Vitronectin binds to the gonococcal adhesin OpaA through a glycosaminoglycan molecular bridge

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Several bacterial pathogens including *Neisseria gonorrhoeae* bind the human serum glycoprotein vitronectin. We aimed at defining the gonococcal receptor for vitronectin. Ligand blots demonstrated that vitronectin bound specifically to the heparin-binding outer-membrane protein OpaA, but that coating OpaA with the sulphated polysaccharide heparin was required for the interaction to occur. Bound vitronectin could be dissociated from OpaA–heparin–vitronectin complexes by the addition of excess heparin, indicating that sulphated polysaccharides provided the main linkage between the two proteins. Binding assays with intact micro-organisms substantiated the requirement of sulphated polysaccharides such as heparin and dextran sulphate for the efficient binding of vitronectin to OpaA$^+$ gonococci. This was underscored by the increased binding of vitronectin to gonococci that had been preincubated with saturating concentrations of dextran sulphate, as opposed to the inhibition of vitronectin binding observed when bacteria were incubated simultaneously with vitronectin and saturating concentrations of dextran sulphate. Binding assays with dextran sulphates of various sizes indicated that vitronectin binding correlated with the size of the polysaccharide rather than with the amount of OpaA produced by the bacteria. The inability of zero-length cross-linking agents to couple vitronectin to OpaA provided further evidence that sulphated polysaccharides formed the linkage between vitronectin and OpaA. Infection experiments demonstrated that proteoglycan-deficient Chinese hamster ovary cells efficiently internalized dextran sulphate/vitronectin-coated gonococci, suggesting that soluble sulphated polysaccharides could substitute for cell surface glycosaminoglycans in the internalization process. On the basis of our results, we propose a novel mechanism of vitronectin binding in which sulphated polysaccharides act as molecular bridges, linking the glycosaminoglycan-binding sites of vitronectin and gonococcal OpaA.

INTRODUCTION

Vitronectin is a multifunctional serum protein that interacts with other serum-derived molecules including glycosaminoglycans [1–3], the complement C5b-7 complex [4], thrombin–antithrombin III [5,6], type 1 plasminogen activator inhibitor [7–10], collagen [11–13] and β-endorphin [14]. Vitronectin has also been shown to bind to a number of mammalian cell receptors including the $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins [15,16] and the urokinase receptor [17]. Binding to integrins is dependent on an Arg-Gly-Asp sequence within the N-terminal portion of vitronectin [18,19]. Depending on the cell type and the experimental conditions, the binding of vitronectin to these receptors can result in cellular attachment, migration or endocytosis of vitronectin. In addition to interacting with mammalian molecules, vitronectin binds to the surface of several bacterial species, including *Escherichia coli*, *Staphylococcus aureus*, *Staph. epidermidis*, *Streptococcus* species, *Enterococcus* species, *Pneumocystis carinii* and *Helicobacter pylori* [20–25]. This binding might contribute to the pathogenicity of these organisms by interfering with complement-mediated bacterial killing and facilitating bacterial adherence. The nature of the molecular interactions between bacteria and vitronectin remains elusive. Vitronectin has been shown to bind to 60 and 72 kDa proteins from *Staph. aureus* but the identity of these putative vitronectin receptors has not been elucidated [21,22].

The bacterial pathogen *Neisseria gonorrhoeae* is the aetiological agent of the sexually transmitted disease gonorrhoea. This bacterium colonizes the human mucosa and can exploit mammalian cell receptors to adhere to and invade human epithelial cells [26–30]. We and others have demonstrated that gonococci producing the OpaA adhesin interact with a variety of mammalian cell types by using membrane-bound heparan sulphate proteoglycans as receptors [31,32]. In some of these cell types this adherence is immediately followed by the efficient internalization of the bacteria. In other cells, such as Chinese hamster ovary (CHO) and HeLa cells, proteoglycan-mediated adherence is not sufficient for uptake to occur, but internalization can be effected by the addition of vitronectin. The interactions between vitronectin, heparan sulphate proteoglycans and OpaA that are required for initiation of the internalization process are unresolved. Both heparan sulphate proteoglycans and vitronectin bind specifically to gonococci producing OpaA, and not to isogenic variants lacking this protein [31,33]. In addition, heparin inhibits the binding of vitronectin to this micro-organism [34]. Unravelling the details of the molecular relationships between these molecules is essential for understanding the complex mechanism behind the vitronectin-mediated invasion of epithelial cells by *N. gonorrhoeae*.

In the present study we investigated a central component of these interactions: the binding of vitronectin to OpaA$^+$ gonococci. Through the use of binding assays with intact micro-organisms and reconstitution experiments with isolated molecules, we demonstrate a novel mechanism of vitronectin binding that involves the formation of a trimolecular complex between gonococcal OpaA, sulphated polysaccharides and vitronectin. Our results are consistent with a model in which the sulphated polysaccharides interact with heparin-binding sites on both OpaA and vitronectin, thus facilitating the formation of a molecular bridge between the two proteins.

Abbreviations used: CHO, Chinese hamster ovary; HRP, horseradish peroxidase.

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EXPERIMENTAL

Bacterial strains and growth conditions

The Opa nomenclature of Swanson et al. [35] is used in the present paper. Variants of *N. gonorrhoeae* strain MS11 producing OpaA, OpaB or no Opa proteins were kindly provided by Dr. John Swanson (Rocky Mountain Laboratories, Hamilton, MT, U.S.A.). All gonococcal strains were non-piliated and expressed LOS type b (lacto-*N*-neotetraose+). Bacteria were routinely maintained on GC phosphate agar plates [composition per litre: 3.75 g of Trypticase peptone (BBL; Becton-Dickinson, Cockeysville, MD, U.S.A.), 7.5 g of Thiotone E (BBL), 4 g of K_2HPO_4, 1 g of KH_2PO_4, 5 g of NaCl, 1 g of soluble starch (BBL) and 1 % (w/v) Bacto-agar (Difco, Detroit, MI, U.S.A.), pH 7.2] containing 1 % (w/v) IsoVitaleX (BBL) at 37 °C in air/CO_2 (19:1). For some experiments the gonococci were cultured on Hepes/agarose plates [composition per litre: 3.75 g of trypticase peptone (BBL), 7.5 g of Thiotone E (BBL), 5.0 g of Hepes free acid (Calbiochem), 2.15 g of Hepes sodium salt (Calbiochem), 5.0 g of NaCl (Sigma), 1.0 g of soluble starch (J. T. Baker) and 8 g of SeaKem agarose lot no. 132893 (FMC)].

Vitronectin binding assays

Vitronectin was purified from fetal calf serum as described [36]. Gonococci to be used in vitronectin binding assays were grown overnight at 37 °C in air/CO_2 (19:1) on either GC phosphate agar plates or Hepes/agarose plates. Bacteria were suspended into 10 ml of Hepes buffer [10 mM Hepes/145 mM NaCl/5 mM KCl/1 mM $CaCl_2$/1 mM $MgCl_2$/5 mM glucose (pH 7.2)], collected by centrifugation (1800 ***g***; 6 min; 20 °C), resuspended in 2–3 ml of Hepes buffer and quantified by absorbance at 550 nm. Unless indicated otherwise, binding of vitronectin was assessed by adding 10^8 gonococci to 200 μl of Hepes buffer with or without polyanions as described for individual experiments. Polyanions were obtained from the following sources: heparin (sodium salt), ICN Biomedicals (Aurora, OH, U.S.A.); heparan sulphate, dermatan sulphate and dermatan disulphate, gifts from Celsus Laboratories (Cincinatti, OH, U.S.A.); salmon sperm DNA, Stratagene (La Jolla, CA, U.S.A.); dextran sulphate (5 kDa), a gift from Professor E. De Clercq (Katholieke Universiteit, Leuven, Belgium) and Dr. John Swanson (Rocky Mountain Laboratories, Hamilton, MT, U.S.A.); dextran sulphate (500 kDa), Sigma Chemical Co. (St. Louis, MO, U.S.A.). After 10 min at 0 °C the bacteria were pelleted (14000 ***g***; 2 min; 4 °C), resuspended in 200 μl of Hepes buffer and washed once more in this manner. The pellets were then resuspended in 200 μl of Hepes buffer containing 5 μg/ml vitronectin, then incubated for 10 min at 0 °C. The bacteria were pelleted (14000 ***g***; 2 min; 4 °C), washed twice with 200 μl of Hepes buffer, resupended in 50 μl of water, transferred to a new tube containing 50 μl of 2 × SDS/PAGE sample buffer [37] and lysed in a boiling-water bath for 5 min. Bacteria-associated vitronectin was detected by SDS/PAGE [12.5 % (w/v) gel] and Western blotting with a vitronectin-specific polyclonal antiserum (1:2000 dilution; Calbiochem), horseradish peroxidase (HRP)-conjugated Protein A (0.1 μg/ml; Sigma) and SuperSignal substrate (Pierce, Rockford, IL, U.S.A.). Documentation and quantification of specific bands were performed with an AlphaImager® 2000 Imaging System (Alpha Innotech, San Leandro, CA, U.S.A.).

Infection assays

The CHO*pgsA*-745 cell line was a gift from Dr. Jeffrey D. Esko. These cells, which have a defect in the xylosyltransferase gene and thus are deficient in the production of both heparan sulphate and chondroitan sulphate [38], were routinely cultured in RPMI containing 5 % (v/v) fetal calf serum (Life Technologies, Gaithersburg, MD, U.S.A.). For use in infection experiments, CHO*pgsA*-745 cells were plated on 12 mm circular glass coverslips in 24-well tissue culture plates and grown to near confluence in RPMI 1640 containing 5 % (v/v) fetal calf serum. Before addition of the bacteria, the monolayers were washed three times with 1 ml of Dulbecco's modified Eagle's medium (Life Technologies). When appropriate, bacteria were preincubated with dextran sulphate and vitronectin as described above. Bacteria (10^7) were added to the CHO cell monolayers (approx. 2×10^5 cells per well) in 1 ml of Dulbecco's modified Eagle's medium. After incubation of the plates for 2 h at 37 °C, the wells were washed three times with 1 ml of Dulbecco's phosphate-buffered saline [140 mM NaCl/2.5 mM KCl/8.1 mM Na_2HPO_4/1.5 mM KH_2PO_4/1 mM $MgCl_2$/1 mM $CaCl_2$ (pH 7.4)], fixed with 2 % (w/v) paraformaldehyde in Dulbecco's phosphate-buffered saline, then stained by immunogold silver staining, which differentially stains intracellular and extracellular bacteria as described previously [39]. Under light microscopy 50 cells per well were chosen at random and the numbers of adherent and intracellular bacteria per cell were determined. The results represent means ± S.E.M. for at least three experiments performed in triplicate.

Ligand blots

Outer-membrane proteins of Opa$^-$, OpaA and OpaB-producing gonococci were prepared by the lithium acetate extraction method as described [40,41]. The outer-membrane preparations were diluted in non-denaturing sample buffer [62.5 mM Tris/HCl (pH 6.8)/20 % (v/v) glycerol/0.1 % SDS] without reducing agent. After solubilization at 37 °C for 10 min, the proteins were separated by SDS/PAGE and electrotransferred to PVDF membranes (Amersham, Arlington Heights, IL, U.S.A.). Towbin transfer buffer was used, except that SDS was replaced with the zwitterionic detergent Empigen (0.1 %) (Calbiochem), which has been shown to maintain the native conformation of neisserial outer-membrane proteins [42,43]. Proteins were visualized with Coomassie Brilliant Blue R-250.

We have found that commercial preparations of BSA (Sigma A-7030) contain minor amounts of both sulphated polysaccharides and vitronectin (T. D. Duensing, unpublished work). To prevent interference in our assays, BSA was passed over heparin–Sepharose (Sigma) and DEAE-Sephacel (Pharmacia) columns in tandem. The flow-through from these columns was used for all subsequent blocking and incubation steps. After transfer, the blots were blocked for 1 h in 3 % (w/v) BSA in PBST [10 mM Na_2HPO_4/10 mM NaH_2PO_4/150 mM NaCl/0.1 % (v/v) Polysorbate (Tween®) 20] and incubated in the presence or absence of 0.1 mg/ml heparin in 3 % BSA/PBST. The blots were then washed three times (2 min each wash) with PBST and incubated with 20 ng/ml vitronectin in 3 % BSA/PBST. After further washing (three times; 10 min each wash), bound vitronectin was detected by using the vitronectin-specific polyclonal antiserum and Protein A–HRP as described above.

Heparin was labelled by incubating 5 mg of heparin with 5 mg of Sulfo-NHS-LC-biotin (Pierce, Rockford, IL, U.S.A.) in 2 ml of 50 mM sodium bicarbonate, pH 8.5 (1 h; 20 °C). Then unreacted biotin was quenched by the addition of Tris/HCl, pH 8.0, and BSA to give final concentrations of 100 mM and 1 % (w/v) respectively. PVDF filters with immobilized outer-membrane proteins (see above) were incubated with biotinylated heparin (0.1 mg/ml) in 3 % BSA/PBST (1 h; 20 °C), washed three times

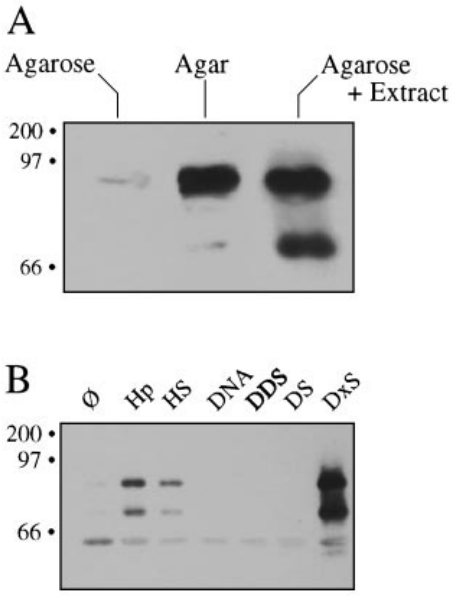

Figure 1 Sulphated polysaccharides enhance the binding of vitronectin to OpaA$^+$ gonococci

Shown are Western blots of bacterial lysates after incubation with 5 μg/ml vitronectin under the conditions described. Vitronectin was detected with a polyclonal vitronectin-specific antiserum, followed by HRP-conjugated Protein A. (**A**) Gonococci producing OpaA were grown overnight on media solidified with agar or agarose and tested for the ability to bind vitronectin. Agarose-grown bacteria were also preincubated for 10 min at 0 °C with an agar extract (agarose + extract) and then tested for the ability to bind vitronectin. (**B**) Agarose-grown OpaA$^+$ gonococci were preincubated with various polyanions for 10 min at 0 °C and washed before testing for the ability to bind vitronectin. The 63 kDa band represents cross-reactivity of the antiserum with a gonococcal protein. Abbreviations: ∅, buffer alone; Hp, heparin; HS, heparan sulphate; DNA, salmon sperm DNA; DDS, dermatan disulphate; DS, dermatan sulphate; DxS, dextran sulphate. The migrations of molecular mass standards are indicated (in kDa) at the left.

(2 min each wash) and bound heparin was detected by enhanced chemiluminescence with HRP-labelled streptavidin (1:1500 dilution; Amersham) in 3% BSA/PBST.

Cross-linking experiments

Vitronectin was allowed to bind to OpaA$^+$ gonococci via dextran sulphate (500 kDa) as described above. The bacteria were then either lysed immediately or incubated for 1 h at 0 °C with 3% (v/v) formaldehyde (EM Sciences, Ft. Washington, PA, U.S.A.) in Hepes buffer before lysis, SDS/PAGE and Western blot. The presence of cross-linked complexes was assessed by probing the blots with the vitronectin-specific antiserum described above and an OpaA-specific polyclonal antiserum (a gift from Dr. R. J. Belland and D. Hogan).

RESULTS

Vitronectin binding to gonococci requires sulphated polysaccharides

Western blot analysis of bacterial lysates with a vitronectin-specific antiserum demonstrated that binding of vitronectin to gonococci varied with the bacterial growth medium employed. Incubation of OpaA$^+$ gonococci with 5 μg/ml vitronectin for 10 min at 0 °C resulted in virtually no vitronectin binding when bacteria were cultured on an agarose-based medium, whereas efficient binding was achieved when the bacteria were grown on an agar-based medium (Figure 1A). Further analysis of this disparity in binding demonstrated that preincubation of agarose-grown gonococci with a soluble extract from agar plates restored vitronectin binding (Figure 1A), suggesting that ingredients in the agar facilitated the binding event. A major difference between agar and agarose is the smaller amounts of highly negatively charged polysaccharides in the latter [44]. This, in conjunction with previous results showing direct binding of sulphated polysaccharides and DNA to OpaA$^+$ gonococci [31,45,46], led us to evaluate the activity of various types of sulphated compounds and DNA in terms of their ability to promote vitronectin binding. As shown in Figure 1(B), preincubation of agarose-grown OpaA$^+$ gonococci with heparin, heparan sulphate and dextran sulphate efficiently supported vitronectin binding, whereas dermatan sulphate, dermatan disulphate and DNA showed no effect. These results strongly suggested that vitronectin binding to gonococci required distinct sulphated polysaccharides.

Vitronectin binds to polysaccharide-coated OpaA proteins

The requirement for sulphated polysaccharides in vitronectin binding was further investigated by defining the bacterial vitronectin receptor. The specificity of vitronectin binding for OpaA$^+$ gonococci [33,34] suggested that OpaA itself, the heparin-like

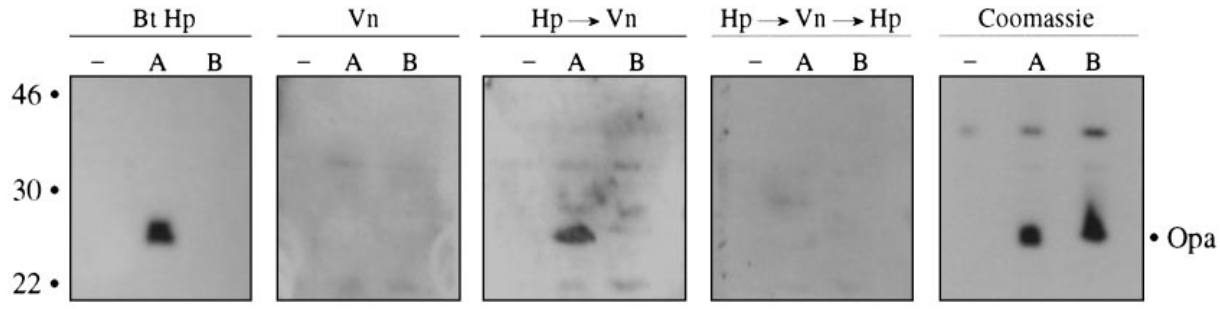

Figure 2 Heparin-mediated binding of vitronectin to isolated OpaA molecules

Outer-membrane proteins from Opa$^-$ (lane —), OpaA-producing (lane A) or OpaB-producing (lane B) gonococci were subjected to electrophoresis and transferred to PVDF membranes. Heparin binding (Bt Hp) was assayed by incubating the blot with biotinylated heparin and streptavidin–HRP. Heparin-dependent vitronectin binding was assayed by incubating blots with vitronectin alone (Vn) or by preincubating the blots with 0.1 mg/ml heparin, followed by incubation with 20 ng/ml vitronectin (Hp → Vn). The ability of heparin to dissociate bound vitronectin was analysed by incubating the blots sequentially with heparin (0.1 mg/ml), vitronectin (20 ng/ml) and an excess of heparin (1 mg/ml) (Hp → Vn → Hp). Vitronectin was detected with a polyclonal antiserum and Protein A–HRP. Protein profiles of the outer-membrane preparations were assayed by Coomassie staining of SDS/PAGE gels (Coomassie). The positions of the Opa proteins are indicated. The migrations of molecular mass standards are indicated (in kDa) at the left.

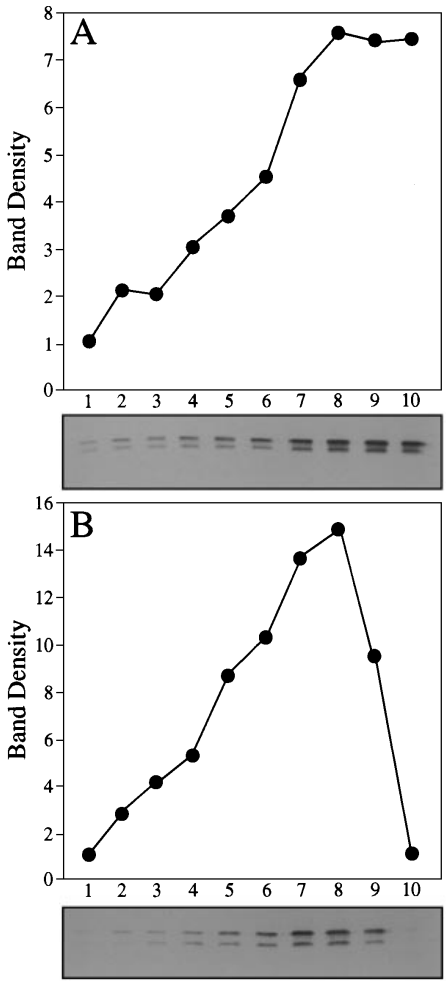

Figure 3 Concentration dependence of dextran sulphate-mediated vitronectin binding

Shown are Western blots of bacterial lysates after incubation with 5 μg/ml vitronectin under the conditions described. Vitronectin was detected with a polyclonal vitronectin-specific antiserum, followed by HRP-conjugated Protein A. In both (**A**) and (**B**), OpaA$^+$ gonococci were preincubated with dextan sulphate for 5 min at 0 °C. (**A**) After preincubation with dextran sulphate, the bacteria were centrifuged and washed before the addition of 5 μg/ml vitronectin. (**B**) After preincubation with dextran sulphate, vitronectin was added to the bacteria without centrifugation and washing. The relative densities of both vitronectin bands in each lane shown in the graphs was assessed by densitometry, with the value of the bands in lane 1 set to 1 for comparative purposes.

compounds bound to OpaA [31,46] or possible polysaccharide-induced alterations in membrane architecture facilitated the binding. To test these options, outer-membrane proteins from gonococci producing OpaA, OpaB or no Opa protein (Opa$^-$) were isolated, separated by non-denaturing gel electrophoresis and transferred to PVDF membranes. Probing of the blots with biotinylated heparin confirmed that OpaA was the predominant heparin-binding protein in gonococcal outer membranes (Figure 2; BtHp). A second set of blots was then incubated with unlabelled heparin for 30 min to allow heparin to bind to the OpaA protein. These blots were then washed to remove unbound heparin and incubated with purified vitronectin. After further washing, bound vitronectin was detected by using the vitronectin-specific antiserum. Vitronectin bound strongly to a protein band co-migrating with OpaA (Figure 2; Hp → Vn); this binding was not observed for blots that had been incubated in buffer lacking heparin (Figure 2; Vn), nor did vitronectin bind to the non-heparin-binding OpaB. The nature of the OpaA–heparin–vitronectin complex was further analysed by testing its stability in the presence of excess heparin. Thus heparin-mediated binding to OpaA was allowed to occur as described above, then blots were incubated with an excess of heparin (1 mg/ml) and tested for the presence of OpaA-associated vitronectin. In this experiment, no binding of vitronectin was observed (Figure 2, Hp → Vn → Hp), suggesting that excess heparin disassembled the ternary complex and that the sustained presence of heparin was required to maintain the interaction of vitronectin and OpaA.

Formation of the OpaA–polysaccharide–vitronectin complex

The molecular interactions between OpaA, sulphated polysaccharides and vitronectin were further examined by analysing the effect that the sulphated polysaccharide concentration had on vitronectin binding. In these experiments, agarose-grown bacteria were preincubated for 5 min at 0 °C with increasing concentrations of dextran sulphate with a molecular mass of 500 kDa (DxS-500), washed, incubated with vitronectin for 10 min at 0 °C, washed again, then analysed for vitronectin binding. As shown in Figure 3(A), vitronectin binding steadily increased with dextran sulphate concentrations up to 2.5 μg/ml. At higher concentrations, this level of vitronectin binding was maintained. Similar results were obtained when dextran sulphate was left present in the assay, with the exception that at concentrations above 2.5 μg/ml there was a sharp decline in the amount of vitronectin bound to the bacteria (Figure 3B). These results indicate that the excess polysaccharide inhibited the formation the OpaA–polysaccharide–vitronectin complex. These findings, together with the observed disassembly of the complex by excess heparin (Figure 2), could be explained if the sulphated compounds were acting as molecular bridges between the glycosaminoglyan-binding sites of vitronectin and OpaA.

Sulphated polysaccharides provide the main linkage between vitronectin and OpaA

The concept that the binding of vitronectin to the bacteria was occurring primarily through sulphated polysaccharides bound to OpaA was explored by evaluating the effect that the size of the polysaccharide had on vitronectin binding. OpaA$^+$ gonococci were preincubated with dextran sulphates with molecular masses of 5 kDa (DxS-5) and 500 kDa (DxS-500) at 5 μg/ml. For both of these compounds this concentration has been found to saturate all of the glycosaminoglycan-binding sites on the surface of OpaA$^+$ gonococci, as indicated by the inhibition of specific [^{3}H]heparin binding to the bacteria (results not shown). Vitronectin binding assays with these bacteria showed substantially more binding to gonococci preincubated with DxS-500 than with DxS-5, whereas the amount of OpaA produced by these bacteria was the same (Figure 4A). Comparison of densitometry values for the OpaA and vitronectin bands revealed that bacteria preincubated with DxS-500 bound approx. 5-fold more vitronectin than did bacteria preincubated with DxS-5.

In search of possible direct interactions between OpaA and vitronectin, we performed experiments with formaldehyde, a zero-length or contact site cross-linking agent that couples amino groups within 2 Å of each other. In this experiment, OpaA$^+$ gonococci were coated with dextran sulphate and vitronectin as described above, and either lysed immediately and prepared for electrophoresis, or incubated on ice for 1 h in the presence of 3 % (w/v) formaldehyde. Bacterial lysates were then prepared and

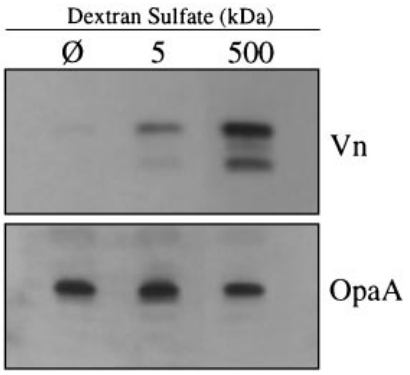

Figure 4 Binding of vitronectin to gonococci preincubated with dextran sulphates of different molecular masses

OpaA$^+$ gonococci were preincubated for 10 min at 0 °C in buffer alone (lane ∅), with 5 μg/ml dextran sulphate with a molecular mass of 5 kDa (lane 5) or with 5 μg/ml dextran sulphate with a molecular mass of 500 kDa (lane 500). The bacteria were centrifuged, washed and incubated with 5 μg/ml vitronectin for 10 min at 0 °C before further centrifugation and washing. Binding of vitronectin (upper panel) was assessed as described in the legend to Figure 1. OpaA was detected in duplicate blots with an OpaA-specific polyclonal antiserum (lower panel).

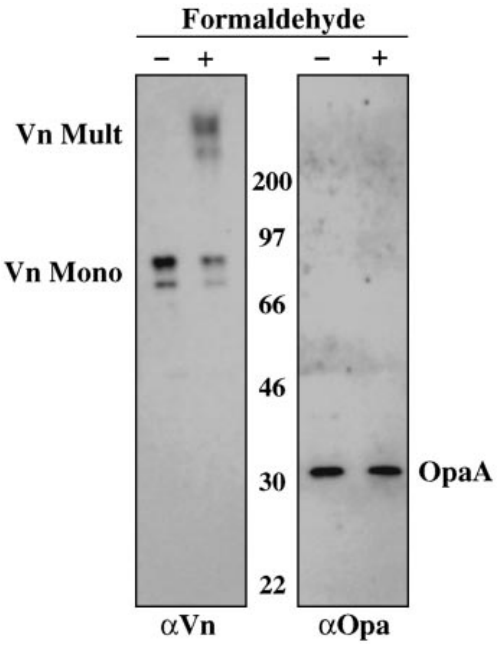

Figure 5 Zero-length cross-linking

Vitronectin was allowed to bind to OpaA$^+$ gonococci via dextran sulphate (500 kDa) as described in the legend to Figure 4. The bacteria were then either lysed immediately (−) or incubated for 1 h at 0 °C (+) with 3% (w/v) formaldehyde before lysis, electrophoresis and Western blotting. The presence of cross-linked complexes was assessed by probing the blots with a vitronectin-specific (αVn) or an OpaA-specific (αOpa) polyclonal antiserum. Abbreviations: Vn Mult, vitronectin multimers; Vn Mono, vitronectin monomers; OpaA, OpaA monomers. The migrations of molecular mass standards are indicated (in kDa) in the centre.

analysed by Western blot for changes in electrophoretic mobility of both OpaA and vitronectin. Vitronectin used in these studies was purified by urea/heparin chromatography and was thus multimeric [36], therefore serving as a convenient positive control for cross-linking. Figure 5 shows that most of the bound vitronectin migrated as multimers. This was concomitant with a substantial decrease in the amount of vitronectin monomers, demonstrating that the cross-linking was effective. Probing duplicate blots with a polyclonal OpaA-specific antiserum revealed that OpaA was not covalently linked to the cross-linked vitronectin complexes. The possibility that high-molecular-mass complexes containing OpaA were present but not detected by the antiserum was unlikely in view of the similar intensities of the OpaA monomeric bands in treated and untreated samples, compared with the marked decrease in the amount of vitronectin monomers in the formaldehyde-treated samples. These results are consistent with polysaccharides being the prime mediators of the binding of vitronectin to OpaA$^+$ gonococci.

Biological relevance of polysaccharide-mediated vitronectin binding

We and others have previously demonstrated that OpaA$^+$ gonococci bind to glycosaminoglycans on CHO cells [31,32] but that vitronectin facilitates bacterial invasion [33]. In view of the current results it is conceivable that, during invasion, the cell-associated glycosaminoglycan side chains bound to OpaA provide a template for vitronectin binding, enabling the bacterial pathogen to exploit vitronectin and its receptors at the epithelial cell surface. We tested this concept by infecting CHO cells deficient in glycosaminoglycan biosynthesis (CHO*pgsA*-745) with gonococci that had been preincubated with dextran sulphate and vitronectin as described above. Determination of the number of adherent and intracellular micro-organisms after 2 h of infection demonstrated efficient internalization of gonococci that carried the dextran sulphate–vitronectin complex, whereas only a few intracellular bacteria were observed when no dextran sulphate was used (Figure 6). Moreover, bacteria preincubated with DxS-500 and vitronectin were internalized much more efficiently than their counterparts coated with DxS-5 and vitronectin, demonstrating that vitronectin-mediated invasion was correlated with the size of the polysaccharide rather than with the amount of Opa protein. These results indicate that the formation of an OpaA–polysaccharide–vitronectin complex might be a key element in the invasion process.

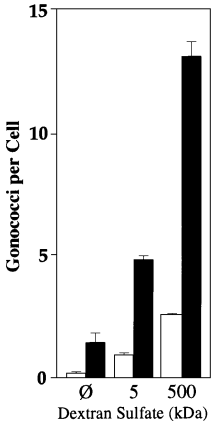

Figure 6 Infection of CHOpgsA-745 cells with dextran sulphate/vitronectin-coated gonococci

Vitronectin was allowed to bind to OpaA$^+$ gonococci via dextran sulphates of different molecular masses as described in the legend to Figure 4; these bacteria were used to infect the proteoglycan-deficient cell line CHO*pgsA*-745. After 2 h of infection, the numbers of extracellular (open bars) and intracellular (filled bars) gonococci were quantified by differential staining as described in the Experimental section. The data are numbers of gonococci per cell, and are means ± S.E.M. for four separate experiments, each performed in triplicate.

DISCUSSION

We examined the molecular mechanism by which vitronectin interacts with the bacterial pathogen *N. gonorrhoeae* producing the adhesin OpaA. Our results point to a novel type of vitronectin binding that involves the formation of a vitronectin–polysaccharide–OpaA complex in which the polysaccharide links the glycosaminoglycan-binding domain of vitronectin to that of OpaA (Figure 7). This conclusion is based on a set of complementary evidence: (1) vitronectin binding to OpaA$^+$ gonococci required heparin or functionally related sulphated polysaccharides; (2) vitronectin could bind to isolated OpaA but only when this protein was coated with heparin (this complex could subsequently be dissociated by the addition of excess heparin); (3) co-incubating the bacteria with vitronectin and an excess of polysaccharide prevented vitronectin binding; (4) vitronectin binding was correlated with the size of the polysaccharide rather than with the amount of OpaA; and (5) no intimate association between vitronectin and OpaA could be demonstrated. The functionality of this mechanism was demonstrated by the efficient OpaA–dextran sulphate–vitronectin-mediated gonococcal invasion of glycosaminoglycan-deficient CHO cells.

A key factor that led to the unravelling of the molecular interaction between vitronectin and OpaA$^+$ gonococci was the perception that sulphated polysaccharides were required to achieve the efficient binding of vitronectin to agarose-grown bacteria but that an excess of polysaccharides prevented the binding. This could be explained if the same polysaccharide molecule were binding to vitronectin and to heparin-binding sites on the bacterial surface. In such an event, maximal vitronectin binding should be observed at concentrations of polysaccharide that saturate all the bacterial binding sites. At higher concentrations polysaccharide–vitronectin complexes should be unable to interact with the bacterial binding sites because the latter would already be occupied by sulphated polysaccharides. The vitronectin binding assays performed in the presence of increasing concentrations of dextran sulphate fully support this concept (Figure 3). The importance of the polysaccharide-binding capability of vitronectin in the interaction was further established by the specificity of binding of vitronectin to bacteria that had been preincubated with various sulphated polysaccharides. Vitronectin bound very strongly to gonococci that had been preincubated with dextran sulphate, less efficiently with heparin, marginally with heparan sulphate, and not at all with dermatan sulphate. These results correlate exactly with the previously described specificity of binding of vitronectin to these polysaccharides [47].

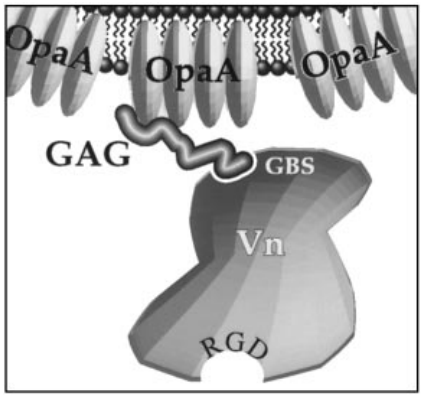

Figure 7 Proposed model for the binding of vitronectin to gonococcal OpaA

In this model, heparin, dextran sulphate and other sulphated polysaccharides interact with the well-described glycosaminoglycan-binding sites of both OpaA and vitronectin, forming a molecular bridge between the two proteins. The observed inhibition of this interaction when OpaA$^+$ gonococci were co-incubated with vitronectin and excess dextran sulphate, and the requirement for sulphated polysaccharides to establish the interaction, lend support to the model. In addition, the dissociation of vitronectin from vitronectin–heparin–OpaA complexes by excess heparin (Figure 2), the increased binding of vitronectin with larger sulphated polysaccharides (Figure 4) and the apparent lack of intimate contact between the two proteins (Figure 5) all suggest that sulphated polysaccharides acted as bridges, rather than as transient templates or stabilizing molecules leading to a more direct vitronectin–OpaA interaction. The polysaccharide-dependent invasion of glycosaminoglycan-deficient CHO cells (Figure 6) emphasizes the potential biological relevance of our findings. This mechanism of binding leaves the Arg-Gly-Asp site available for interaction with integrin receptors on the mammalian cell surface [56].

The ligand-blotting experiments unequivocally implicated the gonococcal OpaA protein as the bacterial receptor for polysaccharide-mediated binding. Biotinylated heparin bound specifically to OpaA; vitronectin bound only to OpaA molecules that had been preincubated with heparin. The latter is consistent with the proposed formation of a polysaccharide bridge between OpaA and vitronectin, although it could be argued that the interaction of heparin with OpaA changed the conformation of this protein, exposing a vitronectin-binding site and allowing direct interaction of the two proteins. However, the dissociation of the reconstituted OpaA–heparin–vitronectin complex by the addition of excess heparin suggested otherwise. This observation indicates that the polysaccharide was the prime linkage between OpaA and vitronectin. The identification of OpaA as the mediator of vitronectin binding is consistent with the reported function of this protein as a bacterial adhesin that binds heparan sulphate proteoglycans [31,32]. It might also explain the observed OpaA specificity of the vitronectin-mediated gonococcal invasion of CHO cells [33].

Two sets of experiments specifically designed to define further the molecular interactions between OpaA, polysaccharides and vitronectin substantiated the model in which sulphated polysaccharides facilitated the interaction of vitronectin with OpaA by acting as molecular bridges rather than by enabling direct interaction between the two proteins. The experiments with dextran sulphates of various sizes revealed a direct correlation between the amount of vitronectin bound and the size of the dextran sulphate with which the bacteria had been preincubated. The concentrations were such that all of the bacterial polysaccharide-binding sites were occupied by dextran sulphate molecules. The most likely interpretation of these results is the ability of larger dextran sulphate to bind more vitronectin molecules than smaller dextran sulphate, which should not occur if OpaA directly participates in the binding event (the number of OpaA molecules produced by the bacteria was the same in each sample). This concept is supported by previous work suggesting that high-molecular-mass dextran sulphate provides a better template than low-molecular-mass dextran sulphate for the alignment of vitronectin molecules before cross-linking by guinea-pig liver transglutaminase [48]. The second piece of evidence came from experiments with the zero-length cross-linking agent formaldehyde. This compound, which couples amino groups arranged within 2 Å of each other [49], did not cross-link OpaA to vitronectin, whereas in the same experiment it did couple vitronectin multimers. These results suggest that there was no intimate contact between vitronectin and OpaA, which is consistent with the proposed mechanism of binding.

Central to the polysaccharide bridge model is the concept that two proteins interact with the same sulphated polysaccharide molecule. Lyon et al. [50] suggested a model for the interaction of transforming growth factor β dimers with heparin in which each monomer binds to distinct domains of the same heparin

molecule. Furthermore it has been postulated that heparin might act as a template on which vitronectin and type 1 plasminogen activator inhibitor interact directly [51], or on which vitronectin multimers can be assembled once bound to type 1 plasminogen activator inhibitor [52]. Heparin has also been shown to serve as a template for the interaction between thrombin and antithrombin III [53,54]. In this system, thrombin and antithrombin III bind independently to the same heparin molecule, forming an intermediate ternary complex. This is immediately followed by the formation of a stable thrombin–antithrombin III complex that is not dependent on the presence of the heparin template. Indeed, heparin is subsequently released and can be recycled [55]. In the template model, the addition of excess heparin should have no effect on the protein–protein interaction. The fact that bound vitronectin could be dissociated from OpaA–heparin–vitronectin complexes by the addition of excess heparin, and the absence of intimate contact between the two proteins, suggest that the enhancement of vitronectin binding to OpaA shown in this work seemed not to involve a temporary glycosaminoglycan template facilitating a direct protein–protein interaction. Thus the interaction between vitronectin and OpaA rather resembles a stable version of the intermediate step in the binding of thrombin to antithrombin III, requiring the physical presence of a sulphated polysaccharide for the association of the two proteins.

The biological significance of our findings is illustrated by the infection experiments with CHO cells. In these experiments, efficient vitronectin-mediated bacterial invasion was obtained only when the bacteria were preincubated with polysaccharide. Furthermore increasing the amount of vitronectin bound to the bacteria by using dextran sulphates of different sizes resulted in increased efficiencies of bacterial internalization, suggesting that vitronectin provided the primary signal for uptake. These experiments were performed with cells lacking the ability to synthesize glycosaminoglycans, which eliminated the cells as a source of sulphated polysaccharide. On the basis of our results it can be envisioned that in cells that carry heparan sulphate proteoglycans, these molecules, which have been demonstrated to interact with OpaA, serve as templates linking OpaA and vitronectin and its receptors.

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REFERENCES

1 Barnes, D. W., Reing, J. E. and Amos, B. (1985) J. Biol. Chem. **260**, 9117–9122
2 Hayman, E. G., Pierschbacher, M. D., Ohgren, Y. and Ruoslahti, E. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 4003–4007
3 Hayashi, M., Akama, T., Kono, I. and Kashiwagi, H. (1985) J. Biochem. (Tokyo) **98**, 1135–1138
4 Podack, E. R., Kolb, W. P. and Muller-Eberhard, H. J. (1978) J. Immunol. **120**, 1841–1849
5 Ill, C. R. and Ruoslahti, E. (1985) J. Biol. Chem. **260**, 15610–15615
6 Preissner, K. T., Zwicker, L. and Muller-Berghaus, G. (1987) Biochem. J. **243**, 105–111
7 Preissner, K. T., Grulich-Henn, J., Ehrlich, H. J., Declerck, P., Justus, C., Collen, D., Pannekoek, H. and Muller-Berghaus, G. (1990) J. Biol. Chem. **265**, 18490–18498
8 Wiman, B., Almquist, A., Sigurdardottir, O. and Lindahl, T. (1988) FEBS Lett. **242**, 125–128
9 Declerck, P. J., De Mol, M., Alessi, M. C., Baudner, S., Paques, E. P., Preissner, K. T., Muller-Berghaus, G. and Collen, D. (1988) J. Biol. Chem. **263**, 15454–15461
10 Seiffert, D. and Loskutoff, D. J. (1991) J. Biol. Chem. **266**, 2824–2830
11 Gebb, C., Hayman, E. G., Engvall, E. and Ruoslahti, E. (1986) J. Biol. Chem. **261**, 16698–16703
12 Izumi, M., Shimo-Oka, T., Morishita, N., Li, I. and Hayashi, M. (1988) Cell Struct. Funct. **13**, 217–225
13 Ishikawa, M. and Hayashi, M. (1992) Biochim. Biophys. Acta **1121**, 173–177
14 Hildebrand, A., Schweigerer, L. and Teschemacher, H. (1988) J. Biol. Chem. **263**, 2436–2441
15 Hynes, R. O. (1992) Cell **69**, 11–25
16 Ruoslahti, E. and Pierschbacher, M. D. (1987) Science **238**, 491–497
17 Wei, Y., Waltz, D. A., Rao, N., Drummond, R. J., Rosenberg, S. and Chapman, H. A. (1994) J. Biol. Chem. **269**, 32380–32388
18 Cherny, R. C., Honan, M. A. and Thiagarajan, P. (1993) J. Biol. Chem. **268**, 9725–9729
19 Zhou, Y. and Sane, D. C. (1993) Biochem. Biophys. Res. Commun. **192**, 575–582
20 Chhatwal, G. S. K., Preissner, K. T., Muller-Berghaus, G. and Blobel, H. (1987) Infect. Immun. **55**, 1878–1883
21 Liang, O. D., Maccarana, M., Flock, J.-L., Paulsson, M., Preissner, K. T. and Wadstrom, T. (1993) Biochim. Biophys. Acta **1225**, 57–63
22 McGavin, M. H., Krajewska-Pietrasik, D., Ryden, C. and Hook, M. (1993) Infect. Immun. **61**, 2479–2485
23 Limper, A. H., Standing, J. E., Hoffman, O. A., Castro, M. and Neese, L. W. (1993) Infect. Immun. **61**, 4302–4309
24 Ringner, M., Paulsson, M. and Wadstrom, T. (1992) FEMS Microbiol. Immunol. **5**, 219–224
25 Zareba, T. W., Pascu, C., Hryniewicz, W. and Wadstrom, T. (1997) Curr. Microbiol. **34**, 6–11
26 Bos, M. P., Grunert, F. and Belland, R. J. (1997) Infect. Immun. **65**, 2353–2361
27 Chen, T., Grunert, F., Medina-Marino, A. and Gotschlich, E. C. (1997) J. Exp. Med. **185**, 1557–1564
28 Gray-Owen, S. D., Dehio, C., Haude, A., Grunert, F. and Meyer, T. F. (1997) EMBO J. **16**, 3435–3445
29 Virji, M., Makepeace, K., Ferguson, D. J. P. and Watt, S. (1997) Mol. Microbiol. **22**, 941–950
30 van Putten, J. P. M. and Duensing, T. D. (1997) Rev. Med. Microbiol. (1997) **8**, 51–59
31 van Putten, J. P. M. and Paul, S. M. (1995) EMBO J. **14**, 2144–2154
32 Chen, T., Belland, R. J., Wilson, J. and Swanson, J. (1995) J. Exp. Med. **182**, 511–517
33 Duensing, T. D. and van Putten, J. P. M. (1997) Infect. Immun. **65**, 964–970
34 Gomez-Duarte, O. G., Dehio, M., Guzman, C. A., Chhatwal, G. S., Dehio, C. and Meyer, T. F. (1997) Infect. Immun. **65**, 3857–3866
35 Swanson, J., Barrera, O., Sola, J. and Boslego, J. (1988) J. Exp. Med. **168**, 2121–2129
36 Yatohgo, T., Izumi, M., Kashiwagi, H. and Hayashi, M. (1988) Cell Struct. Funct. **13**, 211–292
37 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
38 Esko, J. D. (1991) Curr. Opin. Cell Biol. **3**, 805–816
39 van Putten, J. P. M., Weel, J. F. L. and Grassme, H. U. C. (1994) Methods Enzymol. **236**, 420–437
40 Johnston, K. H., Holmes, K. K. and Gotschlich, E. C. (1976) J. Exp. Med. **143**, 741–758
41 Pannekoek, Y., van Putten, J. P. M. and Dankert, J. (1992) J. Bacteriol. **174**, 6928–6937
42 Wedege, E., Bryn, K. and Froholm, L. O. (1988) J. Immunol. Methods **113**, 51–59
43 Mandrell, R. E. and Zollinger, W. D. (1984) J. Immunol. Methods **67**, 1–7
44 Duckworth, M. and Yaphe, W. (1971) Carbohydr. Res. **16**, 189–197
45 Swanson, J. (1991) in Molecular Mechanisms of Bacterial Virulence (Kado, C. I. and Crosa, J. H., eds.), pp. 109–125, Kluwer Academic Publishers, Dordrecht
46 van Putten, J. P. M., Hayes, S. F. and Duensing, T. D. (1997) Infect. Immun. **65**, 5028–5034
47 Tomasini, B. R. and Mosher, D. F. (1988) Blood **72**, 903–912
48 Sane, D. C., Mosser, T. L., Parker, C. J., Seiffert, D., Loskutoff, D. J. and Greenberg, C. S. (1990) J. Biol. Chem. **265**, 3543–3548
49 Kunkel, G. R., Mehrabian, M. and Martinson, H. G. (1981) Mol. Cell. Biochem. **34**, 3–13
50 Lyon, M., Rushton, G. and Gallagher, J. T. (1997) J. Biol. Chem. **272**, 18000–18006
51 Gibson, A., Baburaj, K., Day, D. E., Verhamme, I., Shore, J. D. and Peterson, C. B. (1997) J. Biol. Chem. **272**, 5112–5121
52 Seiffert, D. (1997) J. Biol. Chem. **272**, 9971–9978
53 Griffith, M. J. (1982) J. Biol. Chem. **257**, 7360–7365
54 Olson, S. T. and Bjork, I. (1991) J. Biol. Chem. **266**, 6353–6364
55 Liang, O. D., Flock, J.-I. and Wadstrom, T. (1994) J. Biochem. **116**, 457–463
56 Dehio, M., Gomez-Duarte, O. G., Dehio, C. and Meyer, T. F. (1998) FEBS Lett. **424**, 84–88

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