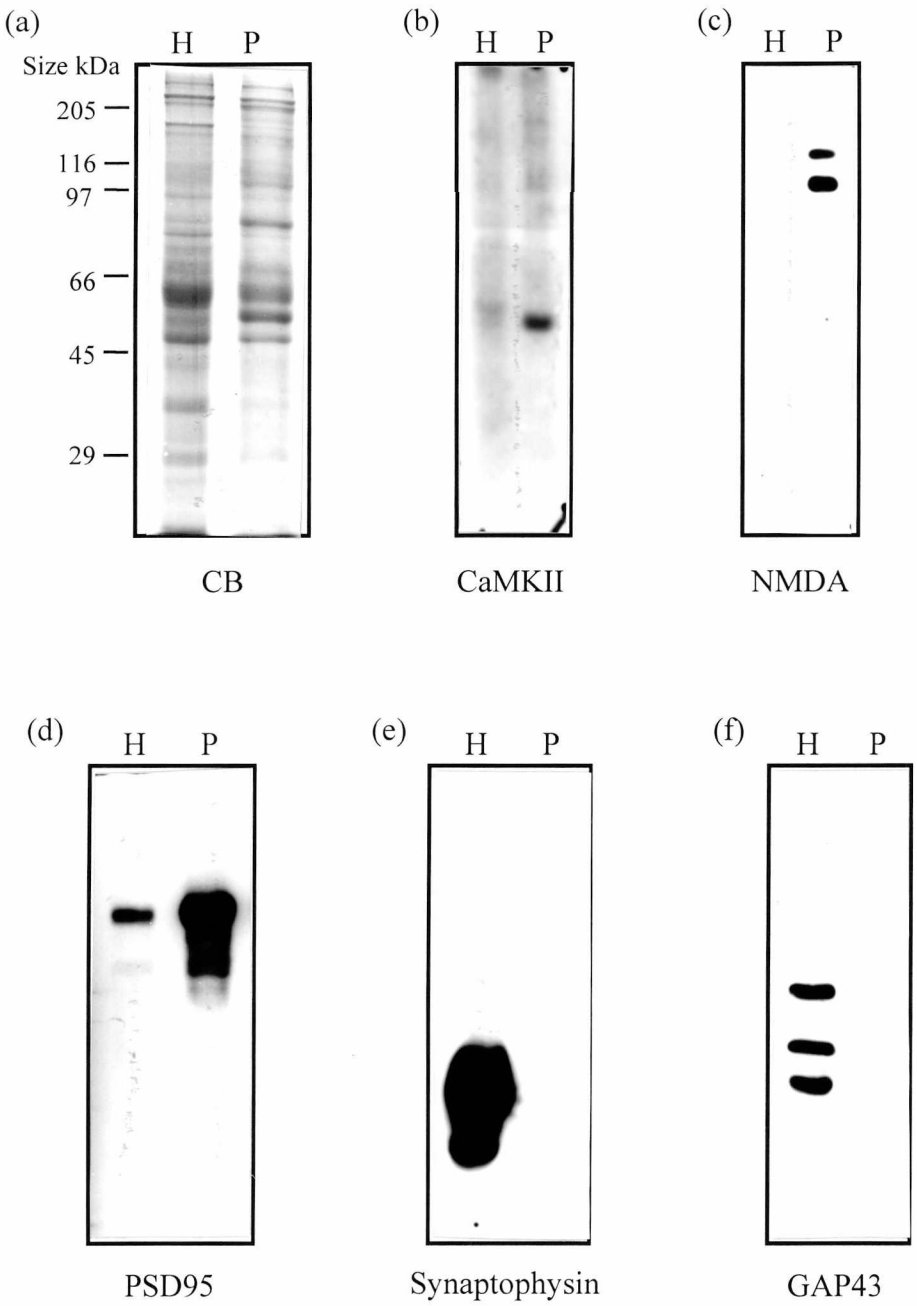


Figure 5.2 Postsynaptic enrichment and de-enrichment of specific neuronal proteins. Pig brain homogenate and PSDs were run on a 9% SDS-PAGE and either Coomassie blue stained to confirm equal protein loadings (a) or immunoblotted with antibodies specific for αCaMKII (b), NMDA receptor NR1 subunit (c), PSD95 (d), synaptophysin (e) and GAP43 (f). PSD fractions were enriched in αCaMKII, NR1 and PSD95 confirming that PSDs had been successfully purified. Depletion of synaptophysin and GAP43 indicated that PSD fractions were not significantly contaminated with presynaptic or axonal proteins.

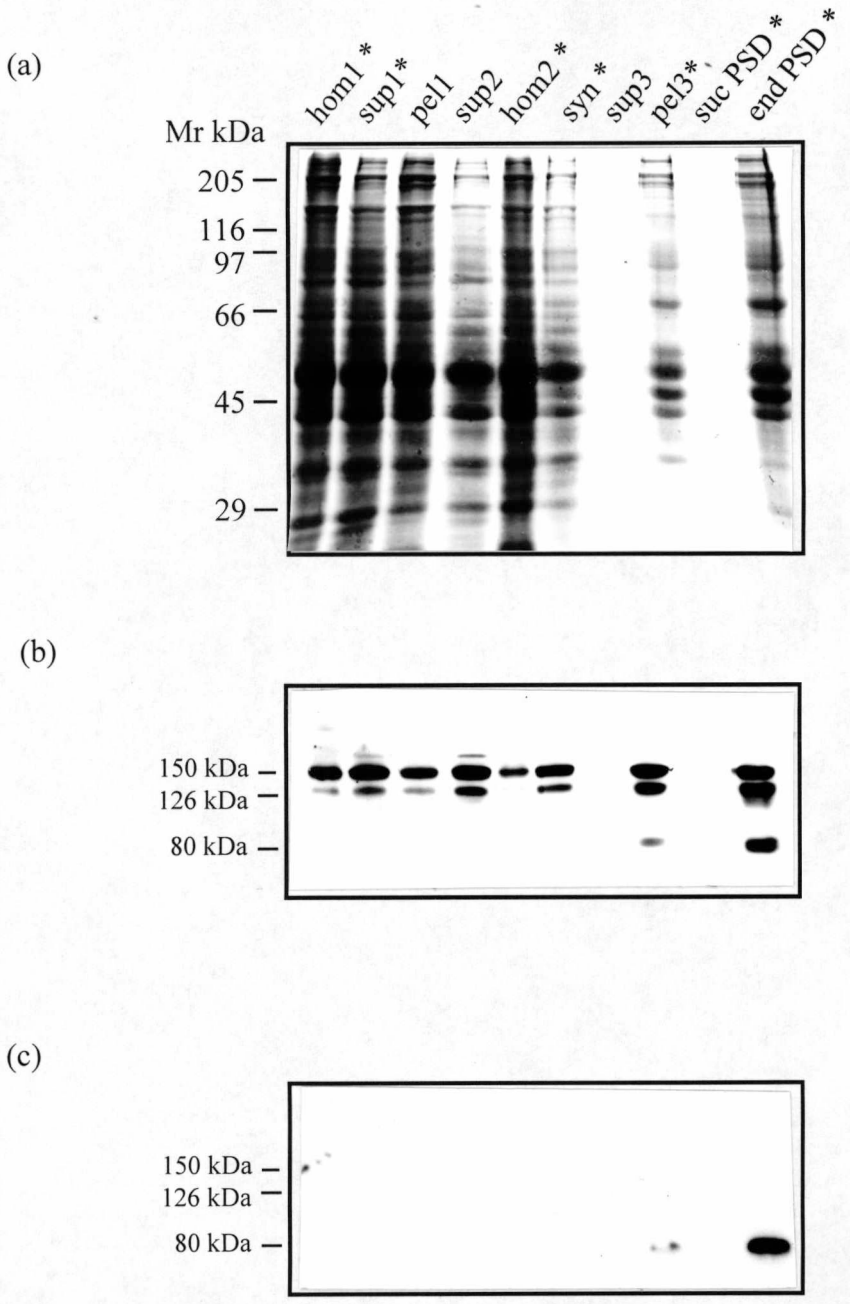


Figure 5.3 Immuno-detection of 4.1 proteins in postsynaptic density fractions. Samples from each step of the PSD preparation were run by SDS-PAGE and stained with Coomassie blue (a), or immunoblotted with anti-4.1R/G/B (b), or anti-*ap*4.1R-CTR (c). Three 4.1 proteins were detected in postsynaptic density fractions. Only the 80kDa protein reacted with the 4.1R specific antibody (panal c). Samples marked with * in (a) were those taken on to the next step of the fractionation process.

fraction; ~150kDa, 126kDa and 80kDa (Figure 5.3). An equivalent blot with anti-*ap*4.1R-CTR detects only the 80kDa band implying that this protein is a product of the 4.1R gene and that the two higher molecular weight species are isoforms of either 4.1B or 4.1G (see 4.1R/G/B specificity Chapter 3, section 5.1). An immunoblot with anti-4.1G demonstrated that neither the 150kDa and 126kDa proteins reacted with anti-4.1G establishing them as isoforms of 4.1B. The 4.1 proteins identified here will be referred to as $4.1R^{80kDa}$, $4.1B^{150kDa}$ and $4.1B^{126kDa}$. This nomenclature will be applied to any 4.1 proteins identified in PSD fractions in future experiments.

Protein 4.1 enrichment in PSD fractions was estimated by immunoblotting equal protein loadings of brain homogenate and PSDs followed by densitometry to quantify the enrichment. Figure 5.4a and b show results obtained when equal protein loadings of brain homogenate and PSDs were immunoblotted with anti-4.1R/G/B and anti-*ap*4.1R-CTR. Quantification of these data indicates that $4.1B^{150kDa}$ is not enriched in the PSD over the original brain homogenate whereas $4.1B^{126kDa}$ and $4.1R^{80kDa}$ are enriched 3 fold and 11 fold respectively. These figures correlate well with the enrichment of αCaMKII (2.5 fold) and PSD95 (14 fold).

Immunoblots with anti-4.1G revealed two isoforms of 4.1G in PSDs; 224kDa and 200kDa respectively (Figure 5.4c). These are present at very low levels and are only detected by anti-4.1R/G/B when the blot is over exposed. Quantification of the over exposed blot indicates that $4.1G^{200kDa}$ is enriched in the PSD fraction 2.6 fold over the original brain homogenate. Anti-4.1N detected four 4.1N isoforms in PSD fractions; 115kDa, 100kDa, 90kDa and 68kDa (Figure 5.4d). Densitometry indicated that each protein was enriched only ~2 fold in the PSD fraction over the original brain homogenate.

2.1. Protein 4.1 Isoelectric Variants in Postsynaptic Densities

IEF of PSDs followed by immunoblotting with anti-4.1R/G/B indicated that four $4.1B^{150kDa}$ isoelectric variants exist (~5.7, 6.1, 6.9 and 7.5 respectively) (Figure 5.5). These variants could represent 4.1B isoforms that differ in their constituent exons but not overall size. Alternatively they may arise from 4.1B glycosylation or methylation.

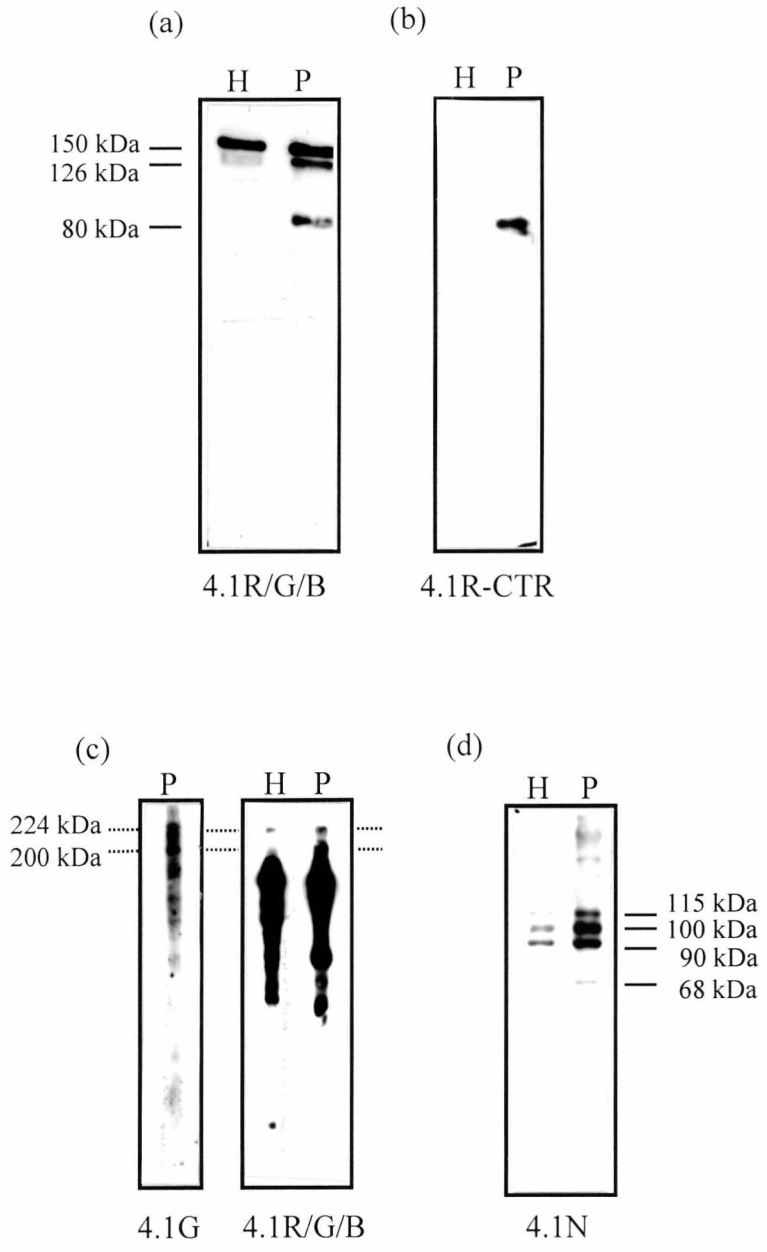


Figure 5.4 Enrichment of 4.1 proteins in postsynaptic density fractions. Equal protein loadings of pig brain homogenate (H) and PSDs (P) were immunoblotted with (a) anti-4.1R/G/B, (b) anti-*ap4*.1R-CTR, (c) anti-4.1G (PSDs only) and (d) anti-4.1N. Various isoforms of 4.1R, G, N and B were enriched in PSD fractions. The extent of this enrichment was determined by densitometry of the immunoblots presented (see text for details). The over exposed anti-4.1R/G/B panel in (c) shows more clearly the enrichment of the 200kDa 4.1G product.

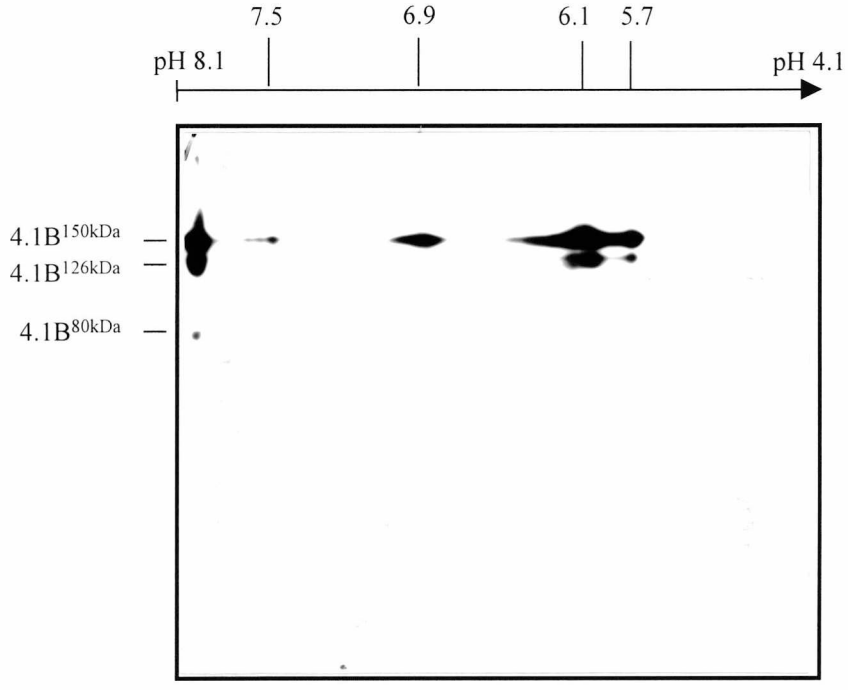


Figure 5.5 Immunoblot with anti-4.1R/G/B on isoelectric focused PSDs. PSDs purifed from pig forebrain were run on two-dimensional IEF gels and immmunoblotted with ani-4.1R/G/B. 4.1B isoforms exist as a number of isoelectric variants. $4.1R^{80kDa}$ was unable to enter the first dimension gel.

The pI difference between each variant is too great to be accounted for by differences in 4.1B phosphorylation. $4.1B^{126kDa}$ exists in two major isoelectric forms, pI 5.7 and 6.1. The similarity in the position and shape of these two species compared to the corresponding $4.1B^{150kDa}$ pI equivalents suggests that each pair is electrostatically similar. The theoretical pI of 4.1B is 5.2 (predicted from the mouse 4.1B sequence (Swissprot Acc: AAD38048) using Swissprot Tools Tag-Ident program). There is no direct correlation between this theoretical value and the values measured *in vitro*. The difference in pI may be due to incomplete focusing of the first dimension gel. Theoretical pI values for known PSD proteins were consistently ~0.6 units lower than the pI value for the same protein determined experimentally (discussed in Chapter 6, section 1.3). 4.1B isoforms focusing at a pI of 5.7 may therefore correspond to the mouse 4.1B protein in the Swissprot database.

Mouse $4.1R^{80}$ (Acc: L00919) is predicted to have a theoretical pI of 6.48. Immunoblotting with anti-4.1R/G/B demonstrated that $4.1R^{80kDa}$ did not enter the IEF first dimension gel (Figure 5.5) and experimental pI determination was not possible. $4.1R^{80kDa}$ may either be bound to PSDs by strong interactions that resist urea disruption or it may exist at the centre of a tight complex where it is inaccessible to IEF solubilisation buffer. To confirm $4.1R^{80kDa}$ did not enter the first dimension, results obtained with anti-4.1 R/G/B were confirmed with anti-*ap*4.1R-CTR (data not shown). Difficulty in solubilising PSDs has been encountered previously (Blomberg *et al.*, 1977; Somerville *et al.*, 1984; Walsh & Kuruc, 1992) and explains why proteinacious material remains at the left side of NEPHGE and IEF gels (equivalent to the top of the first dimension stick).

3. Protein 4.1 in the Postsynaptic Density Core Structure

The non-covalent interactions that exist between PSD proteins differ in strength and stability. Mild detergents such as Triton X-100 disrupt some of these interactions. Others remain intact unless treated with harsh detergents such as sodium N-lauroyl sarcosinate, deoxycholate or lithium diiodosalicylate (Adam & Matus, 1996; Blomberg *et al.*, 1977). This leads to the concept of the core PSD; a postsynaptic junctional

lattice underlying the PSD structure (Chapter 1, section 2.3.3.2). A mixture of 0.5% deoxycholate and 50mM DTT was shown by Blomberg *et al.*, (1977) to release almost all the actin from the PSD structure. Since protein 4.1R is a known actin binding protein there is the possibility that it will be stripped from the PSD at the same time. An immunoblot on the core PSD supernatant and pellet fractions prepared by treatment with deoxycholate and DTT confirmed actin dissociation from PSDs by its presence in the supernatant (Figure 5.6a). Blots with anti-4.1R/G/B and anti-*ap*4.1R-CTR (Figure 5.6b and c) both clearly demonstrate 4.1 in the pellet indicating that 4.1 species are intrinsic components of PSDs and do not accompany actin striped away with deoxycholate and DTT.

4. Post-mortem Effects on Postsynaptic Protein 4.1

A major problem working with PSDs is that not all proteins that co-isolate with them are *in vivo* components. There has been significant debate whether proteins are legitimate PSD components or whether they accumulate artifactually during the purification process. Examples are synapsins, known to be presynaptic from electron microscopy (De Camilli *et al.*, 1983) but always found in PSDs (Walsh & Kuruc, 1992) and tubulin which accumulates at PSDs significantly with increasing time post-mortem (Carlin *et al.*, 1981).

In order to demonstrate that 4.1 proteins do not accumulate post-mortem, PSDs were purified from fresh rat brain. Brains were removed from 15 rats and homogenised within 1 hour of removal for direct use in PSD preparation. As with PSDs purified from pig brain the final product was validated by enrichment of PSD-95 and de-enrichment of GAP43 (Figure 5.7b and c)(this Chapter, section 1.2).

Immunoblots were carried out on equal protein loadings of rat brain homogenate and PSDs using anti-4.1R/G/B and anti-*ap*4.1R-CTR. Results were comparable to those obtained with pig brain PSDs showing three major 4.1 species in the rat PSD fraction (~150kDa, 126kDa and 80kDa respectively) with only the 80kDa species detected with *ap*4.1R-CTR (Figure 5.7d and e).

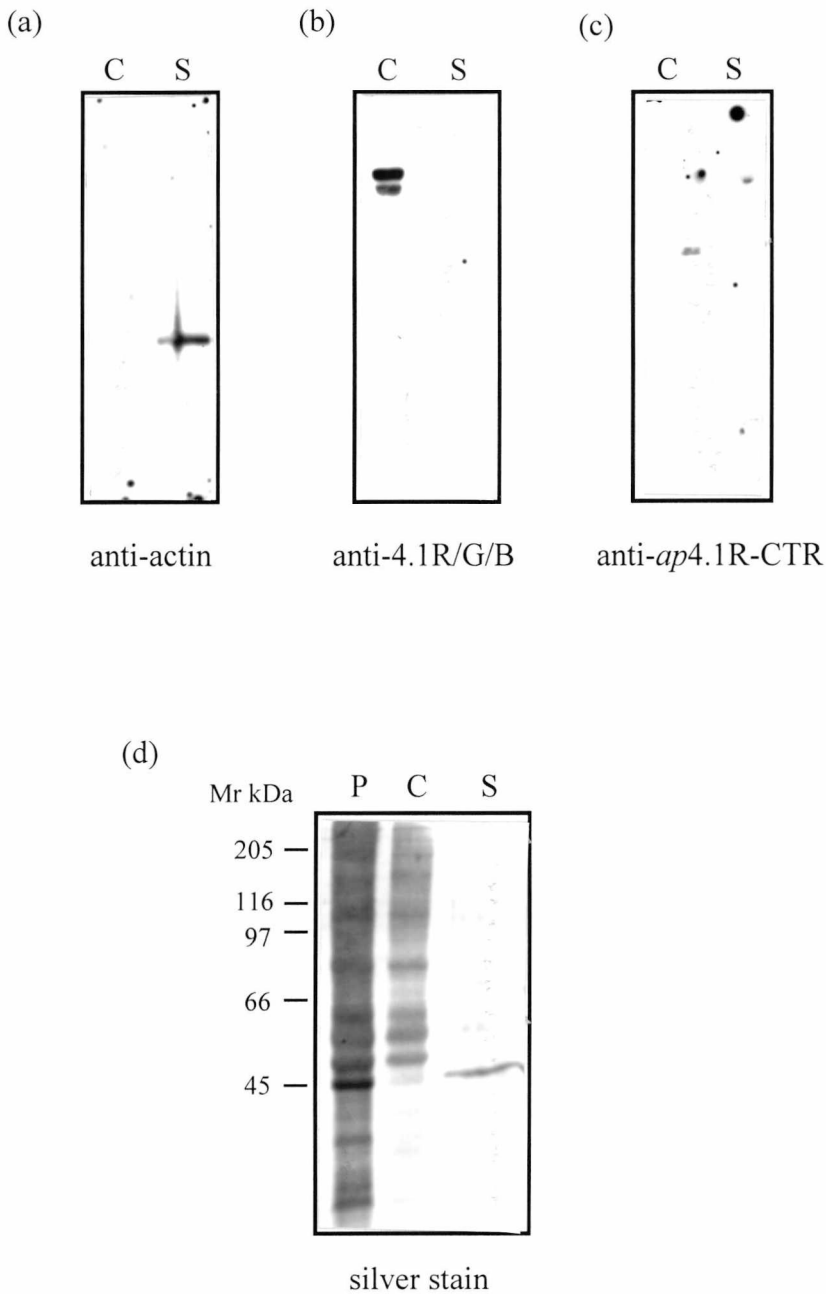


Figure 5.6 4.1 proteins as part of the PSD core. Core PSDs were prepared by treating the PSD fraction with deoxycholate and DTT. The supernatant containing extracted proteins (S) and pellet containing core PSDs (C) were immunoblotted with (a) anti -actin, (b) anti-4.1R/G/B and (c) anti-ap4.1R-CTR. (d) Silver stain of PSDs (P), extracted supernatant and core PSD pellet.

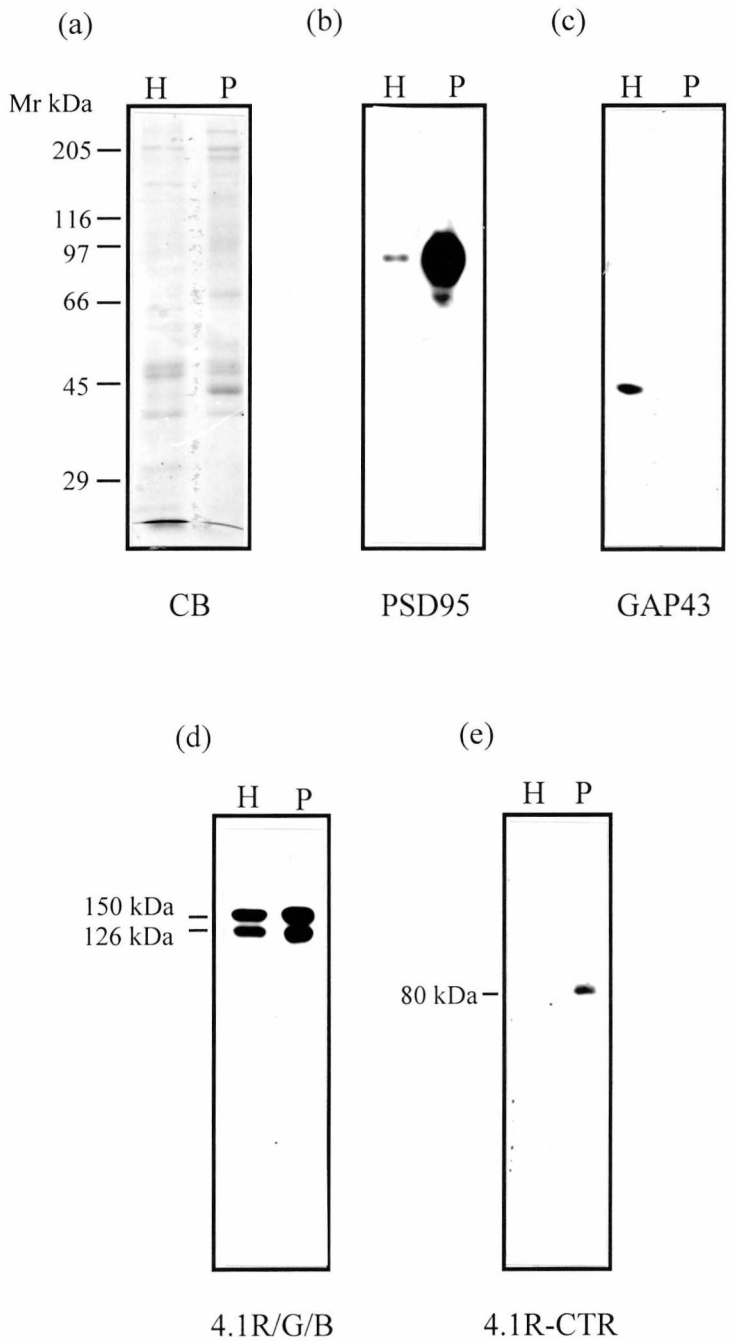


Figure 5.7 Immunoblots on PSDs prepared from fresh rat forebrain. PSDs were prepared according to the method of Carlin *et al.* (1980) using freshly obtained rat forebrain as the starting material. Equal protein loadings of homgenate (H) and PSDs (P) were run on a 9% SDS-PAGE and either stained with Coomassie blue (a) or immunoblotterd with anti-PSD95 (b), anti-GAP43 (c), anti-4.1R/G/B (d) or anti-*ap*4.1R-CTR (e). 4.1B and 4.1R were still detected in the PSD fraction indicating that they do not accumulate at PSDs post-mortem.

These results indicate that 4.1 is not artifactually accumulated at PSDs post-mortem and strengthens the argument that it is a legitimate component of PSDs.

Discussion

A total of nine protein 4.1 species were identified in PSD preparations. Of these, two 4.1B isoforms and one 4.1R isoform purified as intrinsic components of the PSD core and respectively represented the most abundant (4.1B) and most highly enriched (4.1R) 4.1 proteins in isolated PSDs.

Postsynaptic 4.1R

The 11-fold enrichment of $4.1R^{80kDa}$ suggests that this species may be important for PSD function. It is possible that $4.1R^{80kDa}$ represents erythrocyte $4.1R^{80}$ however the exon structure of the PSD product was not determined and an identity between the two proteins cannot be confirmed. Various lines of reasoning support an identity between $4.1R^{80kDa}$ and erythrocyte $4.1R^{80}$: (1) Erythrocyte $4.1R^{80}$ can anchor membrane proteins and form flexible lattice structures. Both of these functions are required at PSDs suggesting that there is a role to be played by $4.1R^{80}$. (2) Both spectrin and actin have been identified at PSDs (Goodman *et al.*, 1995; Ratner & Mahler, 1983). Thus not only are known $4.1R^{80}$ binding proteins present at PSD structures but specifically the proteins involved in $4.1R^{80}$ ternary complex and hence lattice formation.

Postsynaptic 4.1B

$4.1B^{150kDa}$ and $4.1B^{126kDa}$ identified in PSDs are designated according to their reactivity with anti-4.1R/G/B but not with anti-4.1N, anti-4.1G or anti-4.1R (*ap*4.1R-CTR). Full characterisation of the 4.1 protein antibodies (Chapter 3, sections 4 and 5) and peptide competition analysis to confirm the reactivity of anti-4.1R/G/B means that the 4.1B designation is assigned with confidence. The size of the 4.1B products identified in this study agrees closely with 148kDa and 124kDa 4.1B isoforms identified in mouse brain homogenate by Parra *et al.*, (2000) suggesting that these species may be identical. 4.1B proteins are larger than the theoretical 4.1B molecular mass (103kDa) calculated from the full length mouse sequence (Swissprot Acc: AAD38048). This is a common

phenomenon amongst 4.1 proteins (Conboy *et al.*, 1991; Parra *et al.*, 1998; Walensky *et al.*, 1999).

The isoelectric variants of $4.1B^{150kDa}$ and $4.1B^{126kDa}$ are unusual in that they show little, if any difference in molecular mass. Although you would expect alternatively spliced 4.1B isoforms differing in pI to differ in size, it is possible that addition or substitution of small highly charged exons could alter protein pI without shifting molecular mass sufficiently for a difference to be detected on 9% SDS-PAGE. A number of small alternatively spliced exons have been identified with 4.1R (e.g. exon 14; 19 amino acids, exon 15; 14 amino acids, exon 16; 21 amino acids and exon 17a; 17 amino acids) and 4.1G (Keating & Baines, 1999). It is likely that similar sized exons exist in 4.1B.

The variation in 4.1B pI is more plausibly explained by post-translational modifications such as glycosylation, methylation or carbamylation. Carbamylation of lysine residues can occur as an artifact of the IEF procedure and results in multiple protein isoelectric variants. Erythrocyte 4.1R can be carbamylated *in vitro* (Trepanier *et al.*, 1996) however care was taken not to heat PSD samples containing urea above 37°C, the temperature at which urea begins to hydrolyse to isocyanate causing carbamylation to take place. $4.1R^{80}$ has also been shown to contain O-linked N-actyl glucosamine units (Holt *et al.*, 1987; Inaba & Maede, 1989) and along with the MS proteins ankyrin and band 3, is methylated to various degrees *in vivo* (Barber & Clarke, 1983; Galletti *et al.*, 1983; O'Connor & Clarke, 1985). Methylation has been suggested to exist as a mechanism of protein signalling and regulation. For example, nerve growth factor (NGF) induced signal transduction leading to neurite outgrowth in PC12 cells is suggested to involve protein methylation (Cimato *et al.*, 1997). 4.1N has been shown to mediate the anti-proliferative effects of NGF in PC12 cells (Ye *et al.*, 1999) and proteins methylated in response to NGF include species migrating at 120kDa, 94kDa and 68kDa in SDS-PAGE (Cimato *et al.*, 1997). These sizes agree closely with $4.1N^{115kDa}$, $4.1N^{90kDa}$ and $4.1N^{68kDa}$ identified in brain homogenate and PSDs by this study. Methylation of 4.1 proteins may therefore occur *in vivo* and could account for the multiple isoelectric forms of 4.1B.

Postsynaptic 4.1G

The two protein 4.1G isoforms identified in PSDs ($4.1G^{200kDa}$ and $4.1G^{224kDa}$) are both larger than previously reported 4.1G isoforms; ~160kDa, ~140kDa and ~100kDa (Parra *et al.*, 2000; Parra *et al.*, 1998). A 7.3kb 4.1G brain specific mRNA transcript identified by Parra *et al.*, (1998) is theoretically capable of producing proteins of this larger size. The 160kDa, 140kDa and 100kDa 4.1G proteins identified previously in mouse brain homogenate were not detected in this study and may reflect a different isoform specificity of the antibodies involved.

Postsynaptic 4.1N

The 4.1N isoforms identified by this study ($4.1N^{115kDa}$, $4.1N^{100kDa}$, $4.1N^{90kDa}$ and $4.1N^{68kDa}$) exist in the same size range as 4.1N proteins identified previously (207kDa - 66kDa (Ohara *et al.*, 1999; Walensky *et al.*, 1999; Yamakawa *et al.*, 1999). The subset of 4.1N isoforms in brain is currently ambiguous and different authors report 4.1N mRNA and protein species of different sizes (Table 8). Elucidation of the exon structure of the various products will determine if any are identical or whether each represents a specific 4.1N splice form.

BRAIN 4.1N SPECIES IDENTIFIED	REFERENCE
mRNA transcripts – 8.8kb, 7.5kb, 4.1kb Proteins – 165kDa, 135kDa, 100kDa	Walensky *et al.*, (1999)
mRNA transcripts – 8.7kb, 6.2kb, 3.9kb Proteins – 207kDa, 125kDa, 110kDa	Yamakawa *et al.*, (1999)
Proteins – 207kDa, 125kDa, 110kDa, 66kDa	Ohara *et al.*, (1999)
Proteins – 115kDa, 100kDa, 90kDa, 68kDa	This study

Table 8. List of 4.1N mRNA and protein products identified in brain to date.
The mRNA products correlate more closely than the protein products identified. Because 4.1 genes are subject to extensive alternative splicing it is difficult to know if any of the products identified are identical based on their sizes alone.

Differences in the complement of 4.1N isoforms observed in brain could be affected by probe/antibody specificity (Walensky *et al.*, (1999) vs Yamakawa *et al.*, (1999) and this study), the brain region used in the study (Ohara *et al.*, 1999), the exact stage of brain development (Ohara *et al.*, 1999) and the extent to which blots are exposed. Species differences may also be observed.

4.1N and 4.1B both exist at regions of cell-cell contact in the brain (Parra *et al.*, 2000; Walensky *et al.*, 1999). 4.1N has been found to co-localise with PSD95 and GluR1 in cultured hippocampal neurons (Walensky *et al.*, 1999) substantiating the finding that 4.1N is a component of PSD preparations. Recently, Ohara *et al.*, (1999) described a 'brain 4.1' protein that appears to be identical to 4.1N. They localise this protein specifically to presynaptic regions in certain cerebellar neurons. Carlin *et al.*, (1980) noted differences between cerebellar and forebrain (cerebrum/midbrain/hippocampus) synapses, in particular the morphology of their PSDs; cerebellar PSDs are predominantly Gray type II (asymmetric, inhibitory) while forebrain PSDs are Gray type I (symmetric, excitatory). Differences are also noted in the composition of the two types of PSD (Aoki *et al.*, 1985; Carlin & Siekevitz, 1984; Gordon-Weeks & Harding, 1983; Hill *et al.*, 1988; Kim *et al.*, 1992; Trifiletti & Snyder, 1985) highlighted by the fact that αCaMKII, the major PSD protein in forebrain, is virtually absent in cerebellum PSDs (Carlin *et al.*, 1980). Thus synapses in cerebellum and forebrain do not appear to be equivalent and the fact that 4.1N localises to presynaptic sites in cerebellum does not exclude the possibility that it exists at postsynaptic sites in forebrain. PSD95 (also known as SAP90) localises to certain presynaptic nerve terminals in cerebellum (Kistner *et al.*, 1993) but is postsynaptic in forebrain (Hunt *et al.*, 1996). Similarly, the NMDA receptor subunits NR2A and NR2B differentially distribute between forebrain and cerebellum PSDs (Jin *et al.*, 1997). Conversely, the results presented do not discount a role for 4.1 proteins at presynaptic sites. The MAGUK proteins hDlg (also known as SAP97) and CASK appear to distribute to both pre- and post- synaptic nerve terminals in excitatory synapses (Hsueh *et al.*, 1998; Leonard *et al.*, 1998; Muller *et al.*, 1995). Similary, spectrin has been localised to both pre- and post- synaptic sites (Malchiodi-Albedi *et al.*, 1993; Zagon *et al.*, 1986). Diversity among 4.1 proteins

suggests that different isoforms could localise to different subcellular domains in a similar way to the targeting of spectrin and ankyrin isoforms in brain.

4.1 proteins as part of the PSD core

Isolation of the PSD 'core' by stripping more loosely attached proteins with the strong ionic detergent deoxycholate and the reducing agent DTT indicated that the three major 4.1 species were intrinsic components of the PSD. Retention of $4.1R^{80kDa}$, $4.1B^{150kDa}$ and $4.1B^{126kDa}$ under conditions that remove actin (Blomberg *et al.*, 1977) indicates that these proteins associate with other core components of PSDs. It is perhaps surprising that no $4.1R^{80kDa}$ was extractable with actin given that spectrin-actin binding might be an important function of this isoform. $4.1R^{80kDa}$ may have multiple functions at PSDs and in addition to binding spectrin and actin could be important in organising the PSD core. The interactions with core PSD proteins may be strong enough to retain $4.1R^{80kDa}$ at the PSD in spite of actin removal.

A recent report suggests that compact, detergent resistant PSD core structures are a purification artifact resulting from non-specific inter-protein disulphide bond formation during PSD isolation (Lai *et al.*, 1999). Cytoskeletal proteins seem to be particularly susceptible to oxidisation and the presence of free cysteine residues in the MBD of 4.1R and the equivalent region of 4.1B suggests that these proteins could be retained in the PSD core artifactually. In this study PSDs were stripped using deoxycholate in the presence of DTT suggesting that artifactual retention of 4.1 proteins due to disulphide bond formation is unlikely. Isolation of PSDs in the presence of iodoacetamide to maintain only disulphide linkages present *in vivo* should provide a more definitive result.

Postsynaptic density protein artifacts

Postsynaptic densities are notoriously 'sticky' complexes. There has been significant debate as to whether proteins are legitimate PSD components or whether they accumulate artifactually during the purification process. Tubulin has been shown to artifactually accumulate at the PSD with increasing time post-mortem although it is still considered to be an intrinsic component of PSDs at low concentrations. For example,

canine PSDs showed a tubulin increase from 3% of total PSD protein when isolated at 0 hours post-mortem to 23% when isolated 8 hours after death (Carlin *et al.*, 1982). Matus *et al.*, (1980) have also suggested that neurofilament protein and the glial cytoplasmic filament protein GFAP contaminate isolated PSD structures, showing that neither protein is detectable at PSDs in immunoperoxidase stained perfused rat brain. The protein composition of PSDs has also been shown to alter *in vivo* as a result of transient cerebral ischemia (Hu *et al.*, 1998) thus the composition of PSDs isolated may vary according to the exact neuronal state of the animal involved.

4.1 proteins do not appear to accumulate at PSDs as post-mortem artifacts. This is indicated by the fact that PSDs isolated from frozen pig brain and fresh rat brain give identical 4.1B and 4.1R staining patterns. The possibility that 4.1 proteins contaminate PSD preparations as a consequence of the isolation procedure can not be completely ruled out and synaptic vesicle membrane proteins (3.7%), myelin proteins (0.1%) and mitochondrial fraction proteins (6.5%) have all been shown to contaminate isolated PSDs in the past (Cohen *et al.*, 1977). The PSD fractions used in this study were shown to fulfil normal characterisation criteria and were not unusually contaminated by non-PSD proteins (e.g. synaptophysin and GAP43). In addition they were enriched in the postsynaptic density protein PSD95 and contained significant levels of αCaMKII, as identified by immunoblotting. To confirm the localisation of 4.1 proteins at PSDs subsequent research should concentrate on demonstrating this with immunocytochemical techniques (i.e. immunofluorescence on cultured cells and/or immuno-electron microscopy on brain sections).

In summary, the results presented suggest that multiple 4.1 gene products exist at PSDs. The specific PSD related functions of the 4.1 proteins are not known however they could include localisation and anchoring of membrane proteins, formation of lattice structures and neuronal signalling. The latter is inferred by the role 4.1N plays in neuronal responses to NGF (Ye *et al.*, 1999) and the function of DAL1, a 4.1B homolog, as a tumour suppresser (Tran *et al.*, 1999).

Chapter 6

Interactions of Protein 4.1R at Postsynaptic Densities

Introduction

Although the detailed structure of excitatory PSDs has undergone intense study over the last two decades, the molecular organisation remains vague. Large numbers of novel and known proteins have recently been identified as PSD components and although binding partners for most of these have been suggested, the overall picture of how PSDs formulate is unclear (for reviews see Ziff (1997), Kennedy (1998) and Kim & Huganir (1999)). Complicating matters further is the fact that dendritic spines (which contain high concentrations of PSDs) are highly dynamic structures modulating their shape and number in response to excitation and signals thought to confer long term potentiation and depression (van Rossum & Hanisch, 1999). A similar lability must be reflected in PSDs and it is likely that many of the protein-protein interactions that occur at are adaptable and subject to tight regulatory control.

Most proteins accomplish their function by interacting with other proteins. These interactions may be transient or exist on a more permanent basis. If 4.1 proteins are legitimate PSD components they must bind to other PSD proteins to effect a function. The vast enrichment of $4.1R^{80kDa}$ suggests that this isoform in particular plays a fundamental role in PSD structure. The remainder of this study concentrates on identifying binding activities of 4.1R with other PSD proteins. The presence of both spectrin and actin at PSDs (Carlin, Bartelt & Siekevitz (1983), Malchiodi-Albedi *et al.*, (1993), Matus *et al.*, (1982), this study) implies that $4.1R^{80kDa}$ may be involved in ternary complex formation and the production of a lattice-like structure for supporting the neuronal membrane at these sites. In addition, the MBD of 4.1R is known to interact with various membrane proteins (e.g. band 3 (Hemming *et al.*, 1995), GPC (Marfatia *et al.*, 1994), CD44 (Nunomura *et al.*, 1997b), PICIn (Tang & Tang, 1998)) and it is likely that this domain interacts with a similar class of protein at PSDs. Recently identified binding activities of the CTD of 4.1R (e.g. NuMA, ZO-2, EF1α, eIF3, Chapter 1, section 3.1.5.3) suggest that this too has the potential to interact with PSD proteins. C-terminal interactions in conjunction with N-terminal interactions would allow 4.1 to function as an anchoring molecule or as a bridging molecule between proteins needing to be in close proximity.

1. Identification of 4.1R-CTR Binding Interactions with Postsynaptic Density Proteins by Blot Overlay

A postsynaptic role for the C-terminal of 4.1R was investigated by examining the binding activities of this region with proteins present in PSD fractions isolated from pig and rat forebrain. Recombinant 4.1R-CTR (produced as outlined in Chapter 3) was used in one-dimensional and two-dimensional gel electrophoresis blot overlays to assess potential interactions between this region and proteins present at PSDs. The blot overlay technique is a well established tool for analysing cytoskeletal protein interactions (Dransfield *et al.*, 1997; Duval *et al.*, 1995; Edelstein *et al.*, 1988; Igarashi *et al.*, 1992; Kremer *et al.*, 1988; Marfatia *et al.*, 1997; Riederer & Goodman, 1987) and has been used to study neuronal protein associations in the past (e.g. NMDA receptor subunit NR2B-PTPD1 interactions (Lin *et al.*, 1999), GAP43-spectrin interactions (Riederer & Routtenberg, 1999) and binding of syndapin I to dynamin I, synaptojanin, and synapsin I (Qualmann *et al.*, 1999)). It is therefore an attractive technique to use in the study of 4.1R interactions with PSD components.

1.1 Blot Overlays on One-Dimensional SDS-Polyacrylamide Gels

Blot overlays were performed by transferring PSD fractions to nitrocellulose membrane and incubating them with 4.1R-CTR. This allowed specific interactions to take place between the recombinant polypeptide and PSD components containing a 4.1R-CTR binding site. After washing the membrane thoroughly to remove non-specifically bound 4.1R-CTR, specific binding interactions were detected using anti-4.1R-CTR or anti-4.1R/G/B and an appropriate secondary antibody conjugated to horseradish peroxidase. Blot overlays on pig forebrain PSDs with 4.1R-CTR and anti 4.1R/G/B detected up to ten potential 4.1R-CTR binding proteins approximately 316kDa 251kDa, 223kDa, 188kDa, 114kDa, 100kDa, 75kDa, 71kDa, 60kDa and 53kDa in size (Figure 6.1a). An overlay using anti *ap*4.1R-CTR gave similar results with an additional binding protein at approximately 141kDa (Figure 6.1b). This was masked by endogenous 4.1B isoforms in anti 4.1R/G/B overlays. The 251kDa band probably corresponds to brain spectrin, which binds the SAB domain of recombinant 4.1R-CTR (Chapter 3, section 3.2). Immunoblots with a general brain spectrin antibody and an

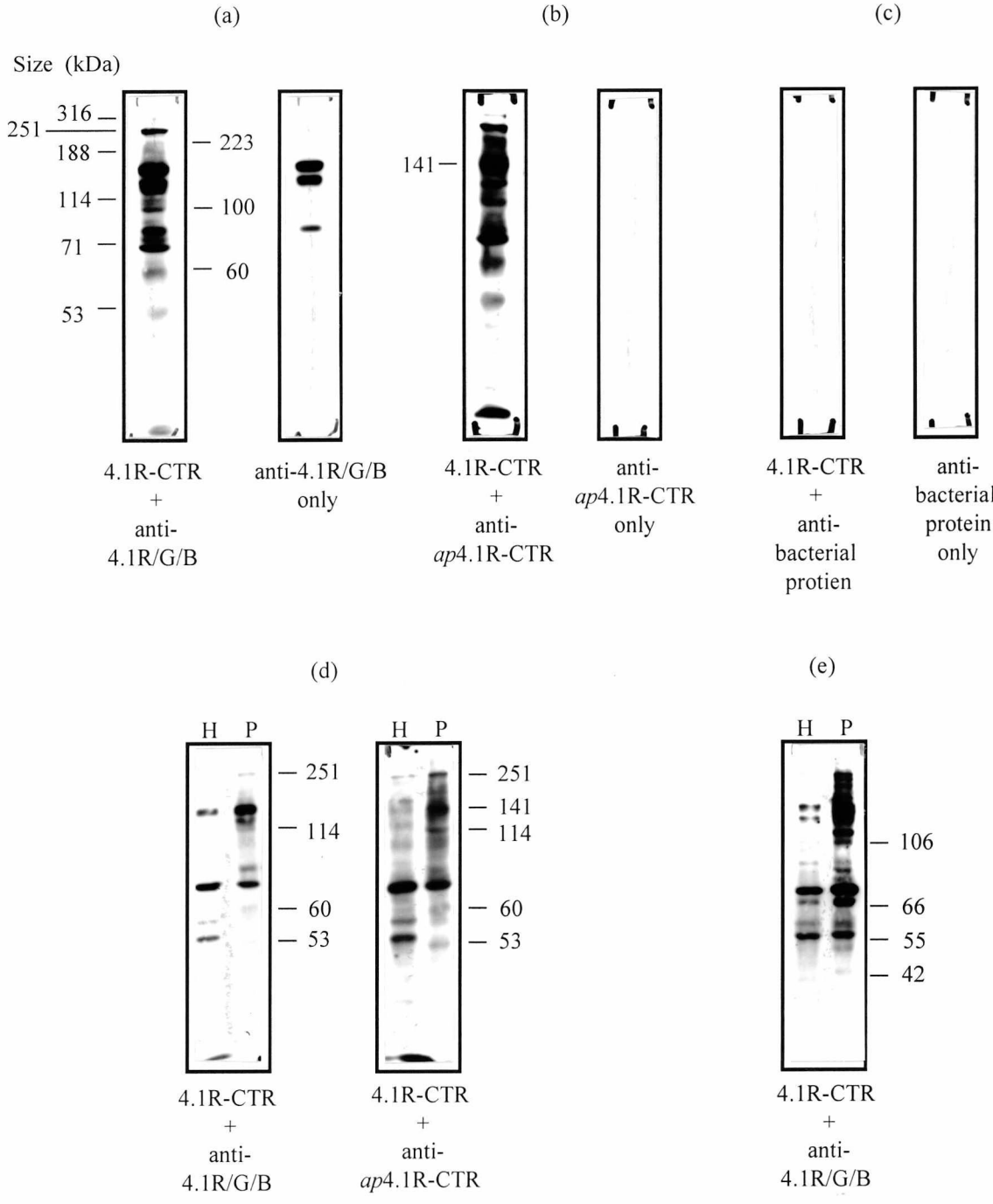


Figure 6.1 Blot overlays on forebrain PSDs with recombinant 4.1R-CTR. PSDs transferred to nitrocellulose were incubated with 4.1R-CTR to determine the complement of PSD binding proteins. Bound 4.1R-CTR was detected with anti-4.1R/G/B or anti-ap4.1R-CTR, several potential 4.1R-CTR binding proteins were identified. (a) and (b) blot overlays on pig PSDs, (c) control overlay for anti-*ap*4.1R-CTR with the bacterial contaminating protein antibody (d) blot overlays on equal protein loadings of pig brain homogenate (H) and PSDs (P), (e) as (d) but with rat forebrain samples. Potential 4.1R-CTR binding protein sizes are shown in kDa.

antibody specific for the βIIΣI subunit confirmed the presence of spectrin in PSD preparations (data not shown). Binding of 4.1R-CTR to spectrin confirms the effectiveness of the overlay technique.

To determine which binding interactions were enriched in PSD fractions, overlays were carried out using equal protein loadings of pig brain homogenate and PSDs. Binding of 4.1R-CTR to the 251kDa, 141kDa, 114kDa, 60kDa and 53kDa proteins was greater in the PSD fraction than in original brain homogenate (Figure 6.1d). Enrichment of binding indicates enrichment of the binding protein and highlights the 4.1R-CTR interactions that are most likely to have physiological relevance. Identical overlays on freshly prepared rat brain PSDs demonstrated additional binding proteins at ~106kDa, 66kDa, 55kDa and 42kDa and an absence of the 316kDa and 100kDa proteins (Figure 6.1e). Over exposed blots to emphasise the fainter bands, revealed that many of the rat PSD protein-4.1R-CTR binding interactions were enriched in the PSD fraction over the original brain homogenate. Enriched binding activities were most prominent for the 66kDa, 71kDa, 114kDa and 141kDa proteins. Differences in the complement of 4.1R-CTR binding proteins in rat and pig brain PSDs may reflect genuine differences between the two species (Gurd *et al.*, 1982). Alternatively they could reflect the processing of pig brains prior to purification and may be a result of, post-mortem degradation of proteins prior to brain freezing, modification of proteins as a consequence of the freezing process or postsynaptic accumulation of protein artifacts (Carlin *et al.*, 1982).

1.2. Verification of Blot Overlay Specificity

Non-specific protein-protein interactions are common *in vitro.* This is particularly true when the tertiary structure of one potential binding partner has been disrupted as in the case of blot overlays. High salt washes and dilution of antibodies in blocking solution can heighten stringency and increase the specificity of the assay but binding artifacts can still remain. An authentic interaction is only demonstrated with this technique if binding is abolished when both binding partners are in a denatured form.

To validate the specificity of 4.1R-CTR binding to PSD proteins a blot overlay was performed using denatured 4.1R-CTR polypeptide. The denatured polypeptide was produced by diluting 4.1R-CTR to the desired concentration and heating the solution to 100°C for 25 minutes. The solution was allowed to cool before using it in overlay assays in place of normal active 4.1R-CTR. All previously observed 4.1R-CTR–PSD protein binding interactions were eliminated in the presence of denatured 4.1R-CTR (Figure 6.2) and only endogenous 4.1 isoforms were detected by anti-*ap*4.1R-CTR and anti-4.1R/G/B. This confirms that binding requires 4.1R-CTR higher order structure and suggests that a specific 4.1R-CTR binding site is involved.

1.3. Blot Overlays on Two-Dimensional SDS-Polyacrylamide Gels

Mass and pI information can help identify proteins provided they have been characterised previously. NEPHGE and IEF are two-dimensional electrophoresis techniques that can be used to separate complex mixtures of proteins and provide mass and pI data on the individual protein components (O'Farrell, 1975; O'Farrell *et al.*, 1977). These techniques were used to obtain further information about the 4.1R-CTR binding proteins identified in PSDs by one-dimension blot overlays.

NEPHGE was the method of choice for PSD protein analysis by Walsh & Kuruc (1992). They suggested that the large number of neutral to basic components made it easier for proteins to enter the first dimension gel and that a better protein distribution was obtained as a result. In the present study NEPHGE used in conjunction with blot overlays gave a result that was difficult to interpret (data not shown). Similar problems were not experienced with IEF and the results obtained were sharper and more reproducible under the conditions used here. Problems with proteins not entering the first dimension gel were not encountered because of the basicity of the proteins but because of the insolubility of PSDs. Difficulty in solubilising PSDs has been encountered previously (Blomberg *et al.*, 1977; Somerville *et al.*, 1984; Walsh & Kuruc, 1992). PSD proteins distributed under NEPHGE and IEF conditions gave a similar, but reversed pattern (Figure 6.3). Named proteins are identified by comparison with Walsh & Kuruc (1992) (Appendix I).

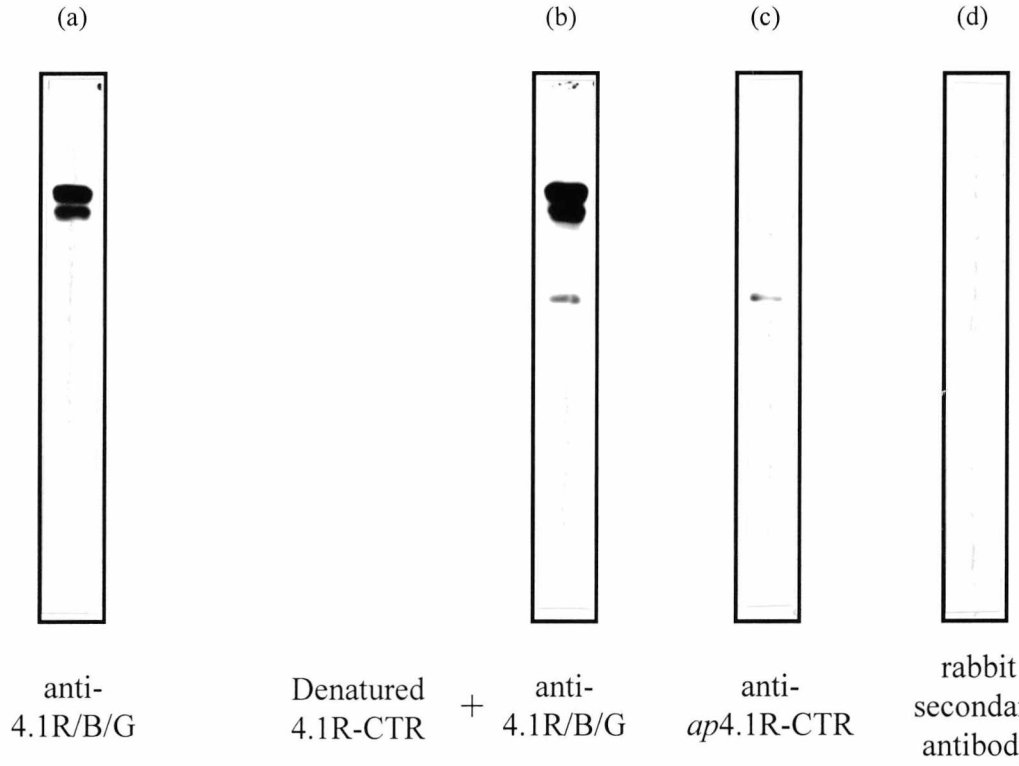


Figure 6.2 Blot overlay on PSDs with denatured 4.1R-CTR. 4.1R-CTR was denatured by heating to 100°C for 25 minutes prior to use in blot overlays. (a) Normal immunoblot on PSDs with anti-4.1R/G/B exposed to detect endogenous 4.1R and B proteins. (b)-(d) Blot overlays on PSDs with denatured 4.1R-CTR plus (b) anti-4.1R-CTR, (c) anti-*ap*4.1R-CTR and (d) rabbit secondary antibody only. No 4.1R-CTR-PSD protein binding interactions were observed with denatured 4.1R-CTR.

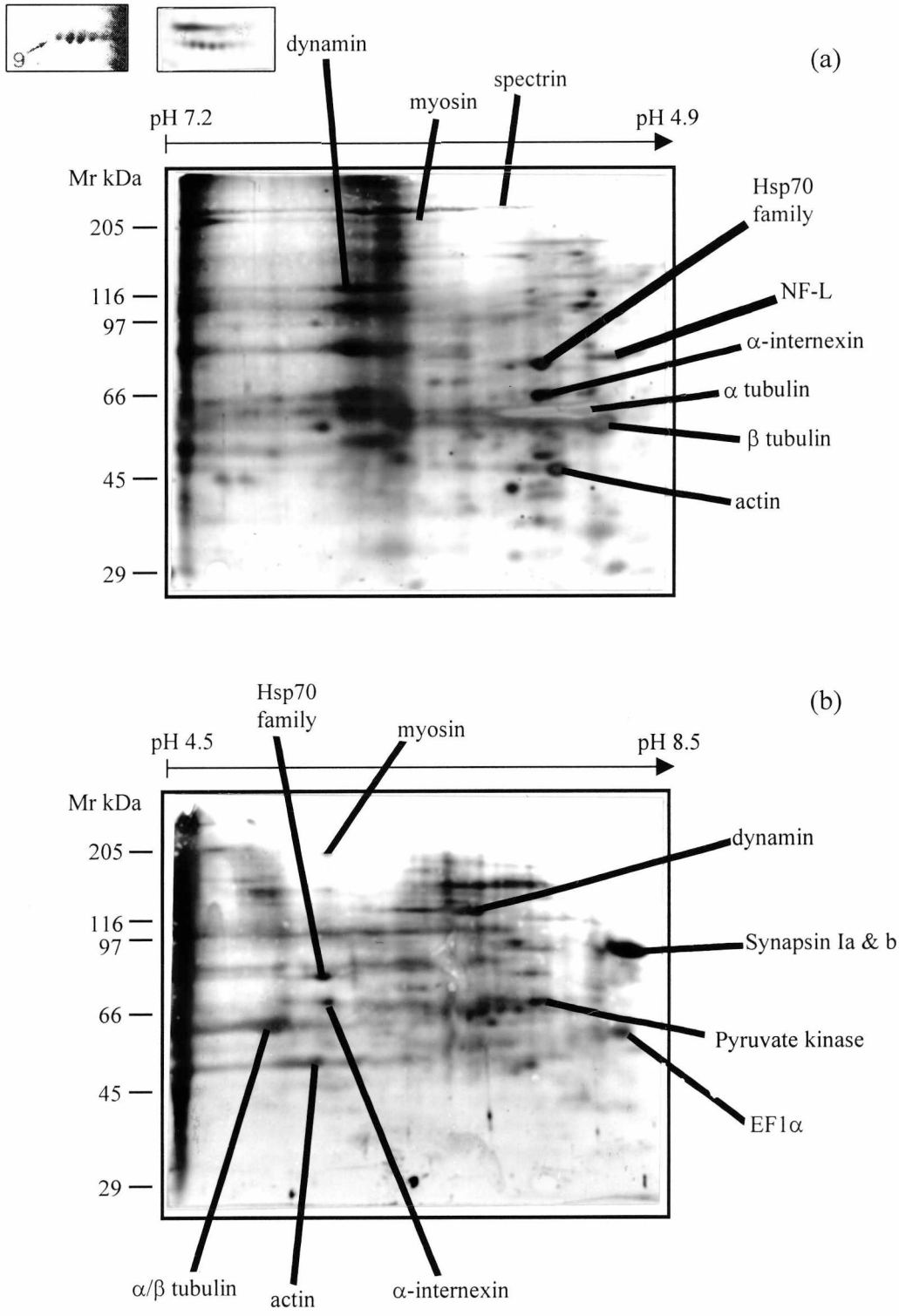


Figure 6.3 Two-dimensional electrophoretic separation of pig forebrain PSDs. (a) IEF, (b) NEPHGE. Gels were Western blotted and stained with colloidal gold. Proteins are tentatively identified by comparison with Walsh and Kuruc, 1992. Inset boxes: Resolution of protein thought to be dynamin on IEF using an immobilised pH gradient (right). The punctate separation of protein phosphoforms compares well to spots identified as dymanin by Walsh & Kuruc (1992) (shown in left panel. Reproduced from Walsh & Kuruc, 1992).

Blot overlays on PSDs separated by IEF were carried out using 4.1R-CTR and anti-4.1R/G/B (Figure 6.4). To aid protein identification the nitrocellulose was stained with colloidal gold on completion of blotting to reveal the total complement of PSD proteins and their distribution. Multiple 4.1R-CTR binding proteins were detected using the two-dimensional blot overlay technique. The sizes of these proteins were consistent with binding proteins identified by one-dimension blot overlays previously. The PSD protein distribution observed in this study was similar to the distribution seen by Walsh & Kuruc (1992) (Appendix I). These similarities were exploited to tentatively identify some of the 4.1R-CTR binding proteins. Experimentally determined molecular mass and pI values were compared with theoretical values determined using SwissProt Tools[3] to further evaluate these identifications. The results from this analysis are summarised in Table 9. Theoretical mass and pI values often differ from values determined experimentally. These differences are often a result of protein post-translational modification *in vivo* as theoretical calculations do not account for these alterations and are based on primary amino acid sequence alone. Both α-internexin and NF-L have been shown to have experimental pI values of 5.8 and 5.3 previously (Pachter & Liem, 1985) and not 5.2 and 4.6 as suggested from their sequences. In addition, NF-L has a relative molecular mass of 68-72kDa (Geisler *et al.*, 1985) not 62kDa as calculated theoretically. These previously determined values for α-internexin and NF-L agree closely with the values recorded in this study (α-internexin pI 5.8, NF-L pI 5.2 and Mr 71kDa) reinforcing the identity of the 60kDa and 71kDa proteins.

Various 4.1R-CTR binding proteins could not be recognised by comparison with Walsh & Kuruc (1992). Identities for these were predicted using the SwissProt Tools program 'Tag-Ident' but no significant identifications were made. Data obtained this way is of limited value because theoretically calculated protein mass and pI values often differ to the values recorded by experimental means.

[3] http://www.expasy.ch/

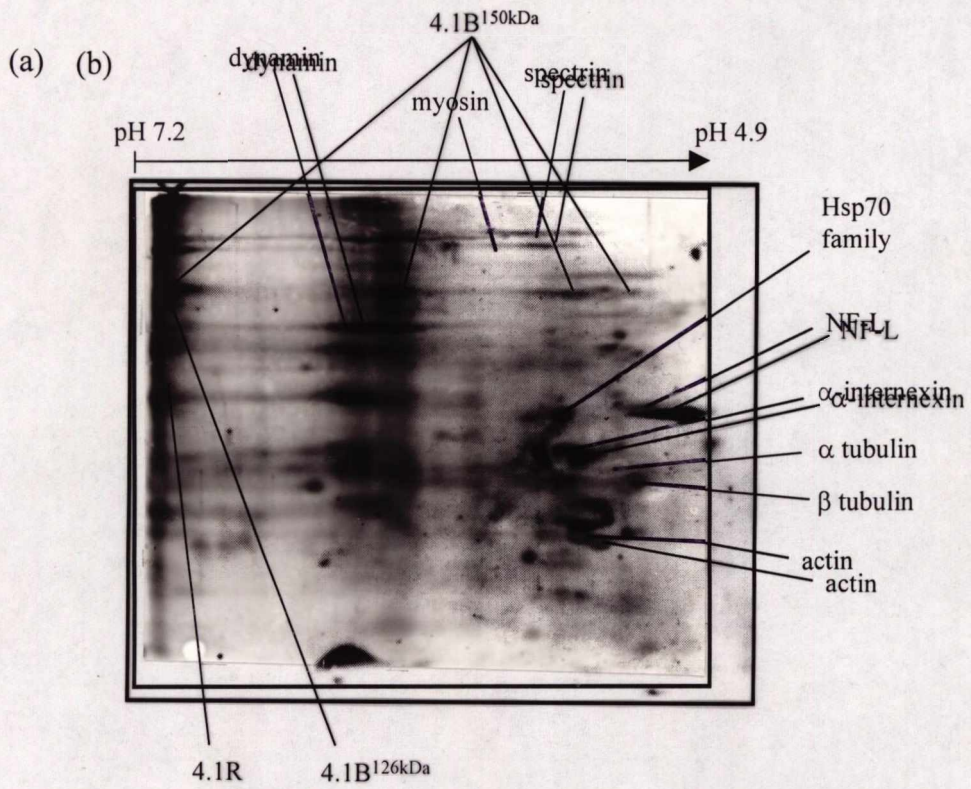


Figure 6.4 Blot overlay with 4.1R-CTR on IEF PSDs. Pig PSDs were separated by two-dimensional electrophoresis, blotted and taken through the 4.1R-CTR blot overlay procedure (b). On completion of the overlay, the nitrocellulose was stained for total protein using colloidal gold. Several PSD proteins appeared to interact with 4.1R-CTR. The identities of these binding proteins are tentatively suggested by comparison with the two-dimensional gels of Walsh & Kuruc (1992).

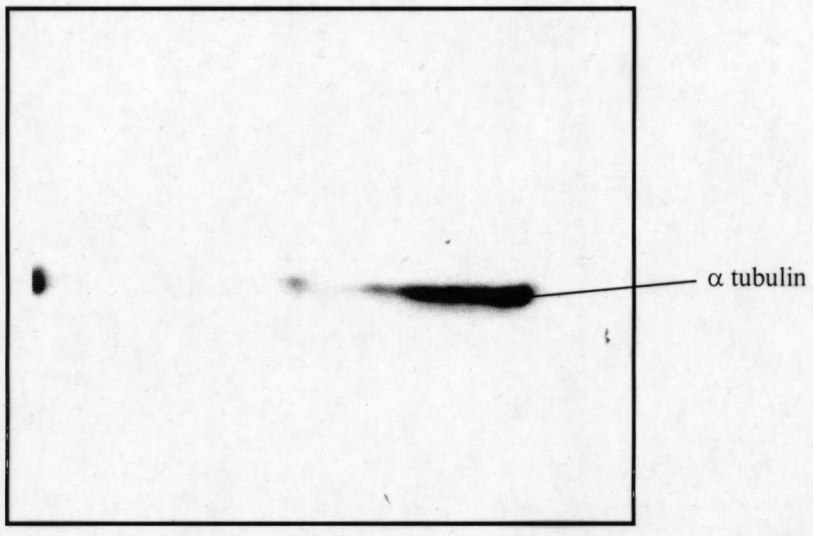
Mr	Experimental pI	Theoretical Molecular Mass	Theoretical pI	dentity	SwissProt Accession Number
251kDa	6.2	246kDa	5.13	Spectrin	P11277
223kDa	6.1	223kDa	5.6	Myosin HC	Q9Y623
114kDa	6.75-6.9	97kDa	6.9	Dynamin	Q05193
71kDa	5.2	62kDa	4.6	NF-L	P02547
60kDa	5.8	56-58kDa	5.2	α-internexin	P23565
53kDa	5.2	50-54kDa	6.9	αCaMKII?	P11798
45kDa	5.6	42kDa	5.3	Actin	P02570

Table 9. Identification of potential candidate 4.1R-CTR binding proteins in PSDs. Mr and pI values were calculated for each gel spot from the IEF blot overlay in Figure 6.4. Proteins were identified by comparing them to NEPHGE data and protein identifications made by Walsh & Kuruc (1992). Identification based on pI was tentative and the pI of various proteins was found to be 0.6 units less then the theoretical value suggesting that the gel may not have focused fully. The theoretical molecular weight and pI were calculated with the SwissProt Tools, 'Compute MW/pI' facility using the protein accession number given.

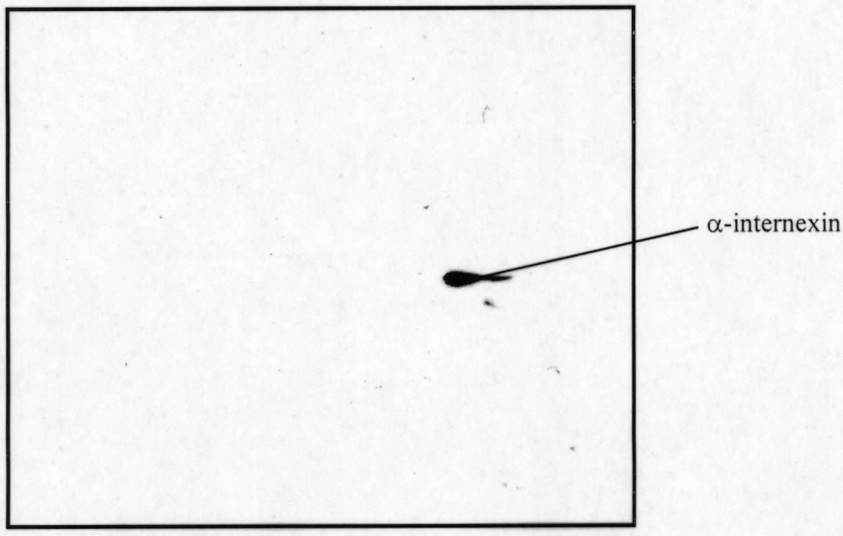
1.3. Confirmation of 4.1R-CTR Binding Proteins: NF-L, α Internexin and Actin

Two-dimension gel analysis suggested that recombinant 4.1R-CTR bound to NF-L, α-internexin and actin. To confirm that the 71kDa, 60kDa and 42kDa 4.1R-CTR binding proteins were NF-L, α-internexin and actin respectively, immunoblots were carried out on PSDs with protein specific antibodies. Alignment of immunoblots with gold stained two-dimension blots and blot overlays demonstrated that each protein shared identity with a 4.1R-CTR binding protein (Figure 6.5). An equivalent blot with anti-tubulin

(f)



(e)



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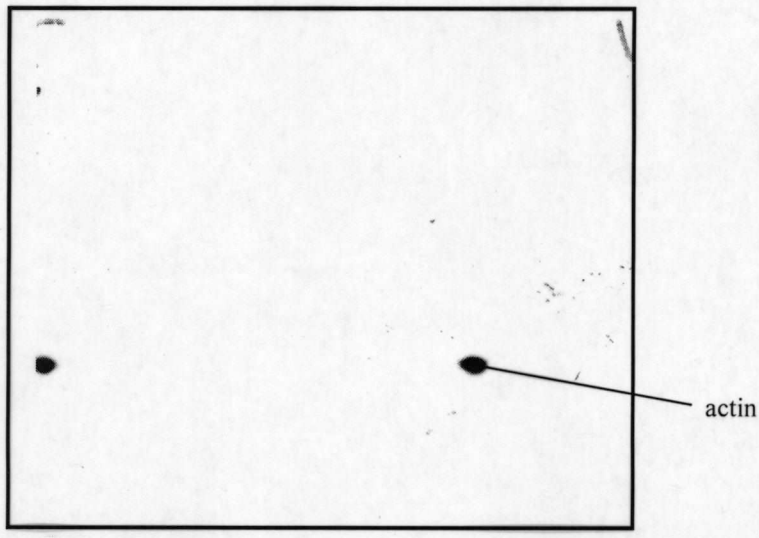
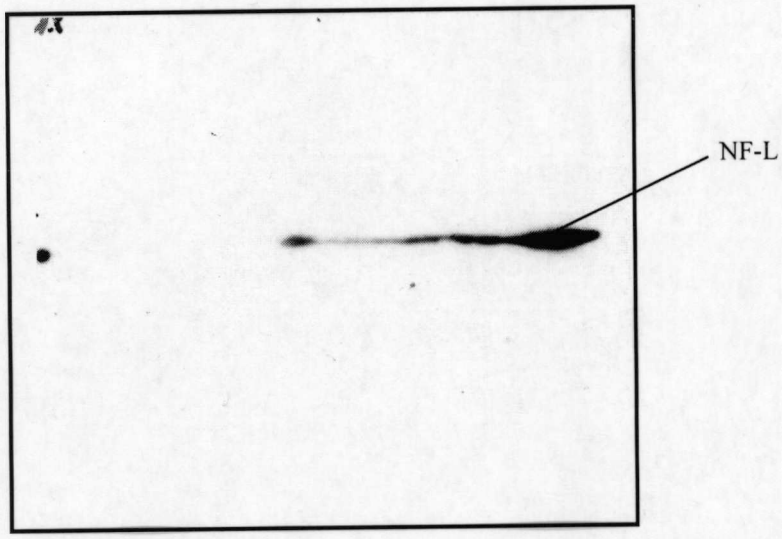
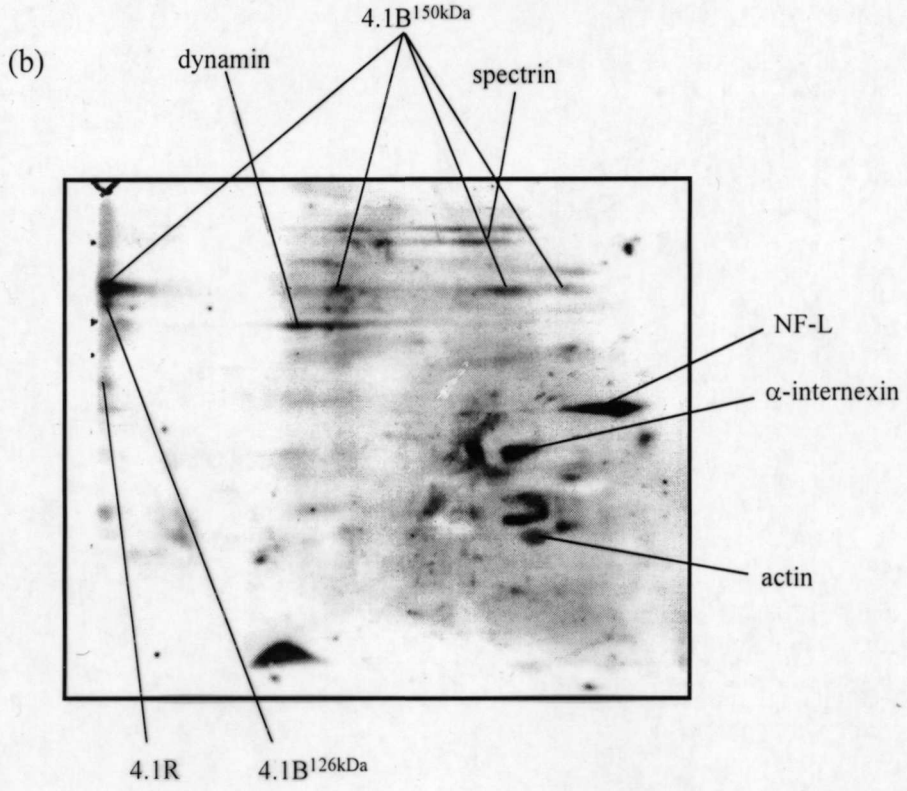


(c)




(b)
4.1B150kDa
dynamin
spectrin
NF-L
α-internexin
actin
4.1R
4.1B126kDa

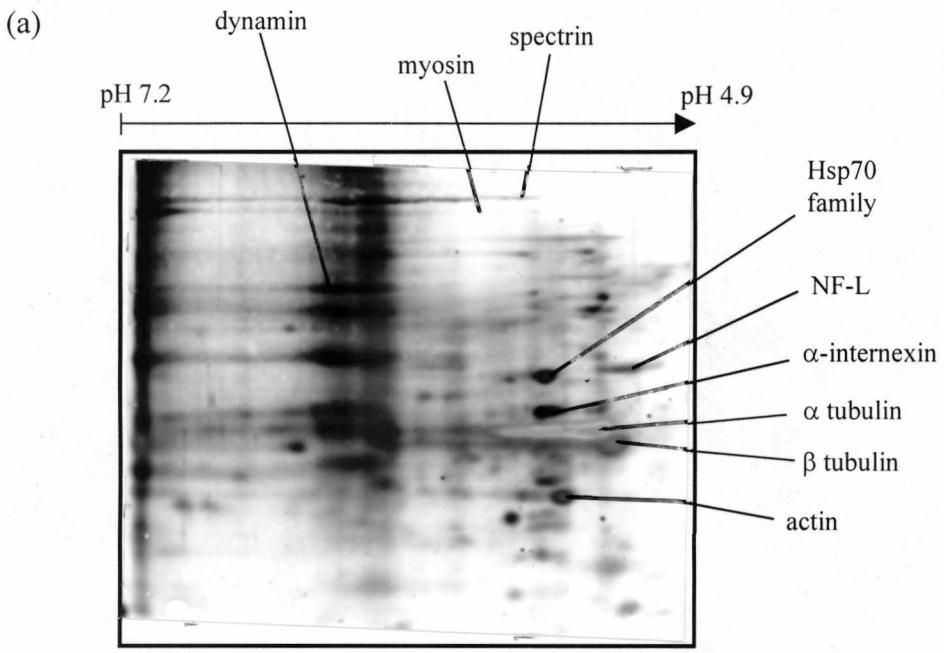


Figure 6.5 Blot overlay with 4.1R-CTR and immunoblots on IEF PSDs with anti-NF-L, anti-actin, anti-α-internexin and anti-tubulin antibodies. Pig PSDs were blotted and stained with collodal gold (a), taken through the 4.1R-CTR blot overlay procedure (b) or immunoblotted with anti-NF-L (c), anti-actin (d), anti-α-internexin (e) and anti-tubulin (f). The immunoblots localise each specific protein in the two-dimensional separation. The blot overlay indicates that 4.1R-CTR interacts with NF-L, actin and α-internexin but not tubulin.

lacked correlation with any 4.1R-CTR binding protein revealing that 4.1R-CTR did not interact with tubulin *in vitro*. The result obtained with tubulin demonstrates the selectivity of 4.1R-CTR interactions.

2. Identification of 4.1R-CTR Binding Interactions with Postsynaptic Density Proteins by Affinity Chromatography

Proteins on SDS-PAGE and IEF blots are denatured. Binding interactions that occur under these conditions may be masked when proteins are folded in their native state. To investigate the binding interactions of 4.1R-CTR under more physiological conditions, purified PSDs were solubilised in buffer containing 1% CHAPS and passed over a 4.1R-CTR affinity column. Bound protein was eluted with buffer containing a high concentration of the chaotropic salt NaBr and visualised by SDS-PAGE. Figure 6.6a and b show gold stained blots of a typical affinity column result. Binding proteins were eluted between fractions 35 and 37 corresponding to 1.3-2 column volumes of elution buffer. Nine PSD proteins were found to bind to 4.1R-CTR. The size and identity of each band is suggested in Table 10. Binding protein identification was based on data determined from one and two dimension gel electrophoresis (this Chapter, section 1).

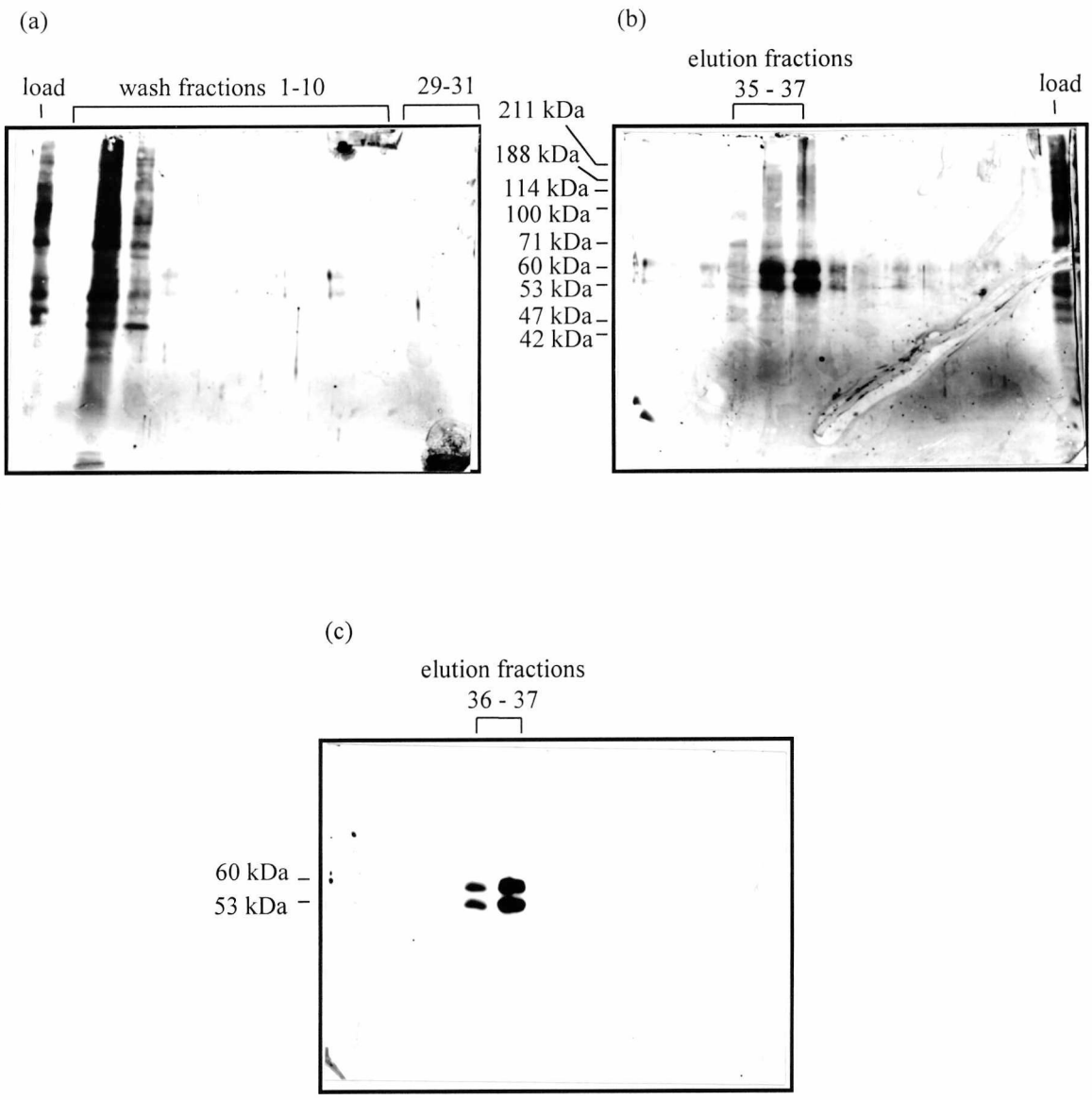


Figure 6.6 Analysis of 4.1R-CTR-PSD protein interaction by affinity chromatography. PSDs were passed over immobilised 4.1R-CTR and the column was washed with 30 column volumes of buffer before eluting specifically bound protein with buffer containing sodium bromide. Fractions were collected throughout and were analysed by SDS-PAGE. (a) Wash fractions, (b) elutions. Binding proteins of a similar size to those identified by immunoblotting were detected in elution fractions 35-37. Blot overlays on column eluates (c) revealed a strong association between 4.1R-CTR and the 53kDa and 60kDa bands to the exclusion of all others.

Mr kDa	Identity
211	Myosin
188	?
114	Dynamin
100	?
71	NF-L
60	α internexin
53	α CaMKII?
47	?
42	Actin

Table 10. Size and possible identification of 4.1R-CTR binding proteins as determined by affinity chromatography. Proteins present in 4.1R-CTR affinity column eluates were sized against protein standards using SDS-PAGE. The identity of each protein is based on its Mr and previous protein identities determined from two dimensional gel analysis.

The 53kDa and 60kDa proteins were particularly enriched in the affinity column elution. A blot overlay on elution samples revealed that 4.1R-CTR interacted with these two proteins to the exclusion of the other seven binding proteins (Figure 6.6c). Two artifact bands appear faintly across all lanes of the gold stained overlays at roughly the same position as the 53kDa and 60kDa binding proteins. These may result from β-mercaptoethanol in the samples as it has been noted previously that SDS polyacrylamide gels in which β-mercaptoethanol is used may develop horizontal lines at approximately 54kDa and 68kDa (Marshall & Williams, 1984; Tasheva & Dessev, 1983). The authenticity of the 53kDa and 60kDa 4.1R-CTR binding proteins is supported by their increased intensity in the 4.1R-CTR affinity column elution fractions containing other 4.1R-CTR binding proteins (as seen on the gold stained blot in Figure 6.6b) and the specific interaction of 4.1R-CTR with the 53kDa and 60kDa bands in just two elution fractions and not with artifact bands across the whole blot.

The specificity of 4.1R-CTR affinity column interactions were also tested by passing PSDs over a BSA affinity column (Figure 6.7). Non-specific interactions between PSD proteins and the BSA affinity column were negligible although binding of 4.1R-CTR to the 211kDa protein may be partly due to non-specific associations as demonstrated by the presence of this protein in the column elution. The BSA affinity column results indicate that 4.1R-CTR affinity column interactions were mainly a result of specific binding between 4.1R-CTR and PSD protein components. The high concentration of salt in the column elution buffer appears to have caused leaching of BSA (seen across elution fractions 20-28). The nature of this band was inferred from its electrophoretic identity with the BSA marker protein and the lack of a strong band this size in the column load.

3. Attempts to Identify the 53kDa 4.1R-CTR Binding Protein

The 53kDa 4.1R-CTR binding protein is similar in size to the α subunit of CaMKII, an important PSD signal transduction protein. To determine if the 53kDa binding protein shared identity with the α subunit of CaMKII, two commercial CaMKII antibodies (anti-CaMKII and anti-α-CaMKII) were used in immunoblots with 4.1R-CTR affinity column elutions (Figure 6.8a and b). Anti-CaMKII showed specific reactivity with 53kDa and 60kDa proteins eluted from the 4.1R-CTR affinity column. Anti-α-CaMKII on the other hand did not react with any eluted protein corresponding to these bands. These conflicting results lead to two opposing conclusions: (1) the 53kDa band is not immunologically related to the α subunit of CaMKII. (2) The 53kDa band is related to the α subunit of CaMKII and co-elutes from the 4.1R-CTR affinity column with the 60kDa β subunit by virtue of their known binding interaction. The result with anti-CaMKII could be a consequence of non-specific antibody-protein interactions. In contrast, the lack of 53kDa protein reactivity with anti-α-CaMKII is difficult to account for given that the specificity of this antibody was confirmed by immunoblots on purified PSD fractions (see Figure 5.2, Chapter 5, section 1.2). The most likely conclusion therefore seems to be that the 53kDa 4.1R-CTR binding protein is not α-CaMKII. To confirm this conclusion and to unambiguously identify the 53kDa protein eluted from 4.1R-CTR affinity columns MALDI (matrix-assisted laser-desorption

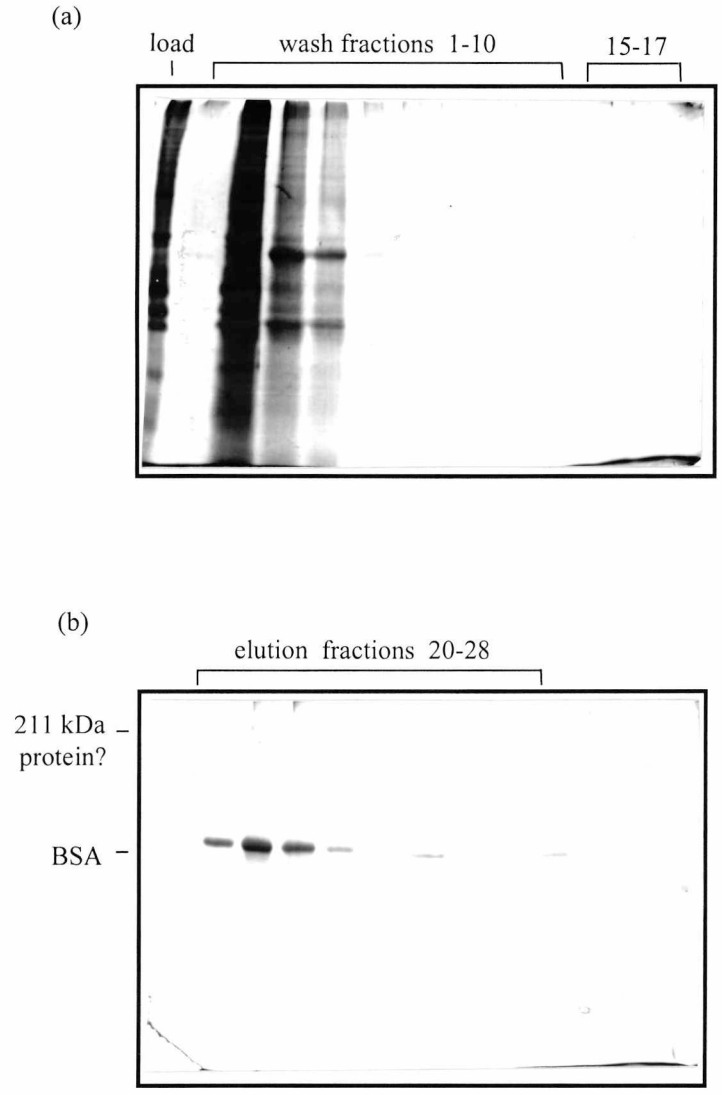


Figure 6.7 BSA affinity chromatography. PSDs were passed over immobilised BSA and the column was washed with 30 column volumes of buffer before eluting specifically bound protein with buffer containing sodium bromide. Fractions were collected throughout and were analysed by SDS-PAGE and silver staining. (a) Wash fractions, (b) elution fractions. A ~211kDa PSD protein appears in the column elution along with a large band thought to result form BSA leeching.

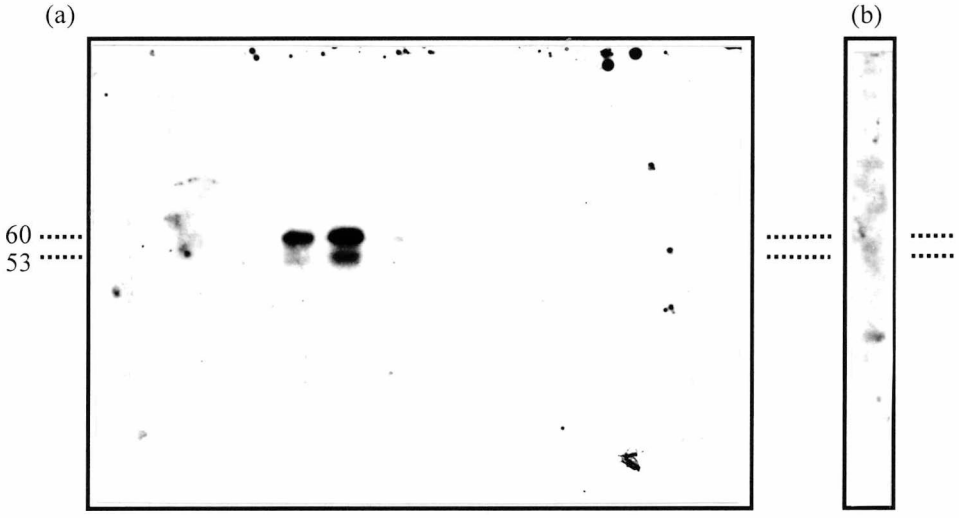


Figure 6.8 Immunoblots on 4.1R-CTR affinity column elutions with anti-CaMKII antibodies. (a) Anti-CaMKII on all 4.1R-CTR affinity column elution fractions, (b) anti-α-CaMKII on a single elution fraction. The anti-α-CaMKII antibody used in (b) was shown previously to detect a protein of the correct size for αCaMKII in PSD fractions (see Figure 5.2, Chapter 5, section 1.2). The lack of reactivity with this antibody on the 4.1R-CTR affinity column elution fraction suggests that the 53kDa 4.1R-CTR binding protein is not αCaMKII. The contrasting result obtained in (a) was presumed non-specific because of the result in (b) (see text for full details).

ionisation) of tryptic peptides was attempted. This method was not successful because sufficient quantities of protein could not be obtained for the analysis. As an additional approach the potential 4.1R-CTR-CaMKII binding interaction was examined by immunoprecipitation from a rat brain high speed supernatant (HSS). HSS production is the first step of the CaMKII purification procedure (Bennett *et al.*, 1983) and generates a pool of soluble cytoplasmic proteins slightly enriched for this protein.

Immunoprecipitation reactions were carried out on the HSS using anti-*ap*4.1R-CTR. Incubation of the antibody with HSS followed by the addition of Protein A resulted in pelleting of endogenous 4.1R along with any 4.1R-bound proteins (Figure 6.9b). Proteins of the following sizes were pelleted in immunoprecipitation reactions along with 4.1R: ~251kDa (spectrin?), 159kDa, 148kDa, 133kDa, 119kDa, 80kDa ($4.1R^{80}$?), 77kDa, 71kDa (NF-L?), 67kDa, 63kDa (βCaMKII?), 50kDa (αCaMKII?), 42kDa (actin?), 39kDa, 32kDa, 28kDa and 27kDa, 25kDa. Addition of 4.1R-CTR to the immunoprecipitation reaction specifically pelleted 4.1R-CTR binding proteins, namely the 251kDa, 71kDa, 67kDa, 63kDa, 50kDa, 42kDa (very faint), 32kDa, 27kDa and 25kDa bands (Figure 6.9c). The ~80kDa band thought to correspond to endogenous $4.1R^{80}$ in Figure 6.9b was not seen in the presence of 4.1R-CTR suggesting that interactions between anti-*ap*4.1R-CTR and endogenous $4.1R^{80}$ are displaced by the high affinity binding of *ap*4.1R-CTR to its antigen. This confirms the identity of the 80kDa band as a 4.1 protein and indicates that immunoprecipitation reactions worked successfully.

Bands identified in the size range of α and β-CaMKII (50kDa and 63kDa bands respectively) were immunoprecipitated from rat brain HSS along with 4.1R but the result is confused by the presence of anti-*ap*4.1R-CTR heavy chain. The fact that these two bands may appear in the 'no HSS' control (Figure 6.9f) brings the authenticity of a 4.1R-CTR interaction into question and reinforces the previous assertion that α-CaMKII and 4.1R-CTR do not interact. Immunoblots with anti-α-CaMKII to verify the nature of these bands were unsuccessful due to cross reactivity of both primary and secondary antibodies with the rabbit IgG used for immunoprecipitation (data not shown).

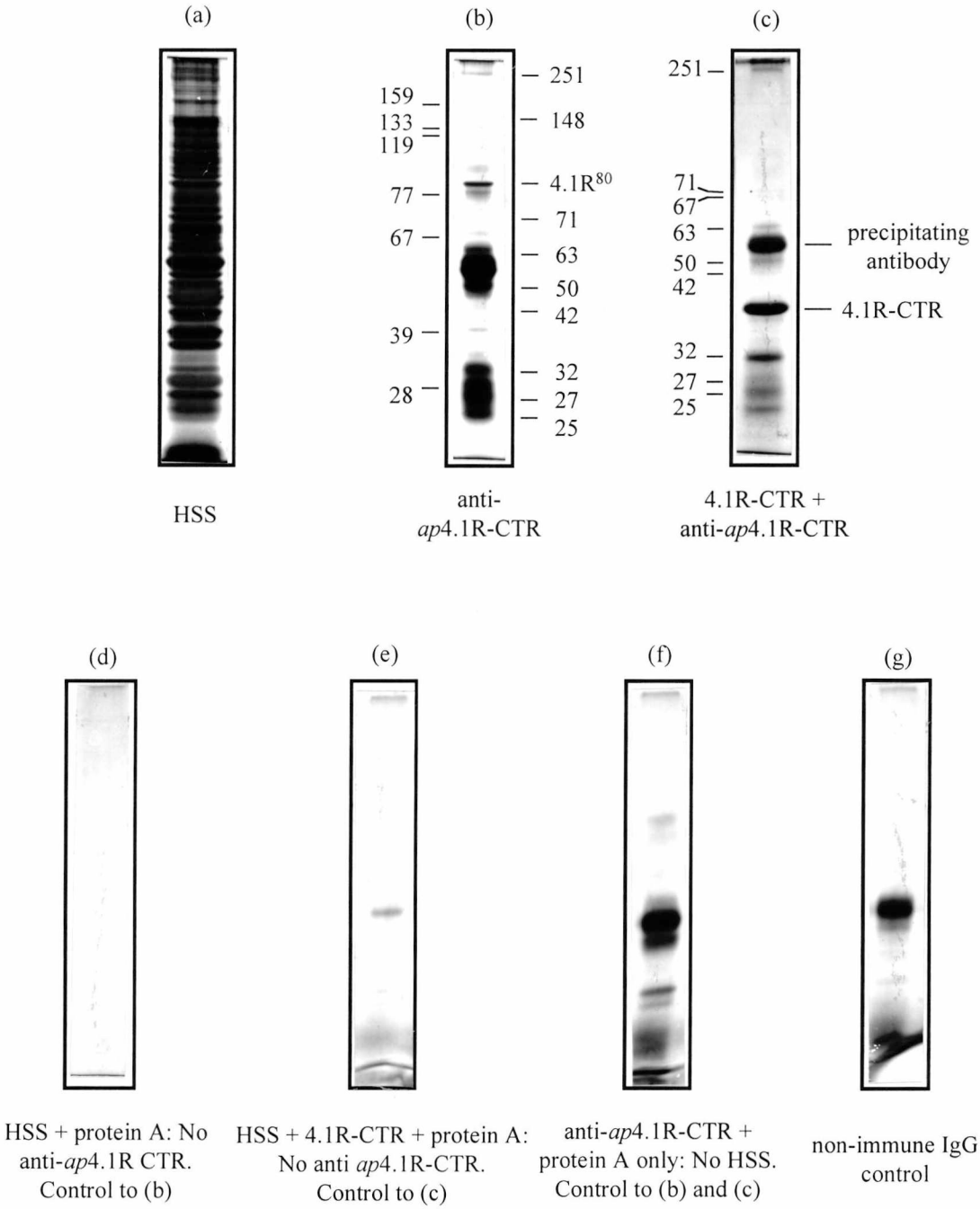


Figure 6.9 Immunoprecipitation of 4.1R potential binding proteins from rat brain HSS. HSS was incubated with 4.1R-CTR and/or anti-*ap*4.1R-CTR to investigate 4.1R binding activities. Protein bound to 4.1R was pelleted with Protein A-sepharose and the pellets obtained were analysed by SDS-PAGE and silver staining. Experimental reactions (b + c) were compared with control reactions (d-g). (a) non-pelleted HSS protein, (b) HSS + anti-*ap*4.1R-CTR + protein A, (c) HSS + 4.1R-CTR + anti-*ap*4.1R-CTR + protein A, (d) HSS + protein A only, (e) HSS + 4.1R-CTR + protein A alone, (f) anti-*ap*4.1R-CTR + protein A only, (g) HSS + non-immune IgG + protein A.

Discussion

The CTR of 4.1R was shown by blot overlay and affinity chromatography to specifically interact with a number of PSD components. The identity of most of these components remains unknown however two-dimensional electrophoresis and immunoblotting revealed that three of these binding proteins were NF-L, α-internexin and actin respectively. 4.1R-CTR did not interact with tubulin as inferred from two-dimensional electrophoresis immunoblots or the α subunit of CaMKII as determined by immunoprecipitation and affinity chromatography.

Unconfirmed interactions of 4.1R-CTR – possible significance to PSD structure and function

4.1R-CTR interactions were tentatively identified with the PSD components dynamin and myosin heavy chain. The identities of the two proteins were inferred from their IEF gel spot shape and position compared with IEF and NEPHGE gels run by Walsh & Kuruc (1992). The multiple punctate spots of dynamin phospho-forms are particularly similar in the IEF gels of the two studies (Figure 6.3, *inset*) and the implications of 4.1R-dynamin and 4.1R-myosin interactions are discussed below. Despite this discussion, it should be stressed that the binding interactions were only suggested by the results observed and have not been confirmed by any other means.

Dynamin is a mechanoenzyme that participates in vesicle budding during endocytosis (for reviews see McNiven *et al.*, (2000) and Urrutia *et al.*, (1997)). It was originally identified in PSD fractions by Walsh & Kuruc (1992) and is involved in AMPA receptor internalisation at postsynaptic membranes (Carroll *et al.*, 1999a). The actin binding protein profilin also interacts with dynamin (Witke *et al.*, 1998) and has been suggested to target it to the membrane, recruit actin and induce clustering of membrane phospholipids to allow vesicle formation. 4.1R may participate in such a function at postsynaptic densities since 4.1R interacts with both actin and phospholipids (Cohen *et al.*, 1988; Morris & Lux, 1995; Pradhan *et al.*, 1989). Regulation of postsynaptic AMPA receptor density by endocytosis occurs in a NMDA receptor activity dependent manner and contributes to LTD in hippocampal cultured neurons (Carroll *et al.*, 1999a;

Carroll *et al.*, 1999b; Luscher *et al.*, 1999). It is tempting to speculate that 4.1R-dynamin interactions could be regulated by elevated levels of Ca^{2+} that accompany NMDA receptor activity, thus forming a functional link between 4.1R and LTD. Various 4.1R MBD and SAB domain interactions are regulated by Ca^{2+} (Lombardo & Low, 1994; Nunomura *et al.*, 1997b; Tanaka *et al.*, 1991). Whether this regulatory mechanism extends to CTD interactions is yet to be seen. In addition to LTD, regulated endocytotic mechanisms involving dynamin are important in intracellular signalling (Schaefer *et al.*, 1999) and the formation and maintenance of specialised membrane domains (Winckler & Mellman, 1999). Cytoskeletal proteins have been implicated in both of these cellular functions. In particular, ankyrin isoforms have been shown to bind the vesicle coat protein clathrin (Michaely *et al.*, 1999) and are suggested to be involved in endocytosis mechanisms at axon initial segments and nodes of Ranvier. 4.1R could participate in similar events at PSDs.

A potential interaction between 4.1R and myosin heavy chain (MHC) is suggested by IEF blot overlay data presented in this study. MHC has been detected in PSD fractions previously (Walsh & Kuruc, 1992) and distributes to the plasma membrane and growth cones in the somatodendritic compartment of CNS neurons (Miller *et al.*, 1992). The results presented are also consistent with a previous report that erythrocyte MHC binds the SAB domain of $4.1R^{80}$ (Pasternack & Racusen, 1989). 4.1R partially inhibits the actin-activated ATPase activity of myosin suggesting that it could function to regulate myosin activity at PSDs. It may also function to localise myosin to the postsynaptic actin cytoskeleton enabling force to be transmitted to the overlying membrane. Actin-myosin contractile forces are activated in response to Ca^{2+} (Rees & Frederiksen, 1981; Szent-Gyorgyi, 1975) and may be involved in the morphological changes accompanying synaptic remodelling and plasticity, such as an increase in the number of dendritic spines and the formation of perforated PSDs (van Rossum & Hanisch, 1999) (Engert & Bonhoeffer, 1999; Maletic-Savatic *et al.*, 1999; Matus, 1999).

Confirmed interactions of 4.1R-CTR – possible significance to PSD structure and function

Results from two-dimensional electrophoresis blot overlays and affinity chromatography implied 4.1R-CTR binding interactions with actin, α-internexin and NF-L. Two-dimensional immunoblotting with protein specific antibodies confirmed the identity of these proteins suggesting a structural role for 4.1R at PSDs. 4.1R interactions with actin may or may not have physiological relevance. The known actin binding activity of 4.1R (Morris & Lux, 1995) is confirmed in the results obtained but may reflect the binding capacity of 4.1R rather than an interaction with functional significance at PSDs. Various actin binding proteins exist at PSDs including spectrin (Carlin *et al.*, 1983; Malchiodi-Albedi *et al.*, 1993), dystrophin (Kim *et al.*, 1992), α-actinin 2 (Wyszynski *et al.*, 1997), adducin (Walsh & Kuruc, 1992), neurabinI/neurabinII (Satoh *et al.*, 1998) and cortactin (Naisbitt *et al.*, 1999). The actin binding capacity of 4.1 is unlikely to be functionally redundant in this case. Actin is suggested to have a major organisational function at PSDs (Adam & Matus, 1996) as well as a role in morphological and functional plasticity (reviewed in Matus (1999)). 4.1R interactions with actin may contribute to the execution of some of these functions. Identification of α-internexin (Suzuki *et al.*, 1997) and NF-L (Terry-Lorenzo *et al.*, 2000) at PSDs suggests that the two proteins may hetero-polymerise in to the ~10nm filaments observed originally by EM (Blomberg *et al.*, 1977; Carlin *et al.*, 1980). While actin is involved in dynamic rearrangements of PSDs and dendritic spines, intermediate filament (IF) proteins (for a review see Lee & Cleveland (1996) may provide a more stable scaffold around which the PSD can be organised.

NF-L may be a contaminant of PSD fractions that accumulates during the purification process (Matus *et al.*, 1980); no NF-L immunoreactivity was detected in rat cerebellar cortex PSDs (Matus *et al.*, 1979). As discussed in Chapter 5, cerebellar PSDs do not always reflect the PSD structures in forebrain and although absent from cerebellum, NF-L may be a component of excitatory forebrain PSDs. This is supported by the results presented here and the study of Terry-Lorenzo *et al.*, (2000). A functional role for NF-L at postsynaptic sites comes from the report that NMDA receptor NR1 subunits bind NF-L and inhibit filament assembly (Ehlers *et al.*, 1998). NF-L is unable to homo-

polymerise and requires another IF protein for filament formation (Ching & Liem, 1993). Localisation of α-internexin at PSDs further substantiates a role for NF-L given that the two proteins have been shown to heteropolymerise *in vitro* and *in vivo* (Balin & Miller, 1995; Ching & Liem, 1993).

Recently, NF-L was co-localised with spectrin and actin in cells lacking cytoplasmic IF (Macioce *et al.*, 1999). Direct association of spectrin and NF-L was not demonstrated and the authors suggest the need for a linker protein to mediate the interaction. The 4.1R-CTR-NF-L interaction reported here indicates that 4.1R could be the linker protein these authors refer to. Non-neuronal IF proteins can bind ankyrin (Georgatos *et al.*, 1987) thereby providing a link with spectrin. 4.1R may perform this role in brain. 4.1R could localise actin with spectrin and NF-L at postsynaptic sites by binding the two former proteins via the SAB domain and the latter via the CTD. Mapping of the NF-L binding site on 4.1R-CTR would address this possibility. In addition to a structural role at PSDs, NF-L could have a role in PSD signalling. NF-L binds protein phosphatase-1 (PP1) and may regulate its activity (Terry-Lorenzo *et al.*, 2000). PP1 regulates the activity of CaMKII (Yoshimura *et al.*, 1999), NMDA receptors (Snyder *et al.*, 1998) and AMPA receptors (Yan *et al.*, 1999) and is concentrated in PSD fractions and dendritic spines (Terry-Lorenzo *et al.*, 2000). The observed interaction between NF-L and NMDA receptors (Ehlers *et al.*, 1998) may localise PP1 in the close proximity of its PSD substrates. In a similar way, interactions between NF-L and 4.1R may recruit PP1 to spectrin-actin junctions which can also be regulated by phosphorylation (Eder *et al.*, 1986; Horne *et al.*, 1990; Ling *et al.*, 1988). One possible scenario is that NF-L forms a firm structural basis for PSDs to which spectrin, actin and associated molecules are linked by 4.1R. Transient dissociation of spectrin, actin and 4.1R (e.g. by phosphorylation) would release the actin based cytoskeleton and allow dynamic molecular changes in response to neuronal activity e.g. NMDA receptor internalisation in response to actin depolymerisation (Allison *et al.*, 1998). Only certain populations of NMDA receptor subunits are bound by NF-L (Ehlers *et al.*, 1998). This limited interaction may function to stabilise a proportion of NMDA receptors at the postsynaptic membrane. When non-NF-L bound NMDA receptor clusters are mobilised by a loss of their link with the actin cytoskeleton, the NF-L bound receptors

may remain at the postsynaptic membrane and maintain a basic structural and functional PSD. IF binding may be responsible for retaining 4.1R at the PSD core when actin is removed (see Chapter 5).

α-internexin is an IF protein expressed in most neurons of the CNS (Kaplan *et al.*, 1990). It localises to both axons and dendrites (Benson *et al.*, 1996; Kaplan *et al.*, 1990) and has been found at the axon hillock (Shea & Beermann, 1999) supporting a role for it at specialised membrane domains. α-internexin is present in PSD fractions even before the period of neuronal synaptogenesis and its expression precedes all other neurofilament proteins (Benson *et al.*, 1996; Fliegner *et al.*, 1990; Kaplan *et al.*, 1990). Final PSD levels are established by postnatal day 14 (Suzuki *et al.*, 1997). Thus α-internexin may play a role in the early development and organisation of PSDs and could recruit 4.1R to these sites. 4.1R has itself been shown to recruit and stabilise spectrin at the MS of erythrocytes (Hanspal *et al.*, 1992; Winardi *et al.*, 1995) and ankyrin is required for targeting and maintenance of NrCAM and neurofascin clusters at nodes of Ranvier (Zhou *et al.*, 1998). The interaction between α-internexin and 4.1R may facilitate the recruitment and stabilisation of spectrin and actin at PSDs. Little is known about the function of IF proteins at PSDs. Extensive research will be required to formulate an accurate model for their function.

CaMKII and Tubulin

Whilst attempting to identify 4.1R-CTR binding proteins in PSD preparations it was demonstrated that CaMKII and tubulin did not interact with 4.1R-CTR. Immunoblots on 4.1R-CTR affinity column eluates with an antibody specific for CaMKII initially suggested that an interaction did occur. This result was later shown to be misleading and it was concluded that there was no direct binding interaction between 4.1R-CTR and the major PSD protein.

An interaction between 4.1R and tubulin has been demonstrated previously (Correas & Avila, 1988) but the binding site was not mapped on 4.1R. The lack of binding with 4.1R-CTR in this study implies that the tubulin binding site does not reside in the SAB or C-terminal domain of 4.1R. Alternatively 4.1R may only interact with microtubule

polymers. Although tubulin appears to be a major component of PSD preparations the levels observed do not accurately reflect the amount *in vivo* due to post-mortem accumulation of tubulin at PSDs (Carlin *et al.*, 1981). *In vivo* tubulin may only be a minor component of PSDs but reports of tubulin-mGluR (Ciruela *et al.*, 1999) and tubulin-NMDA receptor binding (van Rossum *et al.*, 1999) indicate that it is present. NMDA receptors appear to have a preferential affinity for small soluble tubulin forms such as tubulin dimers (van Rossum *et al.*, 1999)). Van Rossum, *et al.*, (1999) propose that NMDA receptors maintain a pool of tubulin within PSD structures that is released and polymerised in response to neuronal excitation (reviewed in Van Rossum & Hanisch (1999)). They also suggest that the microtubule binding protein CRIPT (Passafaro *et al.*, 1999) could interact with newly formed microtubules to enhance their stability. 4.1R could perform a similar function, selectively interacting with polymerised tubulin for this purpose. Microtubule stabilisation could be a common function among 4.1 proteins given that 4.1B was recently identified as a MAP (Bellamy, 1999). Further investigation into 4.1-tubulin interactions will determine if this is the case.

In summary, actin, NF-L and α-internexin present in PSD forebrain preparations were shown to interact specifically with the C-terminal region of 4.1R. These interactions suggest a structural role for 4.1R in the organisation and maintenance of PSDs and possibly in regulating PSD dynamics. The 4.1R-CTR interactions observed could be confirmed using yeast-two-hybrid technology and localisation of 4.1R with these proteins in dendritic spines would demonstrate an interaction *in vivo*. An i*n vivo* interaction could also be inferred by co-sedimenting NF-L, α-internexin and 4.1R-CTR in the SW13 Vim^-, IF lacking cell line. Only binding interactions of the SAB domain and CTD of 4.1R are presented here. 4.1R N-terminal interactions are likely to be just as complex and may reveal associations of 4.1R with postsynaptic membrane proteins.

Chapter 7

Discussion

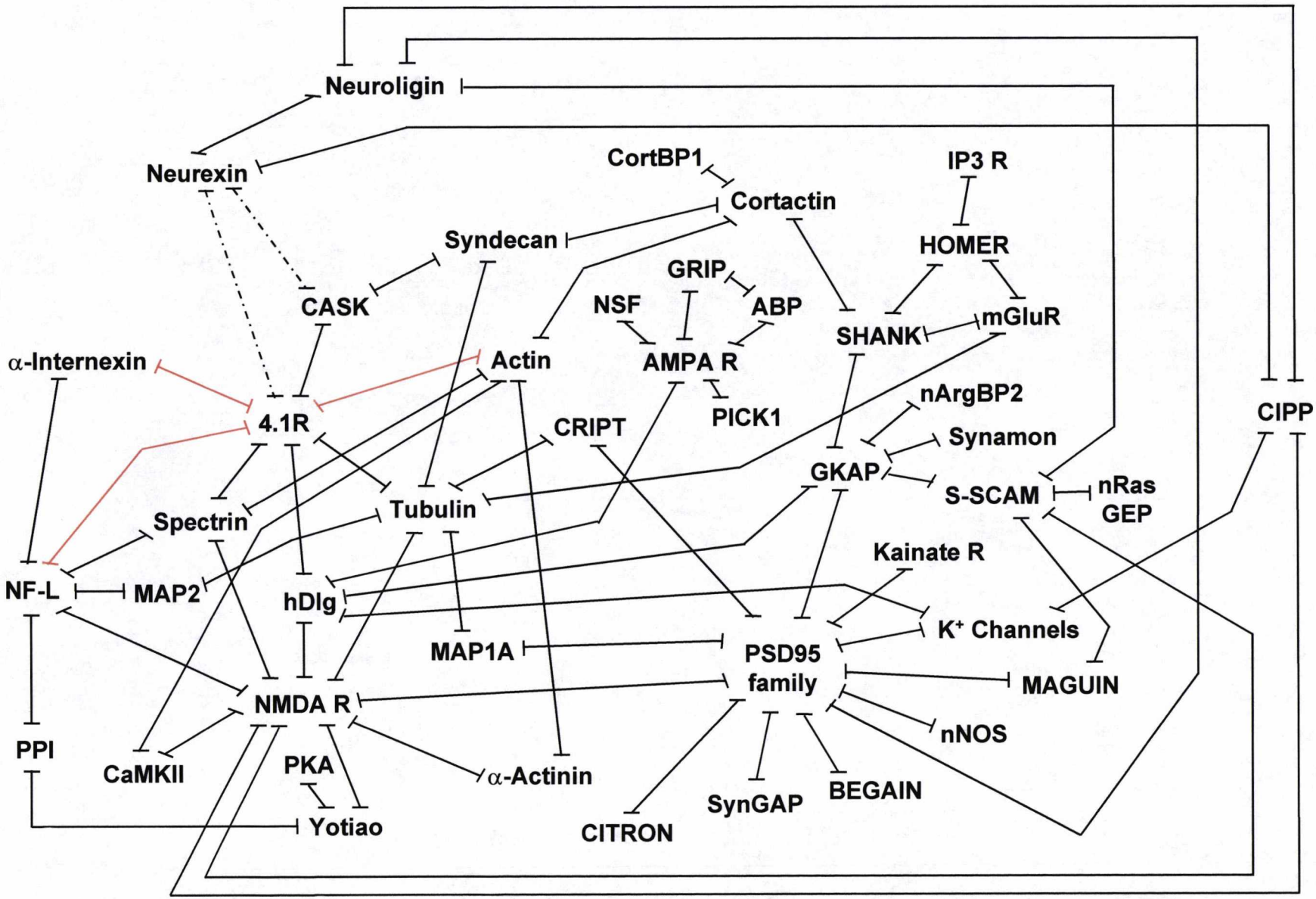
Discussion

4.1 is no longer seen as a simple membrane skeleton anchoring protein. Instead it exists as a family of multi-functional proteins which are expressed diversely across tissues and are important in cellular processes ranging from signalling to mitosis and polarity to transcription. The brain is a major site of 4.1 expression but little is known about the functions of the various gene products in this tissue. Subcellular localisation of 4.1 proteins to PSD fractions prepared from rat and pig forebrain suggests an involvement for these proteins in the establishment and maintenance of this specialised membrane domain, morphological plasticity accompanying neuronal excitation and intracellular signalling. This is supported by the fact that 4.1N mediates anti-proliferative responses to NGF (Ye *et al.*, 1999) and may localise at PSDs awaiting such a signal. Also, 4.1R has for many years been known to stabilise the erythrocyte MS and regulate membrane deformability (Bennett & Gilligan, 1993). Postsynaptic interactions of 4.1R with spectrin, actin, α-internexin and NF-L may reflect a similar function at PSDs.

PSD heterogeneity

A large number of proteins have been identified as components of PSDs using a variety of *in vitro* and *in vivo* techniques e.g. immunoblotting of PSD fractions, immuno-electron microscopy of brain slices and immunofluorescence in neuronal cultures. To support these findings, a plethora of interactions have been identified between individual PSD components, some of which are summarised in Figure 7.1. It is not yet clear where 4.1N, B and G fit into this scheme but interactions of 4.1R have a principal position and may be important for postsynaptic integrity. The presence of so many proteins and interactions that could effectively perform the same function leads to a questioning of how they all actually fit together in the brain. Our knowledge of this whole area is extremely limited although what is slowly being revealed and seems the most likely explanation is the existence of postsynaptic heterogeneity (Rao *et al.*, 1998). Under these circumstances, not all the interactions identified will occur at PSDs at the same time; many interactions will be transient in response to synaptic activity and only a limited number will exist on a more permanent basis to maintain PSD integrity. In a similar context, not all of the PSD proteins identified will localise at all PSDs;

Figure 7.1 Published interactions between proteins identified at excitatory PSDs. The large number of interactions that can occur between components at PSDs highlights the complexity of these structures. How, and to what extent each interaction contributes to the PSD structure is not clear. The red lines are 4.1R interactions identified in this study, dashed lines indicate interactions that may only occur at presynaptic sites. See Appendix II for references.


Neuroligin
Neurexin
CortBP1
Cortactin
IP3 R
HOMER
Syndecan
GRIP
ABP
NSF
CASK
mGluR
SHANK
α-Internexin
Actin
AMPA R
PICK1
nArgBP2
4.1R
CRIPT
Synamon
CIPP
GKAP
S-SCAM
nRas
GEP
Tubulin
Spectrin
Kainate R
NF-L
MAP2
hDlg
K+ Channels
MAP1A
PSD95
family
MAGUIN
NMDA R
nNOS
PPI
CaMKII
PKA
α-Actinin
BEGAIN
SynGAP
Yotiao
CITRON

excitatory and inhibitory PSDs may recruit different subsets of proteins (Craig *et al.*, 1996; Kurschner *et al.*, 1998; Wang *et al.*, 1999) while various components are recruited and lost from PSDs in response to synaptic activity e.g. CaMKII (Strack *et al.*, 1997b) and AMPA receptors (Carroll *et al.*, 1999a). PSDs are not uniform static structures therefore, but highly variable dynamic entities that differ in composition from one neuron to the next or even between the dendritic spines of the same neuron.

Evidence supporting the variable nature of PSDs comes from a study suggesting that α-internexin is found at most but not all PSDs in rat cerebral cortex (Suzuki *et al.*, 1997), the identification of certain 'silent' synapses that lack AMPA receptors (Liao *et al.*, 1999) and the finding that some PSD proteins exist in a variety of forms which could be differentially distributed amongst different PSDs (e.g. NMDA, AMPA and mGlu receptors (for a review see Petralia, Rubio & Wenthold (1998)), Hdlg (Muller *et al.*, 1995), chapsyn 110 (Kim *et al.*, 1996), S-CAM (Hirao *et al.*, 2000), spectrin (Goodman *et al.*, 1995) and 4.1 (Chapter 1, sections 3 and 4). The 'PSD fraction' isolated from brain therefore contains a mixture of PSD populations that differ in their composition and the subset of interactions that are occurring. The result is that a generalised PSD structure is conceived, and although this is sufficient to identify specific components and potential interactions, the exact *in vivo* scenario is somewhat removed.

With this in mind, the distribution of postsynaptic 4.1 proteins may differ amongst PSD domains and not all of the gene products identified may be present at all PSDs. On the other hand, each 4.1 product may have a distinct postsynaptic function and co-exist at these complexes. Immunocytochemical comparison of the 4.1 protein distribution with a relatively ubiquitous excitatory PSD component e.g. NMDA receptors (for a review see O'Brien, Lau & Huganir (1998)) may begin to provide an answer.

The role of 4.1R at PSDs

The interaction of the C-terminal region of 4.1R with the PSD components actin, α-internexin, and NF-L indicates that this 4.1 gene product exists at PSDs in a structural capacity. As part of the PSD core 4.1R may be important in regulating the extent of PSD association and maintaining a minimal PSD during activity dependent

morphological rearrangements (for reviews see Matus (1999) and Van Rossum & Hanisch (1999)). Other known 4.1 binding proteins that localise at synapses include CASK (Hata *et al.*, 1996), neurexin (Missler & Sudhof, 1998) and hDlg (Muller *et al.*, 1995). Each of these will be discussed below.

CASK is a MAGUK protein of slightly unusual nature. In its C-terminal half it is most similar to the erythrocyte MAGUK p55 and contains the 4.1R binding motif suggested by Marfatia *et al.*, (1995). In its N-terminal half it contains a CaMK domain of unknown function. CASK was isolated by virtue of its interaction with neurexins (Hata *et al.*, 1996) and exists at both pre- and post- synaptic sites in brain (Hsueh *et al.*, 1998). An interaction with the MBD of 4.1R was demonstrated in epithelial cells where CASK shows a polarised distribution and localises the cell surface heparan sulfate proteoglycan, syndecan-2 (for a review see Carey (1997)). CASK may therefore be important in targeting syndecans to particular membrane domains and may anchor them to the actin cytoskeleton through interactions with 4.1R. Syndecans bind and mediate the actions of polypeptide growth factors as well as functioning in cell-extracellular matrix and cell-cell interactions. This implies that CASK and 4.1R participate indirectly in these processes also. In brain syndecan-2 plays a critical role in maturation of dendritic spines and may be involved in PSD development (Ethell & Yamaguchi, 1999). This role is reinforced by the observation that CASK and syndecan-2 redistribute from axons in early postnatal brain to synaptic sites during the later stages of synaptic development; the time when most spines morphologically mature (Hsueh & Sheng, 1999). CASK can bind to all syndecan molecules and is unlikely to define the subcellular localisation of syndecan-2. 4.1R on the other hand could specifically target this complex to postsynaptic sites and contribute to PSD development by doing so.

The MAGUK proteins hDlg and chapsyn 110 are also found at postsynaptic sites and contain a conserved 4.1 binding motif. hDlg has been shown to interact with the MBD of 4.1R *in vitro* (Lue *et al.*, 1996; Marfatia *et al.*, 1996) and has been shown to cluster and confer G-proteins sensitivity to certain potassium channels in the postsynaptic membrane (Hibino *et al.*, 2000). hDlg binds AMPA receptors and various studies suggest that it may be involved in regulating receptor and ion channel function through

activity dependant endocytosis mechanisms (Carroll *et al.*, 1999a; Kim & Sheng, 1996; Leonard *et al.*, 1998; Tiffany *et al.*, 2000). An interaction between 4.1R and hDlg could anchor receptors and ion channels to postsynaptic IF or the actin cytoskeleton at PSDs. Alternatively it could promote receptor and ion channel internalisation, an attractive notion given that the C-terminal region of 4.1R may interact with the vesicle budding protein dynamin (Chapter 6, discussion). Immunoblotting does not detect hDlg in PSD fractions (Hsueh *et al.*, 1998; Kim *et al.*, 1996) yet the interactions of this protein strongly suggest it has a function at PSDs. It seems likely that hDlg associates with PSDs transiently, in an activity dependant manner so that only negligible amounts co-purify with PSDs.

Like hDlg, chapsyn 110 contains the MAGUK protein 4.1 binding site and could potentially interact with 4.1R at PSDs. Chapsyn 110 is a member of the PSD95 family of proteins and was originally thought to cluster potassium channels and NMDA receptors at PSDs (Kim *et al.*, 1996). More recent reports have suggested that like PSD95, chapsyn 110 is not involved in postsynaptic NMDA receptor clustering or targeting mechanisms *in vivo* (Migaud *et al.*, 1998; Passafaro *et al.*, 1999). The same may be true for ion channels. Instead, PSD95 has been implicated in NMDA dependent LTP and LTD and the signal transduction pathways that control synaptic strength (Migaud *et al.*, 1998). Interactions of PSD95 with the Ras-GTPase activating protein synGAP (Kim *et al.*, 1998) and the Rho effector citron (Furuyashiki *et al.*, 1999; Zhang *et al.*, 1999), and interactions of PSD95 and chapsyn 110 with nNOS (Brenman *et al.*, 1996) support this role for PSD95 family proteins. Theoretically 4.1-chapsyn 110 interactions could have several postsynaptic functions including anchoring receptors and ion channels to the actin cytoskeleton or an IF network, signal transduction and protein targeting. Whether a functionally significant interaction takes place between these proteins *in vivo* remains to be seen along with the actual functions of PSD95 family proteins.

Protein 4.1 (coracle) also interacts with *Drosophila* neurexin IV (NRX IV) (Ward *et al.*, 1998). Neurexins are presynaptic proteins that establish synaptic contacts through interactions with postsynaptic cell surface adhesion molecules called neuroligins (for

reviews see Missler & Sudhof (1998) and Littleton, Bhat & Bellen (1997) and also Song *et al.*, (1999)). Neurexins contain a protein 4.1 binding site that shares varying degrees of homology with the 4.1R binding site on GPC. Some neurexins (neurexins I, II and III) also bind CASK (Hata *et al.*, 1996). These interactions would allow a neurexin-CASK-4.1 ternary complex to exist at pre-synaptic sites analogous to the GPC-p55-4.1R complex found in erythrocytes. No evidence to suggest such a complex has been found and only an interaction between 4.1 (coracle) and NRX IV has been demonstrated to date. A difference in the binding specificity of *Drosophila* neurexin and neurexins I, II and III may be due to structural differences. The human homologue of NRX IV is not another neurexin protein but the contactin binding protein Caspr (also known as paranodin) (Ushkaryov *et al.*, 1992). NRX IV and Caspr share partial but not complete domain homology with the rat neurexin proteins I, II and III and an overall sequence identity of 21-29%. The two sets of proteins are part of the same superfamily but Caspr and NRX IV are grouped on a more divergent branch (for a review see Bellen *et al.*, (1998)). Functional differences may exist between these two groups of proteins as a result. Binding of 4.1 may be such a difference given that an interaction between Caspr and 4.1R has also been demonstrated *in vitro* (Menegoz *et al.*, 1997).

NRX IV and coracle localise to septate junctions in *Drosophila* epithelia. NRX IV is necessary for recruitment of coracle to these sites while coracle is required to maintain the localisation of neurexin IV (Ward *et al.*, 1998). Severe coracle mutants result in failure of dorsal closure and are lethal (Fehon *et al.*, 1994). This suggests a primary role for coracle in *Drosophila* development and the formation of adult structures. In vertebrates, the paranodal region of myelinated axons is thought to be 'septate-like' and may provide a physical barrier to the diffusion of sodium channels at nodes of Ranvier (for a review see Scherer (1999)). Caspr localises to paranodal regions in myelinated axons and is important in cell-cell contacts and signalling between neurons and glia (namely oligodendrocytes) (Einheber *et al.*, 1997). Caspr redistributes to these sites during myelination and could be recruited and maintained here by protein 4.1 in a similar way to which ankyrin recruits NrCAM and neurofascin to nodes (Zhou *et al.*, 1998). Potassium channels co-localise at juxtaparanodal regions with Caspr2 (Poliak *et al.*, 1999). 4.1 could also function to anchor and localise these proteins to their specific

sites of action in a similar way to which it could anchor receptors and ion channels at PSDs.

The interaction between 4.1 and Caspr provides evidence for 4.1 proteins at other neuronal specialised membrane domains, not just PSDs. Various lines of reasoning support this idea. For example, ankyrin isoforms concentrated at nodes of Ranvier and axon initial segments specifically contain O-linked N-acetylglucosomine residues (Zhang & Bennett, 1996). Similar sugar attachments have been recognised in 4.1R (Inaba & Maede, 1989) but their function is unknown. The finding with ankyrin suggests that proteins containing these modifications are concentrated to neuronal specialised membrane domains and supports a role for 4.1R at nodes of Ranvier and axon initial segments as well as at PSDs. O-linked N-acetylglucosamine residues may participate in protein multimerisation (Zhang & Bennett (1996) and for a review see Snow & Hart (1998)). In the case of 4.1 and ankyrin this would allow them to cluster transmembrane proteins at specific membrane domains. Septate junctions where 4.1 localise in invertebrates, are sites of cell-cell contact important in the formation and maintenance of polarity and growth regulation. They are thought to be functionally analogous to vertebrate tight junctions (Aschenbrenner & Walz, 1998; Willott *et al.*, 1993) implying a role for 4.1 at these sites also. The interaction between the 4.1R and ZO-2 (Mattagajasingh *et al.*, 1999a) supports a function for 4.1 proteins at tight junctions in epithelia and also adherens junctions; ZO-2 is a MAGUK protein that localises to these sites. 4.1R-ZO-2 binding occurs via the CTD of 4.1R suggesting that some of the 4.1-CTR binding proteins identified in PSD fractions may turn out to be MAGUKs. Because parallels such as this can be drawn, the functions of 4.1 proteins at PSDs may be more easily understood by studying the role of these proteins at other cellular domains.

Issues not addressed by this study

Several issues were not addressed by this study. Firstly, the exon composition of the 4.1 proteins identified in PSD fractions was not investigated. Various exons within the 4.1 structure have binding activities associated with them, e.g. exon 16 of 4.1R binds spectrin (Schischmanoff *et al.*, 1995), so too does a similar sequence in 4.1B (Parra *et*

al., 2000). Determining the exon structure of the 4.1 products would allow further analysis of the functions of these proteins at PSDs. In particular it would be interesting to determine the structure of $4.1R^{80kDa}$ and discover whether it is equivalent to erythrocyte $4.1R^{80}$ or whether it contains exons 14 and 15 that are specific to brain (Huang *et al.*, 1993). Exons 14 and 15 presumably confer a tissue specific function on 4.1R and could promote brain protein specific interactions. Alternatively they could disrupt spectin-actin interactions of the 4.1R SAB domain which would suggest that postsynaptic 4.1R functions concentrate in other areas.

The binding sites for α-internexin and NF-L on 4.1R must reside in exons 16–21 as this is the region of 4.1R expressed in 4.1R-CTR. The exact binding sites were not determined in this study but could be deduced with 4.1R constructs containing various exon combinations inferred from our knowledge of CTD splicing in brain (Gascard *et al.*, 1998; Huang *et al.*, 1993; Luque *et al.*, 1999). In its simplest form, the binding capacity of each recombinant protein could be assessed by applying the blot overlay technique used to demonstrate 4.1R-CTR-IF binding in this study. It is particularly important to distinguish whether 4.1R binds IF via the SAB domain due to the implications this could have on 4.1R-spectrin-actin interactions.

The interactions of the N-terminal region of 4.1R were not investigated here either, nor were the interactions of 4.1B, N and G. The N-terminal of 4.1R would be expected to bind a distinct subset of proteins that are not identified by binding studies with 4.1R-CTR. The N-terminal of 4.1R includes the MBD that interacts with a range of transmembrane proteins and MAGUKs (Chapter 1, section 3.1.5.1). Identification of postsynaptic binding partners for the MBD of 4.1R could give considerable insight in to the functions of this protein at PSDs. In addition to 4.1R, 4.1B, 4.1G and 4.1N were also identified in PSD fractions. Each product presumably has a different function at PSDs, however it is not possible to demonstrate this from the work carried out here. Two dimensional blot overlays with recombinant forms of each gene product would highlight differences in the complement of PSD proteins that each product binds. This would either infer specific postsynaptic functions for the different 4.1 proteins or functional redundancy.

Future Prospects

There is a need to demonstrate 4.1 protein localisation at PSDs *in vivo*. Continuing study of 4.1 proteins at PSDs should concentrate on using immunocytochemistry to co-localise each gene product with a known PSD marker in a well characterised system e.g. primary hippocampal cultures. An appropriate PSD marker would be PSD95 or NMDA receptors and although these proteins may not be present at all PSDs, it is well accepted that they are PSD components and exist at a high proportion of excitatory synapses. Immuno-electron microscopy would unambiguously demonstrate a postsynaptic localisation of 4.1 gene products and would clearly reveal any pre-synaptic cross reactivity. Most studies using this technique concentrate on cerebellum sections because of the well-characterised ultrastructure of this brain region. Cerebellum PSDs may not accurately reflect the structures at forebrain excitatory synapses (Chapter 5, discussion) and results could be misleading. De Camilli *et al.*, (1983) used agarose embedded synaptosomes to study the synaptic distribution of synapsin I. Synaptosomes are resealed nerve terminals and retain an association with the postsynaptic membrane and attached PSDs. They can be easily isolated from forebrain tissue suggesting that a similar technique could be used to study the postsynaptic distribution of 4.1 proteins.

The interactions of 4.1R-CTR should also be studied in further detail and confirmed *in vivo*. Co-localisation of 4.1R and IF in the SW13 Vim^- cell line lacking endogenous IF would suggest an *in vivo* association and co-sedimentation of α-internexin and/or NF-L with 4.1R would confirm such an interaction. Localisation of α-internexin and NF-L proteins at PSDs *in vivo* would reinforce the findings of this study however the filamentous nature of these proteins may make this localisation difficult. *In vitro*, the binding sites for α-internexin and NF-L could be mapped and factors affecting these associations could be investigated. The regulation of 4.1-protein interactions by Ca^{2+} may be particularly important to 4.1R functions at PSDs. Little is known about the structures, interactions and functions of 4.1 proteins in non-erythroid tissues and in particular at specific subcellular domains. Consequently there is no shortage of research to be performed on the 4.1 proteins identified at PSDs.

Conclusions

In this study it has been demonstrated that multiple 4.1 gene products are differentially enriched in forebrain PSD fractions. Together with the cytoskeletal proteins spectrin, actin and ankyrin, 4.1 proteins are likely to be important in the formation and maintenance of a variety of specialised membrane domains. 4.1R may be a major structural component of PSDs and binds the intermediate filament proteins α-internexin and NF-L, possibly to maintain a minimal PSD complex during dynamic rearrangements. The high level of enrichment of 4.1R in PSD fractions supports a structural role by implying that 4.1R is found in a high proportion of PSD structures purified and is not transiently associated with PSDs. Mice deficient in 4.1R show deficits in movement, co-ordination, balance and learning (Walensky *et al.*, 1998b). The importance of postsynaptic structures in neuronal signalling and synaptic plasticity suggests that a lack of 4.1R at PSDs may contribute to these abnormalities.

The functions of 4.1 proteins are more diverse than the structural and anchoring function inferred by 4.1R in erythrocytes (Bennett & Gilligan, 1993) and coracle in septate junctions (Ward *et al.*, 1998). A possible interaction with dynamin suggests a role for 4.1R in endocytosis while 4.1N and B are involved in cell signalling and growth (Tran *et al.*, 1999; Ye *et al.*, 1999). 4.1 proteins may also be important in the organisation of the nuclear matrix (Capco *et al.*, 1982; DeCarcer *et al.*, 1995), regulation of pre-mRNA splicing (Lallena & Correas, 1997; Lallena *et al.*, 1998) and structural rearrangements that occur during mitosis (Krauss *et al.*, (1997b), Chapter 1, section 3.1.4.1). The identification of further 4.1 binding proteins at PSDs should help elucidate the functions of 4.1 and provide an insight into the architecture and workings of postsynaptic domains.

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Appendices

APPENDIX I

NEPHGE gel and protein identification table reproduced from Walsh and Kuruc (1992) *Journal of Neurochemistry*, **59**, p667-678.

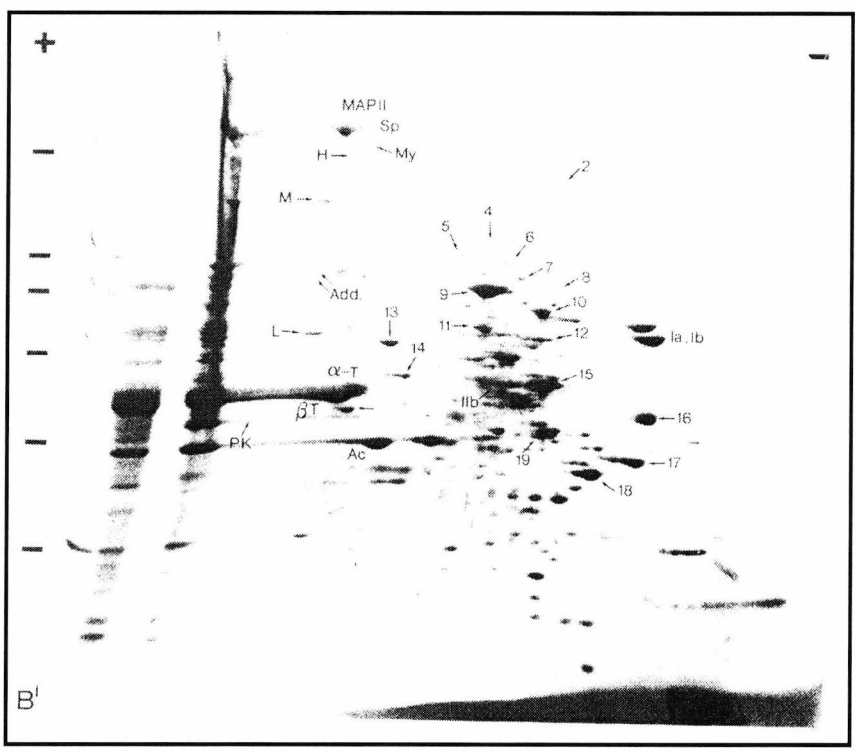

TABLE 1. *Identification of proteins enriched in the PSD fraction*

Protein	Molecular mass[a]	Characterization[b]	ID[c]
MAPII	≈280 kDa	2DE, Ab	—
Spectrin	235, 240 kDa	2DE, Ab	—
1	225, 230 kDa	2DE	—
Myosin	220 kDa	2DE, Ab	—
NF-H	200 kDa	2DE, coE	—
2	190 kDa	p. seq	NP
NF-M	150 kDa	2DE, coE	—
3	140 kDa	—	—
4	125 kDa	p. seq	NP
5	120 kDa	—	—
6	115 kDa	Ab	NP
7	110 kDa	p. seq	NP
8	108 kDa	p. seq	NP
9	100 kDa	p. seq	Dynamin
10	92 kDa	Ab, p. seq	Aconitase
Ia/Ib	85/80 kDa	2DE, Ab	Synapsin Ia/Ib
11	78 kDa	p. seq	NSF
12 (IIa)	76 kDa	Ab, p. seq	Synapsin IIa
NF-L	70 kDa	2DE, coE	—
13	68 kDa	Ab, p. seq	hsp70 family
14	58 kDa	Ab, p. seq	NF-S
15	57 kDa	Ab, p. seq	Pyruvate kinase
α-T	56 kDa	Nt. seq, 2DE	—
β-T	53 kDa	Nt. seq, 2DE	—
GABA-R (α)	52 kDa	Ab	—
GFAP	52 kDa	Ab, coE	—
16	50 kDa	p. seq	Elongation factor 1α
PKII (α-)	50 kDa	2DE	—
Actin	45 kDa	2DE, p. seq	—
19	43 kDa	p. seq	Creatine kinase
17	40 kDa	p. seq	Aspartate aminotransferase
18	38 kDa	p. seq	G3PDH

[a] Molecular masses widely used in the literature are given for well characterized proteins.

[b] Indicates the methods used for spot characterization; Ab, indicates that protein identification was by immunoblotting with specific antibodies; 2DE, indicates that protein identification was facilitated by characteristic 2DE mobility, e.g., the characteristic basic doublet synapsin Ia/Ib; coE, is coelectrophoresis; and p. seq and Nt. seq, indicate peptide sequencing and N-terminal sequencing, respectively.

[c] Other abbreviations: ID, identification; NP, new protein.

APPENDIX II

References accompanying Figure 7.1: Published interactions between proteins identified at excitatory PSDs.

Interaction	*Reference*	*Interaction*	*Reference*
4.1R – spectrin	(Tyler *et al.*, 1979)	NMDA receptor – spectrin	(Wechsler & Teichberg, 1998)
4.1R – hDlg	(Lue *et al.*, 1994)	NMDA receptor – hDlg	(Ehlers *et al.*, 1998)
4.1R – CASK	(Cohen *et al.*, 1998)	NMDA receptor – PSD95 family	(Niethammer *et al.*, 1996)
4.1R – tubulin	(Correas & Avila, 1988)	NMDA receptor – tubulin	(Ciruela *et al.*, 1999)
4.1 - neurexin	(Menegoz *et al.*, 1997; Ward *et al.*, 1998)	NMDA receptor - α-actinin	(Wyszynski *et al.*, 1997)
Actin – spectrin	(Ohanian *et al.*, 1984)	NMDA receptor – yotiao	(Lin *et al.*, 1998)
Actin - α-actinin	(Lazarides, 1976)	NMDA receptor – S-SCAM	(Hirao *et al.*, 1998)
Actin – CaMKII	(Shen *et al.*, 1998)	NMDA receptor - CIPP	(Kurschner *et al.*, 1998)
Actin - cortactin	(Wu & Parsons, 1993)	NMDA receptor – CaMKII	(Strack & Colbran, 1998)
AMPA receptor – ABP	(Srivastava & Ziff, 1999)	PSD95 family – CITRON	(Furuyashiki *et al.*, 1999; Zhang *et al.*, 1999)
AMPA receptor – GRIP	(Wyszynski *et al.*, 1998)	PSD95 family – SynGAP	(Kim *et al.*, 1998)
AMPA receptor – NSF	(Song *et al.*, 1998)	PSD95 family – BEGAIN	(Deguchi *et al.*, 1998)
AMPA receptor – PICK1	(Xia *et al.*, 1999)	PSD95 family – nNOS	(Brenman *et al.*, 1996)
AMPA receptor - hDlg	(Leonard *et al.*, 1998)	PSD95 family - neuroligan	(Irie *et al.*, 1997)
CASK – syndecan	(Hsueh *et al.*, 1998)	PSD95 family – MAGUIN	(Yao *et al.*, 1999b)
CASK – neurexin	(Hata *et al.*, 1996)	PSD95 family – K^+ Channels	(Kim *et al.*, 1996; Kim *et al.*, 1995)
CIPP - K^+ Channels	(Kurschner *et al.*, 1998)	PSD95 family – kainate receptor	(Garcia *et al.*, 1998)
CIPP - neurexin	(Kurschner *et al.*, 1998)	PSD95 family – GKAP	(Kim *et al.*, 1997)
Cortactin – CortBP1	(Boeckers *et al.*, 1999; Du *et al.*, 1998)	PSD95 family – CRIPT	(Niethammer *et al.*, 1998)

Table continued on next page.

Interaction	*Reference*	*Interaction*	*Reference*
GKAP - hDlg	(Hibino *et al.*, 2000; Kuhlendahl *et al.*, 1998)	PSD95 family – MAP1a	(Brenman *et al.*, 1998)
GKAP – nArgBP2	(Kawabe *et al.*, 1999)	SHANK – homer	(Tu *et al.*, 1999)
GKAP – SHANK	(Naisbitt *et al.*, 1999)	SHANK - mGluR	(Tu *et al.*, 1999)
GKAP – S-SCAM	(Hirao *et al.*, 1998)	SHANK – cortactin	(Naisbitt *et al.*, 1999)
GKAP – synamon	(Yao *et al.*, 1999a)	S-SCAM – MAGUIN	(Yao *et al.*, 1999b)
GRIP – ABP	(Srivastava & Ziff, 1999)	S-SCAM – neuroligan	(Hirao *et al.*, 1998)
hDlg - K^+ Channels	(Kim & Sheng, 1996)	S-SCAM – nRasGEP	(Ohtsuka *et al.*, 1999)
Homer – mGluR	(Tu *et al.*, 1999)	Syndecan – cortactin	(Kinnunen *et al.*, 1998)
Homer – IP3 receptor	(Tu *et al.*, 1999)	Tubulin – syndecan	(Kinnunen *et al.*, 1998)
Neuroligan – neurexin	(Nguyen & Sudhof, 1997)	Tubulin – CRIPT	(Passafaro *et al.*, 1999)
Neuroligan – CIPP	(Kurschner *et al.*, 1998)	Tubulin – MAP2	(Tokuraku *et al.*, 1999)
NF-L – spectrin	(Macioce *et al.*, 1999)	Tubulin – MAP1a	(Vaillant *et al.*, 1998)
NF-L - α-internexin	(Balin & Miller, 1995; Ching & Liem, 1993)	Tubulin – mGluR	(Ciruela *et al.*, 1999)
NF-L – NMDA receptor	(Ehlers *et al.*, 1998)	Yotiao – PP1	(Westphal *et al.*, 1999)
NF-L – PP1	(Terry-Lorenzo *et al.*, 2000)	Yotiao - PKA	(Westphal *et al.*, 1999)
NF-L – MAP2	(Frappier *et al.*, 1991)		