Localization and characterization of the ligand-binding domain of the fibrinogen-binding protein (FgBP) of *Streptococcus equi* subsp. *equi*

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The group C streptococcus *Streptococcus equi* subsp. *equi* possesses a 498-residue major cell-wall-associated protein (FgBP) which binds horse fibrinogen (Fg), reacts with convalescent horse serum and protects against lethal *S. equi* challenge in a small animal model. In the present study, analysis of a panel of 17 purified N- and C-terminal FgBP truncates by ligand affinity blotting and SDS-PAGE revealed that the region required for maximum binding of Fg extended over the first half of the mature protein. The C-terminal two-thirds of this domain is predicted to be α -helical coiled-coil and the N-terminal one-third to possess non-coiled-coil single strands. Residues at the extreme N-terminus and within the coiled-coil region are both required for ligand binding. A high incidence of α -helical coiled-coil structure also seems to be responsible in part for the aberrant mobility of FgBP on SDS gels. The efficiency with which FgBP binds Fg from different animal species decreases in the order horse > mouse, pig > rat > sheep, dog, bovine, human. Binding to horse Fg is inversely related to temperature over the range 45–4 °C and is independent of Ca²⁺ ions. MS analysis provided corroborative evidence that FgBP is covalently linked to the cell wall peptidoglycan.

Keywords: *Streptococcus equi* subsp. *equi*, fibrinogen-binding protein, ligand-binding domain

INTRODUCTION

Fibrinogen (Fg) is a major component of plasma, and the ability to bind Fg is common amongst cell wall proteins of pathogenic Gram-positive bacteria (Kehoe, 1994; Patti *et al.*, 1994). Fg consists of three pairs of non-identical chains (α , β , γ) linked by disulphide bonds and has an M_r of 340 000. For the host, Fg has two main functions: polymerization into fibrin and mediation of platelet aggregation. Together, these result in the formation of platelet–fibrin thrombi (Doolittle, 1994). In Gram-positive bacteria, Fg binds to the variable N-terminal/central regions of many cell-wall-associated proteins, an interaction which appears to serve several different functions. For example, although the precise

mechanism by which streptococci resist phagocytosis remains controversial, Fg binding to M protein does appear to play a role in resistance to phagocytosis for several strains of *Streptococcus pyogenes* (Courtney *et al.*, 1997; Kehoe, 1994; Navarre & Schneewind, 1999; Poirier *et al.*, 1989; Whitnack & Beachey, 1982, 1985). On the other hand, Fg binding to *Staphylococcus aureus* has been shown to be important in the initiation of infections of implanted medical devices and surgical wounds by promoting bacterial attachment (Herrmann *et al.*, 1993; Moreillon *et al.*, 1995; Vaudaux *et al.*, 1989, 1995).

Streptococcus equi subsp. *equi*, a group C streptococcus, is the causative agent of strangles, a highly contagious and debilitating upper respiratory tract disease of the family *Equidae*. The disease is initially characterized by nasal discharge and fever followed by swelling and abscess formation in local lymph nodes (Timoney, 1993). In a recent publication, we described the puri-

Abbreviations: CD, circular dichroism; Fg, fibrinogen; FgBP, fibrinogen-binding protein; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.

fication of a fibrinogen-binding protein (termed FgBP) from the cell wall of *S. equi* subsp. *equi*. FgBP was shown to have an apparent M_r 220 000 when analysed by SDS-PAGE, reacted with convalescent horse serum and was protective in a small animal model against lethal *S. equi* challenge (Meehan *et al.*, 1998a). The sequence of the corresponding gene (*fbp*) was determined and was shown to encode a protein of 534 amino acids (M_r 58 344) which possessed some structural and sequence similarities to other streptococcal cell wall proteins. Of note were characteristic signal sequence and cell wall/membrane-spanning domains, two blocks (A and B) of degenerate repeated sequences and a high probability of α -helical coiled-coil structure over about 60% of the molecule. The sequence was identical, with the exception of six amino acids, to the M-like protein (SeM) described by Timoney *et al.* (1997) for a different isolate of *S. equi* subsp. *equi*. There is evidence to suggest that, like streptococcal M proteins, FgBP (SeM) is anti-phagocytic by limiting C3b deposition on the bacterial cell surface, that antiserum to the protein is opsonic and that Fg binding may enhance the ability of *S. equi* subsp. *equi* to resist killing by equine neutrophils (Boschwitz & Timoney, 1994a, b; Timoney *et al.*, 1997).

Previously, we described the overexpression and purification of a recombinant FgBP truncate which lacked the signal sequence and C-terminal cell wall/membrane domain and showed that this recombinant protein behaved in an analogous fashion to wild-type FgBP in terms of Fg binding, seroreactivity and protective immunogenic potential (Meehan *et al.*, 1998a, b). In the present communication, we describe the construction of a panel of FgBP truncates, show that the Fg-binding domain is localized in the N-terminal region of mature FgBP, and that the efficiency of Fg binding is dependent on temperature and source of Fg. We also provide evidence from MS analysis of recombinant and wild-type FgBP that the native protein may be covalently linked at its C-terminus to the streptococcal cell wall.

METHODS

Bacterial strains, plasmids and growth conditions. *Escherichia coli* XL-1 Blue (Stratagene) and plasmid pQE30 (Qiagen) were used for expression of the recombinant FgBP proteins. The virulent isolate, strain TW, of *S. equi* subsp. *equi* was obtained from the late Dr Paul Storm of Intervet International BV, The Netherlands (Meehan *et al.*, 1998a). *E. coli* strains were grown at 37 °C in either LB medium or 2 × YT medium (Lech & Brent, 1997). Where appropriate, media were supplemented with ampicillin (100 µg ml⁻¹) and IPTG (1 mM). *S. equi* subsp. *equi* was grown at 37 °C on Columbia base agar supplemented with 5% (v/v) horse blood or statically overnight in Todd-Hewitt broth supplemented with 0.2% (w/v) yeast extract.

Recombinant DNA techniques. Genomic DNA was isolated from *S. equi* TW by a modification of the method of Yu & Ferretti (1989) as described previously (Meehan *et al.*, 1998a). Plasmid DNA was purified from *E. coli* by a modified alkaline lysis method (Feliciello & Chinali, 1993). DNA restriction digestions, ligations and transformations were carried out by standard methods (Sambrook *et al.*, 1989).

Biochemical procedures. SDS-PAGE was performed using either 12.5% (w/v) or 7.5% (w/v) polyacrylamide separating gels and a 4.5% (w/v) polyacrylamide stacking gel (Laemmli, 1970). Samples were routinely heated for 3 min at 100 °C in Laemmli sample buffer (Laemmli, 1970) prior to electrophoresis. Proteins were detected by staining with Coomassie brilliant blue. M_r values were determined from the relative mobilities of 15 standard marker proteins (Gibco-BRL; BenchMark protein ladder).

Protein concentration was estimated by a modification (Dulley & Grieve, 1975) of the Lowry method using BSA as standard. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS was performed as follows by Dr Len C. Packman, Dept Biochemistry, Cambridge, UK. FgBP samples (0.5 µl) dissolved in dilute (10–50 mM) sodium phosphate buffer (pH 7.2) or 0.1% (v/v) trifluoroacetic acid/50% (v/v) acetonitrile were mixed with 0.5 µl matrix solution [sinapinic acid (10 mg ml⁻¹) in 50% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid] and dried onto sample slides at 25–30 °C in a fan-ventilated drying box. Samples were washed twice with 4 µl water to remove salts, then dried, and a further 0.5 µl matrix was added. As necessary to improve the signal, samples were rewashed and further matrix solution was applied. An empirical dilution series of the protein sample (5–50-fold) was tested to ascertain the sample conditions necessary to give the optimal mass spectral signal. Mass analysis was carried out on a Kratos MALDI 4 time-of-flight mass spectrometer following the manufacturer's recommendations. Masses were determined using rabbit aldolase (M_r 39 211) and horse myoglobin (M_r 16 951) as internal calibrants. Errors were minimized by averaging at least five mass determinations.

Immunological and affinity procedures. Western immunoblotting was performed as described previously (Caffrey *et al.*, 1988) using 5% (w/v) dried skimmed milk as blocking reagent and rabbit anti-FgBP (1:10 000 dilution; Meehan *et al.*, 1998a). Affinity-purified horseradish-peroxidase-labelled goat anti-rabbit IgG H + L (ICN) was used as localizing antibody and 4-chloro-1-naphthol as developing reagent. The procedure for Fg-affinity blotting after electrotransfer from SDS-PAGE was as described by Meehan *et al.* (1998a). For dot Fg-affinity blots, solutions containing FgBP truncates were subjected to doubling dilutions and 150 µl aliquots (containing 100–0.05 pmol) were transferred onto nitrocellulose using the Bio-Rad Bio-Dot apparatus. After the blots had dried, they were blocked, incubated with labelled horse Fg and developed as described for Fg-affinity electroblots (Meehan *et al.*, 1998a).

ELISA tests were carried out using standard procedures (Newell *et al.*, 1988). For analysis of FgBP binding to Fgs from different animal species, wells of microtitre plates were coated overnight with 50 µl Fg solutions, ranging in concentration from 10 to 0.0195 µg per ml 0.1 M sodium carbonate buffer (pH 9.6). Wells were blocked with 4% (w/v) dried skimmed milk, incubated with FgBP (1.25 µg ml⁻¹), followed by rabbit anti-FgBP antibodies (1:5000 dilution) and peroxidase-labelled anti-rabbit IgG (1:5000 dilution; ICN). For analysis of Fg binding at different temperatures and to determine the concentration of ligand resulting in 50% saturation of receptor, wells were coated with 25 ng FgBP1, blocked with 1% (w/v) BSA and probed with doubling dilutions of horseradish-peroxidase-labelled horse/human Fg as appropriate. For these experiments, blocking was performed in two stages: (1) incubation at 22 °C for 30 min followed by (2) incubation for 30 min at one of several temperatures (4, 22, 30, 37 or 45 °C). Subsequent washing and incubation steps were carried out at the chosen temperature. All ELISA tests

Fig. 1. Schematic representation of *S. equi* FgBP and of recombinant FgBP truncates. The upper part of the figure represents the primary structure of FgBP. Open boxes represent the positions of the signal sequence (Ss), the A and B repeats and the wall- and membrane-spanning regions (W/Msr). The lower striped box represents the region of FgBP predicted to have 50–100% probability of possessing an α -helical coiled-coil structure. Below the primary structure of FgBP are scaled representations of N-terminally processed FgBP (N₃₇–N₅₃₄), *S. equi* FgBP after processing at both its N- and C-termini (N₃₇–T₅₀₃) and a panel of 17 recombinant FgBP truncates (FgBP1–17) overexpressed in *E. coli*.

were developed at room temperature using 3,3',5,5'-tetramethylbenzidine as substrate and the A_{450} was measured.

Purification of FgBP. FgBP was purified from *S. equi* as described previously (Meehan *et al.*, 1998a). Briefly, this involved lysis of *S. equi* cells by passage through a French pressure cell (221 MPa) and harvesting of cell envelopes by centrifugation (45 000 g, 1 h, 4 °C). Cell envelopes were then digested overnight with mutanolysin (800 U ml⁻¹) in the presence of a cocktail of protease inhibitors. Following centrifugation (45 000 g, 1 h, 4 °C), proteins, including FgBP, which were released from the cell wall were recovered in the supernatant fraction. FgBP was then purified from this preparation by horse Fg-affinity chromatography (Meehan *et al.*, 1998a).

Expression and purification of FgBP truncates. Oligonucleotide primers (Genosys) complementary to selected *fbp* sequence were used to amplify *fbp* fragments. Restriction enzyme cleavage sites (*Bam*HI or *Xma*I) were engineered into the 5' end of all primers. A stop codon was also engineered directly after the restriction enzyme cleavage site at the 5' end of reverse primers. One hundred microlitre PCR reactions were performed in cloned *pfu* polymerase buffer (Stratagene) containing 5 U cloned *pfu* polymerase, 250 ng forward and reverse primers, 250 μ M dNTPs and 100 ng pFP21 (recombinant pQE30 which expresses truncate FgBP1) or 500 ng *S. equi* genomic DNA. Amplification conditions consisted of 30

cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min/1 kb of product followed by a final extension at 72 °C for 10 min. DNA was then purified using Wizard PCR preps (Promega), cleaved with appropriate restriction enzymes and cloned into pQE30. Recombinant plasmids were transformed into *E. coli* XL-1 Blue.

Hexahistidyl-tagged FgBP truncates were purified by metal chelate affinity chromatography using a modification of the method described by Meehan *et al.* (1998a). *E. coli* containing the recombinant pQE30 plasmids was grown, harvested and lysed by passage through a French pressure cell (221 MPa) as previously described. The soluble cytoplasmic/periplasmic fraction was applied to a nickel-iminodiacetic acid column, which was then washed extensively with binding buffer containing 40 mM imidazole until the A_{280} of the effluent reached zero. Hexahistidyl-tagged FgBP truncates were recovered using an 80–400 mM gradient of imidazole in binding buffer. Most of the purified proteins were then extensively dialysed against several changes of 50 mM sodium phosphate (pH 7.2) containing decreasing concentrations (0.5, 0.2 and 0.1 M) of NaCl. Some of the smaller truncates tended to precipitate at lower NaCl concentrations. Accordingly, these were ultimately dialysed against either 0.2 M NaCl (FgBP6–8) or 0.5 M NaCl (FgBP9). Where necessary, purified proteins were concentrated by ultrafiltration using an Amicon PM10 filter.

RESULTS

Localization of the Fg-binding domain

In order to localize the Fg-binding domain of FgBP, DNA fragments of *fbp* were amplified by PCR and cloned in-frame into the expression vector pQE30. The resultant recombinant FgBP proteins (FgBP1–17; Fig. 1) were purified by metal chelate chromatography in the presence of protease inhibitors and analysed by SDS-PAGE (Fig. 2). The panel of recombinant molecules so

constructed covered truncates lacking the wall/membrane anchor domain (FgBP1), ones lacking the wall/membrane anchor domain and increasing amounts of the putative coiled-coil domain (which includes the A and B repeat regions; FgBP2–9), together with ones lacking the wall/membrane anchor domain and an increasing number of N-terminal residues (FgBP10–17). All purified truncates gave a single dominant band following SDS-PAGE, with the exception of FgBP3, which routinely purified as two components (FgBP3a

Fig. 2. SDS-PAGE of purified hexahistidyl-tagged FgBP truncates. Lanes 1–17, FgBP truncate numbers 1–17, respectively (see Fig. 1). Proteins have been stained with Coomassie brilliant blue, and the positions and M_r values of marker proteins are indicated to the left.

Table 1. M_r values and predicted coiled-coil structure of FgBP truncates

FgBP truncate	Loss of coiled-coil structure (%)	M_r predicted from sequence	M_r estimated by SDS-PAGE
Native FgBP	0	52486	220000
FgBP1	4	49541	200000
FgBP2	18	43861	190000
FgBP3a	39	36317	35500
FgBP3b	48	33111*	29500
FgBP4	60	28434	27500
FgBP5	76	22841	23000
FgBP6	83	20377	16500
FgBP7	85	18868	17000
FgBP8	94	15758	13500
FgBP9	100	13420	12000
FgBP10	18	42915	185000
FgBP11	18	42549	185000
FgBP12	18	41679	185000
FgBP13	18	40121	170000
FgBP14	18	36201	155000
FgBP15	18	32538	110000
FgBP16	30	27224	75000
FgBP17	40	23110	60000

* Estimated by MALDI-TOF MS.

Fig. 3. Fg-affinity blot analysis of FgBP truncates. (a) Reactions obtained following SDS-PAGE and Western affinity blotting of samples depicted in Fig. 2. Only the regions of the blot containing the various FgBP truncates are shown. (b) Affinity dot blot performed with undenatured FgBP truncates. The top (initial) well in each column of doubling dilutions contains 33 pmol truncate. Sample identity is indicated at the top. FgBP7–9 and FgBP14–17 give identical profiles following dot blotting to that shown for FgBP13.

and FgBP3b; see Fig. 2 and Table 1). MS, nucleotide sequencing and affinity binding data confirmed that the lower- M_r species (FgBP3b) was a degradation product lacking a further 30 amino acids from its C-terminus.

All purified truncates (including both FgBP3a and 3b) reacted with anti-FgBP antibodies as assessed by both Western and dot immunoblotting (data not shown). However, only the native protein and FgBP truncates 1, 2, 3a, 3b, 4, 5 and 10 bound detectable levels of Fg in corresponding Western affinity blotting experiments (see Fig. 3a). To place these observations on a more quantitative basis and to obviate demonstrable problems associated with SDS-induced reduction in Fg binding during SDS-PAGE and with less efficient electrotransfer of more tightly focused high- M_r truncates, dot-blotting experiments were performed using undenatured FgBPs (Fig. 3b). These semi-quantitative experiments confirmed the general trend observed during Western affinity blotting and revealed that in the absence of SDS (a) wild-type FgBP, FgBP1 (which lacks the wall/membrane anchor domain) and FgBP2–3a/3b (missing 113 and 182/212 amino acids from their C-termini, respectively) all bound similar levels of Fg to a first approximation, (b) FgBP4–6 (missing 255, 309 and 330 C-terminal residues, respectively) bound approximately 16-, 32- and 128-fold less Fg than FgBP1, (c) N-terminal truncates FgBP10–12 (which have 8, 11 and 19 residues, respectively, deleted from their N-termini) bound about 4-, 16- and 128-fold less Fg than their FgBP2 control and (d) the remaining truncates, i.e. FgBP7–9 and FgBP13–17 (see Fig. 1), bound no detectable Fg (Fig. 3b).

Fig. 4. Efficiency of FgBP1 binding to (a) horse Fg at different temperatures and (b) Fgs from different mammals. For clarity, error bars have been omitted from (b).

Effect of Ca^{2+} , temperature and Fg type on Fg binding

Ca^{2+} has recently been shown to play a regulatory role in the interaction of Fg with several staphylococcal Fg-binding proteins, e.g. ClfA, ClfB and Efb (Ní Eidhin *et al.*, 1998; O'Connell *et al.*, 1998; Palma *et al.*, 1998). However, this does not appear to be the case for *S. equi* subsp. *equi* since preincubation of FgBP with a range of concentrations (in twofold dilutions) of Ca^{2+} (50–0.24 mM), EDTA (20–0.01 mM) or EGTA (20–0.01 mM) did not alter Fg binding to immobilized FgBP in ELISA tests (data not shown). Nor did the sequence of FgBP show the presence of a potential cation-binding EF-hand-like motif reportedly implicated in the binding of Fg to staphylococcal ClfA and ClfB proteins (Meehan *et al.*, 1998a; Ní Eidhin *et al.*, 1998; O'Connell *et al.*, 1998). In contrast, temperature did affect Fg binding to FgBP1 (and to other smaller Fg-binding FgBP truncates), the binding being significantly more efficient at 4 °C than at 37 °C (see Fig. 4a). Other assays performed with Fgs from a number of different mammals (see Fig. 4b) showed that FgBP bound the various molecules with the following approximate efficiencies with regard to horse Fg: viz. mouse and pig Fg, 50 %; rat Fg, 25 %; sheep, dog, bovine and human Fg, 3–8 %. Additional ELISA experiments (not shown) revealed that (a) the concentrations of horse and human Fg resulting in 50 %

saturation of receptor at 4 °C were 40 ng ml⁻¹ and 320 ng ml⁻¹, respectively, and (b) at 45 °C, an approximately ninefold higher concentration of horse Fg was required to cause 50% saturation of receptor than at 4 °C.

Aberrant mobility during SDS-PAGE

Previous studies had shown that the M_r values of native FgBP and recombinant FgBP1 as estimated by SDS-PAGE (220 000 and 200 000, respectively) were greatly at variance with those (M_r 54 597 and 49 541, respectively) predicted from consideration of gene sequence (Meehan *et al.*, 1998a). SDS-PAGE analysis of the current panel of FgBP truncates provides evidence that a high incidence of α -helical coiled-coil structure may be responsible in part for this phenomenon (Table 1). Thus removal of residues from the N-terminus of the protein where there is a low probability of α -helical coiled-coil structure and/or removal from the C-terminus of residues accounting for about 18% of the total coiled-coil structure generated truncates (FgBP1, 2, 10–15) in which the dominant protein species showed an apparent:predicted M_r ratio (4–4.3) almost identical to that observed for wild-type FgBP. Removal of a further 12–22% of predicted coiled-coil structure from the N-terminus caused resultant truncates (FgBP16–17) to migrate with an apparent M_r about 2.6–2.8 times that predicted, whereas removal of 39% or more of predicted coiled-coil structure from the C-terminus caused truncates (FgBP3–9) to migrate on SDS gels with an estimated M_r close to that predicted from a consideration of sequence (see Table 1). Thus it would appear that (a) various FgBP truncates have the ability to migrate as apparent tetramers, trimers/dimers or monomers during SDS-PAGE and (b) this phenomenon can be correlated, at least in part, with the extent of predicted coiled-coil structure in the molecule. Whether these observations have any bearing on the macromolecular structure of FgBP *in situ* remains to be determined (see Meehan *et al.*, 1998a, for further discussion of this issue).

Wall association of FgBP

Several lines of evidence strongly support the view that FgBP is covalently linked to peptidoglycan *in situ*. Firstly, it can only be released from the purified cell walls following digestion of the latter with the muralytic enzyme mutanolysin. Secondly, it possesses an LPSTG cell-wall sorting sequence (Meehan *et al.*, 1998a). In staphylococci, there is formal evidence that such sequences are proteolytically cleaved and that a new amide bond is formed between Thr of the sorting sequence and the uncross-linked peptide crossbridge of peptidoglycan (Navarre & Schneewind, 1999; Ton-That *et al.*, 1997). Finally and importantly, MALDI-TOF MS analysis of wild-type FgBP (purified following mutanolysin digestion) supports this general scenario. Thus the M_r of FgBP1 estimated by mass spectrometry (M_r 49 540 $\pm$ 22) was in excellent agreement with that

predicted from consideration of sequence (49 541). However, the estimated M_r of wild-type FgBP purified following mutanolysin digestion of the cell wall was determined to be 52 440 $\pm$ 90. This is at considerable variance with that predicted for the primary translation product after N-terminal processing of the signal sequence at N₃₇ (M_r 54 597; Meehan *et al.*, 1998a) or after additional C-terminal processing at T₅₀₃ in the cell wall sorting motif (M_r 51 467). On the other hand, it is consistent with a FgBP molecule (predicted M_r 52 486) which had been processed at both its N- and C-termini (i.e. N₃₇–T₅₀₃) and amide-linked at T₅₀₃ to the di-alanyl crossbridge of the putative murein subunit AAK(A)QAMurNAcGlcNAc (Schleifer & Kandler, 1972). However, additional experimentation is required to formally prove this point.

DISCUSSION

Analysis of overlapping truncates has succeeded in defining for the first time the region within *S. equi* FgBP required for Fg binding. Maximum ligand binding requires a considerable proportion (about 52–68%) of the native (wall-associated) protein, and involves a region extending from the vicinity of the N-terminus (residue 37) to beyond residue 279, possibly as far as about residue 352. Within this domain, individual residues at the N-terminus appear to be more crucial to Fg-binding activity than those positioned at the C-terminus. Thus removal of as few as 19 residues from the N-terminus results in dramatic (over 128-fold) loss of binding activity, whereas similar loss of Fg binding is only evidenced after the removal of about 150 residues from the C-terminal aspect of this (316-residue) domain. The presence in the various truncates of the N-terminal hexahistidyl affinity tag did not itself appear to compromise ligand binding as evidenced by the similarity in Fg-binding efficiencies of wild-type FgBP and recombinant derivatives such as FgBP1–3.

An N-terminal localization of Fg binding for FgBP is reminiscent of the reported binding of Fg to other streptococcal proteins such as the M proteins of serotypes 1, 3 and 5, and the FAI protein. However, sequence comparison of the relevant domains of these and other similar proteins has failed to reveal any regions of significant homology or recognized motif structures. It seems that Fg is capable of binding to some of the most variable regions of these proteins (Åkesson *et al.*, 1994; Kehoe, 1994; Reichardt *et al.*, 1997; Talay *et al.*, 1996). In these respects, the mechanism of Fg binding to *S. equi* FgBP is clearly distinct from that displayed by the staphylococcal Fg-binding proteins ClfA, ClfB and Efb. These latter proteins have been shown to bind preferentially to one or more of the three Fg chains in a manner which probably involves Ca²⁺ ions and a cation-binding EF-hand-like motif (McDevitt *et al.*, 1997; Meehan *et al.*, 1998a; Ní Eidhin *et al.*, 1998; O'Connell *et al.*, 1998; Palma *et al.*, 1998).

Of probable relevance to the mechanism of Fg binding

are computer predictions of structure. These suggest that (a) residues 37–45 at the extreme N-terminus of wild-type FgBP (and FgBP1–9) exist in a random coil structure, whilst the balance of the molecule is largely (over 84 %) α -helical; and (b) 60 % of the mature protein (from residues 166 to 475 of the precursor protein) possesses an extremely high probability (95–100 %) of α -helical coiled-coil structure, with zero to very low probability of such structures in the first 110 residues (residues 37–147; Meehan *et al.*, 1998a). Ongoing structural studies of key FgBP truncates by circular dichroism (CD) have confirmed many of these predictions. Thus FgBP1 shows almost 100 % α -helical content by CD using the CONTIN secondary structure estimation procedure (Provencher & Glockner, 1981), and shows a molar ellipticity ($[\theta_{222}]/[\theta_{208.6}] = 1.06$) similar to that observed for other coiled coils (M. Meehan and others, unpublished data). Based on these observations and the ligand-binding profiles detailed above, it is tempting to envisage critical Fg-binding domain(s) located in the less structured non-coiled-coil N-terminal aspect of the FgBP dimer, with α -helical coiled-coil sequences to the flanking (C-terminal) side providing, in part, a stabilizing structure which anchors and places constraints on the relative positioning of the non-coiled-coil single strands. In this model, excessive C-terminal truncation might serve to alter the gross structure of the FgBP molecule, either prior to or following interaction with its macromolecular ligand, leading to loss of effective interaction with Fg. Alternatively, Fg might interact with both the N-terminal and coiled-coil aspects of FgBP. Indeed, the coiled-coil region apparently necessary for full Fg binding (approx. 27 heptads, or 52 helical turns) is substantially greater than the minimum length generally considered necessary for the formation and stabilization of coiled-coil structures (three to four heptads; Su *et al.*, 1994; Lau *et al.*, 1984). Perhaps, as has been suggested for other systems, the coiled-coil structure of FgBP interacts in zipper-like fashion with the coiled-coil structure of Fg (Åkesson *et al.*, 1994; Kehoe, 1994; Reichardt *et al.*, 1997). This might serve to facilitate/stabilize interactions at the distal N-terminal site (or vice versa) and enhance binding efficiency. Both A and B repeated sequences of *S. equi* FgBP are present in the region of predicted coiled-coil structure (see Fig. 1). Of these, the degenerate B repeats are unlikely to be involved in binding since they are absent in truncate FgBP3, which displays wild-type Fg-binding efficiency. In contrast, the A repeats are clearly positioned within the region of the molecule required for full Fg binding. However, the A repeats are neither essential nor sufficient for Fg binding since they are absent in FgBP5, which still retains some, albeit weak, binding activity and are present in truncates (FgBP13–16) which are devoid of detectable activity. Whatever the precise mechanisms involved, it would appear that sites at the immediate N-terminus of FgBP and an extended region within the coiled-coil section of the molecule are both required for optimum Fg-binding activity. Biophysical studies should allow more critical appraisal of the role of coiled-coil structure in binding.

The observation that FgBP binds ligand more efficiently at lower temperatures has also been noted for some class C M proteins of group A streptococci. This in turn has been correlated with the stability of the α -helical coiled-coil structure and a tendency for these proteins to unfold to random coil monomers at higher temperatures (Åkerström *et al.*, 1992; Nilson *et al.*, 1995; Cedervall *et al.*, 1997). A similar explanation seems likely for FgBP since ongoing CD analysis has shown FgBP1 to become progressively more unfolded in response to increased temperature over the range 8–80 °C and to be about 0, 16 and 30 % unfolded at 8, 37 and 45 °C, respectively (M. Meehan and others, unpublished data).

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