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Research paper

Expression of an anti-CD33 single-chain antibody by *Pichia pastoris*

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Abstract

CD33 is a cell surface glycoprotein expressed on cells of myelomonocytic lineage, leukaemic cells, but not haematopoietic stem cells. By virtue of its expression pattern, CD33 has become a popular target for new immunotherapeutic approaches to treat acute myeloid leukaemia. The methylotrophic yeast *Pichia pastoris* strain KM71H was used to produce an anti-CD33 single chain variable fragment (scFv), with the intention of conjugation to a radioisotope, for therapeutic use. To direct secreted expression of the anti-CD33-scFv the α-mating factor secretory signal sequence (α-MF) was used, with constructs containing a complete (CS) and incomplete (INCS) cleavage site to accommodate the potential outcomes of dibasic endopeptidase, Kex2, and dipeptidyl amino peptidase, Ste13, processing. The anti-CD33-scFv was expressed in BMMY cultures using both constructs, with a final yield of 48 mg/l (CS) and 11 mg/l (INCS). N-terminal sequencing showed that the CS-scFv had not been cleaved by Ste13, leaving amino acids EAEA at the N-terminus. The INCS-scFv construct produced a mixture of 50% authentic scFv and 50% with 11 amino acids from the α-MF remaining at the N-terminus. Despite the aberrations in α-MF processing, the anti-CD33-scFv's produced from both constructs were found to be functional. Flow cytometry and Biacore analysis demonstrated binding to target antigen CD33 on the surface of human leukaemic cell line HL-60, and to recombinant soluble CD33 respectively.

Keywords: CD33; scFv; *Pichia pastoris*; AML

Abbreviations: Abs, antibodies; AML, acute myeloid leukaemia; AOX, alcohol oxidase; BMGY, buffered glycerol complex medium; BMMY, buffered methanol complex medium; CFU-GEMM, colony forming units for granulocytes, erythrocytes, monocytes and megakaryocytes; CFU-GM, BFU-E, colony forming units for granulocyte mononuclear phagocytes, and early erythroid progenitors; CS, complete α-MF cleavage site; Da, Daltons; DMI, desipramine; FAB, French–American–British; FACS, fluorescence activated cell sorting; FBS, foetal bovine serum; FITC, fluorescein isothiocyanate; HRP, horse radish peroxidase; IgG, immunoglobulin G; INCS, incomplete α-MF cleavage site; α-MF, α mating factor; MW, molecular weight; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PHA, phytohemagglutinin; PHO1, gene encoding acid phosphatase; PVDF, polyvinylidene fluoride; rsCD33, recombinant soluble CD33; RU, resonance units; ScFv, single chain variable fragment; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; VH, immunoglobulin heavy chain variable region; VL, immunoglobulin light chain variable region; YPDS, yeast extract peptone dextrose medium with sorbitol.

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1. Introduction

CD33 is a 67 kDa transmembrane glycoprotein (Simmons and Seed, 1988) with Ig like extracellular domains and a binding specificity for sialic acid, features that place it in the structurally related group of sialic acid binding Ig related lectins (Freeman et al., 1995; Kelm et al., 1996). CD33 was originally defined in studies with the monoclonal antibody MY9 (Griffin et al., 1984), and with additional studies using antibodies of the CD33 cluster group (MY9, L1B2, L4F3, WM-53, and WM-54) showing that CD33 is an early myeloid differentiation antigen, expressed on colony forming progenitor cells, such as multipotential stem cells (CFU-GEMM), granulocyte, macrophage and erythroid precursors (CFU-GM, BFU-E) (Griffin and Schlossman, 1984; Andrews et al., 1983; Peiper et al., 1988; Favaloro et al., 1987). Expression of CD33 continues along the myelomonocytic pathway until it is downregulated on granulocytes but retained on monocytes and tissue macrophages. Although the function of CD33 has not yet been elucidated, the expression pattern of CD33 and observations from a number of studies suggest that CD33 plays a regulatory role in myeloid cell differentiation/haematopoiesis (Taylor et al., 1999; Paul et al., 2000; Ulyanova et al., 1999; Mingari et al., 2001). CD33 is also expressed by leukaemic blast cells with a density of approximately 10,000 sites per cell (Tanimoto et al., 1989) in 70–90% of acute myeloid leukaemia (AML) patients, and is considered a phenotypic marker for AML, particularly those in the FAB M1–5 classification, where it is consistently expressed (Griffin et al., 1984; Litz and Brunning, 1992; Matutes et al., 1985; Dinndorf et al., 1986). It is, however, not expressed by any non-haematopoietic cells (Peiper et al., 1989) or the pluripotent haematopoietic stem cells (Bernstein et al., 1994; Andrews et al., 1989), responsible for the regeneration of all cells of the haematopoietic system, and therefore has become a popular target for new immunotherapies of AML.

A number of anti-CD33 antibodies (Abs) have already been developed as potential immunotherapeutic agents for AML, used alone (Gibson, 2002; Caron et al., 1998) or infused with cytokines (Caron et al., 1995; Kossman et al., 1999), conjugated to radioisotopes (Jurcic et al., 1995, 2002), immunotoxins (Pagliaro et al., 1998), other antibodies (bispecific Abs) (Balaian and Ball, 2001) and most successfully to the anti-tumour antibiotic calicheamicin, known as Mylotarg™ (Tomblyn and Tallman, 2003). These therapies have concentrated on the use of whole IgG molecules, whereas increasingly, smaller engineered antibody fragments such as the single-chain variable fragments (scFv) are being developed for a number of applications such as: tumour imaging (Begent et al., 1996; Goel et al., 2001), cancer therapy (Kikuchi et al., 2004; Tur et al., 2003), molecular immunolabelling (Malecki et al., 2002), and diagnostics (Peipp et al., 2004).

Single-chain Abs are composed of a variable heavy and light chain joined by a small flexible linker peptide such as $(Gly_4Ser)_3$, a structure that retains the original specificity and full monovalent binding of the intact parent Ab (Huston et al., 1998). The smaller size of 25–30 kDa compared to ~155 kDa of whole IgG Abs confers pharmacokinetic advantages such as faster uptake and better penetration through the vasculature of tumours (Yokota et al., 1992). Single-chain Fv proteins administered intravenously also have a shorter elimination time than whole IgG (Colcher et al., 1990), thereby decreasing the non-specific toxicity to normal tissues/organs associated with the prolonged time in circulation of whole IgG. A shorter elimination time results in high tumour to whole body uptake ratios, which is particularly important when using radiolabelled Abs since increased circulation time exposes normal tissues to an increased radiation dose (Waldmann, 1991; Colcher et al., 1999). Other scFv advantages include little or no immunogenicity, high stability and simplified design of genetically modified constructs for expression of scFv in a range of systems (Bird et al., 1988; Huston et al., 1998; Begent et al., 1996).

In this report we describe the use of *Pichia pastoris* to express an anti-CD33-scFv, with a view to conjugating the scFv to a radioisotope as a possible immunotherapeutic approach to treat AML. Since the use of *P. pastoris* to produce "single-cell-protein" by Philips Petroleum company in the 1980s (Wegner, 1990), many recombinant proteins have been successfully expressed (Cereghino and Cregg, 2000) including other scFv and therapeutic proteins such as anti-desipramine (DMI) scFv for the treatment of DMI over-

dose (Eldin et al., 1997), and an scFv targeted to a tumour associated glycoprotein TAG-72 (Goel et al., 2000b). *P. pastoris* is a methylotrophic yeast whose ability to utilize methanol as a sole carbon source (Lee and Komagata, 1980; Ogata et al., 1969) has been exploited for the expression of recombinant proteins. The enzyme, alcohol oxidase (AOX), which catalyses the first step in the methanol utilisation pathway, is largely encoded by the methanol regulated/inducible AOX1 gene (Cregg et al., 1989; Ellis et al., 1985). The AOX1 promoter has been used in many *P. pastoris* expression vectors and thus recombinant protein genes placed downstream and under the control of the AOX1 promoter are transcribed upon addition of methanol to the expression culture. The highly inducible nature of protein expression in *P. pastoris* combined with the ability to perform post-translational modifications such as glycosylation (Gemmill and Trimble, 1999; Bretthauer and Castellino, 1999), fast and economic growth, ease of scale-up to fermentation, and the option of expression of recombinant protein either intracellularly or secreted into the culture medium (Cereghino and Cregg, 2000) make the system ideal for scFv production.

Here we report the successful expression of an anti-CD33-scFv in *P. pastoris* strain KM71H, and the demonstration of activity in interactions with target antigen CD33.

2. Materials and methods

2.1. Bacteria and yeast strains

Bacterial and yeast strains used were *Escherichia coli* XL1-Blue; *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB* $lac^qZ\Delta M15$ Tn*10* (Tet^r)] (Stratagene, La Jolla, CA, USA) and *P. pastoris* strain KM71H; *aox1*::*ARG4;arg4* (Invitrogen Ltd, Paisley, UK).

2.2. Cell culture

Human leukaemic and T-helper cell lines (HL-60-No. 98070106 and Jurkat E6.1-88042803, European Collection of Cell Cultures, Salisbury, Wiltshire, UK) were maintained in 90% RPMI-1640 (made "in house"), 10% Foetal Bovine Serum (FBS) (Heat-inactivated, Gibco-BRL, Invitrogen) and 2 mM L-glutamine (Gibco-BRL) at cell densities of $1–9 \times 10^5$ and $3–9 \times 10^5$ cells/ml respectively, and incubated at 37 °C with 5% CO_2.

2.3. Cloning of anti-CD33-scFv

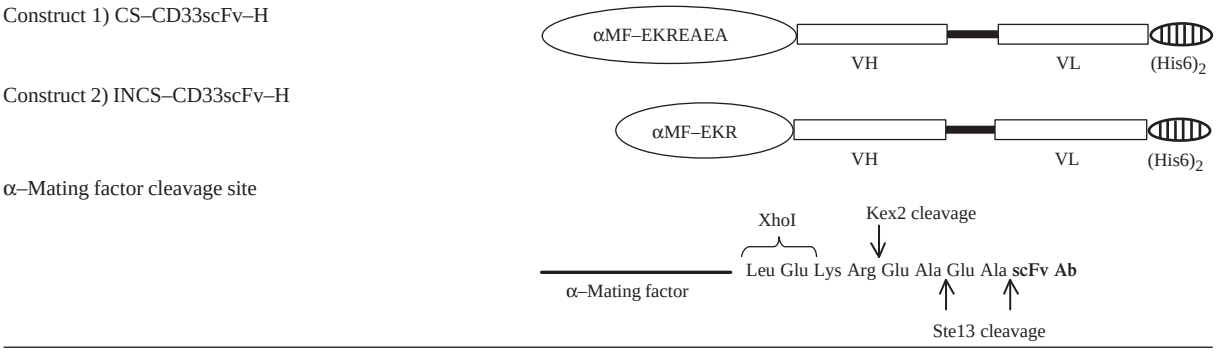
The gene encoding the anti-CD33-scFv was amplified using PCR from an existing construct encoding an anti-CD33-scFv as part of a chimaeric T-cell receptor. This was previously generated in our laboratory (unpublished data) using an anti-CD33-mAb p67. The variable domains of the genes were cloned from the hybridoma using standard techniques (Nicholls et al., 1993).

Primers were designed to facilitate insertion of the construct into *P. pastoris* expression vector pPICZα (Invitrogen), and to generate two constructs with a complete and incomplete α-mating factor signal sequence cleavage site (hereafter referred to as CS and INCS respectively), and a his-tag as shown in Table 1.

The primers used to generate the scFv constructs were: 1) **CSCD33SCFV5**; ATATATCTCGAGAAGAGAGAGGCTGAAGCAGATATCCAGCTCACTCAGAGT and **CD33SCFVHIS**; ATATATGCGGCCGCTCATCAATGGTGATGGTGATGGTGTGAACCGCCACCTCCTGAACCCCCTCCGCCGGATCCACCCC-CGCCATGGTGATGGTGATGATGAGAAGACACTGTCACCAGTGT 2) **INCSCD33SCFV5**; ATATATCTCGAGAAGAGAGATATCCAGCTCACTCAGAGT and **CD33SCFVHIS**. Regions complementary to the scFv gene are underlined, and the remaining regions were included to facilitate cloning. All primers were synthesised by Oswell, Southampton, UK. Components of the PCR for generation of anti-CD33-scFv constructs were 1X TaqPlus buffer, 2.5 units TaqPlus (Stratagene), 50 μM each dNTP (Perkin Elmer, Warrington, Cheshire, UK), 0.25 μM primers, and 160 ng DNA template. Reaction conditions were: 94 °C–1 min, 1 cycle/94 °C–1 min, 60 °C–1 min, 72 °C–1 min, 25 cycles/72 °C–10 min, 1 cycle.

After PCR, the amplified scFv constructs were ligated into pGEMTeasy (Promega, Madison, WI, USA) and gel purified XhoI, NotI digested constructs sub-cloned into the XhoI and NotI sites of the multiple cloning site of pPICZα using a rapid ligation kit (Roche, Lewes, East Sussex, UK). Recombinant pPICZα-INCS/CS-CD33scFv-H was amplified in *E.*

Table 1
Diagrammatic representation of the anti-CD33-scFv gene expression constructs



5′ primers were designed to regenerate the cleavage site of the α-MF secretory signal sequence, such that the cleavage site of the signal sequence would be flush with the N-terminus of the mature recombinant protein. Primers were also used to create complete and incomplete α-MF cleavage sites, as indicated by the large oval. The adjoining black region between VH and VL indicates a $(Gly_4Ser)_4$ linker.

coli strain XL1-Blue and submitted for DNA sequencing to MWG Biotech (Milton Keynes, UK).

2.4. Transformation of P. pastoris

Competent cells of *P. pastoris* strain KM71H were prepared and electroporated with 5 μg SacI linearised pPICZα-INCS/CS-CD33scFv-H DNA according to the manual protocol for electroporation of *P. pastoris* with the BIO-RAD Gene Pulser Xcell™ Electroporation system. Electroporation conditions were $C=25$ μF, PC=200 Ω, V=2.0 kV. Electroporated cells (50 μl) were spread onto YPDS plates containing 100, 250, 500, and 1000 μg/ml zeocin and incubated for 2–3 days at 30 °C. A number of colonies were then re-streaked onto YPDS-zeocin plates to isolate single colonies for PCR analysis and expression studies.

2.5. PCR analysis of positive transformants with AOX1 primers

KM71H positive transformants were analysed for the presence of the pPICZα-INCS/CS-CD33scFv-H constructs using PCR with AOX1 primers (5′AOX1, GACTGGTTCCAATTGACAAGC, 3′AOX1, GCAAATGGCATTCTGACATCC). To obtain template DNA, single colonies from re-streaked YPDS-zeocin plates were resuspended in 50 μl 1 mg/ml lyticase (Sigma-Aldrich, Poole, Dorset, UK), incubated at 37 °C for 30 min, followed by boiling for 5 min. 2 μl of lysed colonies were used for PCR analysis. PCR components and conditions were as follows: 1× Taq polymerase buffer II, 2 mM $MgCl_2$, 0.2 mM each dNTP, 1 Unit Taq polymerase (Perkin Elmer) and 0.5 μM each primer 5′ and 3′ AOX1, 94 °C–1 min, 1 cycle/94 °C–45 s, 60 °C–45 s, 72 °C–1.30 min, 30 cycles/72 °C–7 min, 1 cycle.

2.6. Screening for expression of anti-CD33-scFv

To screen for expression of anti-CD33-scFv, 25 ml buffered glycerol complex medium with zeocin (BMGY; 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base (YNB), 4×10^{-5}% biotin, 1% glycerol) were inoculated with PCR identified positive KM71H-pPICZα-CS/INCS-CD33scFv-H transformants, and grown until an OD_{600} of 20–40 was reached. Cells were then diluted 1 in 10 into 25 ml BMGY+1% Casamino acids, and grown for a further 6–8 h at 30 °C with shaking of 250 rpm. These cultures were centrifuged at $5000\times g$ and the pelleted cells resuspended in 25 ml buffered methanol complex medium (BMMY; as BMGY except that 1% glycerol was replaced with 0.5% methanol and 1% Casamino acids were added). Cultures were grown for 96 h at 30 °C at 250 rpm, with addition of methanol to a concentration of 0.5% v/v every 24 h. Samples were taken at regular intervals for analysis of anti-CD33-scFv expression.

Culture supernatant samples were analysed by electrophoresis on 10% Nu-Page Bis–Tris gels (Invitrogen) followed by either Coomassie staining or

western blotting and immunoprobing. Western blotting was achieved using a semi-dry method with the Trans-Blot® Semi-Dry electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA), with Bjerrum and Schafer-Nielsen buffer (Bjerrum and Schafer-Nielsen, 1986) at 10 V for 30 min. Immunoprobing was carried out using an anti-His-HRP antibody (BD Biosciences Clontech, California, USA) as per manufacturer's protocol.

2.7. Purification of anti-CD33-scFv

To purify the anti-CD33-scFv from KM71H cultures, affinity chromatography was used with a HisTrap™ HP kit using a 1 ml HisTrap™ HP precharged Ni Sepharose™ high performance column (Amersham Biosciences, Chalfont St. Giles, Bucks, UK). The HisTrap column was linked to a peristaltic pump P-1, and operated at a flow rate of 1 ml/min. To prepare the sample, 10 ml of culture medium were centrifuged at 5000×*g* to remove the cells, and the supernatant filtered through a 0.45 μm polyethersulfone filter (Sartorius, Surrey, UK). The manufacturer's optimisation protocol with a stepwise elution gradient was used, to determine the ideal conditions for future purifications of scFv. These were found to be; 1× phosphate buffer pH 7.4 (20 mM phosphate, 0.5 M NaCl) with 100 mM and 300 mM imidazole for equilibration and washes, and elution respectively. Flow-through, washes and elutions were all collected and stored at −20 °C.

2.8. N-terminal sequencing analysis

Purified anti-CD33-scFv was electrophoresed using SDS-PAGE, and the separated proteins transferred to Sequi-Blot™ polyvinylidene fluoride (PVDF) membrane (Bio-Rad) using tris-glycine buffer (20 mM tris, 150 mM glycine, 10% methanol). The semi-dry method used was as described in 2.6. After transfer, the membrane was stained with Coomassie stain (0.1% Coomassie Blue R-250, 40% methanol, 1% acetic acid) for 1 min, followed by destaining with 50% methanol until protein bands were clearly visible. Protein bands corresponding to the expected MW of the anti-CD33-scFv were excised and submitted for N-terminal sequencing analysis. The first 10 amino acids of purified CS and INCS anti-CD33-scFv were sequenced "in house" in the Protein Science Suite using an Applied Biosystems Procise 492 protein sequencer.

2.9. Analysis of cell binding

The binding ability of the anti-CD33-scFv to the target antigen CD33, was analysed using CD33 positive HL-60 and CD33 negative Jurkat cell lines. 1×10^6 cells in 100 μl 1× phosphate buffered saline (PBS) +2% FBS were incubated with a number of antibodies as follows: isotype control IgG1κ-FITC (amount as per manufacturer's protocol), anti-CD33-FITC (20 ng) (BD Biosciences, San Jose, CA, USA), anti-His-tag-FITC (660 ng) (Qiagen), and CS or INCS anti-CD33-scFv (600 ng) followed by anti-His-tag-FITC. Blocking experiments were performed using HL-60 cells with unlabelled anti-CD33-Ab (120 ng) (BD Biosciences) followed by CS or INCS anti-CD33-scFv, and anti-His-tag-FITC. Cells were incubated with antibody at room temperature for 30 min in the dark, followed by two washes with 1 ml wash buffer (1× PBS, 2% FBS) after each antibody incubation, with final resuspension of cells in 1 ml fixing solution (1× PBS, 1% paraformaldehyde). Using a flow cytometer (Becton Dickinson FACSCalibur), unlabelled cells were used to place the cell population of interest on scale, followed by gating of the isotype control labelled cells into the first log phase, to adjust for background fluorescence. The remaining samples were then analysed for the presence of fluorescence.

2.10. Biacore studies

Binding of the anti-CD33-scFv to recombinant soluble CD33 (ectodomain, amino acids 1–242 of the mature protein) (rsCD33) also expressed by *P. pastoris* (Emberson, 2002) was analysed with surface plasmon resonance using a BIAcore 2000 system (Biacore International SA, Stevenage, Herts, UK). Binding studies were performed with both CS and INCS scFv, by immobilizing >10,000RU scFv in 10 mM sodium acetate pH 5.0, onto a CM5 sensor chip using the amine coupling method (Wizard procedure). rsCD33, negative control glycoproteins (a 9 μg/μl mix of human transferrin, ribonuclease B, and human α_1-acid glycoprotein), and rsCD33 mixed with an anti-CD33-Ab (Serotec, Oxford, UK) as a blocking exper-

iment, were then passed over the immobilized scFv in HBS-P buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.005% polysorbate 20 (v/v)) at a flow rate of 10 μl/min at 25 °C. To determine the binding affinity of the CS-anti-CD33-scFv, 750RU of the scFv in 10 mM sodium acetate pH 5.0 was immobilised onto a CM5 sensor chip using the amine coupling method (Wizard procedure). A range of concentrations of rsCD33 were then passed over the immobilised Ab at a flow rate of 30 μl/min at 25 °C. k_a, k_d, and K_D were determined using BIAevaluation version 3.1 software, by fitting data to a 1:1 Langmuir binding model (scFv). HBS-P was used as running buffer, and 10 mM glycine–HCl pH 2.5 was used as regeneration buffer, injected for 30 s at 10 μl/min. All reagents and buffers used were from Biacore.

3. Results

3.1. Anti-CD33-scFv cloning and transformation

Two constructs were generated using PCR to encode an anti-CD33-scFv composed of a variable heavy (VH) and variable light (VL) chain, linked by $(Gly_4Ser)_4$ to allow spatial flexibility (Huston et al., 1991). The two constructs differed in respect to the N-terminus, whereby one construct had a complete α-MF signal sequence cleavage site, and the other an incomplete cleavage site, as indicated in Table 1. The differing signal sequences were included to accommodate the potential outcomes of Kex2 and Ste13 processing of the recombinant scFv. Although it has been suggested that the complete signal sequence is necessary for correct cleavage of the α-MF (Sreekrishna et al., 1997; Raemaekers et al., 1999), there are reports of incomplete cleavage by Ste13 leaving the Glu–Ala repeats at the N-terminus (Goda et al., 2000; Almeida et al., 2001), and also effective cleavage without the Glu–Ala repeats (Rosenfeld, 1999; Ruitenberg et al., 2001). These reports suggest that the complete signal sequence is not always necessary for correct cleavage, and also in some cases leads to incorrect processing of the α-MF. At the C-termini of both anti-CD33-scFv constructs, stop codons and a $(His_6)_2$ tag were included via PCR primers, to prevent the expression of the $(His)_6$ and c-*myc* tags of pPICZαA and replace them with a single $(His_6)_2$ tag. This was done as the $(His_6)_2$ tag was to be used for detection and purification, and thus the c-*myc* tag and additional amino acids between the c-*myc* and His-tag of pPICZα were unnecessary.

The scFv constructs were successfully inserted into the XhoI and NotI sites of pPICZαA, generating the pPICZαA-CS-CD33scFv-H and pPICZαA-INCS-CD33scFv-H plasmids, placing the constructs under the control of the AOX1 promoter, for expression and secretion of the ~30,000 Da anti-CD33-scFv.

After transformation of KM71H, PCR analysis of zeocin-resistant transformants amplified a 1500 bp DNA fragment corresponding to the 850 bp scFv construct and flanking DNA up to the AOX1 primer sites (Fig. 1). This confirmed the integration of pPICZαA-CS-CD33scFv-H and pPICZαA-INCS-CD33scFv-H respectively into 9/9 and 10/10 KM71H clones tested, which were subsequently grown in cultures to screen for expression of anti-CD33-scFv.

3.2. Screening for expression of anti-CD33-scFv

Initially, a number of KM71H-pPICZαA-CS/INCS-CD33scFv-H (hereafter abbreviated as K-CS/INCS-scFv) clones identified as positive transformants by PCR were screened for expression of the anti-CD33-scFv using a protocol described by Gram et al. (1998). Most clones tested expressed the anti-CD33-scFv (data not shown), but further analysis identified one clone of each construct as a high expresser compared to all other clones screened. Time course analyses of BMMY induction cultures of K-CS-scFv clone 4 and K-INCS-scFv clone 4 were performed and samples were analysed by SDS-PAGE followed by Coomassie staining. The results show that a protein with the expected MW of the anti-CD33-scFv (ca. 30 kDa) was present in the culture medium of K-CS/INCS-scFv, but not in that of the parent strain KM71H (Fig. 2a). Further analysis with western blotting/immunoprobing showed that an anti-His-HRP Ab positively bound to protein bands of the same MW in the scFv cultures but again not in the parent strain culture (Fig. 2b). These results suggest that the His-tagged anti-CD33-scFv was successfully expressed in KM71H cultures.

Both Figs. 2a and b show that expression of the anti-CD33-scFv was detectable after just 24 h post

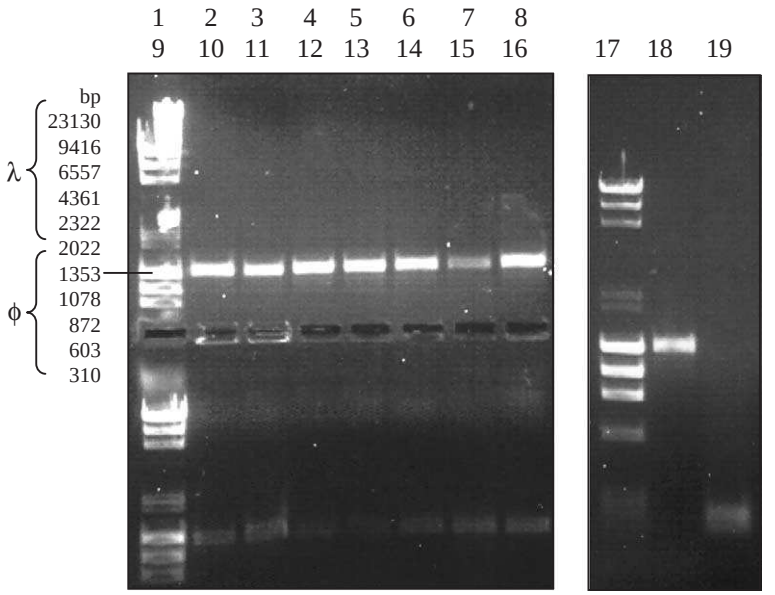


Fig. 1. PCR analysis of KM71H positive transformants using AOX1 primers. Whole PCR (20 μl) was electrophoresed on a 1% agarose gel at 12V/cm for 45–60 min. The gel on the left is a double gel with Lanes 1–8 and 9–16 corresponding to the upper and lower parts of the gel respectively. Lanes 1, 9 and 17 show 0.5 μg λ*Hin*dIII and ϕX174*Hae*III DNA markers, lanes 2–6 show PCR products from 5′ and 3′ AOX1 primer amplification of KM71H pPICZα-CS-CD33scFv-H clones 1–5, lanes 7, 8, 10–16 and 18 KM71H pPICZα-INCS-CD33scFv-H clones 1–10, and lane 19, control reaction with *Pichia pastoris* vector pPIC3.5.

methanol induction and increased over time, although the amount of anti-CD33-scFv was greater in the culture of the K-CS-scFv clone, with the complete α-MF signal sequence, than from the culture of the construct with the incomplete α-MF signal sequence.

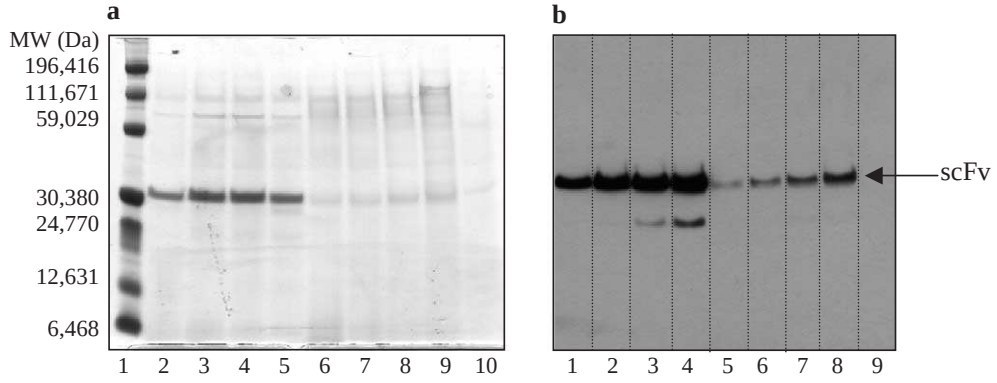


Fig. 2. (a) Time course analysis of BMMY KM71H-pPICZαA-CS/INCS-CD33scFv-H cultures. 30 μl of culture supernatant from 0.5 ml samples taken at 24, 48, 72, and 96 h of BMMY induction cultures were electrophoresed on a 10% Bis–Tris Nu-Page gel (Invitrogen) followed by Coomassie staining. Lane 1 shows 10 μl Bio-Rad kaleidoscope marker, lanes 2–5 and 6–9 show 24, 48, 72, and 96 h samples from KM71H-pPICZα-CS-CD33scFv-H clone 4, and KM71H-pPICZα-INCS-CD33scFv-H clone 4 respectively. Lane 10 shows a 96 h sample from a culture of the parent strain KM71H. (b) Western blot/immunoprobing of time course samples from BMMY KM71H-pPICZαA-CS/INCS-CD33scFv-H cultures. 10 μl of culture supernatant from 0.5 ml samples taken at 24, 48, 72, and 96 h of BMMY induction cultures were electrophoresed on a 10% Bis–Tris Nu-Page gel (Invitrogen) followed by transfer to nitrocellulose, and immunoprobing with 1 in 10,000 anti-His-tag-HRP antibody (BD Biosciences Clontech). Lanes 1–4 and 5–8 show 24, 48, 72, and 96 h samples from KM71H-pPICZα-CS-CD33scFv-H clone 4, and KM71H-pPICZα-INCS-CD33scFv-H clone 4 respectively. Lane 9 shows a 96 h sample from a culture of the parent strain KM71H.

3.3. Purification of anti-CD33-scFv

After confirmation that His-tagged protein was being expressed in K-CS/INCS-scFv cultures, protein purification was performed in order that further analysis and characterisation of the His-tagged protein identified in the time course analysis could be carried out. Affinity chromatography was used with a HisTrap column and protein eluted with 300 mM imidazole.

Using this method of purification, approximately 95–100% of the protein was shown to bind to the Ni-NTA agarose as seen from the lack of scFv in the flow-through (lanes 3 and 7, Fig. 3), and the eluted protein was relatively free of contaminants (lanes 5 and 9, Fig. 3), with a final yield of approximately 48 mg/l and 11 mg/l for CS-scFv and INCS-scFv respectively. The purified CS-anti-CD33scFv has a MW of ~30,000 Da, consistent with the protein seen in the time course analysis, and migrates as a single band, whereas the purified INCS-anti-CD33scFv, although also migrating at the same MW, was present as a doublet of bands seen in lane 9. These bands were of a very similar MW and the relative intensity of each was the same. Electrophoresis of the INCS-scFv on a reducing gel, showed a similar doublet (data not shown), indicating that the apparent difference in size was due to MW rather than different disulphide bonding patterns of mis-folded scFv. This heterogeneity in MW could be due to incorrect processing of the α-MF signal sequence by Kex2. Alternatively or in addition, differences in post-translational modifications such as glycosylation, may give rise to different isoforms of the scFv, as the scFv and α-MF secretory signal sequence contain one and three putative glycosylation sites respectively.

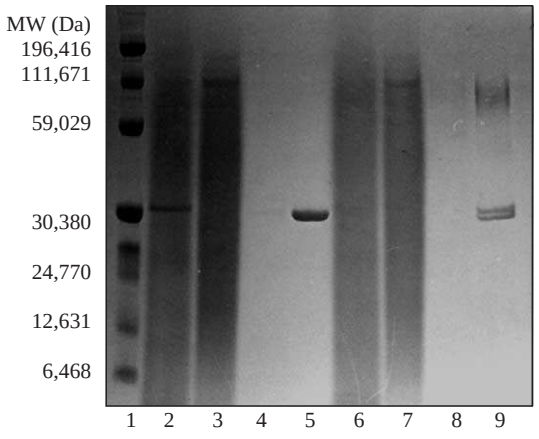


Fig. 3. Coomassie stained Nu-Page gel of purified anti-CD33-scFv. Anti-CD33-scFv was purified on a Ni^{2+} column, and samples electrophoresed on a Nu-Page 10% Bis–Tris gel, followed by Coomassie staining. Lane 1: 10 μl Bio-Rad kaleidoscope marker. Lane 2: CS-scFv, 20 μl, 96 h culture sample (cells removed). Lane 3: CS-scFv, 20 μl 6 ml flow-through concentrated to 0.5 ml. Lane 4: CS-scFv, 30 μl concentrated wash. Lane 5: CS-scFv, 1.5 μg non-reduced purified protein. Lane 6: INCS-scFv, 30 μl, 96 h culture sample (cells removed). Lane 7: INCS-scFv, 20 μl 6 ml flow-through concentrated to 0.5 ml. Lane 8: INCS-scFv, 30 μl concentrated wash. Lane 9: INCS-scFv, 1.5 μg non-reduced purified protein. (The 100 kDa smear seen in lane 9 was evident in some protein purification elutions. It has not been identified, but may be proteins with histidine residues non-specifically binding to the Ni-NTA agarose. Only pure INCS-anti-CD33-scFv was used for further studies.)

3.4. N-terminal sequencing analysis

After successful identification of a His-tagged protein in the MW range expected for the anti-CD33-scFv in the KM71H expression cultures, samples of purified protein were subjected to N-terminal sequencing analysis, to confirm the identity of the proteins. It was apparent from the N-terminal sequencing data (Table 2) that the His-tagged proteins identified in Fig. 2a and b were indeed the anti-CD33-scFv. However, although the Kex2 protease had cleaved the α-MF secretory signal sequence from the CS-CD33scFv, the presence of extraneous amino acids Glu–Ala–Glu–Ala at the N-terminus indicated that Ste13 cleavage had not occurred. Aberrant processing of the αMF had also affected the INCS-scFv as the sequencing data showed that the upper band of the INCS-scFv doublet was most likely to be incorrectly processed anti-CD33-scFv. The amino acids are identical to the last 10 but one of the α-MF secretory signal sequence (C-terminus), showing that cleavage had occurred after Ile–Ala (position 70, 71 of αMF) either by Kex2 or another protease. Although the N-terminal sequencing data obtained did not extend into the scFv region of the recombinant fusion protein, the MW and positive binding of an anti-His-HRP Ab suggests that the upper band of the doublet was INCS-scFv with 11 amino acids from the C-terminus of αMF. These extra amino acids are most likely responsible for the increased MW seen upon migration in an

Table 2
N-terminal sequencing analysis data

INCS–Anti–CD33–sc Fv10 cycles	1	A	K	E	E	G	V	S	L	E	K														
	2																D	I	Q	L	T	Q	S	P	S	T
INCS–Anti–CD33–scFv construct		A	K	E	E	G	V	S	L	E	K	R	–	–	–	–	D	I	Q	L	T	Q	S	P	S	T
αMF secretory signal (Last 15 amino acids)		A	K	E	E	G	V	S	L	E	K	R^K	E	A^S	E	A^S	Anti–CD33–scFv									
CS–Anti–CD33–scFv construct		A	K	E	E	G	V	S	L	E	K	R	E	A	E	A	D	I	Q	L	T	Q	S	P	S	T
CS–Anti–CD33–scFv 10 cycles													E	A	E	A	D	I	Q	L	T	Q				

The table has been designed to radiate out from the middle. Therefore the middle row shows the last 15 amino acids of the α-MF, with the letters in superscript corresponding to the Kex2 (K) and Ste13 (S) cleavage sites. The next two rows going outwards show the native sequence of the N-terminus of the CS and INCS scFv constructs and difference in α-MF sequence. The next rows out show the sequencing data from the CS-scFv (bottom row) and INCS-scFv (top two rows), with 1 and 2 corresponding to the upper and lower band respectively.

SDS-PAGE gel. The lower band of the doublet was correctly processed anti-CD33-scFv, as indicated by the lack of extraneous amino acids at the N-terminus, and 100% match with the native anti-CD33-scFv.

3.5. Cell binding analysis

To determine whether the anti-CD33-scFv expressed in *P. pastoris* was specific for target antigen CD33, the INCS and CS scFv were incubated with CD33 positive cells (HL-60) and a CD33 negative cell line (Jurkat), followed by analysis with a flow cytometer. These cell lines were confirmed to be positive and negative respectively for CD33 expression using an FITC labelled anti-CD33-Ab. As shown in Fig. 4A,B, 99% of HL-60 cells fluoresced in the positive M2 phase, whereas, as expected, only 2.2% of Jurkat cells showed fluorescence in the positive M2 phase, confirming that Jurkat cells express little or no CD33. Analysis of HL-60 and Jurkat cells incubated with CS/INCS anti-CD33-scFv followed by an anti-His-FITC gave similar results, with 99% and 87% of HL-60 cells fluorescing in the positive M2 phase, as compared to 23.5% and 21% of Jurkat cells (CS-scFv and INCS-scFv respectively). Although binding of the anti-CD33-scFv to Jurkat cells was higher than that of the anti-CD33-FITC-Ab (23.5% [CS] and 21% [INCS] as compared to 2.2%), the anti-His-FITC-Ab used as a secondary Ab to detect the scFv, bound significantly to Jurkat cells (16.5%) when used alone, without the scFv. Therefore the apparent positive binding of the anti-CD33-scFv to Jurkat cells is largely accounted for by non-specific binding of the anti-His-FITC Ab to Jurkat cells, rather than specific binding of the scFv.

To confirm the specificity of the positive binding observed with the anti-CD33-scFv to HL-60 cells, a blocking experiment was performed whereby HL-60 cells were incubated with unlabelled anti-CD33-Ab, to block available CD33 antigen sites. Cells were then incubated with scFv, followed by anti-His-FITC. Blocking with unlabelled anti-CD33-Ab reduced the percentage of cells fluorescing in the positive M2 phase from 99% to 22.5% (CS-scFv) and 87% to 10.5% (INCS-scFv), suggesting that the binding of both CS and INCS anti-CD33-scFv to cell surface antigen CD33 was specific.

3.6. Biacore analysis

Biacore studies were performed to assess the ability of the anti-CD33-scFv to bind to the target antigen CD33, in a recombinant soluble form (rsCD33), also expressed by *P. pastoris* (Emberson, 2002). Initial binding studies performed with the scFv (INCS and CS) showed binding to rsCD33, which was reduced when using commercial monoclonal anti-CD33-Ab as a blocking agent (INCS-scFv, Fig. 5a, similar data for CS-scFv, not shown), suggesting that the scFv-rsCD33 interaction was specific. Further Biacore stud-

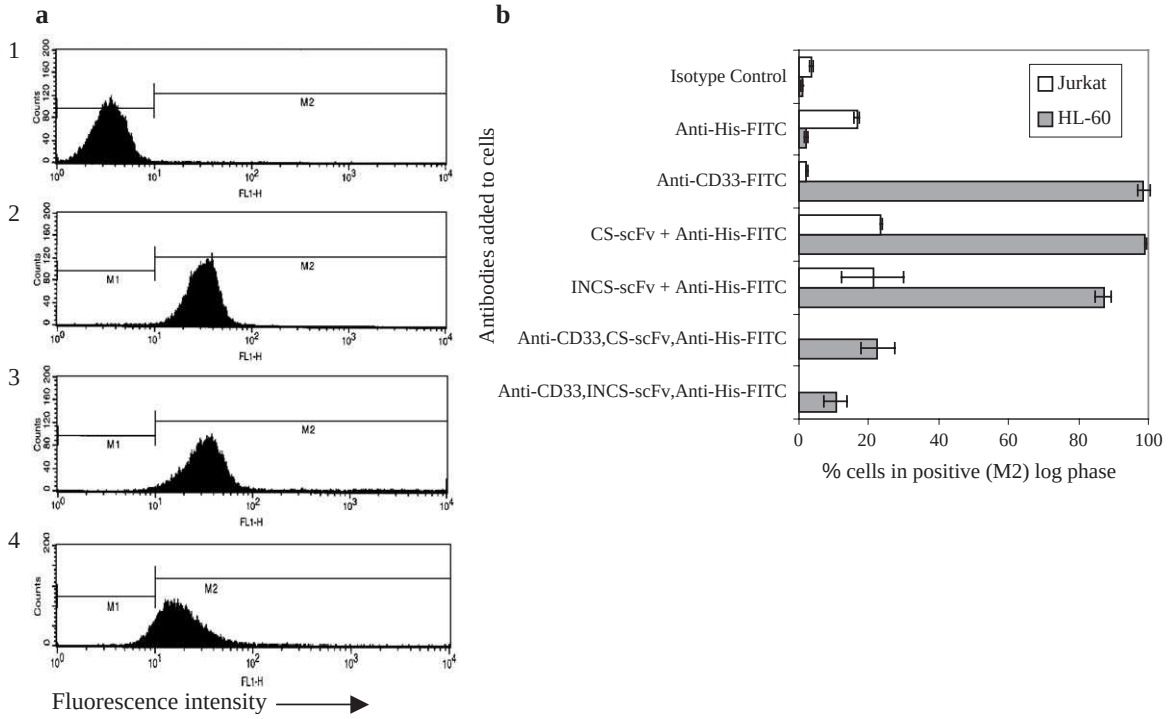


Fig. 4. Binding of anti-CD33-scFv to CD33 positive and CD33 negative cell lines HL-60 and Jurkat. Panel A shows the binding of isotype control (1), anti-CD33-FITC (2), CS-scFv (3), and INCS-scFv (4) antibodies to HL-60 cells, detected by FACS analysis. Panel B shows a plot of the percentage of HL-60 and Jurkat cells in the positive M2 phase of the histogram after incubation with antibodies as stated, analysed using FACS. Error bars represent ± one standard deviation.

ies to determine the kinetics of the interaction between the scFv and rsCD33 were performed only with the CS-scFv because of the heterogeneous nature of the INCS-scFv. A range of concentrations of rsCD33 was passed over 750RU of immobilized CS-scFv (Fig. 5b), and the data fitted to a Langmuir 1:1 model. The k_a and k_d were determined to be 4.57×10^3 M^{-1} s^{-1} and 1.6×10^{-3} s^{-1} respectively, with a K_D of 3.5×10^{-7} M.

4. Discussion

The aim of this study was to produce an anti-CD33-scFv to use as a potential immunotherapeutic agent for treatment of AML. *P. pastoris* strain KM71H was used as it relies on the AOX2 gene for production of alcohol oxidase, and thus grows more slowly with methanol as a carbon source. Strains with AOX1 deleted sometimes express higher levels of protein than wild-type strains, particularly in shake-flask cultures as observed for β-galactosidase, invertase, and hepatitis surface antigen (Tschopp et al., 1987a,b; Cregg et al., 1987) presumably because of lower oxygen demands. As it was planned that the initial screening and expression of scFv for further analysis would be carried out using shake-flask cultures, KM71H seemed the ideal strain to use. In addition, a protocol for scFv expression in *P. pastoris* used a Mut^s strain successfully (Gram et al., 1998).

Anti-CD33-scFv produced using constructs with CS and INCS forms of the α-MF secretory signal sequence (Table 1), were detectable in BMMY cultures after 24 h, increasing in quantity up to 96 h (Fig. 2a,b). The amount of scFv produced in the CS culture was clearly higher than from the INCS culture. This higher level of scFv expression was not clone-specific, since in a comparison of 10 clones of each construct, CS clones consistently expressed

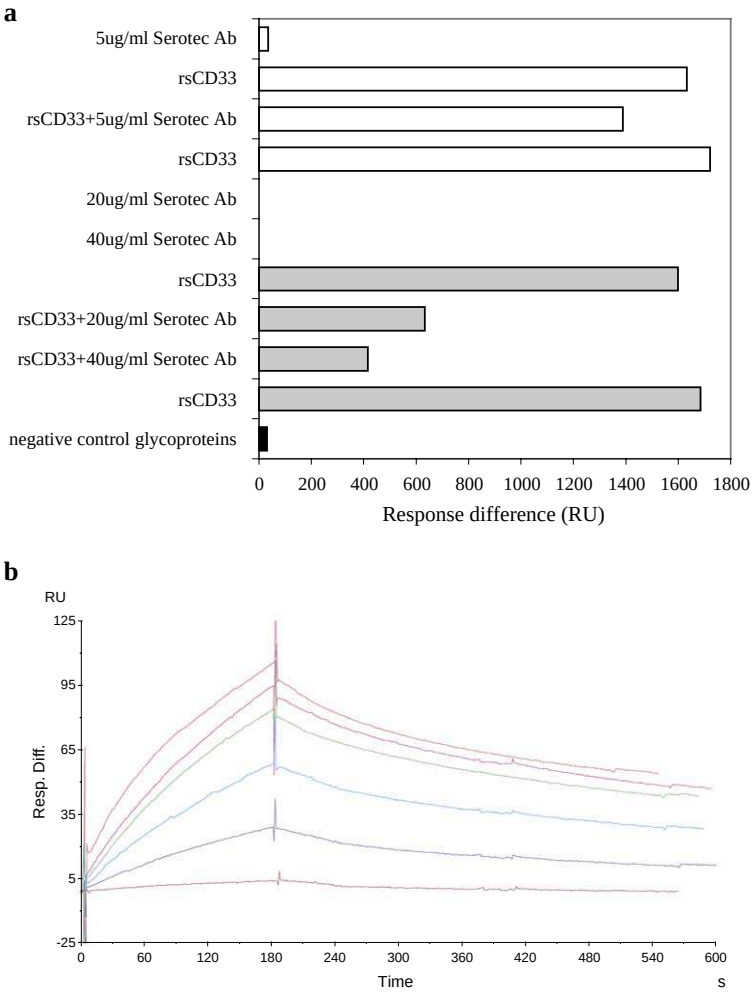


Fig. 5. (a) Plot of interaction analysis between immobilized anti-CD33 scFv Ab (INCS) and rsCD33 alone and with Serotec anti-CD33 Ab. The chart above corresponds to three separate sensorgrams that were obtained by passing rsCD33 or Serotec anti-CD33 Ab alone or in combination, over immobilised INCS-anti-CD33 scFv. These were passed over the chip one after the other in the sequences as shown below with a regeneration step in between to remove the bound analyte. The chart represents the increase in RU seen when each component is passed over the chip, in the order as stated below. Interaction events order: □ Sensorgram 1: 5 μg/ml Serotec anti-CD33 Ab>5 μl regeneration buffer 10 mM glycine–HCl pH 2.5 (RG)>6 μg rsCD33>5 μl RG>6 μg rsCD33+5 μg/ml Serotec anti-CD33 Ab>5 μl RG>6 μg rsCD33>5 μl RG. ▨ Sensorgram 2: 20 μg/ml Serotec anti-CD33 Ab>5 μl RG>40 μg/ml Serotec anti-CD33 Ab>5 μl RG>6 μg rsCD33>6 μg rsCD33+20 μg/ml Serotec anti-CD33 Ab>5 μl RG>6 μg rsCD33+40 μg/ml Serotec anti-CD33 Ab>5 μl RG>6 μg rsCD33>5 μl RG. ■ Sensorgram 3: 5 μg/ml negative control glycoproteins (mix of human transferrin, ribonuclease B, and human α_1-acid glycoprotein)>5 μl RG. (b) Interaction of rsCD33 at concentrations 1220 nM, 1000 nM, 750 nM, 500 nM, 250 nM, and 50 nM (corresponding to curves from top to bottom), with immobilized anti-CD33-CS-scFv to determine binding kinetics.

more scFv than INCS clones. Since both the complete and incomplete signal sequence fusions to the scFv are processed by Kex2, it is possible that inclusion of EAEA in the cleavage site (CS) improves the accessibility of the site and therefore facilitates faster processing.

The His-tagged protein observed in the KM71H cultures was confirmed as the anti-CD33-scFv by N-terminal sequencing (Table 2). Although the scFv was expressed using both the complete and incomplete αMF, aberrant processing of the signal sequence gave rise to three forms of the scFv. The CS construct

produced only one form of anti-CD33-scFv, with EAEA remaining at the N-terminus, whereas the INCS construct produced two forms of anti-CD33-scFv, one with 11 amino acids from the C-terminus of the αMF, and another which was correctly processed scFv.

Although the inclusion of EAEA repeats in the αMF has been recommended to reduce steric hindrance from the folded protein (Sreekrishna and Kropp, 1996), the CS-scFv was processed by Kex2 but not by Ste13, a problem also encountered with other recombinant proteins expressed in *P. pastoris* (Kim et al., 1997; Goda et al., 2000; Almeida et al., 2001). A number of causes have been proposed for the inefficient processing of the Glu–Ala repeats by Ste13. One is that the amount of native Ste13 is insufficient to cleave the Glu–Ala repeats from highly expressed recombinant protein because there is simply too much protein passing through the processing machinery of the cell (Harashima, 1994; Brake et al., 1984). This problem has been overcome in *Saccharomyces cerevisiae* by the inclusion of the Ste13 gene on a multicopy plasmid (Barnes et al., 1982). Another is that the tertiary structure of individual proteins can be such that the Kex2 and/or Ste13 cleavage sites become inaccessible to the proteases necessary for cleavage of the α-MF secretion signal (Cregg, 1999). A third is that glycosylation at a site close to the N-terminus of a protein may also obstruct the protease cleavage sites, as observed by Weiss et al. (1998). Finally, it is thought that the presence of certain amino acids, such as proline, in close proximity to the Kex2 and Ste13 cleavage sites, may inhibit the action of these proteases (Cereghino and Cregg, 2000).

Of these potential inhibitors of Ste13 cleavage, it seems most likely that the protein conformation of the CS-scFv prevents cleavage by Ste13. Although the CS-scFv is highly expressed, if there were not enough Ste13 to process all the scFv correctly, a mixed population of correctly and incorrectly cleaved scFv would be expected. This was the case with expression of soybean root nodule phosphatase, where 60% of protein retained EAEA, 30% EA, and 10% was correctly cleaved (Penheiter et al., 1998). However, N-terminal sequencing clearly shows that Ste13 has not processed any of the scFv. Also, the scFv only has one putative glycosylation site, and as treatment of the CS-scFv with *N*-glycosidase-F did not cause any reduction in MW (data not shown), it is unlikely that glycosylation plays a role. Inhibition of Kex2 and/or Ste13 cleavage by protein conformation can be overcome with the use of a pre rather than a pre-pro signal sequence. The α-MF is a pre-pro signal with the first 19 amino acids (pre) being cleaved while the protein is in extended form by a signal peptidase in the endoplasmic reticulum (ER) (Waters et al., 1988). The pro-region is cleaved by Kex2 and Ste13 in the Golgi apparatus where the protein has taken on its tertiary structure (Cereghino and Cregg, 2000; Julius et al., 1984) potentially protecting the cleavage sites from proteases Kex2 and Ste13. A pre signal sequence is cleaved in the ER while in extended form, eliminating any steric hindrance by the tertiary structure of the protein (Lyman and Schekman, 1996). However, a pre-type sequence PHO1 used in *P. pastoris* (Payne et al., 1995) has also shown variability in processing (Weiss et al., 1995; O'Donohue et al., 1996), showing that use of a pre-type sequence is only a solution in certain cases.

Variation in αMF processing was also observed with the INCS-scFv, with similar amounts of each of the two forms present. Cleavage of the α-MF at position (amino acid) 70–72 has also been observed with expression of aprotinin (Vedvick et al., 1991) and phytohemagglutinin (PHA) (Raemaekers et al., 1999), suggesting that specific protease activity is responsible. Purification of His-tagged protein from the cell pellet of INCS-scFv cultures showed that both scFv forms were present at an intracellular level (data not shown) suggesting that the incorrect cleavage was occurring in the processing pathway, rather than by an extracellular protease. Whether all the INCS-scFv was initially processed this way, and half subsequently cleaved by Kex2, or whether there were two distinct processing pathways, is unclear. Attempts to reduce the amount of protein being expressed to allow for correct processing were performed. However, growth of the induction cultures at 25 °C and 22 °C had no affect on the processing of αMF. Likewise, reduction of methanol concentration from 0.5% to 0.4%, 0.3%, 0.2% and 0.1% merely decreased expression of both forms overall. Addition of the glycosylation inhibitor tunicamycin to cultures had no affect on the processing, suggesting that different glycosylation patterns of the pre-

pro sequence had no affect on Kex2 cleavage (data not shown). Steric hindrance of the folded protein may play a role, since inclusion of EAEA in the scFv construct (CS), and in constructs expressing aprotinin (Vedvick et al., 1991) and PHA (Raemakers et al., 1999) resulted in correct cleavage by Kex2. Since half of the scFv expressed from INCS-scFv was correctly cleaved, we speculate that there is a point in the folding pathway beyond which the Kex2 site becomes inaccessible, but prior to which the protein is in a conformation amenable to cleavage. The rate of protein folding and availability of Kex2 would be limiting factors in this pathway, perhaps accounting for the two forms of INCS-scFv. It is clear from our results and those of others, that signal sequence processing of *P. pastoris* expressed proteins is highly variable and case specific, and therefore it is prudent to generate constructs using different signal sequences to accommodate the various outcomes of signal sequence cleavage.

Despite the aberrant processing of the anti-CD33-scFv, both the CS and INCS forms were found to be functional, binding to the CD33 antigen in soluble form and on the surface of cells. However, because of the heterogeneity of the INCS-scFv, it was impossible to determine whether one or both scFv forms expressed from this construct, bound to CD33. Attempts to separate the two scFv's by ion exchange chromatography have so far been unsuccessful.

Analysis of scFv interaction with native cell surface CD33 on a human leukaemic cell line (HL-60) showed binding to 99% (CS) and 87% (INCS) of cells. This difference in binding of the CS-scFv and INCS-scFv could be due to a concentration effect, since although similar concentrations of scFv were used, the INCS-scFv preparation contained the two forms of the scFv. Only one of these may have been binding to CD33, depending on the functionality of the scFv with the 11 amino acid extension. Also, the affinity of the scFv's may be different, affecting the binding to cell surface CD33. We were able to determine the affinity of the CS-scFv using surface plasmon resonance, but in the case of the INCS-scFv, the heterogeneity prevented any reliable kinetic analysis of the interaction, and therefore, the affinity of the scFvs could not be directly compared. Binding of both the CS and INCS anti-CD33-scFv was significantly blocked by prior incubation with unlabelled anti-CD33-Ab, reducing the number of cells in the positive M2 phase by approximately 80%. Therefore, both CS and INCS scFvs were shown to bind to the target antigen CD33 on the surface of cells, which in both cases could be blocked with monoclonal anti-CD33-Ab, showing that the interaction was specific. Studies using surface plasmon resonance provided further evidence for the functionality and specificity of the anti-CD33-scFvs, showing that both CS and INCS scFvs bound specifically to rsCD33. These results also confirmed that the inclusion of the C-terminal His-tag had not affected the functionality of the scFv in terms of binding to the target antigen. This is important since studies have shown that the inclusion and position of a His-tag on an scFv can significantly affect antigen binding in some cases (Goel et al., 2000a).

Kinetic analysis of the interaction between CD33 and the scFvs was only possible with the CS-scFv because of the heterogeneous nature of the INCS-scFv. Determination of the association and dissociation rate of the rsCD33-CS-scFv interaction showed that the affinity of the scFv was fairly low but in a range expected for monovalent scFvs. No kinetic data is available for the parent anti-CD33-mAb, or a Fab fragment of the Ab, and therefore at present no comparison can be made with the affinity of the anti-CD33-scFv. Although BIAcore was used to analyse the kinetics of the anti-CD33-mAb and rsCD33 interaction, attempts to immobilise rsCD33 have so far been unsuccessful as the methods tested to date affected the stability of rsCD33. This made it unsuitable for repeated interaction analyses, and therefore kinetic analysis of the rsCD33 and anti-CD33-mAb interaction has not been possible. The affinity of the scFv may also be affected by the presence of the additional EAEA residues at the N-terminus of the scFv. Other groups have reported a decrease in affinity with scFv constructs that have extraneous amino acids at the N-terminus, in comparison with a normal scFv (Ping et al., 1993). Further work will include refinement of the antigen binding assay and also a comparison with an alternative binding assay such as Scatchard analysis using FACS. This will be performed with the CS-anti-CD33-scFv and the parent anti-CD33-mAb, using CD33 positive cell line HL-60, which should generate useful interaction ki-

netics data. Also, internalisation studies will be performed to determine whether the scFv is internalised and the rate of internalisation. Since the parent Ab is internalised into leukaemic cells (McGraw et al., 1994), it is likely that the scFv will be also. The importance of affinity of the scFv will partly be determined by whether internalisation occurs, and the rate of internalisation. The kinetic measurements performed to date are intended as a benchmark by which to determine whether future modifications to the scFv, such as addition of a prosthetic group for radiolabelling, will affect the affinity of the scFv, rather than quantitative determination of the interaction kinetics.

In summary, the anti-CD33-scFv was expressed with a complete and an incomplete signal sequence using *P. pastoris*, giving a reproducibly high yield, which was easily purified to high purity, and found to be functional and specific for the target antigen CD33.

They are therefore a potentially useful basis for the development of immunotherapeutic reagents for the diagnosis and treatment of leukaemia.

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