Multiple Interactions of FbsA, a Surface Protein from *Streptococcus agalactiae*, with Fibrinogen: Affinity, Stoichiometry, and Structural Characterization†

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ABSTRACT: *Streptococcus agalactiae* is an etiological agent of several infective diseases in humans. We previously demonstrated that FbsA, a fibrinogen-binding protein expressed by this bacterium, elicits a fibrinogen-dependent aggregation of platelets. In the present communication, we show that the binding of FbsA to fibrinogen is specific and saturable, and that the FbsA-binding site resides in the D region of fibrinogen. In accordance with the repetitive nature of the protein, we found that FbsA contains multiple binding sites for fibrinogen. By using several biophysical methods, we provide evidence that the addition of FbsA induces extensive fibrinogen aggregation and has noticeable effects on thrombin-catalyzed fibrin clot formation. Fibrinogen aggregation was also found to depend on FbsA concentration and on the number of FbsA repeat units. Scanning electron microscopy evidentiated that, while fibrin clot is made of a fine fibrillar network, FbsA-induced Fbg aggregates consist of thicker fibers organized in a cage-like structure. The structural difference of the two structures was further indicated by the diverse immunological reactivity and capability to bind tissue-type plasminogen activator or plasminogen. The mechanisms of FbsA-induced fibrinogen aggregation and fibrin polymerization followed distinct pathways since Fbg assembly was not inhibited by GPRP, a specific inhibitor of fibrin polymerization. This finding was supported by the different sensitivity of the aggregates to the disruptive effects of urea and guanidine hydrochloride. We suggest that FbsA and fibrinogen play complementary roles in contributing to thrombogenesis associated with *S. agalactiae* infection.

Fibrinogen (Fbg[1]), a 340 dimeric glycoprotein, is composed of two identical halves, each consisting of 3 different polypeptide chains designated Aα, Bβ, and γ. It assembles into a three-dimensional fibrin network by the action of thrombin through the removal of fibrinopeptides A and B, which are located at the central domain. The transformation of Fbg in fibrin clot involves the activation of Fbg to originate the fibrin monomer followed by the half-staggered polymerization of fibrin monomers into protofibrils and a side-by-side association of protofibrils to form fibers and a fibrin gel (*1*). Fibrin serves as a template for the activation and localization of the fibrinolytic system that induces the dissolution of the clot (*2*). Fbg and fibrin also bind to a multitude of cell types such leukocytes (*3*), platelets (*4*), and endothelial cells (*5*) to mediate fundamental biological processes including hemeostasis, angiogenesis, inflammation, and wound healing.

The bacterium *Streptococcus agalactiae* (group B streptococcus, GBS) is the major cause of pneumonia, sepsis, and meningitis in neonates. In the adults the spectrum of diseases caused by GBS ranges from infections of urinary tract and soft tissues to arthritis and endocarditis (*6*−*8*). Interactions between bacterial factors and components of the host plasma and extracellular matrix proteins such as Fbg (*9*, *10*), fibronectin (*11*, *12*), and laminin (*13*) may be a prerequisite for *S. agalactiae* infection.

Recently, the isolation of *fbsA* gene, which encodes a Fbg receptor from *S. agalactiae*, has been reported (*14*). The Fbg receptor displays a sequence organization that is similar to that found in many surface proteins of Gram-positive bacteria. An N-terminal signal sequence for secretion is followed by a domain composed of a series of tandem repeats of 16 amino acids each, a wall-anchoring region with a LPKTG motif, a short hydrophobic segment spanning the cellular membrane, and a short C-terminal charged tail. FbsA protein protects streptococcal cells from opsonophagocytosis and promotes adherence of *S. agalactiae* to human epithelial (*15*) and brain microvascular endothelial (*16*) cells. In

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[1] Abbreviations: ClfA and ClfB, clumping factor A and B; DLS, dynamic light scattering; FbsA, fibrinogen binding protein A from *S. agalactiae*; Fbg, fibrinogen; Fn, fibronectin; Fib-1, N-terminal domain of Fn; Fib-2, C-terminal domain of Fn; FnbpA, Fn-binding protein A; mAb, monoclonal antibody; SdrG, serine-aspartate repeat protein G; tPA, tissue-type plasminogen activator.

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addition, clinical strains of *S. agalactiae* bearing FbsA induce a Fbg-dependent platelet aggregation suggesting a central role of FbsA in the pathogenesis of infective endocarditis and thrombus formation (*17*). In a murine model of sepsis and septic arthritis, inoculation with wild type *S. agalactiae* resulted in significantly higher mortality, more pronounced weight decrease, and a more severe arthritis, when compared to inoculation with the *fbsA* mutant isogenic strain (*18*). Thus, FbsA has an obvious role as a virulence factor. Numerous Fbg-binding proteins have so far been identified from different bacterial species, in particular staphylococci (*19*−*23*) and streptococci (*24*, *25*). Among staphylococcal Fbg-binding proteins, ClfA and FnbpA have a potent platelet proaggregating activity (*26*, *27*) and a critical role in experimental endocarditis (*28*). Likewise, M proteins from *Streptococcus pyogenes* bind Fbg and support platelet adhesion and thrombus formation (*29*).

The former studies leave a number of crucial questions still open, in particular for what concerns (a) the specific mechanism of binding of FbsA to Fbg; (b) the size and morphology of the FbsA/Fbg aggregates; (c) the nature and strength of the intermolecular forces stabilizing the aggregates; and (d) the possible effects of FbsA on the formation of fibrin clots induced by the action of thrombin.

The present report is meant to give meaningful, albeit nonexhaustive, answers to the aforementioned questions.

First of all, size-exclusion chromatography is targeted to evaluate the complex stoichiometry, allowing one to pinpoint the FbsA-binding sites in Fbg and fix the FbsA valency for Fbg.

Then, we show that *S. agalactiae* cells shed FbsA in culture medium, and that the released protein triggers the formation of Fbg aggregates. Dynamic light scattering (DLS) proves that FbsA/Fbg association does not simply result in the formation of small oligomeric complexes. Rather, it gives rise to dramatic aggregation effects yielding polymer-like aggregates extending in the micrometer size range. FbsA/Fbg semiflexible polymers self-organize into mesoscopic mesh structures that by scanning electron microscopy (SEM) appear different from fibrin networks and that do not display the finite elastic modulus of a gel.

By comparing the biochemical and immunological properties of the Fbg polymers with those of thrombin-induced fibrin clots, and in particular by examining the effects of different disrupting agents, we investigate the nature of the forces stabilizing FbsA/Fbg complexes, and discuss the potential thrombogenic effects associated with the formation of these aggregates. Finally, we unravel a strong and clinically meaningful synergism between FbsA and thrombin: indeed, the addition of FbsA strongly affects the growth kinetics of fibrin clots, both by speeding up the process and by yielding a more extensively polymerized structure.

MATERIALS AND METHODS

Proteins and Other Reagents. Human Fbg (Calbiochem, San Diego CA) was made free of contaminating fibronectin (Fn) by purification over a gelatin-Sepharose column. For the ELISA binding experiments, prior to coating, Fbg was further purified by size-exclusion chromatography on a Superose 6 HR 10/30 fitted onto an FLPC system. Human factor XIII was from Haematologic Technologies, Inc. (Essex Junction, VT).

The monoclonal antibodies (mAbs) against fibrin 1H1, 5F3, and A11 were generously donated by Dr. P. J. Gaffney (King's College London, London, U.K.). The mAbs 1H1 and 5F3 are specific for E domain of fibrin (*30*, *31*); the antibody A11 recognizes the hexapeptide located at the C-terminus of the α chain of the fibrin (*31*). The mAb 2B1 against the synthetic FbsA repeated unit was produced as previously described (*17*). The polyclonal rabbit anti-mouse and the goat anti-rabbit IgG were from Dako (Glostrup, Denmark). Human plasma Fn was isolated from a gelatin-Sepharose affinity column. The N-terminal and C-terminal fibrin-binding fragments were generated by thermolysin digestion of Fn and purified as reported by Zardi et al. (*32*). Plasminogen, plasmin, bovine thrombin, trypsin, and the synthetic peptide GPRP (gly-pro-arg-pro) were purchased from Sigma (St. Louis, MO). Tissue-type plaminogen activator (tPA) was generously provided by Dr. Daniela Albiero (Boehringer Igelheim Italia, Italy).

Recombinant *Escherichia coli* clones expressing hexahistidine-tagged FbsA fusion proteins, carrying 3, 6, 9, 15, and 19 internal repeats, were generously donated by Dr. D. J. Reinscheid (University of Ulm, Ulm, Germany). The isoforms of FbsA were produced and purified as described by Schubert et al. (*14*).

Recombinant region A of ClfA, ClfB, and FnbpA were a gift of Dr. T. J. Foster (Trinity College, Dublin, Ireland). Region A of SdrG was kindly donated by Dr. M. Höök (IBT, The Texas A&M University System Health Science Center, Houston, TX).

Guanidine hydrochloride (GdmCl) was from Schwarz-Mann Biotech (Cleveland, OH). Urea was purchased from BDH (Poole, Dorset, U.K.).

Preparation of Fibrinogen Fragments. Human Fbg was dissolved in 0.05 M imidazole, containing 0.15 M NaCl and 5 mM $CaCl_2$, pH 7.2 to a concentration of 5 mg/mL and pretreated for 10 min with 5 mM iodoacetamide to inhibit Factor XIII cross-linking. The digestion of Fbg was started by addition of trypsin (enzyme/substrate ratio, 1:100) and allowed to progress for 4 h at 22 °C. The reaction was terminated by addition of 0.03 mg/mL soy bean trypsin inhibitor. Subsequent fractionation of the material was carried out by ion exchange chromatography on a prepacked monoQ HR 5/5 column (Amersham Pharmacia Biothech) fitted onto a FPLC system and equilibrated with 20 mM Tris HCl, pH 8.2. Aliquots of the digest were applied to the column, and the sample was eluted with an increasing salt concentration gradient (0−1 M NaCl). Relevant fractions were then pooled and dialyzed against PBS and stored at −20 °C until used.

Labeling of Proteins with Biotin. Protein (0.5 mg) dissolved in 0.5 mL of PBS was mixed with 0.5 mL of 0.2 M sodium borate buffer, pH 8.0 and 7.5 mg of *N*-hydroxysuccinimidobiotin (NHS-biotin) (Sigma) dissolved in 0.1 mL of dimethyl sulfoxide, and incubated at 22 °C overnight on an end-over-end rotating mixer. The labeling mixture was dialyzed against PBS and stored at −20 °C in small aliquots.

Bacterial Strains and Culture Conditions. The *S. agalactiae* strain 6313 (serotype III) has been described previously (*33*). The FbsA gene was deleted in the chromosome of *S. agalactiae* 6313 as previously described (*14*).

Bacteria were cultivated at 37 °C in brain heart infusion (BHI) (Becton Dickinson, Sparks, MD) containing 1% yeast extract.

ELISAs. Assays were performed as detailed elsewhere (*34*).

Dynamic Light Scattering (DLS). Overnight cultures of *S. agalactiae* 6313 and *S agalactiae* Δ*fbsA* were harvested at 6950*g* for 20 min at 4 °C and the corresponding supernatants filtered through a 0.2 μm syringe filter. Five hundred microliters of each supernatant was mixed with an equal volume of purified 0.6 μM Fbg, and the mixtures were immediately transferred through a 0.2 μm filter into a rectangular microvolume (25 μL) light scattering cell. A mixture made of equal volumes of BHI and 0.6 μM Fbg was also tested by DLS and used as additional control. An overall description of our custom-made DLS setup has been given elsewhere (*35*). Measurements were performed at a scattering angle $\theta = 90°$ using a 70 mW frequency-doubled Nd^+YAG laser operating at a wavelength $\lambda = 532$ nm. The growth of fibrinogen aggregates with time was followed by measuring the time-correlation function $g(t)$ of the scattered intensity. For solutions of particles with the same size, $g(t)$ displays a simple exponential decay with a time constant $\tau = 1/Dq^2$, where D is the particle diffusion coefficient, $q = (4\pi n/\lambda)\sin(\theta/2)$ is the scattering wave-vector, and n is the refractive index of the solution. A first estimate of the size of the scattering objects is given by their hydrodynamic radius $R = k_BT/6\pi\eta D$, where η is the solvent viscosity, to be interpreted as the radius of an effective sphere with the same diffusion coefficient.

Aggregation Kinetics: Turbidity Studies. Fibrin polymerization was initiated by the addition of thrombin (0.05 NIH U/mL) at time zero to 1 μM human Fbg in 20 mM Tris HCl, pH 7.5, containing 150 mM NaCl and 5 mM $CaCl_2$, and polymer formation was measured as a change in turbidity related to the total amount of the scattered light at 350 nm with time in a spectrophotometer (Jasco, UVIDEC-320). The effect of FbsA isoforms or other bacterial Fbg-binding proteins on the assembly of clot was determined by addition of 0.5 μM protein to Fbg solution at the same time with thrombin.

The Fbg aggregation induced by FbsA isoforms was determined by incubating 1 μM Fbg with the indicated concentrations of each protein.

Scanning Electron Microscopy Studies. Fibrin clot was polymerized incubating 0.05 U/mL thrombin and 1 μM purified Fbg, as reported above. FbsA-induced Fbg aggregation experiments were performed by incubation of 0.1 μM FbsA with 1 μM Fbg. In both conditions, Fbg was allowed to polymerize onto glass cover slips for 1 h at 25 °C in 0.15 M NaCl, 20 mM Tris-HCl, 5 mM $CaCl_2$, pH 7.4 in a moist environment. The samples were centrifuged for 5 min at 2000 rpm and, after gentle washings with distillated water, were fixed with a 2.5% (v/v) glutaraldehyde solution in 0.1 M cacodilate buffer pH 7.2 for 1 h at 4 °C, washed with cacodilate buffer, and finally dehydrated through a series of ethanol dehydrations up to 100%. Samples were kept in 100% ethanol for 15 min and then critical point-dried using CO_2. The specimens, mounted on aluminum stubs, were sputter-coated with 10% gold and examined with a Stereo Scan 360 Cambridge microscope at 10 keV acceleration voltage. The samples were prepared in duplicate and were observed at 5000× and 15000× magnification.

Electrophoresis and Blotting. SDS−PAGE was performed using the indicated concentrations of acrylamide, and all the gels were stained with Coomassie Brilliant Blue (BioRad, CA). Plasmin digestion products were fractionated on SDS−PAGE and then electroblotted onto a nitrocellulose membrane (Hybond-ECL) (Amersham Pharmacia Biotech, Uppsala, Sweden). The membrane was treated with a solution containing 10% dried skim milk in 25 mM Tris HCl, pH 7.4, washed, and then incubated with 0.5 μg/mL of a mouse anti-Fbg antibody for 1 h at 22 °C. The membrane was washed 3 × 10 min with PBS and incubated for 1 h with 10% milk containing rabbit anti-mouse IgG horseradish peroxidase conjugate. After several washings in PBST (PBS containing 0,5% Tween 20), the membrane was treated with ECL detection reagents 1 and 2 (Amersham Pharmacia Biotech) according to the procedure recommended by the manufacturer and exposed to an X-ray film for 10−20 s.

Western blotting of Fbg and Fbg fragments was carried out as reported above and binding of FbsA (1 μg/mL) to the membrane detected by incubation with 0.5 μg/mL of the mAb 2B1 followed by addition of 10% milk containing a secondary anti-mouse IgG horseradish peroxidase conjugate.

Table 1: Summary of the Dissociation Constants (K_d) for the Indicated Recombinant FbsA Isoforms[a]

recombinant FbsA isoform	K_d ($\times 10^{-8}$ M)
FbsA-19	2.5 ± 0.14
FbsA-15	3.0 ± 0.54
FbsA-9	5.1 ± 0.13
FbsA-6	5.8 ± 0.20
FbsA=3	13.2 ± 6.30

[a] To calculate the relative affinity constant (K_a) values of each FbsA protein for fibrinogen the following equation was used: $A = A_{max}[\text{FbsA}]K_a/(1 + K_a[\text{FbsA}])$. The reported K_d values were calculated as reciprocal of the association constant. The values are the means ± SDs of at least three independent experiments. The K_d values were determined by ELISA assays.

RESULTS

Saturability and Specificity of FbsA Binding to Fibrinogen. *S. agalactiae* strains express FbsA isoforms including a variable number of tandem repeat units. The repetitive region of FbsA was identified as mediating Fbg binding (*14*). To investigate the binding of FbsA to Fbg as a function of repeat number, FbsA proteins harboring 3 (FbsA3), 6 (FbsA6), 9 (FbsA9), 15 (FbA15), and 19 (FbsA19) repeat units were expressed in *E. coli*. Fbg was coated onto microtiter wells, incubated with increasing concentrations of each recombinant isoform of FbsA, and protein bound to the wells was detected with a mAb recognizing the repeated motif of FbsA. All the FbsA proteins bound to Fbg in a dose-dependent fashion and saturably (data not shown). From the saturation assays we estimated the dissociation constants and found that all isoforms presented similar K_d values for Fbg; however, the K_d value of FbsA3 was four times higher than that of FbsA19 or FbsA15 (Table 1). To assess the inhibitory effect of FbsA repeat numbers on the attachment of bacteria to surface-coated Fbg, we set up a competitive assay where *S. agalactiae* cells were allowed to adhere to immobilized Fbg in the presence of increasing concentrations of FbsA isoforms (Figure 1). In these conditions, soluble FbsA19 and FbsA15 efficiently inhibited adherence of *S. agalactiae* 6313 to Fbg, whereas FbsA9, FbsA6, and FbsA3 were progressively less active. The effect of FbsA on *S. agalactiae* adherence to Fbg was specific because other Fbg-binding proteins such

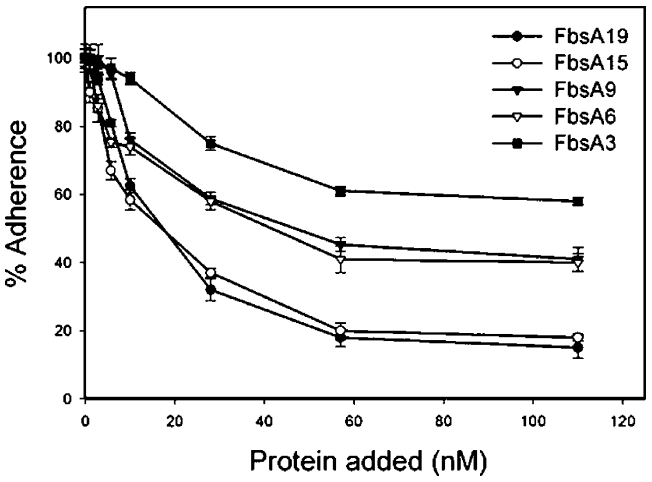

FIGURE 1: Inhibitory effect of FbsA isoforms on *S. agalactiae* adherence to Fbg. Fbg-coated microtiter wells were incubated for 90 min at 22 °C with *S. agalactiae* cells (1×10^8/well) in the presence of increasing concentrations of the indicated competitors. After washing, 500 ng of rabbit anti-GBS IgG was added to each well, followed by an incubation for 90 min. Binding of antibody to the surface-attached bacteria was quantified by addition of peroxidase-conjugated goat anti-rabbit IgG, and the plates were developed with *o*-phenylenediamine. The bars show SDs of triplicate samples.

as FnbpA and ClfA from *Staphylococcus aureus* and SdrG from *Staphylococcus epidermidis* did not affect streptococcal adherence (data not shown). Likewise, no effect was detected when the direct binding of FbsA19 to Fbg was performed in the presence of excess amounts of the same competitors (data not shown). This information indicates that FbsA−Fbg interaction is specific and that the FbsA-binding site in Fbg is distinct from that of ClfA, FnbpA, or SdrG.

Localization of FbsA-Binding Domain in Fbg and Valency of FbsA. To localize the FbsA-binding site in Fbg, the protein was trypsin-digested and the corresponding D and E fragments were isolated by ion exchange chromatography. Intact Fbg and D and E fragments were tested for the binding to surface coated *S. agalactiae* 6313 cells and the binding of the proteins to bacteria detected by addition to the wells of mouse anti-Fbg polyclonal antibodies conjugated with peroxidase. As shown in Figure 2, panel A, Fbg and D fragment but not E fragment bound to immobilized *S. agalactiae* cells. Consistent with this, FbsA19 specifically recognized D fragment in both Western blot and ELISA (Figure 2, panel B). In a solid-phase binding assay FbsA19 showed for D fragment a K_d value of the same order as that exhibited for intact Fbg (data not shown). When intact Fbg was subjected to electrophoresis under reducing conditions and then probed in Western blot with FbsA19, no signal was detected, suggesting that the FbsA-binding site in Fbg is conformational.

Using analytical size-exclusion chromatography, we evaluated the stoichiometry of D fragment binding to FbsA6 molecule. The stoichiometry of the complex was estimated by application of samples made of a constant nanomolar amount of FbsA6 mixed with increasing concentrations of D fragment to a column fitted onto a FPLC system and then monitoring the decrease of the peak area corresponding to the FbsA6 and the increase of the heterocomplex. At saturating concentrations of D fragment (ligand/receptor molar ratio of 6:1, 7:1, and 8:1) stable complexes of 580/600 kDa were recovered from the column. If we assume a molecular mass of 30 and 92 kDa for the FbsA6 and D fragment, respectively, FbsA6 can accommodate six molecules of D fragment (Figure 3). Hence, FbsA is a multivalent protein.

FbsA Released from S. agalactiae Cell Cultures Induces the Formation of Fibrinogen Aggregates. In contrast to other bacterial surface proteins that are expressed predominantly in exponential or stationary phases, FbsA is produced on *S. agalactiae* cells at all stages of the growth cycle (data not shown). When *S. agalactiae* 6313 cells were grown in human serum or BHI in the stationary phase, FbsA was shed from the bacterial surface into the culture medium, possibly by the action of peptidoglycan-degrading enzymes during cell division or cleavage by proteases. In Figure 4, inset A, the shedding of FbsA in human serum and BHI is shown: in these conditions, the same amount of the FbsA was released in the culture independently of the growth culture medium.

Recently, we have shown that the addition of FbsA, even in a very low fractional amount, to human Fbg solutions elicits the formation of large polymer-like Fbg aggregates, and that a similar aggregation process also takes place directly on *S. agalactiae* cells exposed to Fbg (*36*). Here, we provide evidence that Fbg aggregation can be also triggered by the sole addition of the culture medium where *S. agalactiae* strain 6313 colonies had been grown. The main body of Figure 4 shows that the DLS correlation functions decay progressively slower as a function of time after the addition of the *S. agalactiae* 6313 growth medium. As already observed (*36*), the shape of $g(t)$ is not, however, a simple exponential: rather, it is nicely fitted using the distinctive time-dependence (a "stretched-exponential" decay) that has been theoretically predicted for semiflexible polymer networks (*37*) and successfully applied to account for DLS data on many biopolymer networks including fibrin itself (*38*). Conversely (open dots curve, Figure 4), the correlation function for a Fbg solution to which a Δ*fbsA S. agalactiae* culture medium was added fully coincides with $g(t)$ for pure Fbg and does not show any appreciable change with time. Inset B shows that the average aggregate size, which can be evaluated from the average decay time $\langle\tau\rangle$ of $g(t)$, grows about an order of magnitude in less than 2 h, reaching a value of the order of 0.2 μm.

Kinetics of FbsA-Induced Fibrinogen Aggregation. Although DLS measurements allow extracting meaningful values for the aggregate size and structure, they are not sufficiently rapid to follow fast aggregation kinetics, in particular at the earliest stages. This can, however, be accomplished by evaluating the total scattered intensity via turbidity measurements in a conventional spectrophotometer, in particular at short wavelength (350 nm). In Figure 5, panel A, the growth-kinetics for a simple, thrombin-catalyzed fibrin clot is shown. After an initial lag phase, when monomeric fibrin is supposed to associate into long two-stranded protofibrils that have little effect on turbidity, a rapid turbidity increase indicates fiber growth occurring by lateral association and branching of protofibrils; when fibrin assembly is nearly completed, the turbidity reaches a plateau value.

In panel A we also show the effect of several bacterial Fbg-binding proteins on thrombin-catalyzed fibrin polymerization. Adhesins such as ClfA, ClfB, and FnbpA from *S.*

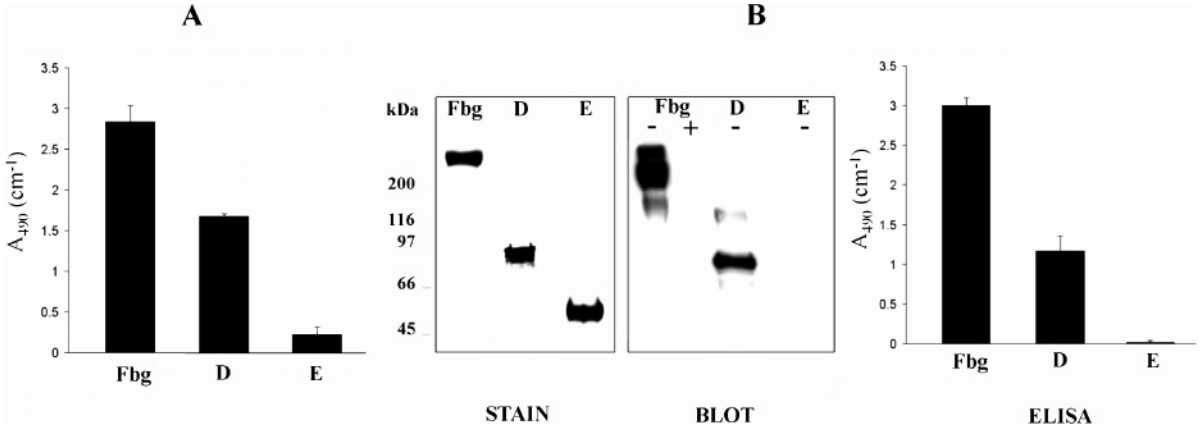

FIGURE 2: (A) Binding of Fbg and Fbg fragments to immobilized *S. agalactiae* cells. Microtiter plates were coated with *S. agalactiae* 6313 cells (5×10^7/well) overnight at 37 °C. The wells were blocked with 2% bovine serum albumin and then incubated with Fbg or D and E fragments (1 μg/well) for 90 min. After extensive washes, 500 ng of mouse anti-Fbg antibodies was added to each well and the binding of antibodies to the wells was detected by addition of a peroxidase-conjugated rabbit anti-mouse IgG and subsequent addition of a chromogenic substrate. (B) Binding of FbsA19 to Fbg fragments. Western blot. Fbg and Fbg fragments were subjected to electrophoresis in SDS−7.5% polyacrylamide gel under nonreducing conditions (−) and then Coomassie stained. Immunostaining was performed by incubating the membrane filter with 1 μg/mL FbsA19 followed by detection with 0.5 μg/mL of the mAb 2B1. No immunostaining was detected when Fbg was subjected to electrophoresis under reducing conditions (+). Molecular mass markers are in kilodaltons. ELISA. FbsA19 was immobilized onto microtiter wells (1 μg/well) and incubated with Fbg and Fbg fragments (1 μg/well). Binding of the ligands to FbsA 19 was determined as reported in panel A.

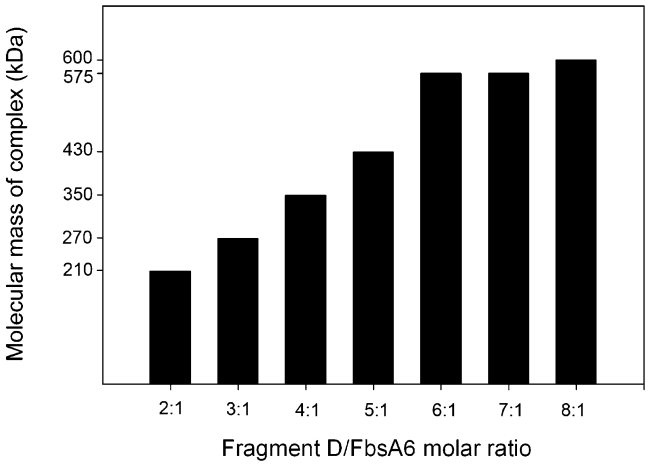

FIGURE 3: Analytical size-exclusion chromatography of FbsA6 with various concentrations of D fragment on Superose 12 HR 10/30. Five nanomoles of FbsA6 was mixed with 3.0, 4.5, 6.0, 7.5, 9.0, 10.5, and 12 nanomolar equiv of D fragment. The column was equilibrated and the samples were eluted with 50 mM NaH_2PO_4 containing 300 mM NaCl, pH 8.0. The flow rate of the column was 1 mL/min. Elution of the samples was monitored by absorbance at 280 nm. The void volume and the total volume of the column were 6.6 mL and 24 mL, respectively. At each molar ratio two runs were performed with similar results.

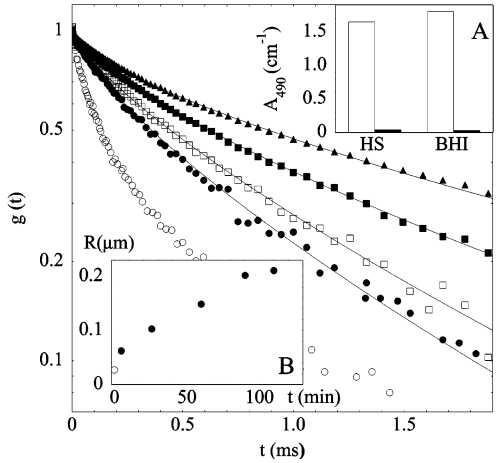

FIGURE 4: Detection of FbsA/Fbg copolymer shed in the growth medium by DLS. Inset A: Shedding of FbsA. The FbsA-positive *S. agalactiae* strain 6313 and its *fbsA*$^-$ mutant were grown in human serum (HS) or BHI overnight at 37 °C. One hundred microliters of the culture supernatant was added to microtiter wells previously coated with 1 μg of Fbg. The wells were incubated for 90 min and, after extensive washing, added with 1 μg of the anti-FbsA monoclonal antibody 2B1. The amount of antibody bound to the wells was detected by addition of peroxidase-conjugated rabbit anti-mouse IgG and subsequent addition of a chromogenic substrate. Main figure: DLS time-correlation functions $g(t)$ obtained 5 (full dots), 27 (open squares), 60 (full squares), and 110 (triangles) min after the addition of the *S. agalactiae* 6313 growth culture medium to a 0.6 μM Fbg solution, fitted to a stretched-exponential decay as explained in the text (full lines). The correlation function for a Fbg solution to which a Δ*fbsA S. agalactiae* culture medium was added, and which essentially coincides with $g(t)$ for pure Fbg, is also shown (open dots). Inset B: Growth of the average polymer hydrodynamic radius R as a function of time. The open dot corresponds to the value obtained with Fbg alone.

aureus and SdrG from *S. epidermidis* actually inhibited the process of fibrin polymerization. These results are consistent with the role of the flexible segment located at the extreme C-terminus of the γ chain of Fbg in both binding ClfA (*39*) or FnbpA (*21*) and fibrin polymerization. In addition, inhibition of fibrin monomer formation and the protofibril assembly by SdrG are probably due to the adhesin binding to the N-terminus of the β chain and the consequent occupation and steric hindrance of the thrombin-binding site (*23*). Conversely, FbsA containing 19 repeats (FbsA19) both accelerates and amplifies thrombin-driven clot formation. Indeed, by incubating Fbg with thrombin in the presence of FbsA19, both the lag time was severely shortened and the plateau turbidity value increased with respect to usual fibrin clot formation. Hence, it is plausible that FbsA does not bind to any of the fibrin(ogen) polymerization sites and that the multivalency of FbsA, due to a multiplicity of tandem Fbg-binding repeat units, allows the formation of complexes

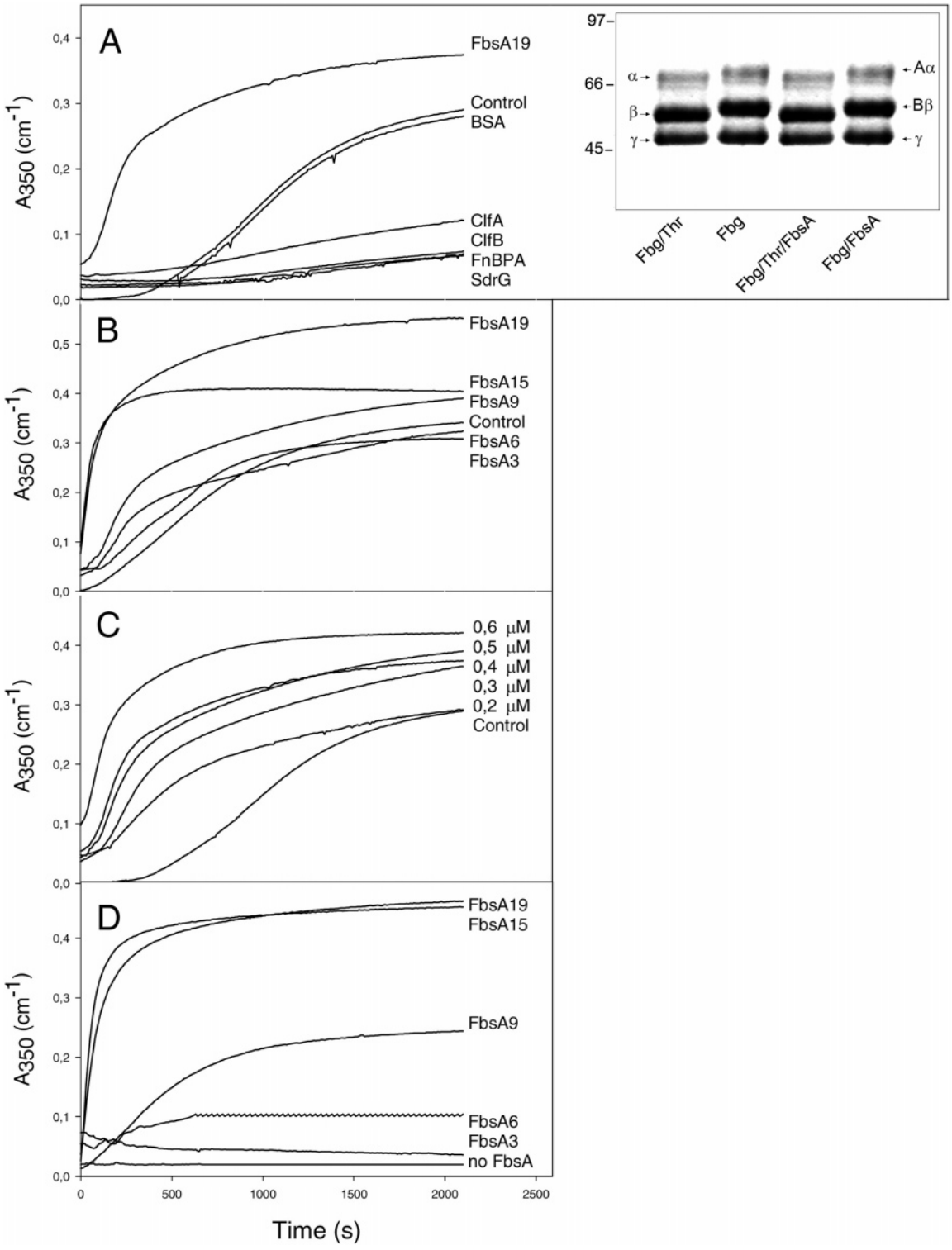

FIGURE 5: Thrombin-dependent clot and FbsA-induced Fbg aggregate formation by turbidimetric analysis. (A) Fibrin polymerization in the absence or presence of bacterial Fbg-binding proteins. Polymerization was initiated by the addition of thrombin (0.05 U/mL) to 1 μM Fbg, and the polymer formation at 25 °C was measured as a change in turbidity at 350 nm with time. The effect of bacterial Fbg-binding proteins on thrombin-induced fibrin polymerization was performed incubating the mixture containing thrombin and Fbg with 0.5 μM FbsA19 from *S. agalactiae*, ClfA, ClfB, FnbpA from *S. aureus* and SdrG from *S. epidermidis*. Polymerization of normal fibrin monomers in the presence of an equimolar concentration of bovine serum albumin at 25 °C, designated as control, was also performed. In the inset the degree of conversion of Fbg to fibrin by thrombin in the presence of FbsA is shown. Fbg (6 μM) dissolved in 20 mM Tris HCl containing 150 mM NaCl and 5 mM $CaCl_2$, pH 7.4, was incubated with 0.1 U/mL thrombin in the presence of 3 μM FbsA for 40 min. Fibrin clot was dissolved in 8 M urea and subjected to 10% SDS−PAGE under reducing conditions. The electrophoretic profiles of Fbg alone and of Fbg incubated with FbsA and with thrombin are reported as controls. On the left side the apparent molecular masses of standard proteins are indicated. (B) Influence of FbsA isoforms (FbsA3, FbsA6, FbsA9, FbsA15, and FbsA19) on the assembly of clot was determined by addition to Fbg solution of 0.5 μM FbsA proteins along with thrombin. (C) Fbg aggregation profiles produced in the presence of increasing concentrations of FbsA19. (D) Effect of FbsA isoforms on formation of Fbg aggregates. Aggregation of Fbg by FbsA isoforms was triggered incubating 1 μM Fbg with 0.5 μM FbsA proteins at 25 °C and measured as reported in panel A.

which prelude and actually promote, as witnessed by the reduced lag time, the fibrin assembly driven by thrombin. To elucidate the nature of the clot structure formed in the presence of FbsA, we evaluated the degree to which fibrinopeptides A and B were released by thrombin during fibrin formation. Consequently, Fbg was incubated with thrombin in the presence of FbsA and after dissolution in 8 M urea the clot was examined by SDS−PAGE under reducing conditions. The inset in panel A shows a complete transformation of Fbg to fibrin, as indicated by the full conversion of Aα and Bβ to α and β chains, respectively. This finding suggests that FbsA does not interfere with thrombin action on Fbg and that FbsA aggregating effects take place also on fibrin monomers.

To evaluate the influence of the repeat number on the assembly of clot, we set up an experiment where a mixture of Fbg was incubated with thrombin in the presence of 0.5 μM FbsA3, FbsA6, FbsA9, FbsA15, and FbsA19. As a matter of fact, Fbg aggregates became measurable entities incubating Fbg with FbsA isoforms bearing a minimum of 9 motifs and reached maximal concentrations in the presence of FbsA15 or FbsA19, suggesting a direct correlation between the aggregation process and the number of motifs in FbsA molecule (Figure 5, panel B).

Turbidity also fully supports the main DLS evidence, namely, that FbsA is able to induce Fbg aggregation even in the absence of thrombin. In Figure 5, panel C, we show the aggregation of Fbg induced by increasing amounts of FbsA19, and compare it to the growth of simple fibrin. Notice that, at variance with the latter, no distinct lag time is observed: aggregate starts growing just after FbsA is added, and both the increase rate and the plateau value of the turbidity rise with increasing FbsA concentration. This remarkable difference between the two kinetics suggests a radically distinct physical nature of these two aggregation processes.

We also tested the effect of equimolar concentrations of FbsA isoforms on Fbg aggregation: isoforms containing 19 or 15 repeat units strongly promoted Fbg assembly; FbsA 9 induced an intermediate increase of turbidity, whereas marginal effects were observed incubating Fbg with FbsA6 and FbsA3 (Figure 5, panel D). Together these data suggest that FbsA alone is capable of promoting Fbg aggregation and the magnitude of the effect depends on the numbers of the repetitive units in FbsA.

Effect of Disruptive Agents on Thrombin-Dependent Fibrin Clot Assembly and FbsA-Induced Fibrinogen Aggregates. To gain insights into the forces that stabilize the Fbg aggregate, mixtures of Fbg and FbsA were incubated and allowed to aggregate for 30 min. The suspensions were then added with indicated concentrations of urea or GdmCl, incubated for 30 min, and examined for turbidity at 350 nm. In these conditions, both agents solubilized more effectively the Fbg aggregate than the regular fibrin clot. Moreover, GdmCl displayed a higher disrupting power than urea on both FbsA/Fbg complex and fibrin clot. On the other hand, NaCl salt even at highest concentrations reduced the turbidity of the mixtures by not more than 15−25% (Table 2).

Either thrombin-driven fibrin clot assembly or FbsA-induced Fbg aggregation experiments were performed in the presence of a buffer containing Ca^{++}; moreover, as indicated by ELISA test (data not shown), Fbg used in our assays

Table 2: Disruption of Fibrin Clot and FbsA-Induced Fbg Aggregate[a]

agent concn (M)	urea		guanidine hydrochloride		NaCl	
	fibrin	Fbg/FbsA	fibrin	Fbg/FbsA	fibrin	Fbg/FbsA
0	100 ± 2.0	100 ± 1.3	100 ± 3.4	100 ± 4.0	100 ± 5.0	100 ± 3.2
0.1	100 ± 1.4	92 ± 3.2	99 ± 2.5	74 ± 2.5	97 ± 3.2	99 ± 4.0
0.5	94 ± 2.6	91 ± 2.0	80 ± 2.7	50 ± 3.8	93 ± 3.6	98 ± 2.1
1	92 ± 1.7	89 ± 3.5	62 ± 1.6	15 ± 1.0	89 ± 1.6	95 ± 5.0
2	86 ± 3.0	73 ± 1.7	49 ± 1.2	1 ± 0.03	88 ± 4.2	87 ± 3.8
3	78 ± 2.2	71 ± 1.3	24 ± 1.8	0 ± 0.01	86 ± 2.8	75 ± 4.3

[a] Polymerization of 1 μM Fbg was initiated by the addition of thrombin (0.05 U/mL) or 0.5 μM FbsA19 at 25 °C, and the cuvettes were incubated until maximum turbidity values at 350 nm were attained (30 min). At that time the mixtures were added with increasing amounts of the indicated solutes or buffer alone (the control) and incubated at 25 °C, and the turbidity was measured after 30 min. The results are presented as percentage of the control turbidity. The values are the average ± SDs of triplicate samples.

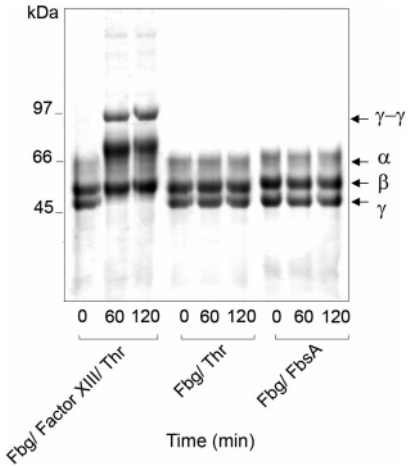

FIGURE 6: Factor XIII-catalyzed cross-links in fibrinogen. Fbg (6 μM) dissolved in 20 mM Tris-HCl, 150 mM NaCl, and 5 mM $CaCl_2$, pH 7.4, was incubated with 3 μM FbsA or thrombin (0.1 U/mL) at 25 °C. At the indicated time points, cross-linking reactions were terminated by addition of 8 M urea and the mixtures analyzed by SDS−PAGE on 7.8% gel under reducing conditions. The cross-linking of fibrin by exogenous factor XIII (15 μg/mL) is also reported as control. The products of fibrin corss-linking reaction are indicated as γγ dimers. On the left side of the gel the molecular masses of standard proteins are reported.

contains trace amounts of factor XIII: these assumptions suggest the possibility that factor XIII-catalyzed cross-linking reactions can occur in the mixtures. To prove that the stronger disrupting effects of urea or GdmCl on FbsA-induced Fbg aggregate compared to fibrin clot could be related to the presence of cross-links due to factor XIII action, we checked out cross-linking reaction products in SDS−PAGE forFbg incubated either with FbsA or with thrombin. As shown in Figure 6, upon incubation of Fbg with thrombin or with FbsA no cross-linking reaction products were detected, suggesting that endogenous presence of factor XIII in Fbg preparation was not enough to trigger a detectable cross-linking reaction or that in the experimental conditions used the transglutaminase activation was not allowed. Conversely, in the reaction where Fbg was incubated with both thrombin and exogenous factor XIII, cross-linking of fibrin resulted in rapid disappearence of the γ chain and concomitant formation of γγ dimers and other multiple new high molecular mass bands. A very similar cross-linking pattern was obtained when the

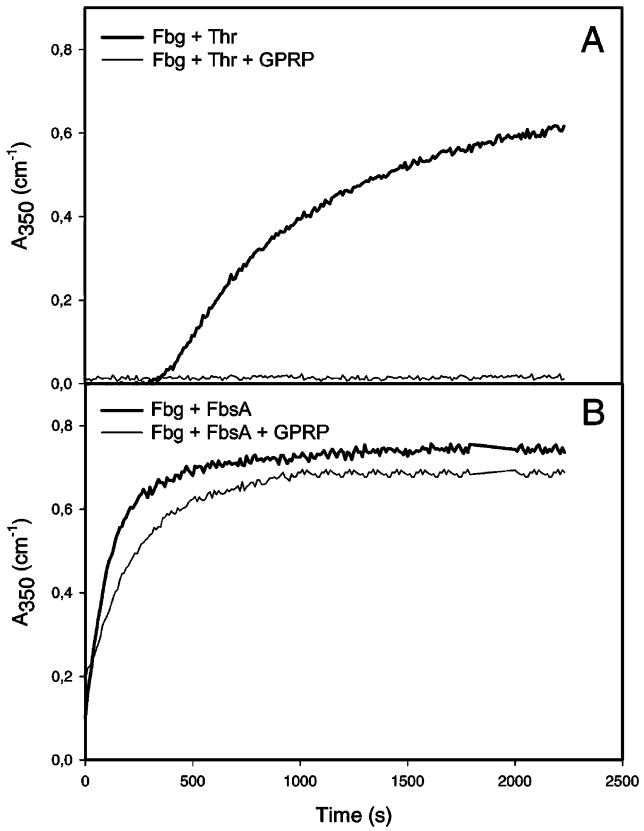

FIGURE 7: Fibrin clot formation and FbsA-driven Fbg aggregation in the presence of synthetic GPRP peptide. Fbg (1 μM) was incubated either with 0.05 U/mL thrombin (panel A) or with 0.5 μM FbsA (panel B) in the absence or presence of 250 μM GPRP, and the aggregation rates were measured at 350 nm.

gel was subjected to transfer to nitrocellulose filter followed by immunostaining with anti-Fbg antibodies (data not shown).

GPRP Peptide Blocks Fibrin Assembly but Not FbsA-Induced Fibrinogen Aggregation. The sequence GPR has been found at the N-terminal of fibrin α chains and can bind to Fbg and effectively prevents the polymerization of fibrin monomers (*40*). When the synthetic peptide GPRP was included in a mixture of Fbg containing thrombin or FbsA, a strong inhibition of thrombin-dependent Fbg clotting was observed, whereas no action of the peptide was noticed on the FbsA-induced Fbg assembly, suggesting that regular fibrin clot formation and Fbg aggregation follow different mechanisms (Figure 7).

FbsA Does Not Modify the Covalent Structure of Fibrinogen. To determine whether FbsA on its own or protease contaminants potentially present in the FbsA preparation could modify Fbg structure, Fbg was incubated with FbsA and the mixtures were examined at the indicated time points by SDS−PAGE. As shown in Figure 8, no change in the electrophoretic mobility of the Fbg Aα and Bβ chains was observed even after 60 min incubation. In addition, fibrinopeptides A and B were not released from the Aα and Bβ chains as verified by N-terminal sequencing of the Fbg chains cut from the gel, thus confirming the integrity of Fbg molecule (data not shown). Thus, while fibrin formation involves molecular cleavage due to the enzymatic action of thrombin, followed by Fbg reorganization in protofibrils and subsequent growth, FbsA probably drives a simpler Fbg

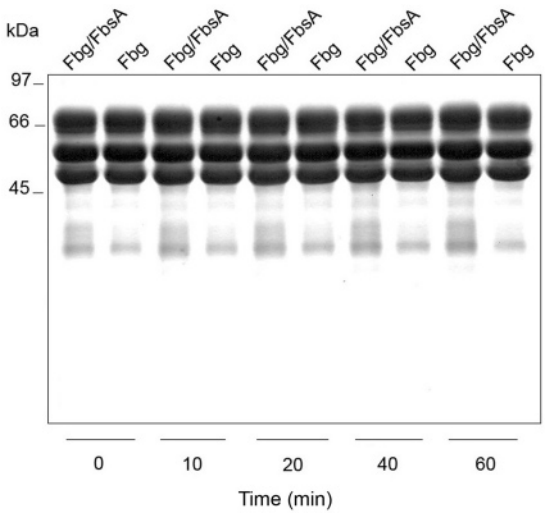

FIGURE 8: Effect of FbsA on the structural integrity of fibrinogen. Fbg (6 μM) dissolved in 20 mM Tris-HCl, 150 mM NaCl, and 5 mM $CaCl_2$, pH 7.4, was incubated for the indicated time points in the absence or presence of 3 μM FbsA. The samples were then subjected to 10% SDS−PAGE under reducing conditions.

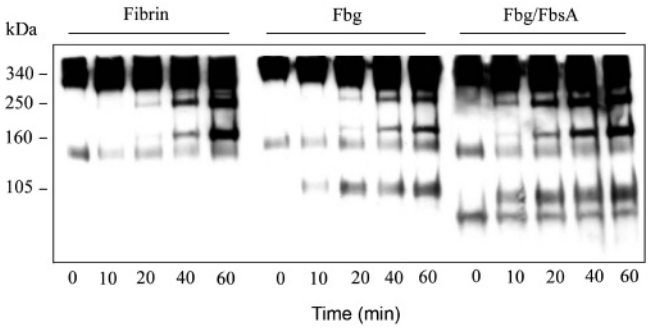

FIGURE 9: Digestion of fibrin clot and FbsA-induced Fbg aggregate with plasmin. Fbg (6 μM) or equivalent concentrations of thrombin-catalyzed fibrin clot or FbsA-promoted Fbg aggregate were each incubated with plasmin (10 nM) in the presence of 5 mM $CaCl_2$. The reactions were stopped at the indicated times by addition of 100 mM Tris-HCl buffer, pH 8.2, containing 100 mM NaCl, 8 M urea, and 2% SDS and boiling for 5 min. The samples were subjected to electrophoresis on a 7.5% polyacrylamide gel under nonreducing conditions, transferred onto nitrocellulose filter, and probed with 0.5 μg/mL of a mouse anti-Fbg antibody followed by addition of a peroxidase-conjugated rabbit anti-mouse IgG.

association process, which does not require preliminary molecular restructuring processes.

Fibrin Clot and FbsA-Induced Fibrinogen Aggregate as Substrates of Plasmin. To further analyze the structure of FbsA-induced Fbg aggregate, we examined the sensitivity of fibrin clot, FbsA/fibrinogen complex, and soluble Fbg to plasmin. The digestion kinetics of Fbg and FbsA/Fbg aggregate appeared almost identical, confirming the concept that when Fbg aggregates in the presence of FbsA, it retains a general structure substantially similar to that of soluble Fbg (Figure 9).

Structural Comparison of FbsA/Fbg Networks to Fibrin Clots. SEM images show that the overall architecture of the meshwork formed by FbsA/Fbg aggregates is rather different from that of thrombin-induced fibrin clots. Fibrin clots show thin fiber strands arranged in bundles (Figure 10, panel A), whereas FbsA-induced Fbg aggregates are organized in a 3D network having a cage-like structure. Moreover, in the

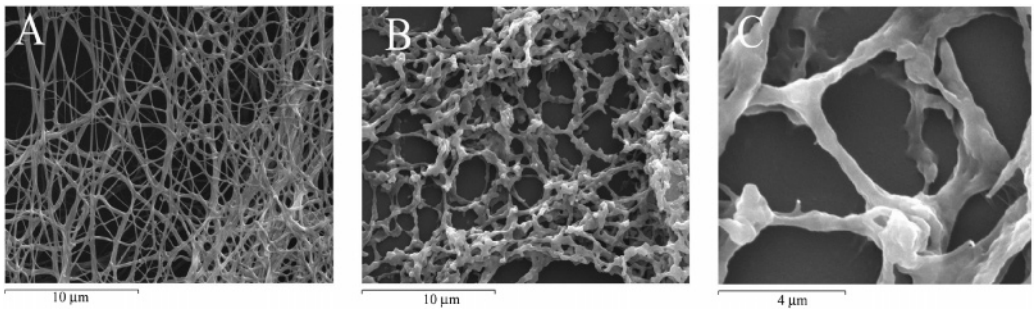

FIGURE 10: Scanning electron micrographs of regular fibrin clot and FbsA-induced Fbg aggregate. A regular fibrin clot was made incubating at 25 °C purified 1 μM Fbg and thrombin (0.05 U/mL) for 45 min (panel A: ×5000). To form macromolecular fibrinogen complexes, FbsA19 and Fbg were incubated at a molar ratio of 1:2 for 45 min (panels B and C: ×5000 and ×15000, respectively).

latter, the constituent fibers are thicker and the overall network structure coarser (Figure 10, panel B). This microscopic difference mirrors into a radically dissimilar macroscopic rheologic solution behavior: while thrombin yields elastic and rather rigid gels, the aggregated FbsA/Fbg solution always shows a fluid-like behavior, even when FbsA is added to Fbg in equimolar ratio (data not shown). Full mobility of the aggregates is further confirmed by DLS data, which do not show any sign of the partially arrested microscopic dynamics typical of a gel phase (for further discussion of this point, see ref *36*). We also point out that Fbg aggregation due to the simultaneous presence of thrombin and FbsA still yields, at least for the FbsA/thrombin concentration ratios we have investigated, solid-like structures, confirming the role of thrombin action in strengthening the overall structure.

Ligand-Binding Properties and Immunological Determinants of FbsA-Induced Fibrinogen Aggregate. As previously reported, native Fn (*41*), N-terminus Fib-1, and C-terminus Fib-2 fragments (*42*, *43*) of Fn preferentially bound to immobilized fibrin clot and to a much lesser extent to Fbg alone. A similar prevalent binding of tPA and plasminogen to fibrin was observed (*44*). To verify the possible formation of fibrin-like determinants in Fbg polymers, we compared the binding of Fn to regular fibrin clot with that to FbsA-promoted fibrinogen polymer (Figure 11, panel A). As expected, Fn, Fib-1, and Fib-2 strongly interacted with fibrin clot, whereas a much weaker binding was detected when the same ligands were incubated with fibrinogen coated wells. Interestingly, Fn and its fragments showed a significant binding to FbsA/Fbg polymer suggesting that Fn-binding sites become somehow exposed for ligand binding upon Fbg network formation. Otherwise, tPA and plasminogen strongly bound to fibrin clot but neither interacted with Fbg or FbsA/Fbg complex.

It has been also reported that epitopes, hidden in Fbg, become accessible to specific mAbs when Fbg is converted to fibrin (*30*, *31*, *45*). Therefore, we analyzed the reactivity of fibrin-specific mAbs 1H10, 5F3, and A11 with FbsA-induced Fbg aggregate. As shown in Figure 11, panel B, all the mAbs recognized fibrin but not FbsA-induced Fbg polymers or Fbg alone, indicating that fibrin specific epitopes are not exposed in the Fbg aggregate.

DISCUSSION

In this study we have tried giving an overall characterization of FbsA-induced Fbg aggregation. First, we have shown that the FbsA isoforms bound immobilized Fbg specifically, dose-dependently, and with similar affinity. As shown by direct binding assays, Fbg interacts with FbsA via its D fragment. Since Fbg consists of two outer D regions each connected to a central E region, then two potential constitutive FbsA-binding sites can be envisaged in Fbg. The results of gel filtration chromatography experiments indicate that in the presence of excess amounts of D fragment the isoform of FbsA containing 6 repeats may associate up to 6 molecules of D fragment. Due to the potential inaccuracy of the estimated stoichiometry of the FbsA/D fragment interaction as determined by gel filtration chromatography we verified the formation of FbsA/D fragment complexes by electrophoresis in a gradient polyacrylamide gel in the absence of denaturating agents (data not shown). In these conditions, the FbsA/D fragment complexes were so smeared out in the gel that no quantitative assessement of their molecular mass was feasible. Thus, a precise quantitative analysis of the valency of FbsA toward the D fragment remains to be determined. However, as supported by DLS data, we feel that FbsA association to Fbg is undoubtedly a multivalent process.

We have then demonstrated that, in accordance with the bivalency of Fbg and the ability of FbsA to interact with several Fbg molecules, FbsA released by *S. agalactiae* cells both in human serum and in BHI medium strongly associates with Fbg. The nature of the FbsA/Fbg aggregates, as shown by DLS and further confirmed by turbidity studies, is that of huge polymer-like aggregates organized into a semiflexible network structurally similar to those formed by other biopolymers such as actin, desmin, or fibrin itself. The FbsA-activated process depends both on FbsA concentration and on the number of the protein tandem repeats. In fact, FbsA-induced Fbg aggregation reaches detectable levels only when Fbg is incubated with FbsA isoforms bearing a minimum of 6 motifs, and is maximal in the presence of FbsA15 or FbsA19. DLS and turbidity results were supplemented by visual inspection of SEM images: while thrombin yielded a fine fibrillar network, FbsA-induced aggregates were thicker fibers (probably due a higher degree of lateral fibril association) organized in a coarser cage-like structure. Similar aggregation kinetics and eventual network structure were observed when human plasma was incubated with FbsA (data not shown).

Another novel, and to our view particularly meaningful, result of this study is the strong synergy between FbsA and thrombin. Unlike staphylococcal Fbg-binding proteins such as ClfA or FnbpA, which interfere with fibrin clot polymerization (*46*, *47*), FbsA actually sped up and

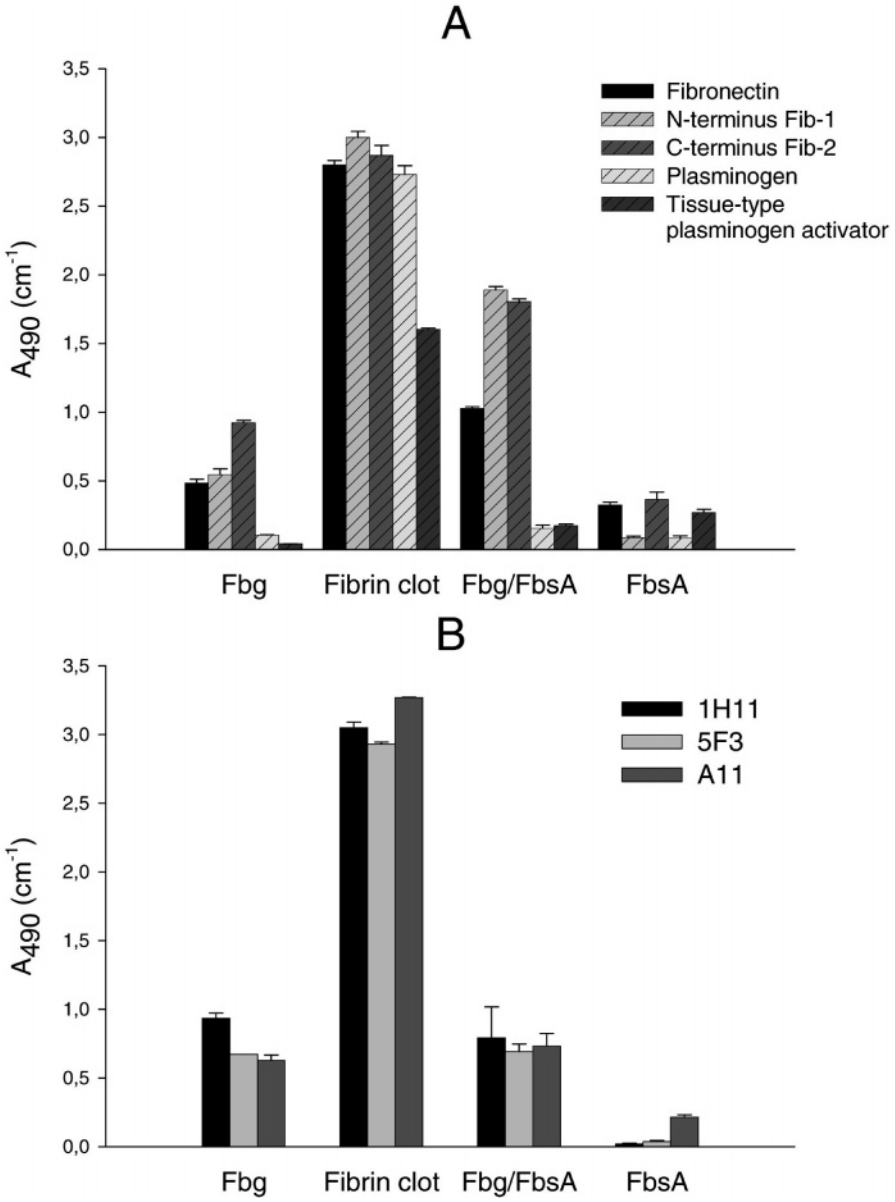

FIGURE 11: Binding of fibrin-specific ligands to FbsA-promoted Fbg aggregates. (A) Microtiter well plates were coated with 1 μM Fbg or 0.5 μM FbsA19 or a combination of both for 1 h at 22 °C. Fibrin clot in the wells was produced by mixing 1 μM Fbg in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 5 mM $CaCl_2$ with 0.05 U/mL thrombin and then overnight dehydration at 22 °C. All the wells were treated for 1 h at 22 °C with 200 μL of PBS containing 2% bovine serum albumin (BSA) and then washed 5 times with PBS containing 0.1% (v/v) Tween 20 (PBST). The wells were incubated with 250 ng of human Fn, the N-terminal or C-terminal fragments, and then added of 500 ng of rabbit antibodies against Fn. The binding of the antibody was detected by incubating the plates with a 1:1000 dilution of goat anti-rabbit peroxidase-conjugated secondary antibody. After washing, binding was quantitated using the substrate *o*-phenylenediamine dihydrochloride and measuring the absorbance at 490 nm. tPA and plasminogen binding to the substrate-coated wells was performed incubating the plates with NHS-biotin-labeled tPA or plasminogen for 90 min. The wells were washed 5 times with PBST, and bound ligand was detected by incubation for 30 min with peroxidase-conjugated avidin diluted 1:2000 and development with *o*-phenylenediamine dihydrochloride. (B) Binding of fibrin-specific mAbs to FbsA-induced Fbg aggregate. Fbg, FbsA, FbsA/Fbg complex, and the fibrin clot were immobilized onto microtiter wells and incubated with 200 ng of the mouse anti-fibrin mAbs 1H10, 5F3, and A11 for 90 min at 22 °C. The binding of antibodies was detected by addition of a peroxidase-conjugated rabbit anti-mouse IgG and subsequent addition of a chromogenic substrate. The bars represent SDs of triplicate samples.

potentiated thrombin-catalyzed fibrin clot formation. This effect is however not due to a direct activating effect of FbsA on thrombin, but rather to the multivalent nature of FbsA. Moreover, due to the exhaustive conversion of Fbg to fibrin by thrombin even in the presence of FbsA (Figure 5, panel A) and taking into account the activating effect of FbsA, one can reasonably assume that FbsA also drives the aggregation of fibrin monomers and in these conditions heterocomplexes made of fibrin clots and aggregates of fibrin may coexist in the mixture. The formation of these complexes when Fbg is coincubated with both thrombin and FbsA remains to be determined by direct microscopic inspection.

To investigate the mechanism of FbsA-induced Fbg aggregation, compare it to the formation of fibrin, and unravel the nature of the FbsA/Fbg binding, we have first shown that GPRP peptide, in contrast to its strong inhibitory effect on fibrin clotting, does not affect Fbg aggregate formation. This evidence clearly suggests that the two processes follow different polymerization mechanisms and

that the initiation of FbsA-driven Fbg aggregation does not necessarily imply a release of fibrinopeptides A and B.

The evidence that inhibitors of serine proteases, such PMSF and TPCK, do not change Fbg aggregation profile (data not shown) and the finding that incubation of Fbg with FbsA does not modify the covalent structure of the Aα and Bβ chains further indicate that FbsA-driven Fbg aggregation does not involve protein cleavage and formation of fibrin monomers.

The similarity of plasmin digestion patterns of soluble Fbg and FbsA/Fbg aggregate also proves that FbsA promotes the formation of a network that essentially keeps the structural integrity of soluble Fbg.

The distinct nature of fibrin clots and FbsA/Fbg aggregates is further highlighted by comparing the effects of chemical agents such as urea and GdmCl on the dissolution of the complexes. These agents promote protein unfolding by favorable interactions with groups exposed upon unfolding. GdmCl is slightly more effective than urea in attenuating hydrogen bond interactions but is considerably more effective against hydrophobic interactions (*48*).

Our finding that urea and GdmCl are more effective in disrupting FbsA/Fbg aggregates than fibrin clots suggests that the former are stabilized by forces that are weaker than those supporting fibrin, which macroscopically mirrors into the fluid, rather than gel-like, nature of the aggregated solution. Furthermore, the better solubilizing activity of Fbg aggregate by GmdCl (compared to urea) indicates that hydrophobic interactions substantially contribute to the stability of the complex. Finally, the absence of detectable traces of cross-linking reaction products in Fbg mixtures incubated with either thrombin or with FbsA suggests that the diverse, disruptive actions of urea or GdmCl on Fbg aggregate and fibrin clots were not attributable to a different degree of intermolecular links of the complexes.

Fbg is rather inert in circulation, but, upon conversion into fibrin, interacts with different proteins and cell types. For instance, the exposure of tPA and plasminogen binding sites upon conversion of Fbg to fibrin has been described (*44*); as a consequence of this binding, the activation of plasminogen by tPA occurs, and fibrin clot dissolves. In contrast, we found that FbsA/Fbg complex neither binds to tPA or plasminogen nor responds to monoclonal antibodies recognizing fibrin-specific epitotes. Consistent with lack of reactivity with tPA and plasminogen, one can predict that a clot composed of insoluble Fbg may exhibit resistance to lysis and a longer persistence in the vascular system.

It has been previously reported that polymeric fibrin, but not Fbg, interacts with Fn (*41*) and Fib-1 and Fib-2 fragments of each Fn subunit (*42*, *43*). Interestingly, in our conditions, Fbg polymer showed a significant Fn-binding capability, suggesting that cryptic Fn-binding determinants became exposed as a consequence of Fbg aggregation in the presence of FbsA. In accordance with this evidence, when FbsA was added to whole human plasma, visible aggregates were formed and Fn was captured and embedded in the precipitate (data not shown).

What are the consequences of Fbg aggregation by FbsA? *Streptococcus pyogenes* and group G streptococci (GGS) express Fbg-binding proteins (*49*, *50*). Protein M from *S. pyogenes* and protein FOG from GGS share with FbsA a few biological properties such as the capability to trigger the formation of Fbg aggregates and resist the phagocytosis (*37*, *49*, *50*). Moreover, M protein or protein FOG in complex with Fbg induces activation of PMNs, release of a heparin-binding protein, and the subsequent vascular leakage. Much like protein M (*51*), either soluble FbsA or *S. agalactiae* cells expressing FbsA interfered with the clotting ability of human plasma. In fact, incubation of human plasma with FbsA (or bacteria) and removal of the precipitates by centrifugation caused a prolonged clotting time of the resulting supernatant (data not shown).

In previous works it has also been demonstrated that *S. agalactiae* strains promoted a Fbg-dependent platelet aggregation (*52*, *53*) and that aggregation is a FbsA-dependent process (*17*). If *S. agalactiae* cells trigger platelet aggregation and soluble FbsA induces Fbg polymerization in vitro, we can speculate that *S. agalactiae* transiently invading the bloodstream could induce signs of thrombosis in the circulation and that FbsA could be a critical prothrombotic agent. In line with this indication, Usui et al. (*54*) reported that intravenous administration of a crude fraction from a clumping factor-positive strain of *S. agalactiae* caused in mice the formation and deposition of thrombi in the glomeruli, heart, lung, liver, and pancreas of the animals. Hence, these authors suggested the possible induction of disseminated intravascular coagulation in the mouse by *S. agalactiae*. In conclusion, FbsA anchored to the streptococcal cell wall as well as FbsA released from bacteria play a different, synergic action in thrombi formation and infective endocarditis. In light of these observations, further investigation into the thrombogenic role of FbsA in animal infection model seems warranted.

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