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Author(s):
Zuberbuehler, Kathrin; Palumbo, Alessandro; Bacci, Camilla; Giovannoni, Leonardo; Sommavilla, Roberto; Kaspar, Manuela; Trachsel, Eveline; Neri, Dario

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A general method for the selection of high-level scFv and IgG antibody expression by stably transfected mammalian cells

Kathrin Zuberbühler[1,4], Alessandro Palumbo[1,4], Camilla Bacci[2], Leonardo Giovannoni[2], Roberto Sommavilla[3], Manuela Kaspar[3], Eveline Trachsel[3] and Dario Neri[1,5]

[1]Institute of Pharmaceutical Sciences, Department of Chemistry and Applied Biosciences, Swiss Federal Institute of Technology Zürich, Wolfgang-Pauli-Strasse 10, CH-8093 Zürich, Switzerland, [2]Philogen SpA, Via Montarioso 11, 53035 Monteriggioni SI, Italy and [3]Philochem AG, c/o ETH Zürich, Institute of Pharmaceutical Sciences, Wolfgang-Pauli-Strasse 10, HCI E520, CH-8093 Zürich, Switzerland

[4]These authors contributed equally.

[5]To whom correspondence should be addressed.
E-mail: dario.neri@pharma.ethz.ch

The isolation of mammalian cell lines capable of high-yield expression of recombinant antibodies is typically performed by screening multiple individual clones by limiting dilution techniques. A number of experimental strategies have recently been devised to identify high-expressing clones, but protocols are often difficult to implement, time consuming, costly and limited in terms of number of clones which can be screened. In this article, we describe new vectors for the expression of recombinant antibodies in IgG format and in other formats, based on the single-chain Fv module, as well as a high-throughput screening procedure, based on the direct staining of antibodies transiting the membrane of a stably transfected cell, followed by preparative sorting using a high-speed cell sorter. This procedure allows, in one step, to deposit single cells into individual wells of a 96-well microtiter plate (thus facilitating cloning) and to preferentially recover those rare cell populations which express dramatically higher levels of recombinant antibody. Using cell cultures followed by affinity purification techniques, we could confirm that the new vectors and the new screening procedure reliably yield high-expression clones and homogenous protein preparations. We expect that these techniques should find broad applicability for both academic and industrial antibody engineering research.

Keywords: CHO/fluorescence activated cell sorting/ recombinant protein production/selection

Introduction

Monoclonal antibodies represent the fastest growing class of pharmaceutical biotechnology products (Walsh, 2005), with sales in 2007 which exceeded 20 billion US dollars (Pavlou and Belsey, 2005). At present, antibody biopharmaceuticals typically consist of antibodies in IgG format (Carter, 2006). However, antibody derivatives [e.g. radiolabeled antibodies (Milenic *et al*., 2004), antibody-cytokine fusion proteins (Reisfeld *et al*., 1997; Neri and Bicknell, 2005) and antibody-drug conjugates (Kovtun and Goldmacher, 2007; Carter and Senter, 2008)] are increasingly being used in pharmaceutical development programs. The facile cloning and high-titer expression of monoclonal antibodies and their derivatives, thus, represent a topic of considerable interest for the development and use of this important class of biopharmaceuticals.

While the expression of full immunoglobulins in bacteria (Simmons *et al*., 2002; Mazor *et al*., 2007) and in yeast (Li *et al*., 2006) has recently been reported, antibodies in IgG format are typically over-expressed in mammalian cells for pharmaceutical applications (Wurm, 2004). In most cases, the heavy and light chains of an IgG molecule are encoded in different plasmids which are co-transfected into recipient Chinese hamster ovary (CHO) cells, and a variety of different vectors have been designed for this purpose (Persic *et al*., 1997; Borsi *et al*., 2002; Li *et al*., 2007). However, co-transfection procedures often lead to relatively low yields of high-expressing clones, and typically several thousand stably transfected clones have to be analyzed using single-cell dilution procedures, in order to identify those rare clones which produce large antibody quantities in a stable fashion. A number of procedures have been proposed which may lead to high antibody-titers and to a higher frequency of transfectants with good expression properties. These include the use of suitable introns in the promoter region of expression vectors (Chapman *et al*., 1991) and the use of survival genes which permit the selection of high expressing clones in selective media. Lonza Biologics has developed a mammalian expression system that makes use of selection via glutamine (GS) metabolism. For cells which do not express sufficient GS to survive without adding glutamine, a transfected GS gene can function as a selectable marker by permitting growth in glutamine-free medium (Bebbington *et al*., 1992). Another expression system uses the dihydrofolate reductase gene as a survival gene (Alt *et al*., 1978). Furthermore, certain sequences flanking the antibody genes (termed 'Matrix Attachement Regions') have been reported to bind isolated nuclear scaffolds or nuclear matrices *in vivo* with high affinity and to reduce the effects of heterochromatin (Zahn-Zabal *et al*., 2001; Wurm, 2004; Girod *et al*., 2005) thus leading to over-expression of the corresponding transfected gene. Transfection techniques have also been reported to substantially affect expression yields (Baldi *et al*., 2007), and there is a growing interest in the definition of insertion sites as well as number of insertion events (Chenuet *et al*., 2008). Researchers are even considering antibody-production processes which rely on the transient transfection of rapidly dividing CHO cells using polyethylenimine or calcium phosphate and disposable plastic shakers (Derouazi *et al*., 2004; Haldankar *et al*., 2006).

In addition to the above-mentioned developments in expression vectors and host cells, pharmaceutical companies and research laboratories are currently focusing on the implementation of advanced screening methods, which may

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allow analysis of several thousand transfected cells, for example, by growing colonies in semi-solid agar with fluorescent detection of the halo of secreted antibodies in the immediate cell proximity (Davis *et al*., 1982; Mann, 2007) or by multiple rounds of fluorescence-activated cell sorting procedures (Ernst *et al*., 1998; Brezinsky *et al*., 2003), sometimes using green fluorescent protein as an indirect reporter of transgene expression (Meng *et al*., 2000).

In this article, we describe a new set of vectors for the mammalian expression of antibodies in IgG format or as scFv-based fusion proteins. Furthermore, we describe for the first time, to the best of our knowledge, a fluorescence-based screening methodology which does not make use of semi-solid agar but, instead, features only a single round of FACS sorting in sterile conditions. The new procedure allows in one step to separate a mixture of cells into individual clones, while at the same time selecting high-expressing clones from a pool of stably transfected cells. We exemplified our procedure using the clinical-stage human monoclonal antibody L19 (Pini *et al*., 1998), which is specific to the alternatively spliced EDB domain of fibronectin, a marker of angiogenesis (Zardi *et al*., 1987; Neri and Bicknell, 2005; Kaspar *et al*., 2006; Schliemann and Neri, 2007). We expressed the L19 antibody both in IgG format and in SIP format (Borsi *et al*., 2002; Berndorff *et al*., 2005; Tijink *et al*., 2006) and confirmed that our FACS-based procedure reliably leads to a substantially higher frequency of over-expressing cell lines, which grow in suspension, are stable over multiple passages and which yield well-behaved protein preparations using GMP-compatible downstream processing procedures.

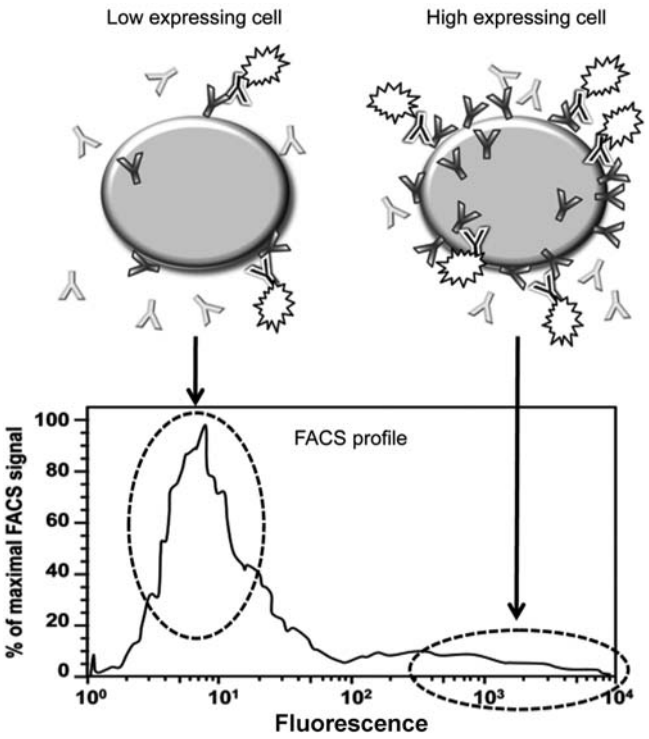

Fig. 1. Schematic representation of the FACS-based selection procedure. Antibodies (depicted in dark grey) secreted from the cell still remain associated to the cell membrane and can be detected with a fluorescently labeled reagent (in black). Fully secreted antibodies (in light grey) are washed away during staining procedure. A cell with a high secretion rate (i.e. many antibodies still attached to the membrane) gives a high FITC signal in the FACS profile and is selected as a high expressing clone.

Results

Figure 1 depicts a schematic representation of the FACS-based selection procedure used for the identification and cloning of high-producing cells, out of a population of stably transfected CHO cells. We reasoned that, during antibody secretion, a non-negligible quantity of antibody molecules could still remain associated to the cell membrane, thus allowing detection using suitable fluorescently labeled affinity reagents (e.g. rabbit polyclonal anti-human IgG antibodies). Unlike the semi-solid agar method, which requires the use of a dedicated imaging and picking instrument and the preparation of agar plates (Mann, 2007), we preferred to use preparative FACS sorting device, as these instruments are commonly available at most biomedical research institutions.

Figure 2 presents a schematic representation of the new vectors developed for stable antibody over-expression, and of the corresponding antibody molecules. Plasmid pBL1 is a derivative of pcDNA3.1, which contains a SIP secretion sequence (Li *et al*., 1997) in frame with the scFv(L19) (Huston *et al*., 1988) fused to the εCH4 domain of human IgE. We have used similar strategies for the cloning, expression and selection of a variety of different scFv-based fusion proteins in the lab, including immunocytokines which would otherwise typically exhibit expression yields at levels below mg/liter (R.S., E.T., M.K., D.N., unpublished result). We chose to use pcDNA3.1 as basic vector for protein expression and to avoid gene amplification strategies, because of the well-documented long-term stability of such expression strategies (Jun *et al*., 2006). Similarly, for IgG expression, we developed two pcDNA3.1-derived plasmids (pKZ1 and pKZ2), which direct the expression of heavy and light chains of the IgG(L19) molecule and which contain different antibiotic resistance genes (hygromycin for the heavy chain and neomycin for the light chain).

Figure 3A shows a representative FACS profile obtained after electroporation of CHO-S cells with the vector pBL1, coding for SIP(L19). While the FACS profile was shifted towards higher fluorescence values compared to a non-transfected cell population, only a minor fraction of transfected cells exhibited a substantially higher fluorescence signal. This population was separated from the main body of transfected cells by FACS sorting, and a representative number of single cells were generated by preparative sorting procedures, then growing the individual clones in microtiter plates. Figure 3B shows that 19/24 FACS sorted cells yielded ELISA values greater than 1, while only 3/24 high expressors were identified in the negatively sorted population. In full analogy, preparative FACS sorting of individual cells doubly transfected for IgG expression resulted in a higher frequency of over-expressing clones (Fig. 3C and D).

Clones generated by preparative FACS sorting, as described in Fig. 3, could be amplified without problems of sterility or of loss of expression up to the 1000 ml volume, growing cells in suspension and allowing the production of banks of cryotubes. For both SIP(L19) and IgG(L19), these cell banks could be used for antibody production in larger scale, using either roller bottles, shaker flasks or fermenters, yielding homogenous protein preparations after purification on Protein-A resin (Fig. 4A and B). Homogeneity of SIP(L19) and IgG(L19) preparation was confirmed by gel-filtration analysis

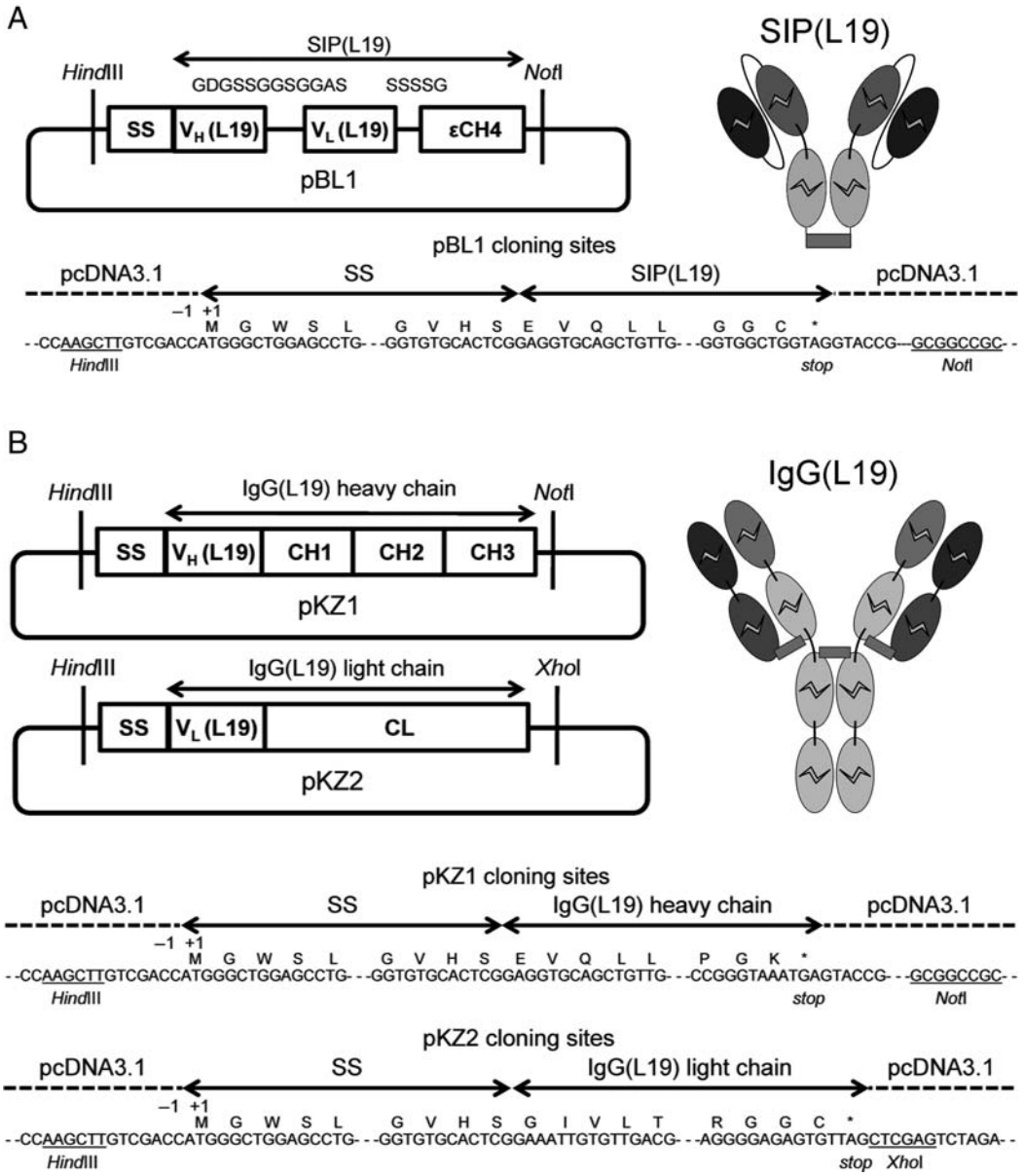

Fig. 2. (**A**) Schematic representation of vector pBL1 directing the expression of SIP(L19). Relevant features of this plasmid are indicated, together with DNA and amino acid sequences, including the sequences of the linker connecting VH and VL in the scFv portion of the molecule (Huston *et al*., 1988) and of the linker connecting the scFv fragment with the C-terminal eCH4 domain of human IgE (Borsi *et al*., 2002). (**B**) Vectors pKZ1 and pKZ2, which direct the expression of the heavy and light chains of IgG(L19). Cloning sites and relevant features of the vector are indicated. SS, secretion sequence.

(Fig. 4C and D). Furthermore, SIP(L19), a non-glycosylated recombinant antibody, was shown to be homogenous using both MALDI-TOF analysis and two-dimensional polyacrylamide gel electrophoresis (Fig. 4E and F).

Highly fluorescent stably transfected IgG clones, after sorting, typically express >10 mg/l in T-flasks using PowerCHO medium in unoptimized conditions, while the same vectors exhibited yields of ~ 1 mg/l in the same conditions upon isolation by limiting dilution (Borsi *et al*., 2002). Similarly, highly fluorescent SIP(L19) clones after FACS sorting exhibited yields in T-flasks in unoptimized conditions >20 mg/l.

Discussion

Using conventional single-cell dilution methodologies, IgG(L19) and SIP(L19) had previously been expressed at the low milligram per liter level (Borsi *et al*., 2002). The new preparative FACS sorting procedure described in this article led to populations of clones which expressed at least 10 times more antibody compared to clones generated by random single-cell dilution. Preliminary fermentation experiments in 30 l fermenters indicate the expression yields for both SIP(L19) and IgG(L19) can be substantially improved through judicious choice of growth medium, fermentation parameters and media supplement. Based on our experience with various formats of the L19 antibody and of other clinical-stage antibodies developed in our lab [e.g. the F16 antibody specific to the alternatively spliced domain A1 of tenascin-C; (Brack *et al*., 2006), and the F8 antibody specific to the alternatively spliced EDA domain of fibronectin (Villa *et al*., 2008)], we believe that the vectors and the preparative FACS sorting procedures described in this article may be of general utility for a variety of different laboratories

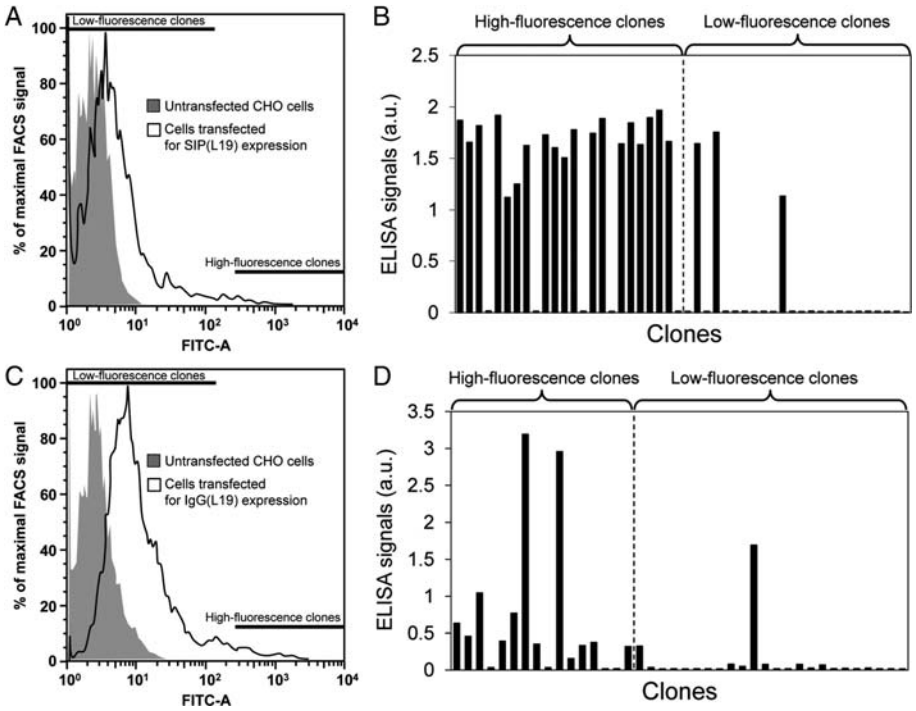

Fig. 3. Key features of the FACS sorting procedure and ELISA results (**A**). Histogram of fluorescent-activated cell sort for SIP(L19) transfected CHO cells. As negative control untransfected CHO cells were used. Horizontal bars indicate the high-fluorescence and low-fluorescence cell populations used for the single cell recovery steps, using FACS in the preparative mode (**B**). Expression levels detected by ELISA of clones selected from high-fluorescent and low-fluorescent populations (**C** and **D**).

active in the development of antibody-based therapeutics. Protein-A-based downstream purification procedures were facilitated by the fact that all antibodies isolated in our laboratories derived from human antibody libraries based on the DP47 heavy chain germline family (Pini *et al*., 1998; Silacci *et al*., 2005), which has long been known to confer protein-A binding also to scFv-based antibody formats (Hoogenboom and Winter, 1992). Furthermore, we have observed that a range of fluorescently labeled detection reagents can be used for the preparative FACS-sorting step, e.g. fluorescently labeled antigen or fluorescently labeled protein-A.

Materials and methods

Plasmids and transfections

Vector pBL1, directing the expression of the recombinant antibody SIP(L19) has previously been described (Borsi *et al*., 2002). Briefly, the SIP format consists of L19(scFv) and the εCH4 domain of the human IgE secretory isoform IgE-S2, which allows dimerization. This plasmid was used to transfect CHO-S cells (Gibco/Invitrogen, Basel, Switzerland) using Amaxa Nucleofector (Amaxa AG, Cologne, Germany) following the manufacturer's protocol. Transfectomas were grown in RPMI supplemented with 10% FCS and selected using 500 μg/ml of Geneticin (G418, Calbiochem, San Diego, CA, USA).

The heavy and light chain gene for the complete IgG(L19) were amplified from the constructs pcDNA3-L19IgG1 and pCMV2Δ-L19-κ (Borsi *et al*., 2002) by polymerase chain reaction using High Fidelity Polymerase (Roche) according to the manufacturer's recommendations. For the complete L19 light chain primers 1_BW_SEC (CCCAAGCTTGTCGACCA TGGGCTGGAGCCT GAT) and 14_FW_hCk (GGCCTCGA GCTAACACTCTCCCCTGTTGAAGCTC TTT), containing HindIII and XhoI restriction sites, respectively, were used. After digestion with HindIII and XhoI, the amplification product was inserted in the vector pcDNA3.1 containing a neomycin resistance gene, to obtain the construct pKZ2.

For subcloning the complete heavy chain in the vector pcDNA3.1 containing a hygromycin resistance gene, primers 1_BW_SEC (CCCAAGCTTGTCGACCA TGGGCTGGAG CCTGAT) and 6_FW_hCG1 (TTTTCCTTTTGCGGCCG CGCTCGGTACCCGGGGAGC) containing the HindIII and NotI restriction sites, respectively, were used. The amplification product was digested and inserted HindII/NotI in pcDNA3.1, yielding vector pKZ1.

Equimolar amounts of these constructs were used to cotransfect CHO-S cells as described above. Transfectomas were selected using 500 μg/ml of Geneticin (G418, Calbiochem, San Diego, CA, USA) and 25 μg/ml hygromycin B (Invitrogen, Carlsbad, CA, USA).

After 14 days of selection in RPMI medium supplemented with 10% FCS and selective antibiotics, cells were brought into suspension, and cultured in PowerCHO-CD 2 (Lonza, Vervier, Belgium).

Staining of recombinant CHO for secreted product and fluorescent-activated cell sorting

Cells were cultured in suspension in T75 cell culture flasks to a density of 5×10^5 cells/ml. For collection of the cells, the suspension was centrifuged 5 min at 1100 rpm, the cells were then resuspended in PBS to a final concentration of about 5×10^6cells/ml. In general, 5×10^5 to 1×10^6 cells were labeled for the sorting procedure. To stain recombinant CHO for secreted product, the following antibodies were used: for the detection of SIP(L19) a rabbit anti-human IgE (Dako,

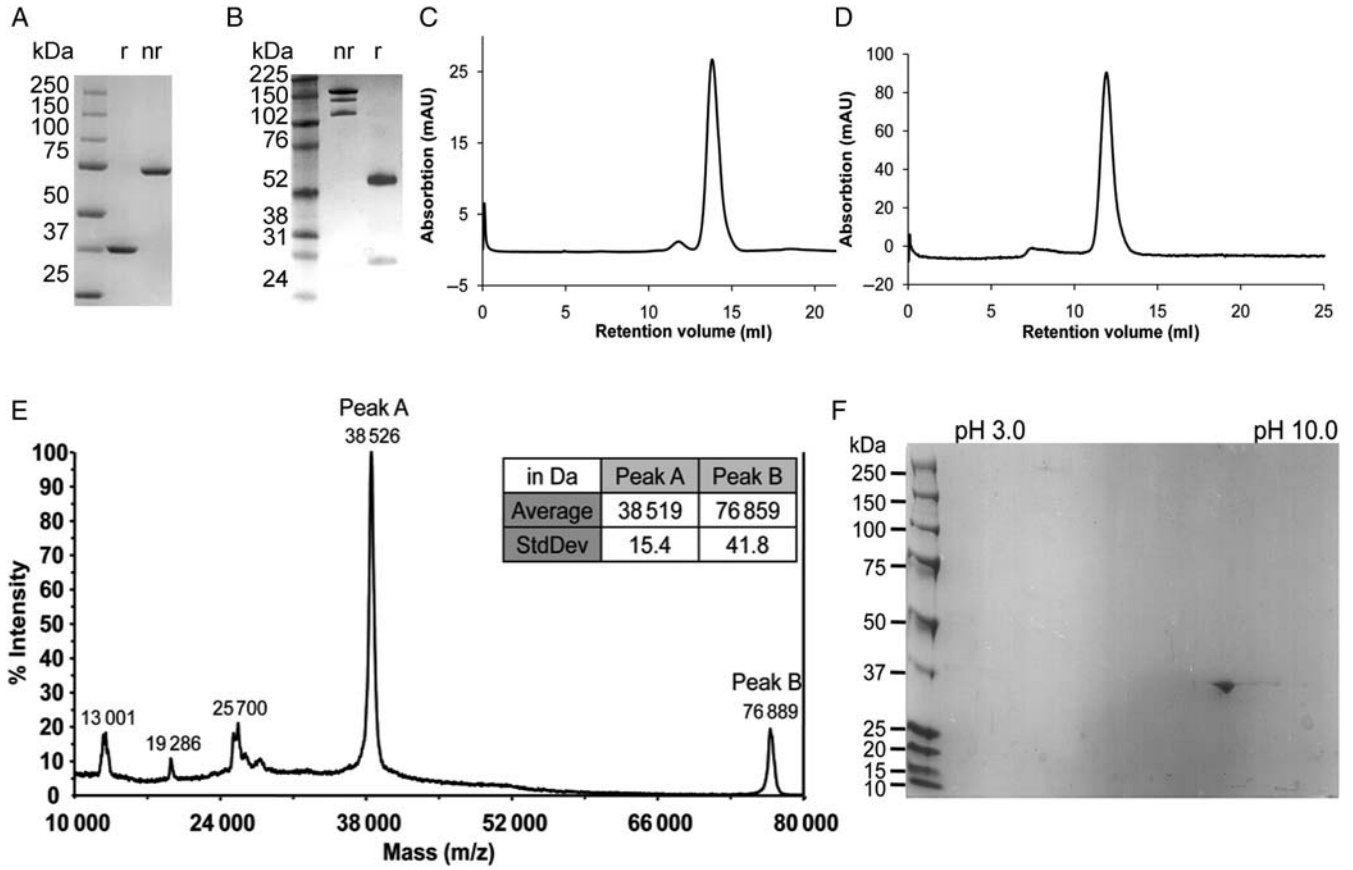

Fig. 4. Quality controls of purified SIP(L19) and IgG(L19). (**A** and **B**) SDS–PAGE analysis of purified SIP(L19) and IgG(L19) under non-reducing (NR) and reducing (R) conditions. (**C**) Size-exclusion chromatography profile of the purified SIP(L19). The retention volume (ml) of the major peak corresponds to the disulfide-linked homodimer. (**D**) Size-exclusion chromatography profile of the purified IgG(L19). (**E**) Mass spectra of SIP(L19). The peak at ~38 530 Da corresponds to the monomeric SIP(L19) as well as to the doubly charged SIP(L19) dimer. The peak at 76 899 ± 41 Da corresponds to the SIP(L19) dimer, in good agreement with the calculated mass of 77 051 Da for the non-glycosylated product. Furthermore, other charge states and a product of dimer fragmentation can be observed. (**F**) 2D-PAGE of SIP(L19) reveals a single spot of unglycosylated protein, using a sample loaded under reducing conditions.

Glostrup, Denmark), diluted according to the manufacturer's recommendations, and in the case of the IgG(L19) a rabbit anti-human IgG (Dako, Glostrup, Denmark). The samples were then incubated with the antibodies at room temperature for 1 h. After washing with PBS, the secondary antibody, an Alexa Flour® 488 conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR, USA), was incubated at room temperature for 1 h in the dark. Next, cells were washed twice with PBS and transferred to a FACS tube and kept on ice. Twenty minutes before sorting, 7-aminoactinomycin (7-AAD) was added in a 1:1000 dilution to stain dead cells. Analytical flow cytometry scans were performed on the FACSCanto (Becton Dickinson, Basel, Switzerland). Data were analyzed with Flowjo software (Treestar, Ashland, TN, USA).

FACS was performed using a BD FACSAria (Becton Dickinson, Basel, Switzerland) equipped with FACSDiva software, an argon laser emitting at 488 nm and an automatic cell deposit unit for plate sorting. The 7-AAD staining was used to identify dead (or dying cells) and PerCP-Cy5.5 emission was detected using a 695/40 bandpass filter. The FITC emission was detected using 530/30 bandpass filter. Dead cells were excluded in a FSC vs. PerCP-Cy5.5 dot blot.

Fluorescently labeled cells positive for FITC and negative for 7-AAD were single cell deposited using FACS into 96-well flat bottom 'tissue culture treated' microplates (Nunc, Roskilde, Denmark) containing 200 μl RPMI supplemented with 10% FCS, G418 and hygromycin B. Clones were incubated at 37°C and 5% carbon dioxide in a humidified incubator for 10–14 days.

ELISA of supernatants

ELISA experiments on the conditioned culture media were performed according to Carnemolla *et al.* (Carnemolla *et al.*, 1996). To detect different clones expressing either SIP(L19) or IgG(L19), the biotinylated ED-B domain of Fibronectin, which includes the epitope recognized by L19, was immobilized on a streptavidin coated plate (StreptaWell, Roche Applied Bioscience, Indianapolis, IN, USA). Horseradish peroxidase conjugated Protein A (GE Healthcare, Buckinghamshire, UK), diluted according to the manufacturer's recommendations, was used as secondary antibody to detect SIPs and IgG1s. In both cases, the immunoreactivity with the immobilized antigen was detected using the substrate BluePOD (Roche Diagnostics, Mannheim, Germany) for peroxidase, and photometric absorbance at 405 nm was measured.

Purification and quality control of antibodies

Both SIP(L19) and IgG(L19) could be purified from cell culture medium by protein A affinity chromatography. After loading, the column was washed with a buffer containing 100 mM NaCl, 0.1% Tween20, 0.5 mM EDTA. Eluted fractions were then dialyzed against PBS, pH 7.4, at 4°C. Batches

of the two antibodies were analyzed using SDS–PAGE under reducing and non-reducing conditions, and by 2D gel electrophoresis using the Invitrogen Page system (product codes ZM0011, ZM0021 and NP0330) following manufacturer instructions. Moreover, Superdex 200 size exclusion column (GE Healthcare, Otelfingen, Switzerland) was used to analyze the gel filtration profiles of the purified antibodies under native conditions using fast protein liquid chromatography (FPLC; Amersham Pharmacia).

Mass spectrometric analysis of SIP(L19)

For MALDI-TOF analysis, purified SIP(L19) samples, dialyzed against PBS, were mixed with matrix (sinapinic acid, 10 mg/ml in 50% ACN and 0.05% TFA), at a 1:4 dilution and spotted onto the MALDI target plate. The analysis of samples was performed on freshly tuned AB4800 MALDI-TOF/TOF mass spectrometer. The calibration of the instrument was performed using the ProteoMass Protein MALDI-MS Calibration Kit from Sigma, using the three calibration proteins Apomyoglobin (16 952 Da), Aldolase (39 212 Da) and Albumin (66 430 Da). For data analysis, the Data Explorer software version 4.8 from Applied Biosystems was used.

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