Expression, purification and biochemical characterization of recombinant murine secretory component: a novel tool in mucosal immunology

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Reconstitution of secretory IgA (S-IgA) by the association *in vitro* of secretory component (SC) and polymeric IgA (pIgA) obtained from hybridomas is a valuable tool in the study of the structure–function relationship in this particular class of antibody. Although dimeric IgA (dIgA) can be obtained and purified from hybridoma clones, SC remains tedious to isolate in sufficient amounts from colostral milk. Several murine models for the study of mucosal immunity are available, which could potentially benefit from the use of cognate IgA antibodies in various molecular forms, including dIgA and S-IgA. We report here on the establishment of two expression systems allowing the production of milligram amounts of pure recombinant murine SC (rmSC) with preserved murine pIgA-binding capability. The first system relies on the use of recombinant vaccinia virus to prompt infected HeLa cells to express the murine SC protein, whereas the second system is based on a stably transfected cell clone exhibiting murine glycosylation. The second source of rmSC will permit the study of the role of its sugar moieties in pathogen–host interactions, and the evaluation of its function in passive protection without risking adverse immune responses. The extensive biochemical characterization conducted in this study demonstrates that rmSC is a dependable and convenient alternative to the natural product, and indicates that the J chain is dispensable in the recognition of pIgA and SC *in vitro*, whereas it is required for proper pIgA–polymeric Ig receptor interaction *in vivo*.

Key words: affinity chromatography, glycosylation, hybridoma, secretory component (SC)–IgA interaction.

INTRODUCTION

IgA has been used successfully for passive protection or therapeutic intervention at mucosal surfaces. Monoclonal IgA antibodies directed against respiratory syncytial virus applied passively to the nasopharyngeal mucosa of mice subsequently prevented initial infection and pneumonia [1]. Passive oral delivery of specific IgA antibodies also protected against bacterial infections including *Shigella* [2], *Salmonella* [3], *Vibrio cholerae* [4] and *Helicobacter felis* [5] in the intestine of mice. However, all these studies were performed with a mixture of monomeric IgA (mIgA) and polymeric IgA (pIgA) devoid of secretory component (SC), because of the lack of active SC to be combined with pIgA. Growing evidence indicates that SC has a crucial role in the biological activity of secretory IgA (S-IgA). SC functions both in protecting pIgA from rapid degradation by proteases in the gastrointestinal lumen [6] and in triggering S-IgA binding to epithelial cells [7]. In addition, SC binds to, and stimulates synergistically, the effector functions of eosinophils [8,9]. Moreover, SC seems to prevent the binding of gastrointestinal bacteria to the epithelium [10]. Therefore, as a first step in the production of homologous murine recombinant S-IgA, we evaluated expression systems as a sustained source of biologically active SC.

Both the classical recombinant vaccinia virus and the hybrid vaccinia virus/T7 RNA polymerase system have been exploited for transient protein expression [11,12]. Numerous studies with the two systems have shown that milligram quantities of recombinant protein can be produced for reseach applications. We have previously achieved the expression of recombinant human SC (rhSC) with specific dimeric IgA (dIgA)-binding capacity and a proper glycosylation pattern [13]. Antigen binding properties of reconstituted SC-dIgA are preserved when compared with dIgA alone [14]. Given the difficulty of recovering sufficient amounts of purified murine SC (mSC) from colostral milk, and the usefulness of the protein in addressing issues related to mucosal immunity, we developed a strategy based on the use of the vaccinia virus/T7 system to produce mSC *in vitro*. The expertise acquired when developing vaccinia virus recombinants expressing rhSC allowed us to optimize conditions rapidly for both expression and purification procedures. The recombinant protein exhibited similar, if not identical, properties to those of the natural product, as judged by the numerous biochemical criteria assayed. These include: specific association with dIgA/pIgA, in comparison with mIgA/IgG; formation of covalent complexes with dIgA/pIgA; preferential binding to J-chain-containing dIgA/pIgA.

The development of an expression system in which recombinant mSC (rmSC) can be glycosylated with a murine pattern is important for a detailed study of the biological role of the protein in pathogen neutralization in mouse models. To this end, we established in parallel a cell line that expresses more than 1 mg of rmSC per 10^8 cells, together with a simple purification procedure, that will allow the study of the function of SC *in vitro* and *in vivo*, both as a free protein and in association with murine

Abbreviations used: dIgA, dimeric IgA; DORA, dot-blot reassociation assay; FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; HRP, horseradish peroxidase; hSC, human SC; LC, long chain; mAb, monoclonal antibody; MALDI, matrix-assisted laser desorption ionization; mIgA, monomeric IgA; mSC, murine SC; pIgA, polymeric IgA; rhSC, recombinant hSC; rmSC, recombinant mSC; SC, secretory component; S-IgA, secretory IgA.

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dIgA. This represents the first source of rmSC exhibiting all the properties of the natural protein.

EXPERIMENTAL

Cell lines and viruses

Human HeLa S3 (American Type Culture Collection CCL2) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal calf serum and antibiotics, at 37 °C under air/CO_2 (19:1). The hybridoma clone IgA71 [15], a gift from Dr. John G. Nedrud (Case Western Reserve University, Cleveland, OH, U.S.A.), was maintained in RPMI 1640 medium supplemented with 10 % (v/v) fetal calf serum, 5 mM Hepes, 1 mM pyruvate, 25 μM folate and 10 μg/ml gentamycin (all from Life Technologies). Clone 2H2 (see below) was cultured under the same conditions. For production purposes, the culture was scaled up into roller bottles (Costar) with the daily addition of medium to a final volume of 800 ml.

The wild-type vaccinia virus strain WR and the ts7 temperature-sensitive strain were obtained from R. Wittek (University of Lausanne, Lausanne, Switzerland). The recombinant vaccinia virus vTF7-3, encoding the bacteriophage T7 RNA polymerase gene under the control of the vaccinia virus 7.5 K promoter, was purchased from the American Type Culture Collection.

Antibodies

pIgA, dIgA and mIgA purified from the murine hybridoma ZAC3 [14] were gifts from Dr. E. Lüllau (École Polytechnique Fédérale, Lausanne, Switzerland). The rabbit antiserum specific for denatured mSC has been described previously [16]. Murine and human J chain were detected by using the cross-reactive rabbit antiserum against human J chain [17]. Biotinylated goat IgG against murine α chain, monoclonal antibody (mAb) against human SC (hSC) and horseradish peroxidase (HRP)-conjugated reagents were obtained from Sigma. Human IgA1, IgA2m(1), and IgA2m(2) were purchased from Nordic Immunological Laboratories. Human myeloma IgA was from Cappel.

Plasmid construction

Enzymes used for cloning were purchased from Boehringer Mannheim and Life Technologies. Deprotected synthetic oligodeoxynucleotides were obtained from Microsynth GmbH (Balgach, Switzerland).

The 3095-bp cDNA encoding murine polymeric Ig receptor [18] was cloned into the *Eco*RI site of Bluescript II KS+ (pBSIIKS+; Stratagene), resulting in plasmid pBSmpIgR. To introduce a hexahistidine tag at the C-terminus, pBSmpIgR was cleaved with *Sac*I, filled in with Klenow, digested with *Eco*RI and cloned into pQE-17 (Qiagen) treated with *Bgl*II site, filled-in with Klenow and cut with *Eco*RI. This construct (pQE17mSC-6 × His) encodes the extracellular portion of murine polymeric Ig receptor down to Arg^{622}, fused to the sequence Asp-Leu-Arg-Ser-His_6, and a stop codon. Insertion plasmid pTM1mSC-6 × His (Figure 1A) was constructed via a three-piece ligation including: (1) vector pTM1 [19] cut with *Kpn*I and *Pst*I; (2) a *Kpn*I/*Kpn*I fragment containing the 3′ end of the encephalomyocarditis virus in pTM1 fused to the 5′ end of mSC, obtained by recombinant PCR [20]; and (3) the *Kpn*I/*Pst*I fragment from pQE17mSC-6 × His.

Because the authentic C-terminus of mSC has not yet been mapped precisely, we chose, on the basis of comparison with hSC [21], to stop the translation of the protein after Arg^{596}. The coding region for transmembrane domain and the cytoplasmic tail in pBSmpIgR was excised with *Sma*I and *Not*I, then replaced by a double-stranded oligonucleotide including a 5′ half-*Sma*I site, the coding sequence for amino acids Pro^{589} to Arg^{596}, a stop codon, an *Eco*RI site and a 3′ half-*Not*I site. The resulting plasmid was named pBSmSC (Figure 1B). The same approach was used to generate the construct pBSmSC-FLAG:Cterm (Figure 1C), with codons for Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (FLAG epitope) replacing the sequence Pro^{589} to Arg^{596} in an otherwise identical oligonucleotide.

Construct pCB6mpIgR(−5′) (Figure 1D) lacked the 5′-untranslated region of the murine polymeric Ig receptor cDNA in expression vector pCB6 [22]. To allow the expression of wild-type mSC and mSC-FLAG, the *Kpn*I–*Eco*RI fragment in pBSmSC and pBSmSC-FLAG:Cterm was introduced into pCB6mpIgR(−5′) cut with the same two enzymes, resulting in the generation of plasmids pCB6mSC(−5′) and pCB6mSC-FLAG:Cterm(−5′) (Figures 1E and F).

Recombinant vaccinia virus and expression of rmSC-6 × His in HeLa cells

The recombinant vaccinia virus vvTM1mSC-6 × His was generated by homologous recombination into the thymidine kinase gene [23]. HeLa S3 cells grown to a density of 6×10^5 cells/ml were centrifuged at 330 ***g***, washed with PBS and resuspended at a density of 10^7 cells/ml in serum-free Dulbecco's modified Eagle's medium before transfer to a 2 litre roller bottle. Cells were infected with recombinant vaccinia viruses VVTM1mSC-6 × His and vTF7-3 at a multiplicity of infection of 5, then incubated for 24 h.

Purification of rmSC-6 × His from cells infected with vaccinia recombinants

Purification of rmSC produced by infected HeLa S3 was achieved through a three-step procedure at 4 °C. Serum-free culture supernatant filtered with a 0.22 μm Nalgene™ unit was loaded on a 5 ml concanavalin A–agarose column (Vector Laboratories) equilibrated in binding buffer [10 mM Tris/HCl (pH 7.5)/150 mM NaCl/1 mM $CaCl_2$/1 mM $MnCl_2$]. After extensive washing, elution was performed after an overnight incubation with binding buffer containing 0.5 M methyl α-mannopyranoside (Sigma). The eluate was diluted with 4 vol. of 10 mM Tris/HCl, pH 7.5, resulting in an NaCl concentration of 30 mM, and then loaded on a 2 ml Q-Sepharose FF (Pharmacia) column equilibrated with 10 mM Tris/HCl containing 50 mM NaCl. After a wash with the starting buffer and a 130 mM NaCl step, mSC was eluted batchwise between 140 and 170 mM NaCl. After concentration with a Centricon-50 cartridge (Amicon), the material was chromatographed on a 27.5 cm × 1.5 cm Superdex 200 (Pharmacia) column equilibrated and run in PBS. NaN_3 was added to 0.02 %; the purified protein was stored at 4 °C until use. When virus-induced cell lysis was minimal, we found it possible to purify the protein to 95 % homogeneity with the concanavalin A–agarose column step alone.

Establishment of a stable cell line (clone 2H2) expressing mSC-FLAG

IgA71 cells (2×10^6) [15] were electroporated with 15 μg of *Pvu*I-linearized pCB6mSC-FLAG:Cterm(−5′) in a total volume of 750 μl of PBS [settings: 400 V, 250 μF, 5 ms, 0.4 cm cuvette (Bio-Rad)]. After the addition of 750 μl of prewarmed medium, the cuvette was incubated for 30 min at 37 °C and cells were spun down and transferred to fresh medium. Selection of stable expressers was performed in the presence of 350 μg/ml of G-418. Out of 192 cells sorted with the FACStar system (Becton

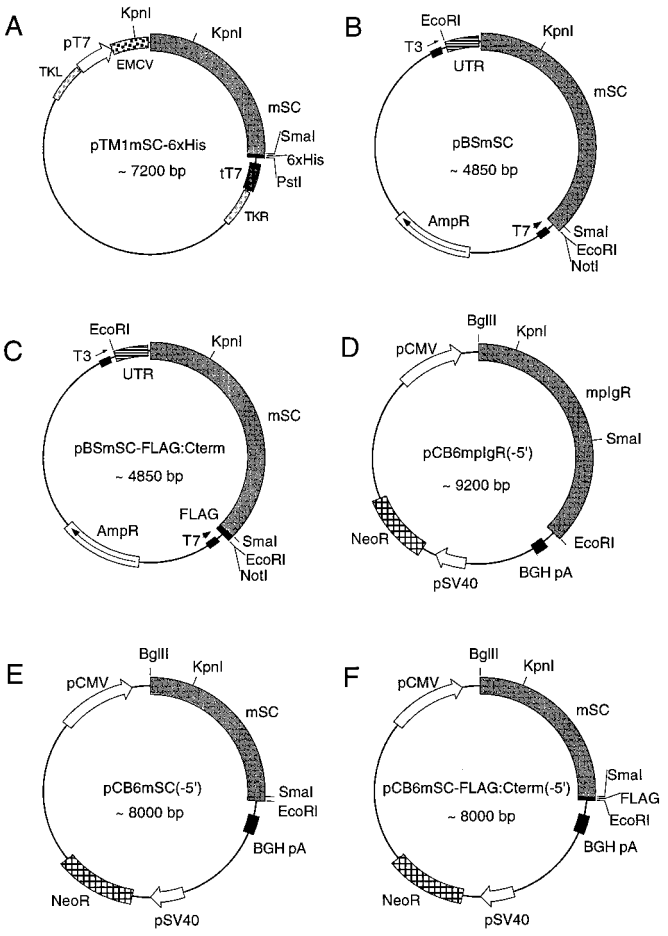

Figure 1 Maps of plasmids used as cloning intermediates and expression vectors

The names and approximate sizes of plasmids are indicated in the centre of each representation. Restriction sites used for cloning are indicated. Abbreviations: TKL, vaccinia virus thymidine kinase gene, left arm; TKR, vaccinia virus thymidine kinase gene, right arm; EMCV, encephalomyocarditis virus untranslated region; tT7, phage T7 terminator; 6 × His, His_6 tag; T3, phage T3 RNA polymerase entry site; T7, phage T7 RNA polymerase entry site; UTR, mSC gene 5′ untranslated region; AmpR, coding sequence for ampicillin resistance; pCMV, cytomegalovirus promoter; BGH pA, bovine growth hormone polyadenylation site; pSV40, simian virus 40 promoter; NeoR, neomycin resistance open reading frame.

Dickinson), seven clones were growing and two of them secreted rmSC. Our initial aim was to isolate clones able to secrete murine S-IgA; however, all clones stopped producing IgA for unknown reasons. Clone 2H2, which secreted rmSC to a high level, was therefore isolated as a continuous source of rmSC.

Affinity purification of FLAG-tagged mSC

rmSC-FLAG was purified from the 2H2 culture medium by affinity chromatography on a 2 ml M2-agarose (Eastman Kodak) column equilibrated in Tris-buffered saline [TBS; 25 mM Tris/HCl/137 mM NaCl/2.7 mM KCl (pH 7.5)]. After extensive washing with TBS, the protein was eluted with 7 column vol. of TBS containing 50 μg/ml FLAG peptide (synthesized at the Institut de Biochimie, Université de Lausanne, Lausanne, Switzerland). The peptide was washed out by repeated passages over a Centricon-50 cartridge (Amicon); the buffer was exchanged for PBS containing 0.02 % NaN_3. The purified protein was stored at 4 °C until use.

To assess the purity and oligomeric state of rmSC-FLAG, 765 μg of protein in 2 ml were injected into a 63 cm × 1.6 cm Superdex 200 column coupled to an FPLC system (Pharmacia) run in PBS at a flow rate of 1 ml/min; 2 ml fractions were collected.

Antiserum and purified IgG against native mSC

Neither polyclonal antibodies against denatured mSC [16] nor against native hSC [13] recognized native rmSC. Therefore 50 μg of purified rmSC-6 × His in PBS was mixed with complete Freund's adjuvant and used to immunize a 3-month-old female New Zealand rabbit. The animal was boosted monthly three times with 50 μg of mSC in incomplete Freund's adjuvant. IgG antibodies were purified by using Protein A–agarose (Pharmacia) [24]. A portion of the antibody was affinity-purified by passage through a column consisting of rmSC-FLAG immobilized on CNBr-activated Sepharose CL-4B beads (1 mg of protein/ml of gel) (Pharmacia). Elution was performed with 0.1 M glycine, pH 3.0, followed by immediate neutralization with 0.05 vol. of 1 M Tris/HCl, pH 8.0. The antibody was concentrated and resuspended in PBS by using a Centricon-50 cartridge.

Matrix-assisted laser desorption ionization (MALDI)-MS

The MALDI mass spectrum was obtained on a Perseptive Biosystems Voyager RP™ mass spectrometer with a 337 nm nitrogen laser, a 25 kV accelerating potential and a delayed extraction time of 300 ns, as will be described elsewhere (details available from the authors on request).

Immunoprecipitation and lectin blotting

Purified rmSC-FLAG or murine colostrum was diluted in cold TBS (final volume 500 μl) and combined in siliconized 2.2 ml tubes with 10 μl of rabbit anti-(native mSC) purified IgGs as described above. After incubation overnight at 4 °C, the antigen–antibody complexes were precipitated with 50 μl of Protein A–Sepharose slurry for 2 h at 4 °C. Immunoprecipitates were washed four times with TENT buffer [50 mM Tris/HCl (pH 7.5)/5 mM EDTA (pH 8.0)/150 mM NaCl/1 % (v/v) Triton X-100] and the beads were combined with SDS/PAGE loading buffer containing 0.1 M dithiothreitol, boiled for 3 min and loaded on an SDS/8 % (w/v) polyacrylamide gel. Proteins were transferred to PVDF membranes and blots were proceeded as described in Cho et al. [25] for detection with biotin-labelled *Griffonia simplicifolia* lectin I (Vector Laboratories), or under the conditions given in Hanasaki et al. [26] for detection with biotin-labelled *Sambucus nigra* lectin or biotin-labelled *Maackia amurensis* lectin II (both from Vector Laboratories).

Biotin-labelling of proteins

To label sialic acid residues specifically, 250 μg of affinity-purified rmSC-FLAG in 0.5 ml of 0.1 M sodium acetate, pH 5.5, was incubated with 2–10 mM sodium periodate on ice in the dark for 30 min [27]. Oxidation was quenched with 15 mM glycerol, which was then removed by several washes with 0.1 M sodium acetate, pH 5.5, with a Centricon-50 filtration unit (Amicon).

Biotin-long chain (LC)-hydrazide in DMSO (Pierce) was added to 5 mM final concentration and incubated at room temperature for 2 h. The buffer was changed to PBS containing 0.02 % NaN_3 by using a Centricon-50 filtration unit. Proteins were stored at 4 °C and used within 2 weeks of biotin-labelling.

dIgA (250 μg) from hybridoma ZAC3 and 2 mg each of rabbit IgG specific for denatured mSC, and rabbit IgG specific for native mSC, were biotin-labelled with sulpho-*N*-hexylsuccinimide-LC-biotin (Pierce) as described by the manufacturer, and recovered and stored as indicated above.

Dot-blot reassociation assay (DORA)

DORA was performed as described previously [13].

Micro-well reassociation assay

The wells of Nunc MaxiSorp ELISA plates were coated with 50 μl of various human and murine IgA preparations at 2 μg/ml dissolved in PBS. Wells were blocked with 0.2 ml of TBS containing 5 % (w/v) non-fat dried milk and 0.05 % (v/v) Tween 20 (Bio-Rad). Purified rmSC-FLAG and hSC-6 × His [13] at 100 ng/ml in 0.1 ml of PBS, or mouse milk diluted 1:2000 in PBS, was incubated for 1 h at room temperature. After three washes with TBS containing 0.05 % Tween 20, either rabbit IgG against native mSC (10 μg/ml; Protein A-purified) or mAb against hSC (1:1000 dilution) was applied for 1 h at room temperature. HRP-conjugated secondary antibodies (1:1000 dilution) were developed with 1,2-phenylenediamine as a chromogene. Reactions were stopped with 1 vol. of 2 M H_2SO_4; the absorbance was measured at 492 nm, with 620 nm as the reference wavelength.

Immunoblot binding assay

Samples were diluted with 1 vol. of gel loading buffer [28] for 3 min and fractionated in non-reducing, denaturing (0.1 % SDS) 6 % or 8 % (w/v) polyacrylamide gels. Blotting to PVDF (BDH, Poole, Dorset, U.K.) was performed with transfer buffer lacking SDS, and the membrane was blocked with TBS containing 0.05 % (v/v) Tween 20 and 5 % (w/v) non-fat dried milk. Membranes carrying various preparations of IgA (1 μg for dIgA and pIgA; 0.5 μg for mIgA) were incubated for 2 h at room temperature with biotinylated rmSC-FLAG (0.2 μg/ml in blocking buffer), washed and revealed with HRP-conjugated ExtrAvidin (1:2000 dilution). The use of biotinylated rmSC was rendered mandatory owing to cross-reactivity observed when using polyclonal anti-mSC antiserum (details available from the authors on request).

Other analytical procedures

Proteins were quantitated with the bicinchoninic acid assay from Pierce [29], with BSA (Pierce) as standard. Silver staining of SDS/PAGE gels was performed by the method of Morrissey [30].

RESULTS AND DISCUSSION

Expression and characterization of rmSC-6 × His produced in vaccinia virus-infected HeLa cells

Culture supernatants of CV-1 cells co-infected with vvTM1hSC-6 × His and vTF7-3 at a multiplicity of infection of 5 were collected at 3, 6, 9 and 23 h; aliquots were separated by SDS/PAGE and immunoblotted with rabbit antiserum against mSC. A polypeptide of approx. 85–90 kDa, which corresponds to the molecular mass of glycosylated mSC, was detected from 6 h after infection onwards (Figure 2A). Similar kinetics was observed for rhSC [23].

In contrast with rhSC-6 × His, rmSC-6 × His did not bind to Ni^{2+}-nitrilotriacetate/agarose matrix and therefore could not be purified with this approach (results not shown). Because we found that mSC-6 × His exposed to 7 M urea can bind to the resin, we hypothesized that the 6 × His tag is somehow in-

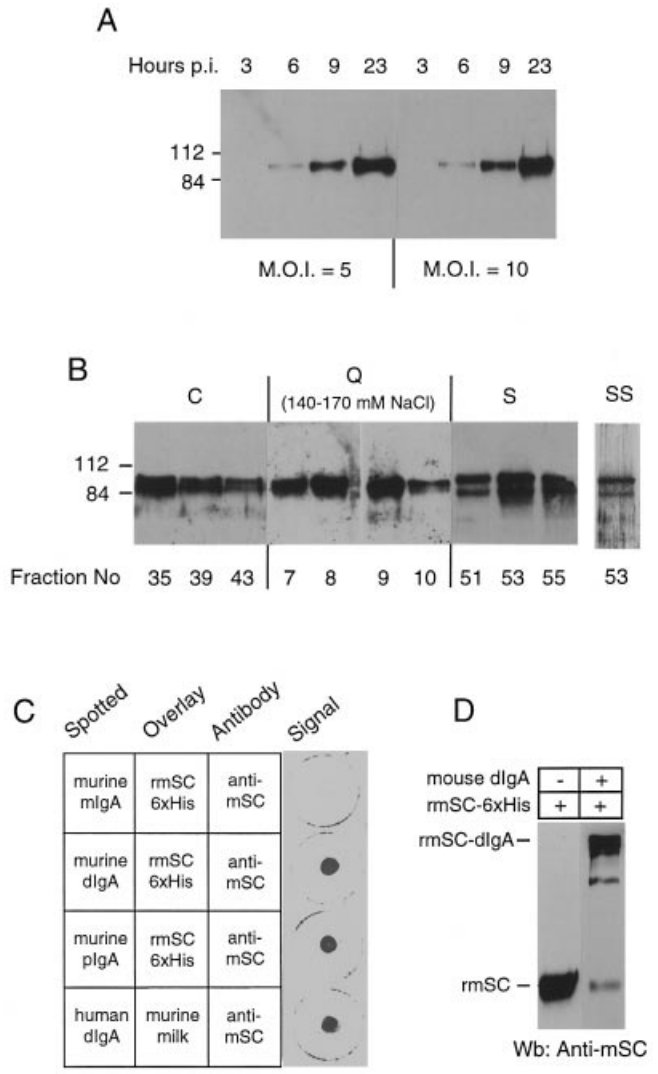

Figure 2 Production and purification of rmSC-6 × His expressed in HeLa cells

(**A**) Time course of rmSC-6 × His expression as a function of time in HeLa cells infected with VVTM1mSC-6 × His and vTF7-3. Abbreviation: M.O.I., multiplicity of infection. Cell culture supernatants recovered 3, 6, 9 and 23 h after infection were separated by SDS/PAGE and analysed by Western blotting with rabbit antiserum against mSC. The positions of molecular mass markers are indicated (in kDa) at the left. (**B**) Purification of rmSC by multi-step chromatography. The content of peak fractions was analysed as in (**A**). Abbreviations: C, concanavalin A–agarose column; Q, Q-Sepharose column; S, Superdex 200 column, SS, silver-staining of fraction 53 in lanes S. (**C**) DORA showing that rmSC proteins possess the capacity to bind specifically to pIgA but not to mIgA. The combination of partners and the order of addition are indicated at the top. (**D**) Covalent reassociation of rmSC-6 × His with dIgA. Samples were separated by SDS/PAGE under non-reducing conditions; specific dIgA–rmSC complexes were detected with anti-mSC antiserum.

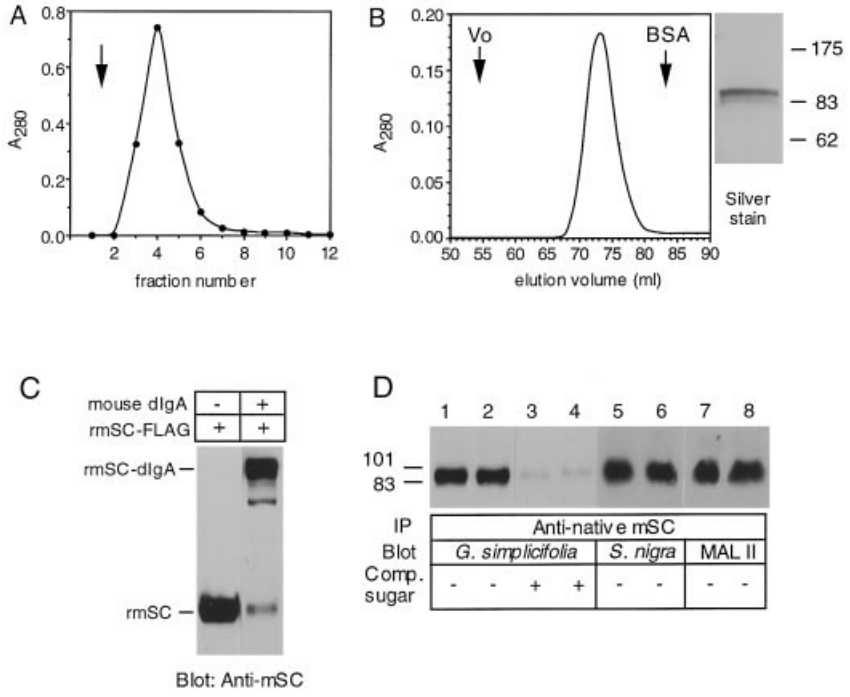

Figure 3 Biochemical characterization of rmSC-FLAG expressed in a murine cell line

(**A**) Purification of secreted rmSC-FLAG by affinity chromatography on agarose-bound M2 mAb. After extensive washes, specific elution was initiated by the addition of the FLAG peptide (arrow) at 50 μg/ml. A_{280} was read and plotted after subtraction of the A_{280} of the free peptide (0.03). (**B**) Left panel: elution profile of size-exclusion chromatography on Superdex 200 of rmSC-FLAG; the void volume (V_0) and the elution volume of BSA are indicated. Right panel: silver-staining of purified rmSC-FLAG separated by SDS/PAGE. (**C**) Covalent reassociation of rmSC-FLAG with dIgA. Samples were separated by SDS/PAGE under non-reducing conditions; specific dIgA–rmSC complexes were detected with anti-mSC antiserum. (**D**) Comparative analysis of sugar structures in recombinant and colostral mSC. rmSC-FLAG and murine colostrum were immunoprecipitated with anti-mSC IgGs and blotting was performed with biotinylated lectins: lanes 1–4, *Griffonia simplicifolia* lectin I in the absence (−) or presence (+) of 0.2 M methyl α-galactoside; lanes 5 and 6, *Sambucus nigra* lectin; lanes 7 and 8, *Maackia amurensis* lectin II (MAL II). Binding of the lectin was detected with HRP-coupled streptavidin and enhanced chemiluminescence. The positions of molecular mass markers are indicated (in kDa) at the left.

accessible in the native protein. We thus developed a strategy combining successive chromatographic steps involving concanavalin A–agarose, Q-Sepharose and Superdex 200. Column fractions containing rmSC were identifed by Western blot (Figure 2B). After silver-staining, the appearance of a doublet at approx. 85–90 kDa after the final chromatography reflected the purity of the material (Figure 2B, lane SS).

We then evaluated the association of purified rmSC with various molecular forms of murine and human IgA. DORA [13] indicated that rmSC binds to murine pIgA and dIgA and to human dIgA, but not to murine mIgA (Figure 2C). The absence of detectable free rmSC from the overlay indicates that binding is quantitative (results not shown). The specificity of association indicates that mSC folds properly and recognizes only pIgA containing the J chain. Up to 80 % covalent association occurs after incubation of rhSC and murine dIgA in the test tube [6,14]. This held true for rmSC-6 × His combined with murine dIgA (Figure 2D). The results indicate that mutual recognition of the two partners can be mimicked *in vitro* and that the accuracy of the interaction extends to the degree of covalency, which reflects the natural situation [31].

Production of rmSC-FLAG in a murine cell line

Although a flexible tool, the vaccinia virus/T7 system does not offer the convenience of a stable cell line. Further, the multi-step purification procedure that we had to design complicated the overall protocol to recover rmSC. In addition, we reasoned that it might be worth obtaining an expression system capable of glycosylating an rmSC with a murine pattern. Initially, in an attempt to create a cell line able to assemble murine S-IgA, we decided to transfect the IgA-secreting hybridoma IgA71 [15] with construct pCB6mSC-FLAG:Cterm (Figure 1E). We obtained evidence that mSC–dIgA complexes were secreted at an early stage after selection (results not shown). Unexpectedly, all positive clones stopped producing IgA as determined by ELISA of culture supernatants. Clone 2H2, secreting the largest amount of rmSC-FLAG, was nevertheless kept as a sustained source of protein. Purification of the protein was performed by chromatography on an anti-FLAG M2 affinity column with gentle elution with the FLAG peptide (Figure 3A), yielding approx. 1.1 mg of mSC per 10^8 cells. Size-exclusion chromatography showed a single peak that eluted at the position of a monomer (Figure 3B). $A^{1\%}_{1\,cm,280}$ was determined as 14.9 litre $g^{-1} \cdot cm^{-1}$ by using protein quantification with bicinchoninic acid [28]. A major band with an apparent molecular mass of approx. 95 kDa, together with a weaker approx. 93 kDa product, were seen after SDS/PAGE and silver-staining (Figure 3B). 'Natural' mSC also migrated as a doublet when purified from milk or bile [31,32]. Similarly to the rmSC-6 × His product, rmSC-FLAG associated covalently with dIgA (Figure 3C).

Murine cell lines and tissues are known to add terminal α1-3-galactosyl residues to *N*-linked carbohydrates, resulting in the structure Galα1-3Galβ1-4GlcNAc-R [25,33]. Further, IgA antibodies secreted by murine hybridomas are known to carry α-galactosylated oligosaccharides [34–36]. The presence of α1-3-

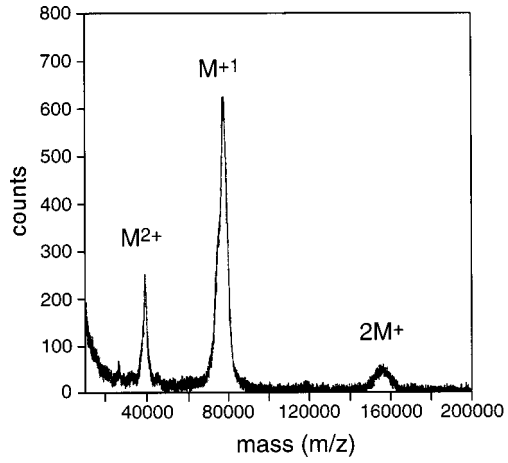

Figure 4 MALDI-MS of purified mSC-FLAG produced by clone 2H2

The spectrum was obtained as described in the Experimental section. M^{2+}, monomeric rmSC doubly ionized; M^{+1}, mono-ionic monomeric rmSC, $2M^{+}$, mono-ionic dimeric rmSC.

galactosyl residues in mSC secreted by clone 2H2 was demonstrated by positive detection with *Griffonia simplicifolia* lectin I on blot (Figure 3D, lane 1). Colostral mSC isolated by immunoprecipitation with the purified rabbit IgG against native mSC yielded an identical result (Figure 3D, lane 2). In addition, Neu5Acα2-6Galβ1-4GlcNAc-R and Neu5Acα2-3Galβ1-4GlcNAc-R structures are also present on both rmSC and colostral mSC, as reflected by the positive signals observed on detection with *Sambucus nigra* lectin and *Maackia amurensis* lectin II (Figure 3D, lanes 5–8). Both linkages of sialic acid residues have also been found in murine IgA [35]. The presence of substantial amounts of sialic acids is also indicated by the selective oxidation and biotinylation of these residues (see the Experimental section), and reflects proper terminal glycosylation of the recombinant protein. Thus clone 2H2 represents the first reported line capable of expressing rmSC displaying murine glycosylation features present in the 'natural' protein.

To determine its size and purity with more accuracy, the purified protein was analysed by MALDI-MS (Figure 4). Monomeric mSC showed a molecular ion peaks at 80 kDa for the M^{+} ion and 40 kDa for the M^{2+} ion. The carbohydrate heterogeneity is reflected in the spectrum. Given a calculated molecular mass of 66447 Da for the mSC-FLAG polypeptide, this result indicates that carbohydrates contribute approx. 17% of the mass. The additional signal at 158 kDa represents dimeric mSC, which was not detected by size-exclusion chromatography (Figure 3B) because it did not form at the concentration loaded on the column (approx. 4.5 μM). The tendency for homodimerization at a high concentration might reflect a physiologically relevant function because SC can reach a concentration of 25 μM (2 mg/ml) in human milk [37]. In the presence of dIgA, polymeric Ig receptor is found as dimers in the plasma membrane [38].

Recombinant SC binds dIgA containing the J chain and pIgA forms containing or lacking the J chain

Using DORA, we have shown previously that both rhSC and rmSC interact specifically with murine dIgA ([13], and the present study), even though one partner protein is immobilized on a membrane support. To make the assay quantitative, we adapted the procedure to ELISA plates. Micro-well reassociation assays performed with rmSC, colostral mSC and rhSC proteins can be used as an alternative to DORA, and reflect accurately the specific interaction taking place between SC and IgA molecular forms. Commercial IgA preparations or our own purified IgA molecular forms exhibited similar recognition by either recombinant or colostral SC (Figure 5A). However, purified pIgA are much more potent binders, suggesting that commercial preparations might prevent a proper evaluation of SC proteins. Heterogeneity of IgA molecules might represent a valuable explanation of differences in binding specificity (Figure 5B). Certainly, the importance of species-independent features in binding specificity is demonstrated by the series of experiments in the present study. The immunoblot in Figure 5(C) illustrates the correlation existing between binding observed in Figure 5(A) and the presence of J chain, further demonstrating the accuracy and power of this simple assay as well as the integrity of rmSC-FLAG.

DORA does not permit the identification of selective molecular features involved in specific interaction unless purified proteins are used. We therefore developed another assay based on the initial resolution of protein by non-reducing SDS/PAGE, blotting and finally overlay incubation with the binding partner. When we tested the binding of biotinylated mSC-FLAG to IgA preparations that had been subjected to SDS/PAGE and electrotransfer, all the J-chain-containing species were detected by HRP-conjugated streptavidin (Figure 5D). Interestingly, in the pIgA lane, an additional band not containing J chain (marked with an asterisk) was also recognized by rmSC-FLAG. This band and all the other bands recognized by rmSC-FLAG consisted of α chains that were covalently joined together, as shown by immunoblotting (Figure 5B).

In human dIgA, J chain was shown to link only two of the α chains covalently, the other two α chains being covalently linked together [39]; hence these two pieces will dissociate in SDS. Therefore, although the presence of J chain in a native IgA molecule somehow promotes the formation of SC-binding sites during biosynthesis, these are exposed *in vitro* in α-chain oligomers with no J chain covalently attached. Brandtzaeg and Prydz [40] showed that the adsorption of immunoglobulin polymers on polymeric Ig receptor-expressing epithelial cells depends solely on a J-chain-determined binding site, but Tomasi and Czerwinski [41] described two myelomas producing pIgA lacking J chain that were still able to bind SC *in vitro*. Consistently, the presence of J chain in pIgA seems not always to guarantee binding of SC [42]. In J chain-deficient mice, SC cannot be found associated with IgA [16] and IgA antibodies from these mice are not transported by the polymeric Ig receptor [17]. Brandtzaeg [43] showed that purified J chain affords only marginal blocking of SC binding to dIgA and IgM, but the possibility of direct non-covalent interactions between polymer-associated J chain and SC could not be excluded. An antibody against J chain was recently shown to block the assembly of SC with IgA *in vitro* and to inhibit the polymeric Ig receptor-mediated biliary and epithelial transport of human pIgA [44]. Accordingly, transepithelial transport of pIgA lacking the J chain was precluded [45]. In contrast with our observation with murine J chain-deficient pIgA and mSC, human pIgA lacking the J chain did not combine with rat SC [40]. Taken together, these results indicate that the J chain is dispensable in the reassociation of pIgA and SC *in vitro* in a species-specific manner, whereas it is required for proper pIgA–polymeric Ig receptor interaction *in vivo*. In mIgA, the lack of J chain precludes the recognition by SC/polymeric Ig receptor both *in vitro* and *in vivo*.

In conclusion, this study represents the first demonstration that rmSC with preserved biological and biochemical properties

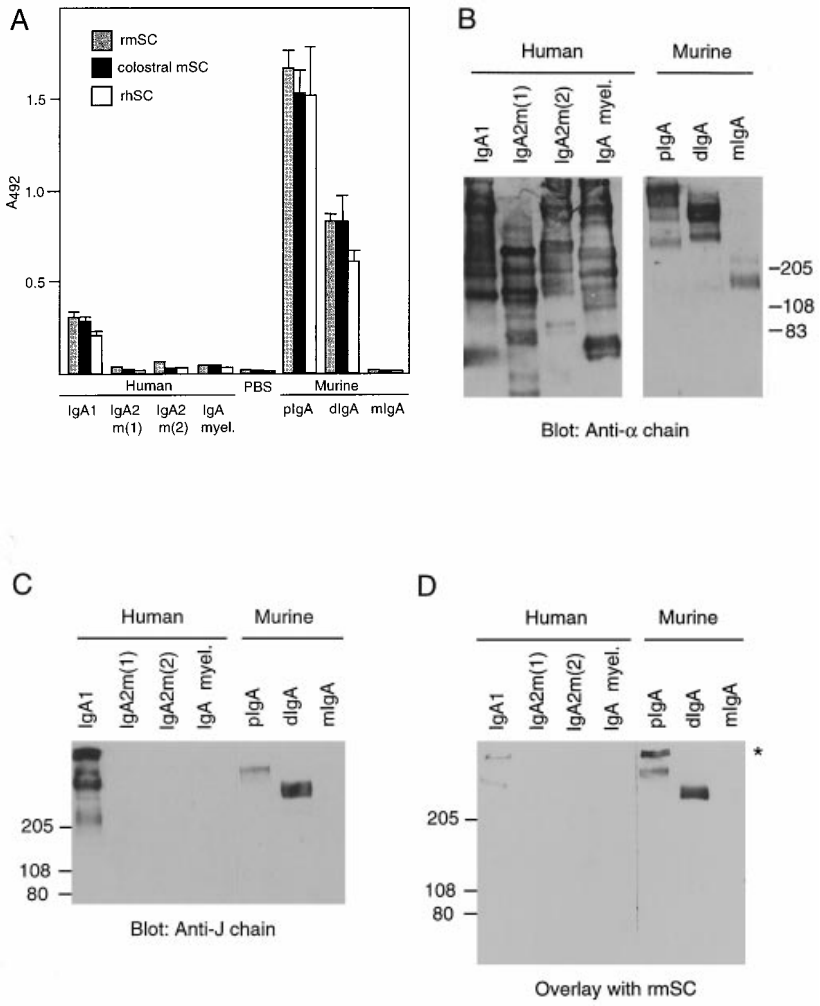

Figure 5 Comparative binding specificities of rmSC, milk mSC and rhSC to various molecular forms of IgA

(**A**) The correlation between the binding capacity of various SC preparations and the presence of J chain in human and murine IgA was determined by micro-well reassociation assay. Results are means ± S.D. for triplicate wells. (**B**) Pattern of IgA preparations analysed by immunoblotting with antibodies against human or murine α chain. The positions of molecular mass markers are indicated (in kDa) at the right. (**C**) Western blot analysis of IgA preparations fractionated in a non-reducing 6% (w/v) polyacrylamide gel, with a rabbit antiserum recognizing both the human and the murine J chains. The positions of molecular mass markers are indicated (in kDa) at the left. (**D**) Molecular features involved in SC–IgA interaction examined by overlay assay. IgA preparations were separated by SDS/PAGE under non-reducing conditions, blotted on membranes and incubated with biotinylated rmSC followed by detection with HRP-coupled streptavidin and chemiluminescence. Positive detection reflects specific association between partners. The band not containing J chain is marked by an asterisk.

can be produced in sufficient amounts to address the structure–function relationship of the protein in S-IgA complexes. The use of mSC together with murine IgA in mice will allow the further evaluation of the role of IgA compared with S-IgA antibodies in the mechanisms of mucosal neutralization [46]. When polymeric Ig receptor knock-out mice can be generated, the immediate availability of mSC will allow the exploration of novel functional aspects of the protein, including the involvement of SC in epithelium recognition, targeting of S-IgA for reuptake, and competition for attachment of micro-organisms.

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