Functional and structural characterisation of human colostrum free secretory component

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\textbf{A R T I C L E  I N F O}

Article history:
Received 24 November 2008
Accepted 30 December 2008
Available online 23 February 2009

Keywords:
Analytical ultracentrifugation
Immunoglobulin A
Mucosal immunity
X-ray scattering

\textbf{A B S T R A C T}

Secretory component (SC) in association with polymeric IgA (pIgA) forms secretory IgA (SIgA), the major antibody active at mucosal surfaces. SC also exists in a free form in secretions, with innate neutralizing properties against important pathogens. IgA-bound SC and free secretory component (FSC) are both produced by proteolytic cleavage of the polymeric Ig receptor whose function is to transport IgA and IgM across mucosal epithelia. Although the proteases have not been characterised and the site(s) of cleavage of the polymeric Ig receptor has been debated, it has been assumed that bound and free SC are produced by cleavage at the same site. Here we show by SDS-PAGE analyses that FSC is slightly smaller than SIgA1- or SIgA2-bound SC when purified simultaneously. The FSC preparation was functionally active, shown by binding to dimeric and polymeric IgA, and by its ability to trigger a respiratory burst by binding to ‘SC receptors’ on eosinophils. We also show that FSC from different human secretions have different molecular sizes. The solution structure of FSC from colostrum was studied by analytical ultracentrifugation and X-ray scattering. The sedimentation coefficient of 4.25 S is close to that for recombinant FSC. The X-ray scattering curve showed that FSC adopts a compact structure in solution which corresponds well to the J-shaped domain arrangement determined previously for recombinant FSC which terminates at residue Arg585. The smaller sizes of the FSC forms are attributable to variable cleavages of the C-terminal linker region, and may result from the absence of dimeric IgA. The FSC modelling accounts for the lack of effect of the C-terminal linker on the known functions of FSC.

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1. Introduction

The mucosal epithelia of the gastrointestinal, respiratory and urogenital tracts represent the largest surface in contact with the external environment, with a total surface area of 400 m\textsuperscript{2} in comparison to only 1.5 m\textsuperscript{2} for skin (Brandtzaeg, 2007). Consequently, mucosal surfaces are constantly exposed to foreign antigens and must therefore be efficiently protected to prevent possible invasion by pathogens. Secretory immunoglobulin A (SIgA) is one of the first lines of mucosal immune defence, where SlgA exerts its function of antigen neutralization and opsonisation. IgA is produced locally as polymeric IgA (pIgA), which consists of usually two (or sometimes three or four) IgA molecules linked covalently by a \( \alpha \) chain. The pIgA (and to a lesser extent pentameric IgM) is transported across the epithelium by the polymeric Ig receptor (pIgR), which is expressed on the basolateral surface of epithelial cells. The pIgR–pIgA complex is transcytosed across the cell, and at the luminal surface the pIgR is cleaved by protease within a 42-amino acid region adjacent to the cell membrane thus releasing SlgA into the lumen (Hughes et al., 1997). The cleaved extracellular portion of pIgR remains bound to pIgA and is now termed secretory component (SC) (Fig. 1). pIgR can also be transported into the mucosa even if pIgA is not bound to it, thus most exocrine fluids contain SC both bound within SlgA and also free (Phalipon and Corthésy, 2003). In vitro, purified free SC (FSC) is able to bind non-covalently to pIgA or IgM (Crottet and Corthésy, 1998). In secretions and serum, human IgA exists as two subclasses, IgA1 and IgA2. SC binds to both SlgA subclasses, although recently it has been shown that SC is bound covalently to SlgA1, but mostly non-covalently to SlgA2 (Almogren et al., 2007). FSC is known to have important anti-microbial functions, and its carbohydrate chains have been considered necessary for its interactions with numerous bacterial pathogens (Perrier et al., 2006; de Oliveira et al., 2001; Giugliano et al., 1995; Hammerschmidt et al., 1997, 2000). FSC can also bind to eosinophils but not to neutrophils via a 15 kDa protein receptor with lectin activity. This ‘SC receptor’ triggers degranulation and release of eosinophil peroxidase to protect the host further from pathogens (Lamkhioued et al., 1995).
SC has a molecular mass of 79.6 kDa and contains five immunoglobulin (Ig) variable (V)-type domains (D1–D5) of between 100 and 110 residues each, together with the unstructured 42 amino acid, C-terminal linker region that joins the extracellular domains to the membrane (Fig. 1) (Kaezelt, 2005; Piskurich et al., 1995). Each domain (D1–D5) contains an internal conserved disulphide bond. A second internal disulphide bond is found in D1, D3, D4 and D5. A third Cys502 residue is present in D5, which covalently links SC to one of the α-heavy chains of SIgA through Cys311 (Fallgreen-Gebauer et al., 1993). SC is highly glycosylated, with seven putative N-linked glycosylation sites representing approximately 22% of its molecular mass (Fig. 1) (Hughes et al., 1999; Eiffert et al., 1984, 1991). The exact cleavage site(s) that releases SC from plgR, and the proteases that are involved have not been characterised. It is not known whether the same cleavage occurs for free or ligand bound plgR (Kaezel, 2005). In one study, FSC from pooled human colostrum was shown to have a C-terminal residue varying from Ala550 to Lys559, but predominantly at Ser552 (Eiffert et al., 1995). Each domain (D1–D5) contains an internal conserved disulphide bond. A second internal disulphide bond is found in D1, D3, D4 and D5. A third Cys502 residue is present in D5, which covalently links SC to one of the α-heavy chains of SIgA through Cys311 (Fallgreen-Gebauer et al., 1993). SC is highly glycosylated, with seven putative N-linked glycosylation sites representing approximately 22% of its molecular mass (Fig. 1) (Hughes et al., 1999; Eiffert et al., 1984, 1991). The exact cleavage site(s) that releases SC from plgR, and the proteases that are involved have not been characterised. It is not known whether the same cleavage occurs for free or ligand bound plgR (Kaezel, 2005). In one study, FSC from pooled human colostrum was shown to have a C-terminal residue varying from Ala550 to Lys559, but predominantly at Ser552 (Eiffert et al., 1984, 1991). A later study of FSC purified from milk from a single donor identified the cleavage site as Arg585 (Fig. 1; Hughes et al., 1997). We are unaware of any C-terminal sequence data on SC isolated from SIgA. Since SC and SIgA by nature are found in secretions rich in proteases, further cleavage of SC when free in solution and not protected by SIgA is a clear possibility. A number of polymorphisms have been identified in the plgR genes, some of which have been associated with disease (Hirunsatit et al., 2003). One polymorphism of note is in the linker region, which might alter the susceptibility of the plgR to proteolytic cleavage at mucosal linings (Obara et al., 2003).

Recently, the solution structure of human recombinant FSC expressed in Chinese hamster ovarian cells was determined (Bonner et al., 2007). Recombinant FSC possessed an unexpectedly compact structure, adopting a J-shaped conformation in which the D4 and D5 domains are folded back against the D2 and D3 domains. In contrast, the solution structures of the two major SIgA1 and SIgA2 isomers show that SC adopts an extended domain arrangement along the edge of the Fc–Fc dimer within SIgA1 and SIgA2 (Bonner et al., 2009a, 2009b). During studies on the proteolysis of colostrum FSC and SC bound in SIgA1 and SIgA2 (Almogren et al., 2007), we observed unexpectedly that FSC appeared to be smaller than the bound form. It is possible that the absence of dimeric IgA facilitates further cleavages in FSC. In this paper, we confirm and extend this observation by showing that the FSC preparation that was purified from a single pool of human colostrum is functionally active, this being able to bind to plgA but not to monomeric IgA, and to eosinophils to stimulate an oxidative burst. We also show that this colostrum FSC has a similar compact J-shaped, five-domain structure in solution to that previously determined for recombinant SC (Bonner et al., 2007). We conclude that cleavage of the C-terminal linker region at different positions in FSC appears to have little effect on the structure and function of the molecule.

2. Materials and methods

2.1. Simultaneous purification of FSC, secretory IgA1 and secretory IgA2 from colostrum

Colostrum samples were collected, with ethical approval, from healthy multigravidae Saudi women attending the King Khalid University Hospital, Riyadh, Saudi Arabia. A single pool of colostrums was used in this study. The secretory immunoglobulins (polymeric and dimeric SIgA1, SIgA2, secretory IgM and FSC) were separated from a clarified colostrum preparation using thiophilic-gel chromatography, jacalin–agarose chromatography, and Sephacryl S-300 gel filtration. The FSC-containing fractions from the gel filtration were pooled, concentrated and further purified to homogeneity by affinity chromatography on agarose bound with serum plgA1 (Almogren et al., 2007). The purified proteins were analysed by SDS-PAGE, Western blotting and gel filtration FPLC. Molecular weight markers used in SDS-PAGE analysis were from New England BioLabs, Hitchin, UK (mol. wt. 175, 80, 58, 46, 30, 25, 17 and 7 kDa) or from Bio-Rad, Hemel Hempstead, UK (pre-stained broad range mol. wt. 250, 150, 100, 75, 50, 37, 25, 15 and 10 kDa).

2.2. Deglycosylation of free and bound SC

FSC, SIgA1 and SIgA2 (2 mg/ml) were incubated with recombinant N-glycanase (Glyko, Europa Bioproducts, Ely, UK) according to the manufacturer’s instructions. Deglycosylation was confirmed by SDS-PAGE analysis.

2.3. Characterisation of the association of FSC with different forms of IgA

To assess the capability of colostrum FSC to associate with each form of IgA in vitro, an ELISA microtitre plate was coated with FSC (5 μg/ml in coating buffer) by incubation overnight at 4 °C before washing the plate three times. Then mlgA1, mlgA2m(1), dIgA1, tri-IgA1 or plgA2, each adjusted to a concentration of 20 μg/ml, were added in triplicate to the coated plate and incubated for 1 h at 25 °C. After the plate had been washed three times, anti-IgA (α-heavy chain specific) antibody alkaline phosphatase conjugate was added and incubated for 1 h at 25 °C. The plate was then washed three times, substrate was added and the plate was incubated whilst the absorbance was measured at 405 nm.

2.4. Preparation of isolated eosinophils, neutrophils or monocytes

Eosinophils were purified from the peripheral blood of patients with eosinophilia, kindly supplied by the Department of Haematology, Ninewells Hospital, Dundee. Ethical approval had been obtained. A mixture of eosinophils and neutrophils were first obtained by differential centrifugation using discontinuous density gradient of Lymphoprep and Ficoll-Hypaque. Then a negative selection procedure was performed to remove contaminating neutrophils. Briefly, 1 volume of anti-FcyRIII mAb 3G8 supernatant was mixed with 19 volumes of magnetic polymer beads pre-coated with sheep anti-mouse IgG (Dynal, Invitrogen, Paisley, UK) and incubated overnight at 4 °C on a rotary mixer. The beads were then washed three times in Hanks’ buffered salt solution (HBSS) (Gibco Invitrogen, Paisley, UK) containing 0.1% bovine serum albumin and retrieved magnetically according to the manufacturer’s instructions. Neutrophils express large amounts of FcγRIII so they bind to the beads, whereas eosinophils do not express the receptor and remain in the supernatant. Eosinophil-enriched supernatant cell fractions were washed twice in HBSS and resuspended in HBSS/0.1%
bovine serum albumin to which 3G8-coated beads were added (at a bead-to-neutrophil ratio of 1:1), and the mixture was incubated again for 30 min at 4 °C on a rotary mixer. Multiple rounds of magnetic extraction were carried out until the eosinophils were 99% pure (Pless et al., 2007). Cytospin preparation slides were judged by Eosin/thiazine staining (Diff-Quik, Baxter Diagnostics, Switzerland). Neutrophils were purified by the method of English and Andersen (1974) as described previously (Stewart and Kerr, 1990). Monocytes were purified over Nycodenz monocyte medium as described by the manufacturers (Axis-Shield, Kimbolton, UK). Luminol-enhanced chemiluminescence was measured in 96 well plates coated with FSC or other proteins as described by Almogren et al. (2003).

2.5. Analysis of FSC from different body fluids

The following samples were characterised: unfracionated colostrum (above); urine and clarified saliva from laboratory volunteers; endotracheal aspirate and amniotic fluid were superfluous “normal” clinical samples submitted to the Departments of Microbiology and Immunology, Ninewells Hospital Medical School, Dundee, UK. One sample of endotracheal aspirate was treated with mucolytin.

2.6. Structural studies of FSC in solution

X-ray scattering data were obtained in two sessions on the Beamline ID02 at the European Synchrotron Radiation Facility, Grenoble, France, operating at 6.03 GeV (Narayanam et al., 2001). To reduce the incident flux, the experiments were performed in single-bunch mode with storage ring currents ranging between 16.4 and 12.3 mA. The sample-to-detector distance was 3.0 m, which yielded a Q range from 0.08 nm⁻¹ to 2.24 nm⁻¹ (Q = 4π sin θ/λ; 2θ = scattering angle; λ = wavelength). FSC samples from colostrum dialysed into Dulbecco’s phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.5) were measured in static cell holders at concentrations of 0.07, 0.12, 0.27 and 0.42 mg/ml. Data were acquired using ten time-frames of 1 s and 2 s to establish the absence of radiation damage. Other details, including calibrations and data reduction, and the Guinier analyses to determine the radius of gyration Rₒ, are described previously (Boehm et al., 1999; Furtado et al., 2004). Constrained modelling of the X-ray data to give a solution structure for the SC domains utilised the same two sets of 5,000 FSC models as previous (Bonner et al., 2007). These were created either using four unconstrained linkers between the five domains, or holding the D1–D2–D3 and D4–D5 regions fixed in their conformation and varying only the long linker between D4 and D5 (Fig. 1).

Analytical ultracentrifugation was performed at 20 °C on a Beckman XL-I instrument, equipped with an AntiS0 rotor. Sedimentation velocity data for FSC from colostrum in the same phosphate buffer were acquired over 16 h at rotor speeds of 20,000 rpm, 25,000 rpm and 30,000 rpm with column heights of 12 mm. FSC was studied at three concentrations from 0.12 mg/ml to 0.27 mg/ml. SEDFIT c(s) distribution analyses based on the non-interacting species model were performed as previously described, in which the frictional ratio and baseline were initially floated, then held fixed for the final fits and using the buffer density and partial specific volume from previous (Bonner et al., 2007).

3. Results

3.1. Purification of FSC

SlgA1, SlgA2 and FSC were purified in sequence from the same colostrum pool as described previously (Almogren et al., 2007). This technique has a number of advantages over earlier procedures. The initial thiophilic chromatography step removes lactoferrin which does not bind to the resin. Lactoferrin is a recognised problematic contaminant in previous SIgA and SC preparations. Efficient removal was important since lactoferrin, which is present in larger amounts than FSC in colostrum, migrates in SDS-PAGE gels at a similar position to FSC, often distorting the SC band in impure preparations. Early work in our laboratory had suggested that FSC did not bind to commercial thiophilic resin. However, FSC bound strongly to a more highly derivatised resin (Almogren et al., 2007).

Improved purification techniques for the different forms of IgA using thiophilic resin chromatography as a starting step have previously enabled us to produce stable preparations of serum and secretory IgA in sufficient quantities for structural studies (Almogren et al., 2007). Furthermore our purification of stable myeloma IgD using the same technique (Sun et al., 2005) suggests that the thiophilic resin does not bind proteases that can often contaminate immunoglobulin preparations from serum and other fluids resulting in the cleavage of the immunoglobulins. Many previous publications had commented on the difficulty of preparing stable IgD (Sun et al., 2005). This procedure therefore appears to limit the proteolytic degradation of immunoglobulin samples from human sources, and our FSC and SlgA preparations were stable for months at 4 °C and at 37 °C overnight (Almogren et al., 2007; Almogren and Kerr, unpublished data).

3.2. Comparison of FSC and bound SC released from SIgA1 and SlgA2

We have recently shown that, unexpectedly, the SC of SlgA2 (but not SlgA1) was released under non-reducing conditions, giving a band on the gel with mobility very similar to FSC, suggesting that there was significant non-covalent binding between SC and dimeric IgA2 within SlgA2. In contrast, all the SC was covalently bound to dimeric IgA1 in SlgA1 (Almogren et al., 2007). We also showed that, when SlgA1 or SlgA2 was incubated with Proteus mirabilis protease, the SC bound within SlgA1 was only cleaved very slightly to yield a fragment of approximately 76.5 kDa, whilst SC bound within SlgA2 was more significantly cleaved to yield multiple fragments of around 76.5 kDa, 64 kDa and 32–34 kDa (Fig. 2, lane 1) (Almogren et al., 2007). Further analysis of SC bound within SlgA1 and SlgA2 and FSC, with molecular weight markers run adjacent to each SC sample, suggests that FSC is smaller in molecular mass than SC bound to either SlgA1 or SlgA2 (dashed line in Fig. 2). FSC is, in fact, similar or identical to the 76.5 kDa fragment produced from SIgA by P. mirabilis proteolytic cleavage (Fig. 2). In the SDS-PAGE gel analyses, the 80 kDa marker always ran below the band for SC derived from the two SlgA isoforms, but above the band for FSC (Fig. 2).

3.3. The effect of deglycosylation of FSC and bound SC

SC contains up to seven N-linked carbohydrate residues that represent approximately 22% of its molecular mass (Fig. 1). To investigate whether the apparent size difference between FSC and bound SC released from SlgA1 and SlgA2 was due to differences in glycosylation or the polypeptide length, the N-linked carbohydrate moieties of these three SC preparations were removed by incubation with each with recombinant N-glycanase. The molecular mass of native bound SC released from the SlgA isoforms was reduced from around 80 kDa to 62 kDa after deglycosylation (Fig. 3), which is consistent with the N-linked carbohydrate residues contributing 22.5% of the molecular mass of SC. FSC showed a similar reduction in molecular mass after deglycosylation. Although difficult to assess accurately by SDS-PAGE, the loss of carbohydrate appeared to reveal a slightly lower molecular mass for the protein component of FSC compared to bound SC (dashed line in Fig. 3).
Fig. 2. SDS-PAGE analysis of FSC and SC released from SIgA1 and SIgA2 and the effect of Proteus mirabilis proteolytic cleavage. FSC and SC released from SIgA1 or SIgA2, samples were analysed in the presence (lane 1) and absence (lane 2) of protease Proteus mirabilis. The samples that were subjected to proteolytic cleavage were incubated with Proteus mirabilis for 24 h at 37 °C. Molecular weight markers are shown (M). The dashed line corresponds to FSC in the absence of protease (lane 2).

3.4. Binding of FSC to different forms of IgA

Purified FSC associates in vitro with serum pIgA and IgM (Brandtzæg, 1975). Indeed, in our purification procedure, FSC was purified using affinity chromatography based on binding to serum pIgA1. To assess the associative capabilities of FSC for different forms of IgA, an ELISA microtitre plate was coated with FSC and this was incubated with monomeric IgA1 and IgA2m(1), dimeric IgA1, pIgA1 and pIgA2, with IgG and human serum albumin as controls. Binding of IgA and FSC was detected at optical density of 405 nm using an anti-γH9251-heavy chain IgA antibody alkaline phosphatase conjugate. FSC readily associated with three polymeric forms of IgA of both isotypes with similar affinity, yet did not bind to the two monomeric IgA isoforms (Fig. 4). This shows that FSC retained its functionality after purification in selectively binding to the J-chain containing pIgA isoforms as expected.

3.5. Binding of native and deglycosylated FSC and SIgA to eosinophils, monocytes and neutrophils

SIgA1 and SIgA2 stimulate strong respiratory bursts from neutrophils, monocytes and eosinophils, since each cell type expresses the FcεRI receptor (CD89) (Pleass et al., 2007; Monteiro et al., 1993). A unique ‘SC receptor’ which binds free and bound SC has also been identified on eosinophils, although little is known about its specific function. In order to demonstrate the functionality of the colostrum FSC preparation used in this study, the ability of FSC to trigger a respiratory burst in eosinophils was investigated using chemiluminescence. Purified FSC was coated onto chemiluminescence microtitre plate strips and eosinophils, monocytes or neutrophils were added to the plates in the presence of luminol. FSC only stimulated respiratory bursts in eosinophils, and did not stimulate monocytes or neutrophils, for which the chemiluminescence reading was the same as the background level (Fig. 5a). This shows that our FSC preparation is functionally active and able to bind to the ‘SC receptor’ to trigger the eosinophil respiratory burst. It has been proposed that the eosinophil ‘SC receptor’ is a lectin that recognises the carbohydrate moieties on SC (Lamkhioued et al., 1995). We therefore investigated whether deglycosylated FSC could stimulate eosinophils, by removing its carbohydrate moieties through incubation with N-glycanase (Fig. 3, lane 2). Deglycosylated FSC was not able to elicit a chemiluminescence response, suggesting that the FSC carbohydrates are essential for its interaction with the eosinophil ‘SC receptor’ (Fig. 5b). The comparison of the amplitude of the eosinophil respiratory burst when triggered by FSC and SC bound within SIgA1 and SIgA2 showed that the chemiluminescence response stimulation by SC within the SIgA isoforms is over five-fold greater than that by native FSC (Fig. 5c), and that for SIgA1 is over two-fold greater than that for SIgA2.

Fig. 3. SDS-PAGE analysis of native and deglycosylated FSC and SC released from SIgA1 and SIgA2 and the effect of Proteus mirabilis proteolytic cleavage. The FSC and SC released from SIgA1 and SIgA2 samples were incubated in the absence (lane 1) or presence (lane 2) of recombinant N-glycanase. Deglycosylated FSC and SC released from SIgA samples were then subjected to proteolytic cleavage by incubating with Proteus mirabilis protease for 24 h at 37 °C (lane 3; compare with Fig. 2, lane 1). Molecular weight markers are shown (M). The dashed lines correspond to SC from SIgA1 and SIgA2 in the absence and presence of N-glycanase.

Fig. 4. Binding of FSC to different forms of serum IgA. ELISA plates were coated with FSC, and five different forms of IgA, with IgG or human serum albumin as negative controls, were added at concentrations of 20 μg/ml. FSC binding to the proteins was detected using alkaline phosphatase conjugated anti-IgA α-chain specific antisera, where the OD was measured at 405 nm. Each value is the mean of triplicates.

3.6. Characterisation of the mass of SC in different body fluids

Both SIgA and FSC are found in many different body fluids such as milk, urine, saliva, bronchial wash, endotracheal aspirate, nasopharyngeal aspirate and amniotic fluid. Using SDS-PAGE and Western
Fig. 5. Native and deglycosylated FSC respiratory burst stimulation. 96-well microtitre plates were coated with 10 μg/ml of FSC. Luminol enhanced chemiluminescence was measured at 37 °C for 60 min after the addition of purified cells. The traces show the mean values of triplicate samples. (a) Eosinophil, monocytes or neutrophil cells were added. (b) Eosinophils were added to native or deglycosylated FSC (as characterised in Fig. 3, lane 2). (c) Eosinophils were added to SiGA1, SiGA2 or mucolycin (lane 5) and untreated (lane 6) are shown.

Fig. 6. Western blot to show the varying sizes of human FSC from different body fluids. Samples of unfractionated fluids were separated by SDS-PAGE (not shown) and blotted using anti-SC antibody. FSC from colostrum (lane 1), amniotic fluid (lane 2), urine (lane 3), clarified saliva (lane 4) and endotracheal aspirate treated with mucolycin (lane 5) and untreated (lane 6) are shown.

blotting, we observed heterogeneity in the mass of FSC from different fluids (Fig. 6). SC in urine had the highest molecular mass (Fig. 6, lane 3), followed by SC in the amniotic fluid (Fig. 6, lane 2). The size of SC in saliva, endotracheal aspirate and nasopharyngeal aspirate were similar to each other (Fig. 6, lanes 4–6). The lowest molecular weight SC was found in colostrum (Fig. 6, lane 1). In a large number of SDS-PAGE analyses of fractions from chromatography columns, no difference was observed between the molecular mass of FSC in colostrum and at various stages of its purification (results not shown).

3.7. Structural analyses of FSC by analytical ultracentrifugation and X-ray scattering

Sedimentation coefficients 

\[ s_{20,w} \]

monitor macromolecular elongation. Sedimentation velocity runs were used to determine the 

\[ s_{20,w} \]

value of colostrum FSC and to assess any sample heterogeneity due to degradation or aggregation, which would be revealed by additional peaks in the size distribution analyses \( c(s) \) (Bonner et al., 2007; Nan et al., 2008). The fitted boundaries showed good agreement with the experimental ones (Fig. 7a,c). The resulting \( c(s) \) plots showed that FSC has an \( s_{20,w} \) value of 4.25 S (interference) and 4.3 S (absorbance) (Fig. 7b,d), and the single peak showed no evidence of other species. The \( s_{20,w} \) value of recombinant FSC was comparable with this, but slightly less in a range of 3.9–4.2 S depending on the method used to measure this (Bonner et al., 2007). The conversion of the \( c(s) \) analyses into mass distribution analyses \( c(M) \) showed that the FSC peaks in Fig. 7(b,d) correspond to molecular weight values of 78 (±1) kDa. This is slightly less than the sequence-derived value of 79.6 kDa, based on the termination of FSC at Arg585 (Hughes et al., 1997) and seven biantennary complex-type oligosaccharides (Bonner et al., 2007). The \( s_{20,w} \) value leads to the frictional ratio \( f/f_0 \), where \( f_0 \) is the frictional coefficient of the sphere of volume equal to that of the hydrated protein. Many globular proteins show \( f/f_0 \) of 1.2 to 1.3. For FSC, the \( f/f_0 \) ratio was determined to be 1.44, which is slightly less than that of 1.55 for recombinant FSC (Bonner et al., 2007). This agreement shows that the domain structure of FSC is not extended and is similar to that seen for recombinant FSC (Bonner et al., 2007). The slight decrease in the \( s_{20,w} \) and \( f/f_0 \) values would be consistent with a reduction in the length of the 42-residue C-terminal linker. This was confirmed by calculation of the sedimentation coefficient of FSC using HYDROPRO, which gave values of 4.06 S and 4.38 S for the FSC models with and without the 42-residue C-terminal linker respectively.

Solution scattering is a diffraction technique that studies the overall structure and size of biological macromolecules in random orientations (Perkins et al., 2008). X-ray scattering experiments were used to determine the overall domain arrangement of FSC. Data were acquired at FSC concentrations between 0.07 mg/ml and 0.42 mg/ml (Section 2). Comparison of the \( I(Q) \) data for FSC for a single time frame and that averaged over ten consecutive frames showed satisfactory signal–noise ratios (although noisy at large \( Q \) values) and no radiation damage was detected (Fig. 7e). The ten merged time frames were used for analyses. Trace amounts of aggregates were detected at the lowest \( Q \) values (data not shown), which are commonly observed in highly glycosylated glycoproteins (Boehm et al., 1999). These preclude accurate Guinier radius of gyration \( R_g \) analyses at low \( Q \) that monitor macromolecular elongation. At larger \( Q \) values, analyses of \( I(Q) \) against \( Q^2 \) yielded the \( R_g \) of the cross-section, \( R_{XS} \) (Fig. 7e). A mean X-ray \( R_{XS} \) value of 1.97 (±0.20) nm (six values) was determined, which is within error of the \( R_{XS} \) value of 1.76 (±0.08) nm for recombinant FSC (Bonner et al., 2007). This \( R_{XS} \) agreement suggested that FSC has a similar compact arrangement of its five domains as that previously determined for recombinant FSC.

In order to determine the solution arrangement of the five domains in FSC (Fig. 1), constrained modelling of the X-ray scattering data was performed. The first search utilised 5000 randomised FSC domain arrangements with four unconstrained linkers joining the five domains, and the second search utilised 5000 FSC mod-
Fig. 7. Sedimentation velocity and X-ray scattering analyses of FSC. (a–d) Sedimentation velocity analysis of FSC using SEDFIT. (a, c) The black circles represent the experimental scans and the continuous white lines represent the fits. Interference (a) and absorbance (c) scans are shown for FSC at 0.267 mg/ml and at 20,000 r.p.m. (interference) and 30,000 rpm (absorbance), both showing every sixth boundary of 120 scans. (b, d) The corresponding c(s) plots from (a, c) are shown, from which the sedimentation coefficient for FSC was determined to be 4.2 S (interference) and 4.3 S (absorbance). (e) X-ray fit of the FSC experimental solution scattering curve with the best-fit recombinant SC model curve. The black circles represent the FSC experimental I(Q) curve and the continuous black line represents the best-fit recombinant FSC model curve. The Guinier R_G fit range of Q values was 0.2–0.4 nm^{-1} and that for the R_XS fit was 0.42–0.86 nm^{-1}. (f) The best-fit recombinant FSC model (PDB code: 1xed) is shown as a black ribbon trace, with the D1–D5 domains, residues V544, S552 and R585 in the C-terminal linker, and the C- and N-termini labelled as shown. The seven carbohydrate chains are shown in grey.

Models created by only varying the D3–D4 linker in all orientations and utilising the solution structures of the D1–D3 and D4–D5 fragments (Bonner et al., 2007). As the C-terminal residue was not known in colostrum FSC, the 42-residue C-terminal linker was retained in all these models. The filtering of the models for compatibility with the number of spheres in each model, the R_XS value and the goodness-of-fit R factor showed that 156 models in the first search and 73 models in the second search agreed with the experimental data. Interestingly, these models included the best-fit recombinant FSC structure from previous (Bonner et al., 2007). The best R factor values ranged between 16.3% and 20.0%, with the best-fit recombinant FSC structure giving 20.0% (Fig. 7e). The removal of the 42-residue C-terminal linker caused a minimal change in the R factor. Even though the R factor is optimally less than 10% for good fits, the higher R factor values obtained here is attributable to a higher signal-noise ratio than is usually the case. Visualisation of the best-fit models with the lowest R factors of 16.3–16.5% on molecular graphics software showed that all the best-fit models had a compact arrangement of their domains, in which D4 and D5 were bent back against D2 and D3 to form a U-shape or J-shape. The modelling confirms that FSC has a compact domain structure in solution, similar to that seen for recombinant FSC (Fig. 7f).
4. Discussion

During study of the binding of secretory component to human colostral SlgA1 and SlgA2 (Almogren et al., 2007), we demonstrated surprisingly that when analysed by SDS-PAGE under non-reducing conditions SC was released from SlgA2, in contrast to SlgA1 where SC remained bound. This suggested that SC binds differently to the two major SlgA isoforms and showed different sensitivity to proteolytic cleavage. During the further studies reported here, it became increasingly clear that FSC was smaller than bound SC released from SlgA1 and SlgA2. We now show that colostrum FSC has a molecular mass of approximately 76.5 kDa compared with approximately 80 kDa for bound SC (Fig. 2). If the 42-residue C-terminal linker is removed, this corresponds to a sequence-predicted mass of 75.0 kDa, this being reduced by 4.6 kDa starting from a sequence-predicted mass of 79.6 kDa. FSC appears to be very similar if not identical to the first fragment produced on cleavage of bound SC using a protease from Proteus mirabilis (Fig. 2). Further SDS-PAGE analyses of deglycosylated free and bound SC showed that the size difference was attributable to a smaller peptide chain rather than to a change in glycosylation level (Fig. 3). A number of studies have commented on the sensitivity of FSC to proteolysis, with a similar degradation pattern of cleavage of SC into D1–D3 and D4–D5 fragments followed by further cleavage of D1–D3 into individual domains (Bonner et al., 2007; Almogren et al., 2007). Although the FSC preparation used in the present study showed that this pattern of cleavage by added protease, cleavage was not observed in the absence of added protease. There are no reports of cleavage of the 42-amino acid linker region in vitro though its lack of secondary structure would suggest it to be highly sensitive to proteases. Although it is difficult to be certain whether the cleavage of FSC in our preparation occurred in vivo or during purification, western blotting of FSC in colostrum and during purification provided no evidence of cleavage in vitro.

To confirm the functional integrity of the FSC sample, we carried out binding studies using a qualitative ELISA, which showed that purified FSC associated with serum dimeric IgA and plgA regardless of the isotype, but not to the monomer (Fig. 4). This indicated the requirement of J chain for association to take place, and is consistent with our structural models for SlgA1 and SlgA2 that indicate that SC and J chain interact with each other on the Fc–Fc dimer edge (Bonner et al., 2009a, 2009b). This association confirms previous reports of the ability of isolated FSC to selectively bind to polymeric immunoglobulins (Kaetzel, 2005). The functional nature of the FSC was further demonstrated by its ability to bind to and trigger a respiratory burst from purified human eosinophils, but not from neutrophils or monocytes (Fig. 5a). This is consistent with reports of a unique 15 kDa protein receptor for SC expressed on the cell surface or secreted by eosinophils (Lamkhioued et al., 1995). This SC cell surface receptor is able to trigger superoxide production and the degranulation of eosinophils (Motegei and Kita, 1998). In keeping with the proposed lectin-like properties of the receptor, we demonstrate that deglycosylation of FSC removes its ability to bind to and stimulate eosinophils (Fig. 5b). We have compared directly the respiratory burst induced by FSC and that induced by secretory IgA, which also binds to the cells through the FcαRI (CD89). The binding of aggregated IgA (serum or secretory) to the eosinophil FcαRI receptors and ability to trigger cellular effector functions, has been studied extensively (Abu-Ghazaleh et al., 1989; Monteiro et al., 1993; Pleass et al., 2007). Our results confirm and extend observations by Motegei and Kita (1998) who showed that the very small eosinophil respiratory burst induced by FSC adds comparatively little to the much larger burst induced by IgA binding to the FcαRI. However, FSC did synergize with other eosinophil stimulants. Our own results suggest that FSC coated on microtitre plates is able to trigger eosinophils in the absence of other stimuli. We have recently determined solution structures for human recombinant FSC and likewise for dlgA1, SlgA1 and SlgA2 using constrained X-ray and neutron solution scattering modelling and analytical ultracentrifugation (Bonner et al., 2007, 2008, 2009a, 2009b). By this, recombinant FSC which was functionally active, formed a compact J-shaped structure, with domains D4 and D5 bent-back upon D2 and D3 (Perrier et al., 2006; Bonner et al., 2007). This resembles a compact FSC structure deduced many years ago by ultracentrifugation (Beale, 1985). The most recent structures for SlgA1 and SlgA2 show that, starting from the same randomised family of 5000 models, the five SC domains open into an extended conformation that binds along the edge of the Fc–Fc dimer in both SlgA isoforms (Bonner et al., 2009a, 2009b). In the present study, the constrained modelling of the colostrum FSC scattering data showed that FSC adopted a similar compact J-shape structure to that of recombinant FSC. This validates the outcome of the work with recombinant FSC. The final step of colostrum FSC purification utilised the binding capability of FSC to serum plgA that was attached to the affinity chromatography column (Section 2). Given our knowledge of the SlgA1 and SlgA2 structures, this indicates that SC binding to plgA will involve the SC domains unfolding themselves from its J-shaped arrangement in order to become extended along the plgA Fc–Fc region. After its removal from plgA, the FSC structure reverts to a compact J-shape, showing that the opening up and compaction of the SC structure is reversible. A compact SC structure that is covered by oligosaccharides may be less susceptible than an extended SC structure to proteolytic cleavage in the harsh external environment of the mucosa.

The modelling of recombinant FSC with a C-terminal Arg585 residue (Fig. 7f) suggested that the C-terminal linker peptide (residues 544–585) exhibited no particular folding (Bonner et al., 2007). The C-terminal residue of recombinant FSC was deduced from Hughes et al., (1997), who purified SC from a single donor. However earlier studies using pooled colostrums suggested a slightly smaller SC molecule with ragged C-termini around residue 552 (Effert et al., 1984; Fig. 7f). It should be stressed that human colostrum and milk are rich in both proteases and protease inhibitors (Lindberg, 1979; Lindberg et al., 1982). The ratio of inhibitor to protease defines whether active protease is present. The ratio changes markedly with the time after birth and appears to differ in different individuals. Since the FSC C-terminal linker peptide is highly susceptible to proteases, it might well be the case that there is no “correct” C-terminus for colostral and milk FSC. The stability of the D5 immunoglobulin-like domain will define the smallest possible FSC.

To our knowledge, there has been no study of the C-terminal sequence of SC bound to SlgA. Although it has been assumed that it is the same as that of FSC (based on various biochemical and immunological techniques, but not SDS-PAGE), our findings suggest that this need not be the case. The most detailed structural comparison of free and bound SC did suggest two additional tryptic peptides in bound SC (Cunningham-Rundles et al., 1974). Given the suggested lack of structure in the C-terminal linker region, it is very possible that additional proteolysis can occur after cleavage of the plgR. The fact that FSC from different mucosal fluids appear to have slightly different molecular weights might suggest that proteolysis does occur in vivo after release of FSC from plgR. This study suggests that the cleavage of the linker region neither significantly affects the structure nor the function of the molecule. This long linker may facilitate the plgR interaction with dimeric IgA in the lamina propria during transcytosis, and its release in the lumen. The generally larger size of SC in SlgA1 and SlgA2 compared to FSC may result from the presence of dimeric IgA in the former, which may shield the C-terminal linker of SC when the plgR is cleaved after transcytosis. This shielding by dimeric IgA would be absent when FSC is cleaved in similar circumstances, and
may explain our observations of slightly reduced FSC sizes in this study.

Acknowledgements

We thank the Biology and Biotechnology Sciences Research Council and the Wellcome Trust for financial support. We thank Mr J. Gor for excellent assistance with the analytical ultracentrifuge, Dr A. Robertson and Dr P. Whitty for assistance with X-ray data collection, and Dr S. Finet (ESRF, Grenoble) and Dr K. Heenan and Dr S.M. King (ISIS, Rutherford-Appleton Laboratory, Oxford) for instrumental support.

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