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Common Antigenic Domains in Transferrin-Binding Protein 2 of *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *Haemophilus influenzae* Type b

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There is now considerable evidence to show that in the *Neisseria* and *Haemophilus* species, membrane receptors specific for either transferrin or lactoferrin are involved in the acquisition of iron from these glycoproteins. In *Neisseria meningitidis*, the transferrin receptor appears to consist of two proteins, one of which (TBP 1) has an M_r of 95,000 and the other of which (TBP 2) has an M_r ranging from 68,000 to 85,000, depending on the strain; TBP 2 binds transferrin after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotting, but TBP 1 does not do so. The relative contributions of these two proteins to the binding reaction observed with intact cells and to iron uptake are presently unknown. However, they are being considered as potential components of a group B meningococcal vaccine. Analogous higher- and lower-molecular-weight proteins associated with transferrin binding have been found in *N. gonorrhoeae* and *Haemophilus influenzae*. Previous work with polyclonal antibodies raised in mice with whole cells of iron-restricted *N. meningitidis* showed that the meningococcal TBP 2 exhibits considerable antigenic heterogeneity. Here, we report that antiserum against purified TBP 2 from one strain of *N. meningitidis* cross-reacts on immunoblotting with the TBP 2 of all meningococcal isolates examined, as well as with the TBP 2 of *N. gonorrhoeae*. This antiserum also cross-reacted with the TBP 2 of several strains of *H. influenzae* type b, thus showing the presence of common antigenic domains among these functionally equivalent proteins in different pathogens; no cross-reaction was detected with a purified sample of the human transferrin receptor.

Pathogenic bacteria which multiply successfully in body fluids to establish extracellular infections have evolved high-affinity uptake systems which allow them to compete effectively with the host iron-binding glycoproteins, transferrin or lactoferrin, for essential iron (6, 8, 17, 35). The best-understood systems whereby bacterial pathogens assimilate iron from these iron-binding proteins are those which depend on the production of siderophores. However, some pathogens, such as *Haemophilus* and *Neisseria* species, use siderophore-independent receptor-mediated iron uptake systems which involve a direct interaction between the bacterial cell surface and the iron-binding protein. These systems are now attracting considerable attention, and they are distinguished from the known siderophore-mediated mechanisms by their high degree of specificity for the iron-binding glycoproteins (19, 20, 27, 28). This has implications for explanations of host specificities of these pathogens and for the development of animal models (25).

Although the molecular mechanism of iron uptake in such organisms is not understood, there is considerable evidence to show that saturable membrane receptors specific for either transferrin or lactoferrin are involved (4, 18, 22, 27, 28, 32). These transferrin and lactoferrin receptors are distinct entities, and their expression has been shown to be regulated by iron. However, the biochemical identities of the receptors remain unclear, although lactoferrin- and transferrin-binding proteins have been identified among the several iron-regulated proteins found in the envelope of *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *Haemophilus influenzae* (3, 12, 20, 24, 26–28, 32). In *N. meningitidis*, the transferrin receptor appears to consist of two proteins, both of which can be isolated from intact membranes by affinity procedures with biotinylated human transferrin (23, 26, 28). One of the receptor-associated proteins, which has an M_r of about 95,000, is unable to bind transferrin after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and has recently been referred to as transferrin-binding protein 1 (TBP 1) (23), although there is still a need to prove conclusively that it can bind transferrin. The other protein is capable of binding to human transferrin after SDS-PAGE and electroblotting and corresponds to the protein discussed in the present paper; for consistency, we will call this protein transferrin-binding protein 2 (TBP 2). The relative contributions of these two proteins to the binding reaction observed with intact cells are unknown. Of interest is the considerable molecular and antigenic heterogeneity of TBP 2 in different clinical isolates of *N. meningitidis* (2, 7, 10). Most strains examined have a TBP 2 with an M_r of between about 78,000 and 85,000, but a few have a binding protein with an M_r of about 68,000. The size of the protein seems unrelated to the serogroup or serotype of the organism. Analogous higher- and lower-molecular-weight proteins associated with transferrin binding have been found in *N. gonorrhoeae* and *H. influenzae*.

There is currently considerable interest in incorporating transferrin-binding and other iron-regulated membrane proteins into protein-based vaccines against group B meningococcal disease (3). If these proteins are to play a useful role in such vaccines, and especially, if the vaccines are to be broadly cross-protective, then their design must be based on a thorough understanding of the antigenic structures and

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TABLE 1. Strains of *N. meningitidis* examined in this study[a]

Serogroup or serotype	Total no. examined
B15P1.16	6
B15P1.15	1
B4P1.15	1
B nontypeable P1.15	1
B nontypeable	1
B2a P1.2	3
B2a	3
B2b	3
C2b	4

[a] Strains were obtained from D. M. Jones, Public Health Laboratory, Withington Hospital, Manchester, United Kingdom; R. A. Wall, Clinical Research Centre, Northwick Park Hospital, Harrow, United Kingdom; I. Thangkhiew, Pathology Department, St. Cross Hospital, Rugby, United Kingdom; and C. E. Frasch, Center for Biologics Evaluation and Research, Bethesda, Md.

expression of the protein antigens involved. Previous work with polyclonal antibodies raised in mice to whole cells of iron-restricted *N. meningitidis* showed TBP 2 to exhibit antigenic heterogeneity (10), which appears to make it a less than promising vaccine candidate. In the present study, we isolated TBP 2 from one strain of *N. meningitidis* by preparative SDS-PAGE and used the antiserum raised to this denatured preparation to seek common antigenic domains among the transferrin-binding proteins from other meningococcal strains; possible cross-reactivity with analogous proteins in *N. gonorrhoeae* and *H. influenzae* was also investigated.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains of *N. meningitidis* used in this study are listed in Table 1. The *N. gonorrhoeae* strain used (strain 2211) was provided by I. Brown, St. Mary's Hospital, London, United Kingdom. This strain was a recent clinical isolate. The strains were stored at −70°C in Mueller-Hinton broth containing glycerol (10% [vol/vol]). Iron-regulated membrane proteins were induced in *N. meningitidis* by growing the bacteria in Mueller-Hinton broth containing ethylenediaminedihydroxyphenylacetic acid (EDDA) (40 μM; Sigma) as described before (10). *N. gonorrhoeae* was grown in the following medium: proteose peptone no. 3 (15 g/liter; Difco Laboratories), K_2HPO_4 (4 g/liter), KH_2PO_4 (1 g/liter), NaCl (5 g/liter), and soluble starch (1 g/liter). Following autoclaving, the medium was supplemented with $NaHCO_3$ (0.42 g/liter), glucose (0.5% [wt/vol]) and IsoVitaleX (1% [vol/vol]; BBL Microbiology Systems). Iron-regulated membrane proteins were induced by growing the bacteria in the liquid medium described above containing desferrioxamine mesylate (25 μM) (Desferal; CIBA Laboratories). The growth conditions used were as described previously for *N. meningitidis* (10), except that the incubation in medium containing Desferal was carried out for 22 h.

The *H. influenzae* type b strains RM926 (NCTC 8468 or ATCC 9335) (14), Eagan, and RM7004 used in this study were kindly provided by J. S. Kroll, Department of Pediatrics, University of Oxford, John Radcliffe Hospital, Oxford, United Kingdom. Strains NU20 and NU698 were obtained from K. J. Towner, Department of Microbiology and PHLS Laboratory, University Hospital, Nottingham, United Kingdom. These organisms were originally isolated from patients with meningitis in the United Kingdom (NU20 and NU698), the Netherlands (RM7004), and the United States (Eagan and RM926). Haemophili were grown routinely on chocolate agar plates and stored at −70°C in brain heart infusion broth containing glycerol (10% [vol/vol]). For the isolation of transferrin-binding proteins, *H. influenzae* was grown overnight at 37°C, with aeration, in brain heart infusion broth supplemented with 2.0 μg of NAD and 0.5 μg of protoporphyrin IX per ml. Iron restriction was achieved by the addition of 100 μM EDDA (19).

Extraction and electrophoresis of total membrane proteins. Cell envelopes from *N. meningitidis* and *N. gonorrhoeae* were isolated as described by Griffiths et al. (10), but with the addition of an extra centrifugation step (3,000 × *g*, 4°C, 15 min) following formation of the spheroplasts to remove any remaining whole cells. The protease inhibitor Benzamidine (Sigma) was included at a final concentration of 10 mM during preparation of the spheroplasts. The meningococcal membrane proteins were solubilized as described by Laemmli (15) at 100°C for 5 min, and SDS-PAGE was performed as described by Lugtenberg et al. (16). As described before, electrophoresis was carried out for an extended period of time to improve resolution of the iron-regulated membrane proteins (11). Following electrophoresis, the proteins were transferred to nitrocellulose paper by electroblotting (29). Blots were used either for the detection of antibody-antigen interaction (immunoblotting) or for the detection of the human transferrin-binding protein or were stained to detect transferred proteins with the AuroDye forte kit (Janssen Life Science Product). Staining with AuroDye was carried out according to the manufacturer's instructions. When more than one of these procedures were carried out on proteins transferred from the same SDS-PAGE gel, the nitrocellulose paper was cut into the appropriate pieces following electrophoretic transfer by using pinking shears. This allowed easier matching of the pieces after treatment (11).

For examination of lipooligosaccharide (LOS), whole-membrane preparations were digested with proteinase K (Sigma) at a membrane protein mass/enzyme ratio of 10:2 in SDS-PAGE solubilizing buffer (60°C, 1 h); after digestion, the preparation was incubated at 100°C for 10 min prior to electrophoresis and transfer to nitrocellulose paper.

Affinity isolation of transferrin-binding proteins. Transferrin-binding proteins were isolated from *H. influenzae* essentially as described by Schryvers (24). Briefly, haemophili were sonicated on ice and whole-cell envelopes were collected by centrifugation (38,000 × *g* for 60 min) and resuspended to give approximately 5 mg of protein per ml in 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl (TN100). A 25-μl volume of a 1-mg/ml solution of human transferrin labelled with *N*-hydroxysuccinimido-biotin as described previously (20) was added to 1 ml of cell envelope preparation and incubated with gentle shaking for 60 min at 37°C. Cell envelopes were recovered by centrifugation and resuspended in 0.9 ml of TN100, to which 100 μl of 20% (wt/vol) Sarkosyl (Sigma) containing 100 mM EDTA was added. Following incubation at room temperature for 10 min, insoluble material was removed by centrifugation and a 1:2 dilution of streptavidin-agarose beads (Sigma) was added to the supernatant which contained the transferrin-binding protein-biotinylated transferrin complexes. After 60 min of incubation at room temperature with gentle shaking, the beads were removed by centrifugation, collected, and washed twice with each of the following buffers: (i) 50 mM Tris-HCl (pH 8.0) containing 1 M NaCl, 0.5% (wt/vol) Sarkosyl, and 2.5 mM EDTA; (ii) 50 mM Tris-HCl (pH 8.0) containing 1 M NaCl; and (iii) TN100. The agarose beads

were finally resuspended in 40 to 60 μl of SDS-PAGE sample buffer without 2-mercaptoethanol, incubated for 30 min at 37°C, and collected by centrifugation, and the supernatants were loaded onto SDS-10% (wt/vol) polyacrylamide gels.

Affinity isolation of transferrin-binding proteins from *N. meningitidis* and *N. gonorrhoeae* was performed as described above, except that for the initial step, 25 μl of a solution containing 1.6 mg of Biotin-SP-conjugated ChromPure Human Transferrin (Jackson Immunoresearch Laboratories) per ml was added to 1 ml (1 to 3 mg of protein per ml) of a suspension of cell envelopes. These were prepared as described earlier, except that TN100 buffer was used both for the final wash and for resuspending the final cell envelope preparations. SDS-PAGE was performed as described before for total-membrane proteins of *N. meningitidis*.

Detection of antigen-antibody complexes. Immunoblotting of *Neisseria* antigens was performed as described previously (29), except that in some experiments skim milk powder (Marvel) at 1 to 5% (wt/vol) in Tris-buffered saline (20 mM Tris-HCl [pH 7.6], 137 mM NaCl) (TBS) was used for blocking. TBS was also used for the washing steps. The antigen-antibody complexes were detected by using: ^{125}I-labelled second antibody [goat anti-mouse F(ab')$_2$, kindly supplied by R. Thorpe, National Institute for Biological Standards and Control, or goat anti-rabbit immunoglobulin G, Miles Laboratories) as described previously (29) or by using a horseradish peroxidase (HRP)-conjugated second antibody (HRP anti-mouse immunoglobulin G [whole molecule] or HRP anti-rabbit immunoglobulin G [whole molecule]; Sigma). Following incubation in blocking solution (1 to 5% milk powder [wt/vol] in TBS) containing the HRP-conjugated second antibody (anti-mouse, 1:2,000 dilution; anti-rabbit, 1:5,000 dilution) for 4 h at room temperature, the nitrocellulose paper was washed over a period of 1 h with several changes of TBS and then developed for 10 min in a solution of 4-chloro-1-naphthol (0.5 mg/ml; Sigma) in 0.014% (vol/vol) hydrogen peroxide (BDH)–25 mM Tris-HCl (pH 7.4). *Haemophilus* antigen-antibody complexes were detected with protein A-peroxidase (2.5 μg/ml; Sigma). Blots were developed as described above.

Detection of transferrin-binding protein. Following electrophoretic transfer, the transferrin-binding protein was detected either, as described previously (10), with ^{125}I-labelled human transferrin or by using peroxidase-conjugated human transferrin (Jackson Immunoresearch Laboratories). In the latter method, the nitrocellulose paper was blocked by incubation for 1 to 2 h in 5% (wt/vol) skim milk powder in TBS. This solution was then discarded, and fresh blocking solution containing the peroxidase-conjugated human transferrin was added (2.5 μg/ml). Following overnight incubation at room temperature, the nitrocellulose paper was washed over a period of 1 h with several changes of TBS and then developed by using 4-chloro-1-naphthol as described in the previous section.

Preparation of antisera. Antiserum raised against live iron-restricted whole cells of *N. meningitidis* 70942 (B15P1.16) or B16B6 (B2a P1.2) was as described previously (10). Antiserum against the purified 68-kDa TBP 2 of strain B16B6 was raised in a female Dutch rabbit by giving 3 subcutaneous injections (two sites) of the protein in Freund's incomplete adjuvant at intervals of 3 weeks. Material obtained from three gels (SDS-PAGE) was used for each injection. The antiserum was obtained 13 days following the third injection and stored at −20°C. It was adsorbed with envelope preparations of *N. meningitidis* B16B6 grown in Mueller-Hinton broth.

Human transferrin receptor. A sample of the purified human (placental) transferrin receptor was kindly provided by J. Williams, Department of Biochemistry, University of Bristol, Bristol, England. The sample gave a single band in the expected position of a 180-kDa protein on electrophoresis under nondenaturing conditions.

RESULTS

Purification of the meningococcal 68-kDa transferrin-binding protein. One strain of *N. meningitidis* group B2a P1.2 (B16B6, from C. E. Frasch) was chosen as the source of TBP 2, since the protein in this strain (M_r, 68,000), being well removed from obvious contaminants following electrophoresis, appeared to be the most accessible for extraction from an SDS-PAGE gel. The antigen used was prepared from total-membrane proteins of strain B16B6 grown in Mueller-Hinton broth containing EDDA as described previously (10). The 68-kDa protein was isolated by preparative SDS-PAGE essentially as described by Chart and Griffiths (5). Membranes were solubilized by heating at 60°C for 30 min in buffer consisting of 0.06 M Tris (pH 6.8), SDS (3% [wt/vol]), glycerol (10% [vol/vol]), and bromophenol blue (0.001% [wt/vol]). The mobility of the 68-kDa protein was not altered by the solubilization conditions. Total-membrane protein preparations (approximately 1-mg amounts) were applied to a well extending the whole width of the gel, and electrophoresis was carried out for an extended period of time. Benzamidine (10 mM) was included in the buffer used to elute the protein from the acrylamide macerate.

Traces of breakdown products of the isolated 68-kDa protein were visible on AuroDye staining, following electrophoretic transfer from an SDS-PAGE gel onto nitrocellulose paper (data not shown). In one preparation of the isolated protein, a trace of a protein of around 70 kDa was also visible. The protein preparations were used to raise antibodies in a rabbit.

Anti-68-kDa serum cross-reacts with TBP 2 from other strains of *N. meningitidis* and from *N. gonorrhoeae*. Previous work had shown that antiserum raised in mice with whole cells of iron-restricted *N. meningitidis* group B15P1.16, strain 70942 (with TBP 2 with an M_r of 78,000) (10, 34), contained antibodies which reacted strongly with the TBP 2 of the homologous strain. These antibodies also cross-reacted with several but not all TBP 2 molecules of the same size; there was no reaction at all with the 68-kDa TBP 2 of strain B16B6. Similarly, antibodies raised to the whole cells of strain B16B6 (TBP 2 of 68 kDa) reacted with TBP 2s of similar sizes from other group 2a strains but failed to react with any of the TBP 2 molecules of higher molecular weights (M_r, 78,000 to 83,000) that were tested.

In contrast, we now report that antibodies raised to the purified, denatured TBP 2 of strain B16B6 reacted well with affinity-purified TBP 2s of both the homologous strain and *N. meningitidis* 70942 (Fig. 1, lanes 6 and 3, respectively), thus indicating the presence of conserved epitopes between the two proteins. No cross-reactivity was observed with the TBP 1 of either strain (Fig. 1). To see whether cross-reactivity was common, the anti-68-kDa serum was reacted with total-membrane protein preparations from 21 other isolates of *N. meningitidis* grown under iron-restricted conditions. The results showed that the antibodies reacted with the TBP 2s in each case, even when antibodies raised to the native protein had not cross-reacted (data not shown). Some

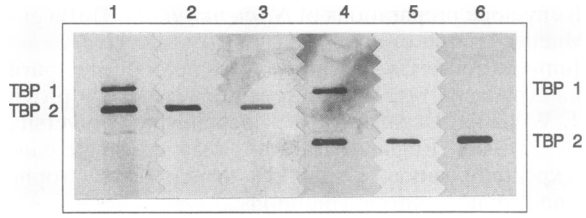

FIG. 1. Electrophoretic blot showing the reaction of HRP-human transferrin and anti-68-kDa antiserum with the affinity-purified membrane proteins (15 μl per lane) (TBP 1 and TBP 2) obtained from *N. meningitidis* 70942 (lanes 1 to 3) and B16B6 (lanes 4 to 6) grown under iron-restricted conditions. Lanes 2 and 5 were reacted with HRP-human transferrin and lanes 3 and 6 were reacted with anti-68 kDa antiserum (30 μl per lane). Lanes 1 and 4 (stained with AuroDye) show the profile obtained following electrophoretic transfer of the affinity-isolated membrane proteins. Electrophoresis was carried out for an extended period of time. Only the region of the gel containing the affinity-isolated proteins is shown.

cross-reactivity was observed with proteins other than TBP 2 when whole envelope preparations were used for immunoblotting. Adsorption of the antiserum with membrane proteins from iron-replete cells reduced cross-reactivity with proteins other than TBP 2. No cross-reaction was obtained with meningococcal LOS obtained by proteinase K digestion of whole-membrane preparations from two strains of *N. meningitidis* which showed strongly cross-reacting TBP 2 proteins.

Analogous higher- and lower-molecular-weight proteins associated with transferrin-binding were found in *N. gonorrhoeae* by the affinity isolation procedure detailed above. Figure 2 (lane 3) shows the protein profile obtained following affinity extraction of one strain of *N. gonorrhoeae* (strain 2211) grown under iron-restricted conditions: the protein analogous to meningococcal TBP 2 binds human transferrin following SDS-PAGE and transfer to nitrocellulose paper (Fig. 2, lane 2), although the binding seemed weaker than that observed with the TBP 2 of *N. meningitidis*. The TBP 2 expressed by *N. gonorrhoeae* also cross-reacted on immunoblotting with the anti-68-kDa antiserum (Fig. 2, lane 1), although the reaction seen was weaker than that observed with the TBP 2 of *N. meningitidis*. The antiserum did not cross-react with the gonococcal protein analogous to TBP 1.

Cross-reactivity of the anti-68-kDa antiserum with TBP 2 of *H. influenzae* type b. Affinity isolation of iron-regulated

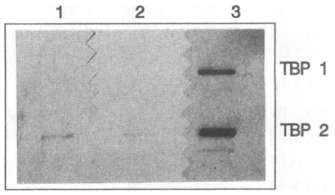

FIG. 2. Electrophoretic blot showing the reaction of anti-68-kDa antiserum and HRP-human transferrin with the affinity-purified membrane proteins (15 μl per lane) (TBP 1 and TBP 2) obtained from *N. gonorrhoeae* 2211 grown under iron-restricted conditions. Lanes 1 and 2 were reacted with anti-68-kDa antiserum (30 μl) and with HRP-human transferrin, respectively. Lane 3 (stained with AuroDye) shows the profile obtained following electrophoretic transfer of the affinity-isolated membrane proteins. Electrophoresis was carried out for an extended period of time. Only the region of the gel containing the affinity-isolated proteins is shown.

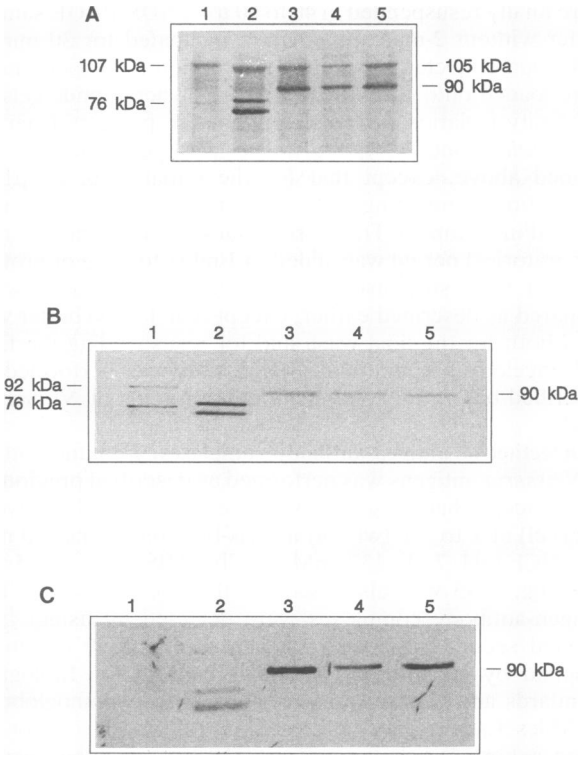

FIG. 3. Electrophoretic blot showing the reaction of HRP-human transferrin and anti-68-kDa antiserum with affinity-purified membrane proteins (10 to 15 μl per lane) obtained from different strains of *H. influenzae* type b grown under iron-restricted conditions. The profile was obtained following staining with AuroDye (A) and reaction with HRP-human transferrin (B) and with the anti-68-kDa antiserum (diluted 1:400) (C). The strains used were Eagan (lane 1), RM926 (lane 2), RM7004 (lane 3), NU698 (lane 4), and NU20 (lane 5). Only the upper part of each gel is shown.

proteins associated with the binding of transferrin in *H. influenzae* type b produced a protein profile analogous to that of *N. meningitidis*. Figure 3 shows the results obtained with five different strains; RM7004, NU698, and NU20 had TBP 2s of 90 kDa (Fig. 3A and B, lanes 3, 4, and 5), whereas in strain Eagan (Fig. 3A and B, lane 1), transferrin-binding activity was associated primarily with a protein with an M_r of 76,000 but with weaker binding to a protein with an M_r of 92,000; the upper band was not always seen. *H. influenzae* type b RM926 (Fig. 3B, lane 2) showed the presence of two transferrin-binding proteins with M_rs of 72,000 and 80,000. All strains expressed a protein with an M_r of approximately 105,000 (Eagan strain, 107,000; Fig. 3A, lane 1) which did not bind HRP-transferrin on electroblotting but which, nevertheless, was always coextracted with the transferrin-binding proteins by the affinity extraction procedure; this is considered to be equivalent to TBP 1 in *N. meningitidis*.

Figure 3C shows an immunoblot of the *H. influenzae* affinity-isolated transferrin-binding proteins probed with the anti-68-kDa antiserum. Strong cross-reaction with proteins analogous to TBP 2 was found in four of the strains (Fig. 3C, lanes 2 to 5). In the case of strain RM926 (Fig. 3C, lane 2), both of the transferrin-binding proteins cross-reacted. Some cross-reactivity was also noted with a protein with an M_r of approximately 45,000 in strains RM7004, NU698, and NU20 (data not shown); weak binding of HRP-transferrin to this protein suggests that it might be a breakdown product of the

higher band. In contrast, no cross-reactivity was observed between the antiserum and either the 76-kDa or the weaker 92-kDa transferrin-binding proteins present in strain Eagan (Fig. 3C, lane 1). Likewise, no cross-reactivity was seen between the antiserum and the protein in *H. influenzae* type b believed to be analogous to the TBP 1 of *N. meningitidis*.

No cross-reactivity with the human transferrin receptor. The anti-68-kDa antiserum failed to react on immunoblotting with a purified and denatured sample of the human transferrin receptor (placental) (data not shown). This protein had an M_r of approximately 90,000 on SDS-PAGE in the presence of 2-mercaptoethanol and consists of half of the native receptor, which is made up of two identical subunits of the protein.

DISCUSSION

The data presented in this paper provide clear evidence for the existence of common antigenic domains among the molecularly heterogenous TBP 2s of *N. meningitidis*. It remains to be seen whether any of these common epitopes are readily exposed on the surface of the intact organisms and can participate in protective immune responses. However, the discovery that antiserum raised to the meningococcal 68-kDa TBP 2 also reacted with functionally equivalent proteins in *N. gonorrhoeae* and *H. influenzae* type b strongly indicates a molecular relationship among all TBP 2 molecules and raises interesting questions regarding their origin. It also raises the exciting possibility that if common, surface-exposed protective epitopes can be identified in these molecules, then a single vaccine might be designed to protect against the majority of infections caused by all of these pathogens. The possibility that the cross-reactions observed with some strains may be due to certain shared epitopes whereas cross-reactions with other strains may be due to another epitope will be addressed by the use of monoclonal antibodies. Previous work showed that whole cells of iron-restricted *N. meningitidis* elicited anti-TBP 2 antibodies in mice which had only limited cross-reactivity. Whether the same situation applies in humans remains to be seen. It is already known that human convalescent sera contain antibodies which react strongly with the TBP 2 of the infecting strain of *N. meningitidis* (1). Similarly, for *H. influenzae* type b, antibodies to both TBP 1 and TBP 2 have been observed in the convalescent sera of infants recovering from meningitis caused by *H. influenzae* (13).

An important area for future investigation, and indeed for concern, is the possible cross-reactivity of anti-TBP 2 antibodies, or anti-bacterial transferrin receptor antibodies, with the human transferrin receptor. Work reported here shows that the anti-68-kDa antiserum failed to react on immunoblotting with a purified sample of the human transferrin receptor protein. In addition, a monoclonal antibody to the human transferrin receptor (Dakopatts, Copenhagen, Denmark) did not react with whole cells or with electroblots of the *H. influenzae* type b Eagan TBPs (37). This does not, of course, exclude the possibility that similar conformational epitopes exist in the native forms of the human, meningococcal, gonococcal, and *Haemophilus* transferrin receptors, perhaps at or close to the transferrin-binding site. Some but not all monoclonal antibodies to the human transferrin receptor can interfere with receptor function, block iron uptake, and lead to the inhibition of cell proliferation (30, 31). Such antibodies have been considered for therapeutic purposes (9).

The polyclonal antibody preparation described in this paper cross-reacts with proteins of variable molecular weights in *N. meningitidis*, *N. gonorrhoeae*, and *H. influenzae* type b, all of which were isolated by affinity processes based on transferrin and all of which bound transferrin following SDS-PAGE and electroblotting. This is the first report of gonococcal transferrin-binding proteins reacting with transferrin following SDS-PAGE and electroblotting, although the interaction has been sought previously (26). The binding of transferrin with the gonococcal TBP 2 observed in the present study was weak, as was the cross-reaction seen with the anti-68-kDa antiserum. This is likely to be due to difficulties in sufficiently renaturing the gonococcal TBP 2 following SDS-PAGE and transfer to nitrocellulose. The reaction of HRP-human transferrin with the *Haemophilus* TBP 2 has also been reported to be difficult to detect by this procedure (24). In the present work, milder solubilization conditions than those used originally for meningococcal proteins were used. A recent report by Ferreiros et al. (7) draws attention to the effect of different sample preparation protocols on the ability to detect transferrin binding after SDS-PAGE and electroblotting with the TBP 2s from different strains of *N. meningitidis*, again showing that although there are similarities among these proteins, there are also clearly some differences. Although some cross-reactivity of the anti-68-kDa antiserum was noted with proteins other than TBP 2 when whole-envelope preparations of *N. meningitidis* were used for immunoblotting, the object of the present study was specifically to examine antibodies cross-reacting with the TBP 2s of different organisms. Some of this cross-reactivity could have been due to contamination of the preparation used for immunization with traces of other proteins. Adsorption of the anti-68-kDa antiserum with membrane preparations from iron-replete meningococcal cells reduced this cross-reactivity but still allowed the reactions with different TBP 2 proteins to be observed. No cross-reaction was observed with the LOS from two strains of *N. meningitidis*, which showed a strongly cross-reacting TBP 2 protein. Likewise, no cross-reactions were observed with *H. influenzae* LOS (strain RM7004) (37).

The differences between the TBP 2 of the Eagan strain of *H. influenzae* type b and those of the other strains with regard to molecular mass and reaction with the anti-68-kDa antiserum are also of interest and need further investigation. Alloenzyme electrophoretic typing analysis has revealed that the population structure of encapsulated *H. influenzae* is clonal. Two major phylogenetic divisions (I and II) have been proposed, with division I containing the major clonal groups of type b strains responsible for the majority of disease cases in North America and Europe (21). Eagan and RM926 are North American strains which belong to different phylogenetic divisions (I and II, respectively) (14) and interestingly show considerable variation in their TBP molecular masses and cross-reactivities. The TBPs from the three European isolates examined, of which strain RM7004 is known to represent the majority of disease-causing isolates in Western Europe (33, 36), showed the same TBP molecular masses and cross-reactivities with the anti-68-kDa serum but differed in size from those of the North American strains. Clearly, the TBPs of many more isolates need to be examined to ascertain the relatedness within the major clonal groups of type b organisms responsible for invasive disease.

Further progress in this field will now depend critically upon obtaining the primary amino acid structures of the TBP 2 molecules and on identifying both conserved epitopes among proteins from different strains of *N. meningitidis* and

from *N. gonorrhoeae* and *H. influenzae*, which might be of potential use for a vaccine, and the variable domains responsible for the differences among them. The elucidation of the molecular structures of these proteins is also essential for understanding their role in transferrin binding and the limitations these structures might impose on host specificity and on iron uptake.

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REFERENCES

1. **Ala'Aldeen, D. A.** 1991. Personal communication.
2. **Ala'Aldeen, D. A., R. A. Wall, and S. P. Borriello.** 1990. The 70-kilodalton iron-regulated protein of *Neisseria meningitidis* is not the human transferrin receptor. FEMS Microbiol. Lett. **69:**37–42.
3. **Banerjee-Bhatnagar, N., and C. E. Frasch.** 1990. Expression of *Neisseria meningitidis* iron-regulated outer membrane proteins, including a 70-kilodalton transferrin receptor, and their potential for use as vaccines. Infect. Immun. **58:**2875–2881.
4. **Blanton, K. J., G. D. Biswas, J. Tsai, J. Adams, D. W. Dyer, S. M. Davis, G. G. Koch, P. K. Sen, and P. F. Sparling.** 1990. Genetic evidence that *Neisseria gonorrhoeae* produces specific receptors for transferrin and lactoferrin. J. Bacteriol. **172:**5225–5235.
5. **Chart, H., and E. Griffiths.** 1985. Antigenic and molecular homology of the ferric-enterobactin receptor protein of *Escherichia coli*. J. Gen. Microbiol. **131:**1503–1509.
6. **Crosa, J. H.** 1989. Genetics and molecular biology of siderophore-mediated iron transport in bacteria. Microbiol. Rev. **53:**517–530.
7. **Ferreiros, C. M., M. T. Criado, M. Pintor, and L. Ferron.** 1991. Analysis of the molecular mass heterogeneity of the transferrin receptor in *Neisseria meningitidis* and commensal *Neisseria*. FEMS Microbiol. Lett. **83:**247–254.
8. **Griffiths, E.** 1987. The iron-uptake systems of pathogenic bacteria, p. 69–137. *In* J. J. Bullen and E. Griffiths (ed.), Iron and infection: molecular, physiological and clinical aspects. John Wiley and Sons, Chichester, England.
9. **Griffiths, E., and J. J. Bullen.** 1987. Iron and infection: future prospects, p. 283–317. *In* J. J. Bullen and E. Griffiths (ed.), Iron and infection: molecular, physiological and clinical aspects. John Wiley and Sons, Chichester, England.
10. **Griffiths, E., P. Stevenson, and A. Ray.** 1990. Antigenic and molecular heterogeneity of the transferrin-binding protein of *Neisseria meningitidis*. FEMS Microbiol. Lett. **69:**31–36.
11. **Griffiths, E., P. Stevenson, R. Thorpe, and H. Chart.** 1985. Naturally occurring antibodies in human sera that react with the iron-regulated outer membrane proteins of *Escherichia coli*. Infect. Immun. **47:**808–813.
12. **Holland, J., K. J. Towner, and P. Williams.** 1991. Isolation and characterization of *Haemophilus influenzae* type b mutants defective in transferrin-binding and iron assimilation. FEMS Microbiol. Lett. **77:**283–288.
13. **Holland, J., and P. Williams.** 1991. Unpublished data.
14. **Kroll, J. S., and E. R. Moxon.** 1989. Genetic and phenotypic variation in the capsulation and virulence of culture collection strains of *Haemophilus influenzae* type b. Microb. Pathog. **7:**449–457.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **256:**495–497.
16. **Lugtenberg, B., J. Meijers, R. Peters, P. van der Hoek, and L. van Alphen.** 1975. Electrophoretic resolution of the major outer membrane proteins of *E. coli* K12 into four bands. FEBS Lett. **58:**254–258.
17. **Martinez, J. L., A. Delgado-Iribarren, and F. Baquero.** 1990. Mechanisms of iron acquisition and bacterial virulence. FEMS Microbiol. Rev. **75:**45–56.
18. **McKenna, W. R., P. A. Mickelsen, P. F. Sparling, and D. W. Dyer.** 1988. Iron uptake from lactoferrin and transferrin by *Neisseria gonorrhoeae*. Infect. Immun. **56:**785–791.
19. **Morton, D. J., and P. Williams.** 1989. Utilization of transferrin-bound iron by Haemophilus species of human and porcine origins. FEMS Microbiol. Lett. **65:**123–128.
20. **Morton, D. J., and P. Williams.** 1990. Siderophore-independent acquisition of transferrin-bound iron by *Haemophilus influenzae* type b. J. Gen. Microbiol. **136:**927–933.
21. **Musser, J. M., J. S. Kroll, D. M. Granoff, E. R. Moxon, B. R. Brodeur, J. Campos, H. Dabernat, W. Frederiksen, J. Hamel, G. Hammond, E. A. Hoiby, K. E. Jonsdottir, M. Kabeer, I. Kallings, W. H. Khan, M. Kilian, P. Knowles, H. J. Koornhof, B. Law, K. I. Li, J. Montgomery, P. E. Pattison, J. C. Piffaretti, A. K. Takala, M. L. Thong, R. A. Wall, J. I. Ward, and R. K. Selander.** 1990. Global genetic structure and molecular epidemiology of encapsulated *Haemophilus influenzae*. Rev. Infect. Dis. **12:**75–111.
22. **Niven, D. F., J. Donga, and F. S. Archibald.** 1989. Responses of *Haemophilus pleuropneumoniae* to iron restriction: changes in the outer membrane protein profile and the removal of iron from porcine transferrin. Mol. Microbiol. **3:**1083–1089.
23. **Padda, J. S., and A. B. Schryvers.** 1990. N-linked oligosaccharides of human transferrin are not required for binding to bacterial transferrin receptors. Infect. Immun. **58:**2972–2976.
24. **Schryvers, A. B.** 1989. Identification of the transferrin- and lactoferrin-binding proteins in *Haemophilus influenzae*. J. Med. Microbiol. **29:**121–130.
25. **Schryvers, A. B., and G. C. Gonzalez.** 1989. Comparison of the abilities of different protein sources of iron to enhance *Neisseria meningitidis* infection in mice. Infect. Immun. **57:**2425–2429.
26. **Schryvers, A. B., and B. C. Lee.** 1989. Comparative analysis of the transferrin and lactoferrin binding proteins in the family *Neisseriaceae*. Can. J. Microbiol. **35:**409–415.
27. **Schryvers, A. B., and L. J. Morris.** 1988. Identification and characterization of the transferrin receptor from *Neisseria meningitidis*. Mol. Microbiol. **2:**281–288.
28. **Schryvers, A. B., and L. J. Morris.** 1988. Identification and characterization of the human lactoferrin-binding protein from *Neisseria meningitidis*. Infect. Immun. **56:**1144–1149.
29. **Stevenson, P., and E. Griffiths.** 1985. Detection of antibodies and antigens by immunoblotting, p. 457–464. *In* M. Sussman (ed.), The virulence of *Escherichia coli*. Academic Press, London.
30. **Taetle, R., K. Rhyner, J. Castagnola, D. To, and J. Mendelsohn.** 1985. Role of transferrin, Fe and transferrin receptors in myeloid leukemia cell growth: studies with an antitransferrin receptor monoclonal antibody. J. Clin. Invest. **75:**1061–1067.
31. **Trowbridge, I. S., and F. Lopez.** 1982. Monoclonal antibody to transferrin receptor blocks transferrin binding and inhibits human tumor cell growth *in vitro*. Proc. Natl. Acad. Sci. USA **79:**1175–1179.
32. **Tsai, J., D. W. Dyer, and P. F. Sparling.** 1988. Loss of transferrin receptor activity in *Neisseria meningitidis* correlates with inability to use transferrin as an iron source. Infect. Immun. **56:**3132–3138.
33. **Van Alphen, L., L. Geelen-van den Broek, and M. van Ham.** 1990. *In vivo* and *in vitro* expression of outer membrane components of *Haemophilus influenzae*. Microb. Pathog. **8:**279–288.
34. **Wall, R. A., H. A. Davies, and S. P. Borriello.** 1989. Epitopes of serogroup B *Neisseria meningitidis* analysed *in vitro* and directly from cerebrospinal fluid. FEMS Microbiol. Lett. **65:**129–136.
35. **Weinberg, E. D.** 1989. Cellular regulation of iron assimilation. Q. Rev. Biol. **64:**261–290.
36. **Weiser, J. N., D. J. Maskell, P. D. Butler, A. A. Lindberg, and E. R. Moxon.** 1990. Characterization of repetitive sequences controlling phase variation of *Haemophilus influenzae* lipopolysaccharide. J. Bacteriol. **172:**3304–3309.
37. **Williams, P.** 1991. Unpublished observation.