Recognition of Saccharides by the OpcA, OpaD, and OpaB Outer Membrane Proteins from *Neisseria meningitidis*

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The adhesion of the pathogen *Neisseria meningitidis* to host cell surface proteoglycan, mediated by the integral outer membrane proteins OpcA and Opa, plays an important part in the processes of colonization and invasion by the bacterium. The precise specificities of the OpcA and Opa proteins are, however, unknown. Here we use a fluorescence-based binding assay to show that both proteins bind to mono- and disaccharides with high affinity. Binding of saccharides caused a quench in the intrinsic fluorescence emission of both proteins, and mutation of selected Tyr residues within the external loop regions caused a substantial decrease in fluorescence. We suggest that the intrinsic fluorescence arises from resonance energy transfer from Tyr to Trp residues in the β-barrel portion of the structure. OpcA bound sialic acid with a K<sub>d</sub> of 0.31 μM and was shown to be specific for pyranose saccharides. The binding specificities of two different Opa proteins were compared; unlike OpcA, neither protein bound to monosaccharides, but both bound to maltose, lactose, and sialic acid-containing oligosaccharides, with K<sub>d</sub> values in the micromolar range. OpaB had a 10-fold higher affinity for sialic acid-containing ligands than OpcA as a result of the mutation Y165V, which was shown to restore this specificity to OpaD. Finally, the OpcA- and Opa-dependent adhesion of meningococci to epithelial cells was shown to be partially inhibited by exogenously added sialic acid and maltose. The results show that OpcA and the Opa proteins can be thought of as outer membrane lectins and that simple saccharides can modulate their recognition of complex proteoglycan receptors.

The human pathogens *Neisseria gonorrhoeae* and *Neisseria meningitidis* are the causative agents of gonorrhea and meningococcal meningitis, respectively (1, 2). Models for the colonization of mucosal cells by *Neisseria* suggest that primary adhesion occurs through pili but that this encounter is followed by a second, closer range contact between outer membrane proteins and host cell surface receptors (3–5). There is evidence that two integral outer membrane proteins, Opa and OpcA, play a role in this process (5–7). OpcA (formerly called Opc) has a molecular weight of ~28,000 but is unrelated in sequence to the Opa proteins (8–11). It is expressed at high levels in some, but not all, meningococcal isolates and is a potent antigen (12). OpcA has been shown to mediate the invasion of epithelial and endothelial cells by nonencapsulated meningococci (13–16). It is able to bind to heparin in vitro and also to proteoglycan on the surface of epithelial cells (17). OpaB has been shown to bind in a complex with vitronectin and α<sub>1</sub>β<sub>3</sub> integrin (15), although the details of this interaction are not well understood. The function of OpaB as an adhesin is, therefore, well established. Homologues of OpaB have been identified in *N. gonorrhoeae* and the commensal bacterium *Neisseria polysaccharea*, although their functions in these organisms are not well defined (18, 19).

Multiple chromosomal copies of *opa* genes occur in *N. meningitidis*, *N. gonorrhoeae*, and other *Neisseria* species (5, 8, 9, 20, 21). Unlike OpcA, which has limited sequence variability (22), the Opa proteins are highly polymorphic (10). The sequence variability lies predominantly within two hypervariable (HV) regions and one semivariable (SV) loop region, which are predicted to be exposed on the outer surface of the bacterium. Two distinct classes of ligands have been identified for the Opa proteins (5). Binding of certain Opas to heparan sulfate glycosaminoglycans is associated with adhesion and uptake of *N. gonorrhoeae* into epithelial cells (5, 23–31); this interaction has been shown to be mediated by syndecan-1 and syndecan-4 (32). A gonococcal Opa protein, MS11-Opa<sub>300</sub>, can be bound and eluted from heparin-agarose in a similar fashion to OpaB (23). The second class of Opa ligands is the human CEACAM family of cell surface molecules, which are also specific for certain Opa variants (7); this is a protein-protein interaction that is not dependent on the glycosylation state of the CEACAM molecules (33) and is, therefore, distinct from glycosaminoglycan binding.

Although the binding of OpcA and Opa proteins to glycan ligands has been well described at the cellular level, there is little information currently available concerning the specificities of individual proteins for particular saccharide ligands. The crystal structure of OpcA has shown that the protein adopts a 10-stranded β-barrel structure, with extensive loop regions that protrude above the surface of the outer membrane (34). The five external loop regions pack together to form a deep crevice ~8 Å wide, 22 Å long, and 11 Å deep, which could form a binding site for saccharide ligands. There is currently no crystal structure of an Opa protein available, but the structure of a homologue, NapA, has been determined (35). The Opa proteins are predicted to form an 8-stranded β-barrel structure, with the hypervariable sequence regions corresponding to the surface-exposed loops. At present it is unclear how Opa proteins recognize oligosaccharide ligands or what effect the se-
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sequence polymorphism of the opa gene family might have on binding specificity. To address these questions we have developed a binding assay for OpaC and Opa proteins that makes use of the intrinsic fluorescence of each protein and have used this method to compare binding specificities for different saccharide ligands.

EXPERIMENTAL PROCEDURES

Materials—Saccharides were obtained from Sigma. LDAO (N,N-dimethyldecylamine-N-oxide) was from Fluka, n-Octylpentaoxyethylene (C₈E₅) was from Fluka, n-Octyl-β-D-glucopyranoside (BOD) was from Molecular Dimensions Ltd. Bacterial—The meningococcal derivatives used in adhesion experiments were from serogroup A strain C751 (Z2491) and included OpaB, OpaA, Opa’, and Opa’ (13). Epithelial Cells—Chang conjunctiva epithelial cells were cultured as described previously (36). Bacterial Growth Conditions and Measurement of Association with Epithelial Cells—Bacterial culture conditions and determination of their association with epithelial cells was carried out as described in Virji and Everson (37) and Virji et al. (36), with the exception that, to determine the effects of saccharides on the association of the meningococcal derivatives with conjunctiva epithelial cells, 15 cycles of 15 s bursts was used.

Expression of OpaA, OpaD, and OpaB—Selection of the N terminus of recombinant OpaC was guided by the crystal structure of the native protein (34); clear electron density was visible for the sequence starting at the sequence QTAN. Consequently, an oligonucleotide was designed to incorporate an initiator Met plus an NdeI restriction site, 5′-GGAGATCCGATATGCAAAAGCTTATGTTT-3′. The second primer comprised a BamHI site, 5′-GCCGATGCTGATCTTCTTAGATGATGGTTCATATCA-3′. The coding sequence for N. meningitidis OpaC was amplified from the original expression vector (38) and ligated into the NdeI and BamHI sites in PET22b. Coding sequences for OpaC from N. meningitidis and N. polysaccharea were amplified from a template kindly provided by Dr. P. Zhu (18, 19). The OpaC coding sequence was amplified by PCR from MC58 genomic DNA using the primers 5′-GGATCCGATATGCAAAAGCTTATGTTT-3′ and 5′-GGAAATCCGAGGCGGCCGAGCCCGACGGTG-3′ and 5′-GGAAATCCGAGGCGGCCGAGCCCGACGGTG-3′, which incorporate NdeI and XhoI restriction sites at the 5′ and 3′ ends, respectively. The primers were designed to remove the signal sequence and CTATT polymeric tract at the 5′ end of the gene. The PCR product was then digested with NdeI/XhoI and ligated into PET22b (Novagen). The same procedure was used for OpaB, except that the starting template DNA was a PCR product containing the OpaB coding sequence and amplified from genomic DNA from strain Z2491 using the method described by Hobbs et al. (39) (a kind gift from Dr. M. Callaghan). N. meningitidis OpaD was amplified from genomic DNA by PCR with primers incorporating a BamHI site, 5′-GAGATCCGCATATGCAAAAGCTTATGTTT-3′ and 5′-GATGATTTCAAATCA-3′. The PCR product was ligated into the NdeI and BamHI sites in PET22b (Invitrogen).

Purification of OpaA, OpaD, and OpaB—OpaC was refolded and purified as described by Prince et al. (38). Both OpaD and OpaB proteins were expressed as inclusion bodies in E. coli BL21*(DE3) (Invitrogen).

Purification of OpaA, OpaD, and OpaB—OpaC was refolded and purified as described by Prince et al. (38). Both OpaD and OpaB proteins were expressed as inclusion bodies in E. coli BL21*(DE3) (Invitrogen) in DYT medium. DYT medium consists of 16 g of tryptone, 10 g of yeast extract (both from Duchafla Biochem), and 5 g of NaCl per liter. Expression was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside to a concentration of 1 mM. The cells were harvested by centrifugation at 2700 × g for 30 min, both OpaD and OpaB were refolded by rapid dilution of the clarified supernatant into 50 mM Tris-HCl (pH 9.0) and 5% (v/v) LDAO and dialyzed overnight against 50 mM Tris-HCl (pH 9.0) and 5% (v/v) LDAO. The suspension was sonicated (15 cycles of 15 s bursts) and then stirred for 1 h at 4 °C. Inclusion bodies were isolated by sedimentation at 18,000 × g for 15 min, and the pellet was washed once with 50 mM Tris-HCl (pH 9.0). The pellet, containing insoluble OpaD or OpaB, was taken up in 16 μM guanidine HCl, in 50 mM Tris-HCl (pH 9.0). Insoluble material was removed by centrifugation at 30,000 × g for 30 min. Both OpaD and OpaB were refolded by rapid dilution of the clarified supernatant into 50 mM Tris-HCl (pH 9.0) and 5% (v/v) LDAO and dialyzed overnight against 50 mM Tris-HCl (pH 9.0) and 5% (v/v) LDAO. The dialyzed solution was applied directly to a HiPrep 16/10 Heparin FF (Amersham Biosciences) fast protein liquid chromatography column equilibrated in 50 mM Bis Tris propane (pH 7.0) with 0.1% (v/v) LDAO; the Opa proteins were eluted by a salt step using 50 mM Bis Tris propane (pH 7.0), 0.1% (v/v) LDAO, and 1 mM NaCl. OpaD or OpaB protein was precipitated by the addition of ethanol to 80% (v/v) to pooled fractions from the heparin column and stored at −20 °C for at least 4 h. The precipitate was then resuspended by centrifugation at 18,000 × g for 15 min. The protein pellet was dried in a vacuum desiccator for 1 h before being resublimed in 50 mM Bis Tris propane (pH 7.0), 5% (v/v) Zwittergent 3-14 (Calbiochem). A final purification step involved binding and elution from a HR 5/5 Mono S fast protein liquid chromatography column (Amersham Biosciences). The column was equilibrated in 50 mM Bis Tris propane (pH 7.0), 0.1% (v/v) Zwittergent 3-14, and Opa proteins were eluted by application of a salt gradient from 0 to 1 M NaCl in equilibration buffer. Fractions containing the Opa proteins were pooled and concentrated in a YM-50 Centricon (Millipore), and the protein was precipitated by the addition of ethanol to 80% (v/v). OpaD was shown to be folded by circular dichroism (not shown). The identities of purified OpaD and OpaB were confirmed by protease digestion and fragment analysis by matrix-assisted laser desorption at m/z with an on-line-offline spectroscopy. Protein concentration was determined by a modified Lowry assay against bovine serum albumin standards (40).

Site-directed Mutagenesis—Site-specific mutations were introduced by using the QuikChange mutagenesis kit (Stratagene) and following the manufacturer’s instructions.

Fluorimetric Titration Assay—The intrinsic fluorescence of OpaC, OpaD, and OpaB was used to develop a useful and robust fluorescence-based assay to measure the binding of different saccharides. Fluorescence spectra were produced using a Varian Cary Eclipse fluorescence spectrophotometer. Protein (OpaC, OpaD, or OpaB) was added to a final concentration of 10 μM/ml in 50 mM Bis Tris propane (pH 7.0) and 0.1% (v/v) octyl-β-D-glucopyranoside in a total volume of 3 ml (except where stated otherwise). The samples were excited at 280 nm, with the emission monitored at 306 nm with an excitation slit width of 5 nm and emission slit width of 2.5 nm. In all cases saccharide binding was observed as saturable quenching of the emission fluorescence intensity. None of the ligands studied had a significant absorbance at 280 nm within the concentration ranges used, and hence, the inner filter effect was negligible. A 5-min delay was included after each addition of ligand to allow uniform mixing of the solutions. The temperature was maintained at 25 °C using a Cary single cell Peltier accessory with continuous stirring.

Analysis of Fluorescence Quench Data—Data obtained from the assay were processed and fitted assuming the single-site equilbrium, L + R ↔ LR, where L, R, and LR are ligand, protein (OpaC/OpaA), and the ligand-protein complex, respectively. The model assumed that only a fraction of the observed fluorescence was accessible to quench by added ligand. The values of the equilibrium dissociation constant (K_D) and the fluorescence value at infinite ligand concentration (F_0) were fitted to experimental values of fluorescence and ligand concentration by a non-linear least squares regression method, as implemented in the program DYNAPFIT (41). Fluorescence quench can be analyzed by using the Stern-Volmer expression, F/F_0 = 1 + K_SV [Q], where K_SV is the Stern-Volmer quenching constant (lipid, in this case). In cases where a proportion of the observed fluorescence is not susceptible to quenching, a modified Stern-Volmer expression can be used, F/F_0 = 1/[Q][K_0] + 1/f_s, where f_s corresponds to the fraction of fluorescence that is susceptible to quench.

RESULTS

Changes in the intrinsic fluorescence of proteins have been widely used to measure the binding of small ligands. To examine whether this method was applicable to this group of outer membrane proteins, we set up an experiment to record the emission spectra of purified OpaC and OpaD from N. meningitidis. With an excitation wavelength of 280 nm, the emission spectrum has peaks at around 340 nm for both OpaC and OpaD from N. meningitidis strain MC58 (Fig. 1). Similarly, spectra were obtained with the OpaB protein from N. meningitidis strain Z2491 (not shown). The experiment was highly sensitive, with microgram quantities of protein providing a readily detectable signal for each of the outer membrane proteins examined. The spectra were sensitive to the addition of small saccharide ligands, with a partial quench detectable with titration of the ligand for each protein (Fig. 1). A variety of conditions were examined to identify conditions for optimal response to the binding of small ligands. The experiment was more sensitive when using an excitation wavelength of 280 nm rather than...
295 nm, which would favor Trp fluorescence. Titration of OpcA with sialic acid produced a reduction in total fluorescence emission, which could be fitted to a single, saturable equilibrium binding site model (Fig. 2A). Similar behavior was observed for the OpaD protein on titration with maltose (Fig. 3A). Both calculated equilibrium dissociation constant ($K_d$) values were below 1 μM, indicating high affinity binding. The stoichiometry of binding was investigated by replotting the data as log($F_o/F$) versus log[ligand] after the method of Chipman et al. (42) ($F_o$, $F_{\text{inf}}$, and $F$ correspond to the fluorescence values at zero, infinite, and all other ligand concentrations, respectively). Repplotting the data in this way produced a straight line, which was fitted by linear regression to give gradients of 0.96 and 1.1 for the OpcA and OpaD titrations in Figs. 2A and 3A, confirming a 1:1 stoichiometry in each case (not shown). Stern-Volmer plots of $F_o/F$ versus concentrations of ligand were non-linear for both the OpcA/sialic acid and OpaD/maltose data (Figs. 2B and 3B). Non-linearity arises because a proportion of the fluorescence is not susceptible to quenching under these conditions. Use of a modified Stern-Volmer expression showed that graphs of $F_o/F$ versus $1/[Q]$ were linear for both the OpcA and OpaD titration data (Figs. 2C and 3C), and the values of $f_s$ were deduced to be 0.44 and 0.43 for OpcA and OpaD, respectively.

A possible problem with the use of BOG as detergent in these assays could arise from competition for binding between the detergent and the saccharide ligands used. The $K_d$ values for sialic acid (for OpcA) and maltose (for OpaD) were, therefore, measured at several different concentrations of BOG. Measurements were also made using the polyoxyethylene detergent, C$_8$E$_5$, instead of BOG. $K_d$ values for sialic acid binding to OpcA did not rise with increasing BOG concentration, as would be expected if BOG were competing with sialic acid for binding. Furthermore, the $K_d$ for sialic acid binding to OpcA in 0.1% C$_8$E$_5$ was 1.2 μM, a value in the same range as that recorded in BOG. Similarly, the $K_d$ for maltose binding to OpaD in 0.1% C$_8$E$_5$ was 1.9 μM. It was, therefore, clear that the low concent-
In an attempt to identify the origin of the observed fluorescence from OpcA, the crystal structure was examined for Trp and Tyr residues that could plausibly account for a ligand-dependent fluorescence response. Of the four Trp residues in OpcA, three (137, 153, and 189) form part of the aromatic "girdle" of side chains that protrude into the hydrophobic portion of the lipid bilayer (Fig. 4A). The fourth (158) is also part of the β-barrel but points inward toward the water-filled channel within the protein. None of the side chains from these residues would be likely to form a close contact with a ligand binding to the exterior surface of the protein. We, therefore, considered the possibility that one or more Tyr residues could contribute to the ligand-responsive fluorescence signal from the protein. Of the seven Tyr residues in OpcA, five are associated with the β-barrel portion of the structure, in a similar fashion to the Trp residues. The remaining two, Tyr-169 and Tyr-218, constitute more likely sites for interaction with ligands (Fig. 4B). A closer examination of the environment surrounding these two Tyr residues reveals that they form part of a pocket of hydrophobic residues formed by association of Leu-66, Leu-81, Pro-82, Ile-123, Leu-171, and Ile-237 from loops 2–5 (Fig. 4C) (34). To assign the source of the fluorescence from OpcA, Y169F and Y218F mutations were introduced into OpcA, and the fluorescence spectra of these mutated proteins were compared with wild type (Fig. 5A). Y218F produced a fluorescence emission signal reduced by about 30% compared with wild type protein. The effect of the Y169F mutation was much greater, however, at about 64%. Repetition of the same fluorescence binding experiment using the OpcA homologues from N. gonorrhoeae and N. polysaccharea (18, 19) produced a fluorescence response that was essentially identical to the Y169F mutant protein. Positions 169 and 218 are occupied by Leu and Phe in the gonococcal OpcA, and the low fluorescence emission from this protein failed to respond to the addition of ligand. OpcA from the commensal organism N. polysaccharea also has non-Tyr residues at these positions but has a Tyr residue at position 217 in the sequence, and it was possible to measure ligand-dependent quenching of the fluorescence from this protein (see below).
Tyr fluorescence emission is usually observed in proteins without Trp residues and peaks at a shorter wavelength than Trp fluorescence, around 303 nm (43, 44). In Trp-containing proteins, Tyr fluorescence is generally quenched by neighboring Trp residues. In some cases resonance energy transfer between Tyr and Trp has been observed, with the result that the fluorescence emission peaks at a wavelength typical of Trp residue emission, at about 340 nm (45). The efficiency of energy transfer depends, among other parameters, on the orientation of the two dipoles and the Förster distance between them, which would be ~10–15 Å for Tyr and Trp (45). The distances from the Cβ atom of Tyr-169 to the Cβ atoms of Trp-137 and Trp-189 are 15.2 and 16.6 Å, respectively. For Tyr-218, the equivalent distances are 13.5 and 8.8 Å. Trp-137 and Trp-189 lie at the top of the β-barrel and are, therefore, closest to the external loop regions. On distance criteria alone, it is plausible to invoke a resonance energy transfer process between these groups of residues to explain the fluorescence emission observed at 340 nm. The apparent quench in fluorescence observed on titration of OpcA with sialic acid and other saccharides could be attributed either to a change in the efficiency of the resonance energy transfer process or a direct quench of the fluorescence of the individual Tyr residues.

In these experiments it was found to be essential to use concentrations of BOG below its reported critical micelle concentration of 0.7% (46) to observe a fluorescence response to ligands as seen in Figs. 2 and 3. The fluorescence emission intensity of OpcA was examined in response to increasing concentrations of BOG; little change was seen up to a value of 0.5%, but the fluorescence increased steadily with BOG concentrations thereafter (data not shown). Our interpretation of this observation is that formation of BOG micelles around OpcA introduces a more hydrophobic environment for the Trp residues which protrude outwards from the β-barrel.

In a similar approach to that adopted for OpcA, selected Tyr residues in the OpaD protein were mutated to Phe. Although no crystal structure for an Opa protein has been reported, the structure of a homologue, NspA, has been determined (35). From the predicted secondary structural arrangement of OpaD, several candidate Tyr residues were identified, mutations were introduced, and the effects on fluorescence were evaluated (Figs. 5, B and C). The effect of the mutation Y165V was examined because Val occurs at this position in the majority of opa gene sequences. It is interesting that, although Y165V shows a reduction in fluorescence of more than 80%, the effect of the Y165F mutation is much more modest, with a reduction of around 40%. Mutation of Tyr-32, Tyr-49, and Tyr-88 to Phe produced reductions of 30–40% in the fluorescence emission peak, but the largest effect was for Y189F, which showed a 70% reduction. The situation with regard to the origin of fluorescence in OpaD is, therefore, somewhat more complicated than for OpcA. We suggest that the side chains of all the Tyr residues are structured so that mutation of any one affects the packing of the other loop regions, and therefore, the fluorescence emission from the other Tyr residues. The greater effect of the Y165V mutation compared with Y165F could be explained if Tyr-189 and Tyr-165 lie in close proximity within the three-dimensional structure, a proposal that is supported by observations on the effects of these mutations on binding specificity.

The binding affinities for the sialic acid (2–8) dimer (Neu(2–8)Neu) and maltose are compared in Fig. 6 for wild type OpaD, OpaB, and the range of Tyr mutant proteins generated from OpaD. OpaD has a lower affinity for the sialic acid (2–8) dimer than for maltose, but for OpaB the situation is reversed; the $K_d$ for maltose is the same within experimental error, but affinity for the sialic acid (2–8) dimer is ~10-fold higher. A comparison of the sequences of OpaD and OpaB showed that they differed by 25 residues within the HV1 and HV2 regions and by a further 8 residues outside these regions. The fact that position 165 is more frequently occupied by a Val than a Tyr residue in opa gene sequence alignments led us to analyze the effects of

![FIG. 5. Fluorescence emission spectra of OpcA and OpaD with specific mutations in selected Tyr residues. A, OpcA mutants. B and C, OpaD mutants. Protein concentrations were 10 µg/ml for all spectra. Detector voltages were set at 1000 V for OpcA experiments and 950 V for Opa experiments. wt, wild type.](http://www.jbc.org/content/372/26/31493.full)
the Y165V mutation on OpaD. Interestingly, this mutation was found to confer the higher binding affinity for the sialic acid (2–8) dimer on OpaD, which is observed for OpaB (Fig. 6). This result demonstrates that a single site mutation can modify the binding specificity of an Opa protein for a chemically defined saccharide ligand. Maltose binding affinities were largely unaffected by these sequence changes and are presumably dependent on interactions with residues that are conserved between OpaD and OpaB.

The specificity of meningococcal OpaA was examined for a variety of monosaccharide ligands. It was clear that OpaA is specific for pyranose sugars; fructose had a $K_d$ of 2.3 mM, but D-glucose is able to bind with a reasonably high affinity ($K_d = 13 \mu M$). D-Glucosamine, D-galactose, and D-fucose had $K_d$ values in the range 13–30 $\mu M$, but the specificity of the interaction was indicated by the fact that D-glucose bound with a $K_d$ value of 63 $\mu M$, −5-fold weaker than D-glucose. Interestingly, OpaA from the commensal organism N. polysaccharea bound D-glucose, D-fucose, and sialic acid much more weakly than the meningococcal protein ($K_d$ values of 75, 1200, and 55 $\mu M$, respectively). This observation suggested that at least some of the residues involved in saccharide recognition in OpaA are located in regions of the protein that differ in sequence between the meningococcal and polysaccharea proteins; loop 2, for example (18).

The binding of a range of analogues of sialic acid was also examined, including N-acetyl-2,3-dehydro-2-deoxyneuraminic acid ($K_d \approx 4.0 \mu M$); 2-O-methyl sialic acid ($K_d = 3.3 \mu M$), and the N-acetylmethyl ester ($K_d = 3.1 \mu M$); the results indicate that recognition of sialic acid is likely to be mediated by a range of functional groups.

Previous studies on saccharide binding by OpaC and Opa proteins have focused on their binding to complex oligosaccharides, such as heparin and heparan sulfate. The binding of a range of heparin-derived ligands (e.g. α-ΔUA- (1–4)-GlcN-6S) were measured using the intrinsic fluorescence assay described here. They were found to bind with $K_d$ values in the micromolar range, although binding affinity did not depend on the degree of sulfation (data not shown). To examine this binding phenomenon further, a fluorescamine-coupled disulfated heparin disaccharide ligand was synthesized using the method of Cesur et al. (47). The coupled ligand was shown to bind to OpaC and OpaD by measurement of the fluorescence emission at 480 nm of the coupled fluorescamine when excited at 390 nm. The fluorescence emission intensity was found to rise with increasing protein concentration, with saturation occurring in the same concentration range as observed with the intrinsic fluorescence assay (data not shown). Competition of the bound fluorescamine-coupled ligand was attempted with increasing concentrations of sialic acid up to 1 $\mu M$, but no inhibition of fluorescence was observed. Furthermore, attempts to elute OpaC or OpaD with sialic acid or maltose from a heparin-Sepharose matrix were unsuccessful. The most likely explanation for these results is that heparin-like ligands, with one or more sulfate groups, bind non-specifically or to multiple sites on OpaC and OpaD.

The crystal structure of OpaA identified a crevice-like feature, caused by association of the external loop regions of the protein, which constituted a plausible binding site for proteoglycan ligands. Mutation of residues in and around the crevice (K27A, K29A, K31A, K61A, V85A, K239A) produced only modest rises in the $K_d$ for sialic acid in the corresponding mutants (less than 3-fold; data not shown). If these residues contribute to sialic acid binding, their contributions to the binding energy are relatively modest, at least individually. The mutations Y169F and Y218F also resulted in modest rises in $K_d$ values of around 3-fold, suggesting that the phenolic hydroxyl groups do not play a major role in sialic acid recognition.

A comparison of the binding affinities of OpaA, OpaD, and OpaB for a range of oligosaccharide ligands is shown in Fig. 7. Maltose and related ligands such as maltotriose (Glc (1–4)Glc) and maltotetrose (Glc (1–4)Glc) bound with binding constants in the micromolar range to all three proteins. Although OpaA was able to bind pyranose saccharides with remarkably high affinity, we were unable to identify any monosaccharides that elicited a response in fluorescence from OpaD or OpaB. Galactobiase and lactose were good ligands for OpaC and OpaD, and their binding affinities were not greatly dependent on the nature of the linkage between the two monosaccharide units. The principal difference between the binding specificity of OpaB and OpaD lies in the affinity for sialic acid-containing oligosaccharides. OpaA appears to bind any oligosaccharide that terminates in a sialic acid unit irrespective of the nature of the linkage to the adjacent saccharide in the chain. This is not the case for OpaD, however; sialyl-(2–6)-lactose (Neu (2–6)Gal(1–4)Glc) had a high affinity for all three proteins, but OpaD bound more weakly to sialyl-(2–3)-lactose (Neu (2–3)Gal(1–4)Glc). Both OpaD and OpaA bound to more complex oligosaccharides such as the blood group saccharides A-trisaccharide, B-trisaccharide, H-trisaccharide, sialyl Lewis a, and sialyl Lewis x, with $K_d$ values from 0.8 to 5 $\mu M$ (not shown). $K_d$ values of between 8 and 39 $\mu M$ were also recorded for binding of OpaD to the antibiotics gentamycin, kanamycin, and hygromycin, presumably mediated by recognition of the saccharide portion of these molecules.

The results reported above show that mono- and disaccharides can bind to OpaC and OpaA proteins with high affinity. If this interaction originates from the same binding site as that for cell surface proteoglycan, it should be possible to reduce the adhesion of meningococci to epithelial cells competitively by the addition of particular saccharides. Previous work has shown that OpaC- and Opa-dependent binding to epithelial cells can be competitively displaced by heparin (17, 48). To determine the extent to which this interaction is mediated by simpler saccharide ligands, we investigated the effect of sialic acid, which binds to OpaA with high affinity, and fructose, which does not appear to bind well, on adhesion of meningococci to epithelial cells. Little effect on adhesion was seen at low concentrations of sialic acid, but at 10 and 20 $\mu M$, up to 50% inhibition of binding was observed (Fig. 8A). Binding was unaffected by fructose within the same concentration range (Fig. 8B). In other experiments the effects of maltose and glucose on Opa-dependent binding to Chang cells were compared (Fig. 8, C and D). Lower concentrations of maltose gave an initial rise in...
**FIG. 7.** Comparison of equilibrium dissociation constants for binding of a range of complex oligosaccharides to OpcA (black), OpaD (white), and OpaB (gray). Error bars represent 95% confidence limits, as calculated by DYNAFIT (41).

**FIG. 8.** Effect of different saccharides on OpcA- and Opa-dependent adhesion of meningococci to epithelial cells. Adhesion of *N. meningitidis* C751 OpcA⁻Opa⁻ derivative (A and B) or OpaB⁺OpcA⁻ (C and D) to Chang cells is shown at different saccharide concentrations. Saccharides used were sialic acid (A), fructose (B), maltose (C), and glucose (D). Results shown are from one typical experiment (n = 3 ± S.E.).
adhesion, perhaps due to the use of the saccharide as a metabolic substrate that stimulated growth. Higher concentrations of maltose showed a similar pattern of inhibition to those observed for OpaA/sialic acid. Adhesion was unaffected within the limits of experimental error for the control saccharide glucose-6-phosphate. Various lines of evidence suggest that electrostatic interactions do not play an important part in the binding of ligands by OpaB. The results presented in Fig. 7 suggest broad-based specificities for all three proteins for GlcNAc and Gal-containing oligosaccharides, to a large extent independent of linkage between adjacent saccharides and not greatly dependent on N-acetylation or sulfation. This observation carries the obvious caveat that the ligand binding behavior of a detergent-solubilized outer membrane protein may be different from its counterpart located in situ within the outer membrane. Nevertheless, the results establish that the apparently complex binding behavior of OpaA is reducible to the recognition of simple monosaccharides. In particular, the high affinity binding of sialic acid is in good agreement with the observation of de Vries et al. (17), that sialylation of LPS can modulate attachment of meningococci to soluble proteoglycan receptor. Our results provide further evidence for this hypothesis by demonstrating that OpaA is able to bind to a wide range of saccharides that have a terminal sialic acid. Interestingly, the affinity of more complex saccharides does not increase with the size of the ligand, suggesting that the binding site may only accommodate one sialic acid moiety.

Our observations have pointed to the involvement of Tyr-165 and Tyr-189 in saccharide recognition by OpaD; these residues are predicted to be located at the base of the HV2 loop in a model of an Opa structure based on the crystal structure of the homologue NspA (35). Grant et al. (31) showed that transfer of the HV1 region of OpaA from N. gonorrhoeae to MS11mk was sufficient to confer proteoglycan binding on to a non-binding OpaB from the same organism. Our results do not preclude an important role for HV1, although Tyr-165 and Tyr-189 are located in HV2. Tyr-165 appears to be important for sialic acid recognition rather than maltose binding, so mutation at this site may not alter binding to heparin and related ligands. Although Tyr-189 appears to be important for the intrinsic fluorescence emission of the protein, we were unable to ascertain unambiguously whether mutation of this residue caused a reduction in binding affinity because the corresponding mutant protein (Y189P) exhibited low fluorescence emission that was unresponsive to the addition of any ligands.

The observation that OpaD and OpaB differ in their saccharide binding specificities, albeit in a subtle manner, suggests that Opa sequence variation could at least in part contribute to different specificities of Opa proteins. It is well established that different Opas can differentiate between different CEACAM molecules (5, 48). It is presumed that Opa sequence variation is driven by immune selection, a process that is better understood in the case of the Neisseria porins PorA and PorB (49, 50). The example of the Y165V mutation, which appears to confer a higher binding affinity for sialic acid on OpaD, demonstrates that specific sequence changes can indeed modulate saccharide binding specificity and, therefore, could possibly also influence tissue adhesion tropism.

The $K_d$ values of OpaA and the Opas for mono- and disaccharides lie in the micromolar range, values that can be considered relatively high affinity compared with, for example, the fimbrial adhesins, where dissociation constants of around 1 mM have been reported (51). Interestingly, the affinity of the bacterial tetanus toxin Hc fragment for ganglioside receptors lies within the micromolar range; in this case the effect of multivalency or “cross-linking” of a ligand between adjacent subunits of the toxin has been proposed to explain the much higher affinity observed for these ligands when studied in whole cells (52). One possibility is that multiple copies of Opa or OpaA proteins within the outer membrane could provide the same effect by binding to the same, extended polysaccharide chains.

The results in Fig. 8 show that mono- and disaccharides can influence OpaA- or Opa-dependent adhesion of meningococci to epithelial cells. It was not possible to completely inhibit adhesion by competition, however, as is the case with heparin (17, 48). The results suggest that the binding of heparin to OpaA and OpaB may have two components, one of which is to a small, high affinity saccharide binding site located within the external loop regions of either protein. The presence of other sites for heparin binding is also suggested by the observation that we were unable to compete heparin binding to OpaA effectively with exogenously added sialic acid. Clearly, elucidation of these secondary sites will be required for a complete description of saccharide binding by OpaA and the Opa family of outer membrane proteins.

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