

Binding and Surface Exposure Characteristics of the Gonococcal Transferrin Receptor Are Dependent on Both Transferrin-Binding Proteins

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Neisseria gonorrhoeae is capable of iron utilization from human transferrin in a receptor-mediated event. Transferrin-binding protein 1 (Tbp1) and Tbp2 have been implicated in transferrin receptor function, but their specific roles in transferrin binding and transferrin iron utilization have not yet been defined. We utilized specific gonococcal mutants lacking Tbp1 or Tbp2 to assess the relative transferrin-binding properties of each protein independently of the other. The apparent affinities of the wild-type transferrin receptor and of Tbp1 and Tbp2 individually were much higher than previously estimated for the gonococcal receptor and similar to the estimates for the mammalian transferrin receptor. The binding parameters of both of the mutants were distinct from those of the parent, which expressed two transferrin-binding sites. Tbp2 discriminated between ferrated transferrin and apotransferrin, while Tbp1 did not. Results of transferrin-binding, affinity purification, and protease accessibility experiments were consistent with the hypothesis that Tbp1 and Tbp2 interact in the wild-type strain, although both proteins were capable of binding to transferrin independently when separated in the mutants. The presence of Tbp1 partially protected Tbp2 from trypsin proteolysis, and Tbp2 also protected Tbp1 from trypsin exposure. Addition of transferrin to wild-type but not mutant cells protected Tbp1 from trypsin but increased the trypsin susceptibility of Tbp2. These observations indicate that Tbp1 and Tbp2 function together in the wild-type strain to evoke binding conformations that are distinct from those expressed by the mutants lacking either protein.

With few exceptions, microorganisms require inorganic iron as a cofactor for various metabolic processes, including RNA synthesis, electron transport, and oxygen detoxification (11). The vast majority of iron available in a mammalian host is found intracellularly in the forms of heme, hemosiderin, or ferritin or complexed with metalloproteins (56). Extracellular iron stores include transferrin, lactoferrin, and hemoglobin, the last of which results from erythrocyte lysis and is ordinarily complexed with the serum protein haptoglobin. Sequestration of iron by these iron-binding proteins and compounds effectively lowers the concentration of available soluble ferric iron to a level insufficient to allow bacterial growth. To circumvent this phenomenon, which has been termed nutritional immunity (56), pathogenic microorganisms have evolved at least four mechanisms to acquire iron from these compounds. Reduction of ferric iron to ferrous iron by secreted reductases, resulting in release of iron from the iron-binding compound, is a strategy employed by some bacteria, including *Listeria monocytogenes* (17, 18). *Pseudomonas aeruginosa* secretes proteases that, in conjunction with neutrophil-derived products, are capable of modifying transferrin and lactoferrin such that they unload their iron (12). Many microbes synthesize or utilize exogenously made siderophores, low-molecular-mass compounds that can scavenge the iron from host iron-containing compounds. The synthesis of siderophores and their high-affinity acquisition systems located in the cell envelope are regulated by the availability of iron in the environment (6, 37). Several

bacterial pathogens make no identifiable siderophores but instead have evolved systems whereby they can scavenge the iron directly from host iron-binding compounds by a high-affinity, receptor-mediated mechanism. Of these pathogens, *Neisseria* spp., *Haemophilus* spp., and *Actinobacillus pleuropneumoniae* have some of the best-characterized, iron uptake systems (2, 5, 15, 20–23, 30, 34–36, 38, 47, 48, 57).

The pathogenic *Neisseria* spp. can obtain iron directly from the glycoproteins transferrin and lactoferrin as well as from heme and hemoglobin (33). The mechanism of iron acquisition from transferrin has been the most thoroughly studied (for a review, see reference 16). Binding of transferrin for the pathogenic *Neisseria* spp. is saturable and species specific in that the neisserial receptor is capable of recognizing only human transferrin (9, 28, 48, 54). Transferrin is not internalized but is deferrated at the cell surface by an unknown mechanism, and the iron enters the cell in an energy-dependent fashion (4, 32, 50). Affinity purification was utilized to identify two proteins, transferrin-binding protein 1 (Tbp1) and Tbp2, which have subsequently been shown, biochemically and genetically, to be involved in transferrin receptor function (2, 9, 15, 23, 30). Mutants lacking Tbp1 exhibit diminished transferrin binding, cannot internalize transferrin-bound iron, and are incapable of growth on transferrin as the sole iron source (15, 23). The predicted protein sequence of Tbp1 suggests that it is a member of the family of TonB-dependent outer membrane receptors (15, 30), some of which are required for entry of iron complexes in enteric bacteria (10, 26). Tbp2 is acetylated and is not a member of the TonB-dependent family (2). Gonococcal Tbp2⁻ mutants are capable of growth on transferrin-derived iron, but the efficiency of iron uptake from transferrin is decreased compared with that of wild-type levels (2). In contrast, both meningococcal Tbp2⁻ and Tbp1⁻ mutants are in-

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capable of growth on transferrin-bound iron (23), although in other respects the gonococcal and meningococcal transferrin iron uptake systems are very similar.

These observations have been incorporated into a model of iron acquisition from transferrin by these organisms (for a review, see reference 16) based primarily on the analogy with TonB-dependent uptake of siderophores and vitamin B₁₂. In this model, Tbp1 forms an energy-dependent, gated, pore (25, 31, 44) or pump (41) through which the transferrin-derived iron crosses the outer membrane, while Tbp2 is suggested to increase the efficiency of the receptor. Other aspects of a transferrin iron utilization system include Fbp, which is suggested to be the periplasmic binding protein component of high-affinity iron uptake from transferrin and citrate (13); the *thu* gene product, which is required for iron utilization from transferrin and lactoferrin (9); TonB, which has not yet been identified in the pathogenic *Neisseria* spp. but has been suggested to exist in all gram-negative bacteria to energize outer membrane receptors (40); a cytoplasmic membrane permease (1) to allow the ferric iron to cross the cytoplasmic membrane; and a reductase (29) to convert ferric iron, the form bound to Fbp, to ferrous iron, the form found in the cytoplasm.

The purpose of this study was to define more precisely the roles that Tbp1 and Tbp2 play in transferrin binding. We have used a combination of liquid-phase and solid-phase (dot blot) transferrin-binding assays to determine the relative contributions that Tbp1 and Tbp2 make to the affinity and specificity of the receptor. Additionally, we present data from affinity purification and protease accessibility experiments suggesting that the presence of Tbp1 affects the conformation or exposure of Tbp2 while the presence of Tbp2 alters the protease exposure of Tbp1.

MATERIALS AND METHODS

Strains and growth conditions. The gonococcal strains used in this study were previously described (2, 15, 34). Gonococci were routinely maintained on Bacto GC medium base (Difco) plates containing Kellogg's supplement I (24) and 12 μ M Fe(NO₃)₃ in a 5% CO₂ atmosphere at 35°C. To induce iron-stressed growth, bacteria were grown in CDM-0 (58) which was rendered iron-free by treatment with Chelex-100 (Bio-Rad, Richmond, Calif.). During the course of this study, we determined that the gonococcal strain FA6819 expressed a stable, truncated version of Tbp2 to which transferrin did not bind (data not shown). We constructed another mutant in which the 3' 2,058 bases of the 2,127-base Tbp2 open reading frame were deleted. This mutant did not make a stable Tbp2 peptide and was identical to FA6819 in iron uptake experiments, transferrin-binding experiments, and protease protection experiments (data not shown).

Preparation and labeling of human transferrin. Human transferrin (Calbiochem, San Diego, Calif.) was ferrated as previously described (9). Ferrated transferrin (94% saturated as measured by a ferrozine assay [51]) was iodinated using Iodogen (Pierce, Rockford, Ill.) according to the manufacturer's recommendations. ¹²⁵I label was separated from the iodinated transferrin by passage over a desalting column (Econo-Pac; Bio-Rad).

Liquid-phase, transferrin-binding assay. Nonpiliated, transparent gonococci were grown overnight on Bacto GC medium base plates and then inoculated into liquid CDM-0. After one mass doubling, cells were diluted 1:4 in fresh CDM-0 and grown 3 h. In experiments to measure transferrin binding to sialylated gonococci, cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-NANA; Sigma, St. Louis, Mo.) was added to a final concentration of 5 μ g/ml with the addition of fresh CDM-0, and then cultures were grown 3 h to induce iron stress and sialylation. Approximately 1 \times 10⁷ to 5 \times 10⁷ CFU in growth medium was mixed with various amounts of ¹²⁵I-transferrin in the presence of 10 mg of bovine serum albumin per ml in individual wells of a Multi-Screen microtiter dish (0.45- μ m-pore-size filter; MAHV N45; Millipore, Cambridge, Mass.). Transferrin was allowed to bind to cells for 20 min at room temperature, and unbound transferrin was removed by filtration followed by three washes with CDM-0. Filters at the bottom of the Multi-Screen microtiter dishes were punched out and counted with a gamma scintillation counter. In order to standardize binding assays to total cellular protein, wells containing cells only were similarly processed, except that they were washed with phosphate-buffered saline, and assayed for protein content by the bicinchoninic acid (BCA) assay (Pierce). Total transferrin bound was then normalized to micrograms of total cellular protein. Three identical experiments, including growth and iron stress experiments, were conducted. Each datum point represents the mean of the three independent

experiments. In individual binding assays, each concentration was tested in duplicate. Nonspecific binding was also measured at each concentration by addition of excess cold, 94% saturated transferrin (3.8 μ M). Specific binding was calculated by subtracting nonspecific binding from total counts for each transferrin concentration. The specific activity of iodinated transferrin was 5.0 \times 10⁵ to 5.5 \times 10⁵ cpm/ μ g. Copy number and *K_d* estimates were obtained by using Lunden-1 (Lunden Software, Inc.; Cleveland, Ohio), which applies a nonlinear, curve-fitting algorithm to binding data.

A competitive, liquid-phase, transferrin-binding assay was conducted essentially as described above except that the unlabeled competitor concentration varied from 0 to 1.5 μ M and the iodinated transferrin concentration was held constant at 100 nM. Each dilution of competitor contained ferrated (94% saturated) transferrin or apotransferrin in addition to 0.8 mM deferoxamine mesylate (Desferal; CIBA Pharmaceuticals).

Competitive solid-phase, transferrin-binding assay. Approximately 1 \times 10⁷ to 5 \times 10⁷ CFU of iron-stressed gonococci was applied to nitrocellulose filters and allowed to dry. Filters were then probed with a mixture of unlabeled competitor transferrin (95% saturated transferrin or apotransferrin; Sigma) and horseradish peroxidase (HRP)-labeled transferrin (Jackson ImmunoResearch; West Grove, Pa.) essentially as described previously (14). The commercially available HRP-labeled human transferrin was partially saturated with iron and prepared by conjugation of activated peroxidase to amino groups on human transferrin. Serial 1:2 dilutions were made in the presence of 0.8 mM deferoxamine mesylate.

Batch affinity purification of transferrin-binding proteins from gonococcal mutants. Membrane proteins and biotinylated transferrin were prepared as described previously (15). Batch affinity purification was performed essentially as indicated previously (15) except that 5 mg of total membrane protein was used as starting material. Either 0.5% Sarkosyl (*n*-lauroylsarcosine) or 0.1% Triton X-100 (Pierce) was used to solubilize membrane proteins in the presence of 1 mM EDTA. Specificities of the binding reactions were determined by performing mock-affinity assays in the absence of biotinylated transferrin. Wash conditions included 100 mM, 500 mM, or 1 M NaCl for purification of transferrin-binding proteins from FA19 with Sarkosyl, as indicated in the legend to Fig. 6. Wash solutions for proteins isolated with Triton X-100 from FA19 contained 1 M NaCl, from FA6747 contained 500 mM NaCl, and from FA6819 contained 1 M NaCl.

Protease accessibility of Tbp1 and Tbp2. Iron-stressed gonococci were grown as described above for the transferrin-binding assays and then treated with trypsin (Sigma) at a concentration of 2.5 μ g/ml for 0 to 30 min. Protease treatment was stopped by addition of 0.6 trypsin-inhibiting units of aprotinin (Sigma), and this was followed by centrifugation, resuspension of the bacterial pellet in loading buffer (27), and boiling for 2 min. Protease-treated whole-cell lysates were separated on 7.5% polyacrylamide gels (27), and proteins were blotted to nitrocellulose filters (53) for 16 h at 40 mA. Western blots (immunoblots) were probed with anti-Tbp1 antiserum (15) or with HRP-labeled transferrin to detect Tbp2 (2, 15). Blots probed with HRP-labeled transferrin were visualized with ECL reagents (Amersham, Arlington Heights, Ill.) and exposed to Kodak X-Omat film. For protease accessibility experiments in the presence of transferrin, transferrin was added to a final concentration of 100 nM before cells were treated with trypsin. Protease-treated cells were subsequently processed as indicated above.

RESULTS

Transferrin binding to gonococcal mutants. We compared the transferrin-binding characteristics of a panel of gonococcal strains including FA19 (Tbp1⁺ Tbp2⁺), FA6747 (Tbp1⁻ Tbp2⁺), FA6819 (Tbp1⁺ Tbp2⁻), and FA6815 (Tbp1⁻ Tbp2⁻). The *tbpB* and *tbpA* loci of these mutants, which encode Tbp2 and Tbp1, respectively, are shown in Fig. 1. A polar transposon insertion mutation in *tbpB* generated FA6815, which did not express Tbp1 or Tbp2 (2) and did not bind transferrin in solid-phase dot blot (data not shown) or in liquid-phase (data not shown) transferrin-binding assays. As shown in Fig. 2, the strain that expressed only Tbp1 bound significantly less transferrin than did the wild-type strain or the strain that expressed only Tbp2.

Binding data from these experiments were analyzed with the program Lunden-1, which uses a nonlinear, curve-fitting algorithm to estimate copy numbers and *K_d*. The predictions made by the nonlinear models best fit the binding data from all strains. Binding data from FA19, when plotted according to the Scatchard-Rosenthal method (7, 43, 45), yielded a curve, as shown in Fig. 3A. The dotted lines in Fig. 3A represent the two binding components estimated by Lunden-1 that best fit the binding data. The apparent *K_d* estimates in the two-site model shown in Fig. 3A were *K_{d1}* = 0.8 nM and *K_{d2}* = 16 nM. The

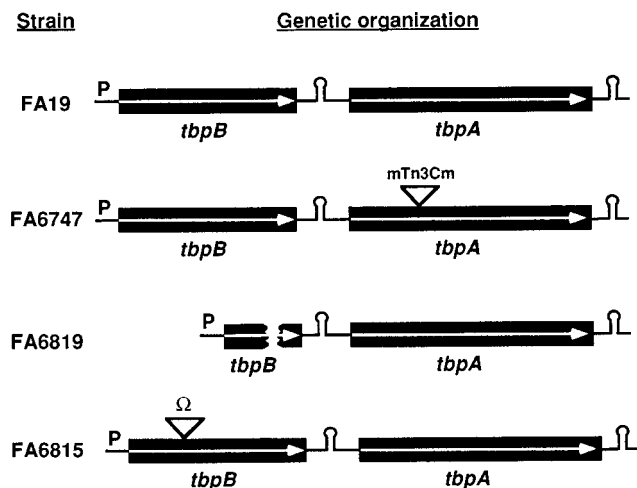


FIG. 1. The *tbpB* and *tbpA* loci of gonococcal mutants. The *tbpB* gene encodes Tbp2; the *tbpA* gene encodes Tbp1. P indicates the approximate location of a putative promoter upstream of the *tbpB* and *tbpA* genes. The arrows indicate the direction of translation of the genes. The inverted triangles indicate the approximate positions of transposon insertions in *tbpA* and *tbpB*. The jagged ends of the box representing *tbpB* in FA6819 indicate that most of the structural gene has been deleted. The *tbpA* gene is flanked by inverted repeats represented by hairpin symbols.

copy number estimates of the two binding components were approximately 1.2×10^9 and 7.2×10^9 molecules per μg of total cellular protein for the first and second components, respectively. This analysis assumes that these represent two separate, noninteracting binding sites. However, without kinetic data we cannot differentiate between the presence of two discrete sites and the presence of multiple sites that interact noncooperatively. Binding data from FA6747 (Tbp1⁻ Tbp2⁺) and FA6819 (Tbp1⁺ Tbp2⁻) were consistent with the model of a single transferrin-binding component in each of these strains.

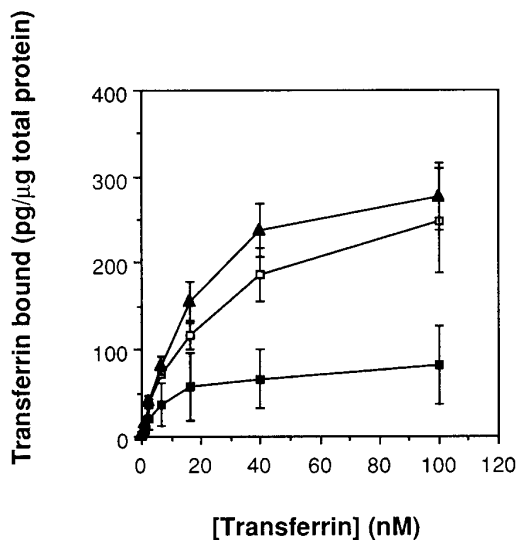
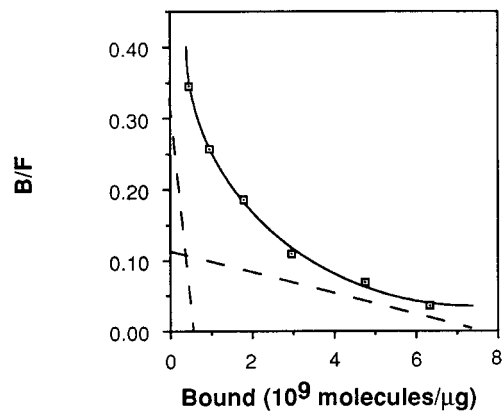
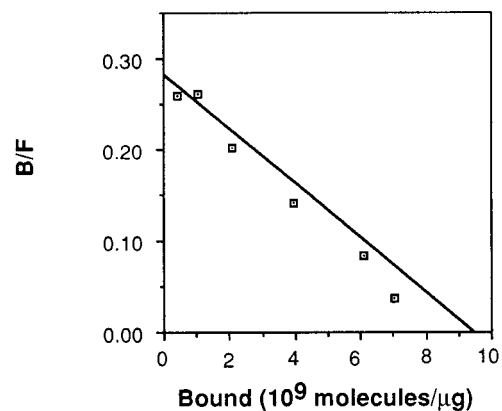


FIG. 2. Isotherm of transferrin binding to mutants. The plot shows the amount of specifically bound transferrin as a function of transferrin concentration. Open squares indicate binding to FA19 (wild type), closed triangles indicate binding to FA6747 (Tbp1⁻ Tbp2⁺), and closed squares indicate binding to FA6819 (Tbp1⁺ Tbp2⁻). Datum points represent means of three individual experiments. Error bars indicate standard deviations within each triplicate set.

A.



B.



C.

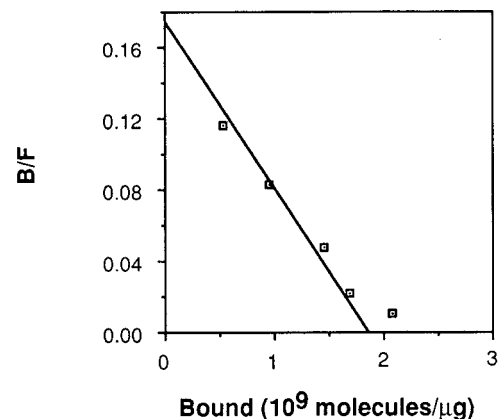


FIG. 3. Scatchard-Rosenthal plots of binding data. Analyses of binding data from FA19 (wild-type) (A), FA6747 (Tbp1⁻ Tbp2⁺) (B), and FA6819 (Tbp1⁺ Tbp2⁻) are shown. The dotted lines in panel A represent the binding sites predicted by a two-site model (Lundon-1). These two binding sites correspond to the K_d and copy number estimates: K_{d1} , 0.8 nM; K_{d2} , 16 nM; N_1 , 1.2×10^9 molecules per μg ; N_2 , 7.2×10^9 molecules per μg . K_d s and copy numbers predicted by the single-site model (Lundon-1) for FA6747 (Tbp1⁻ Tbp2⁺) (B) were 7.4 nM and 9.4×10^9 molecules per μg , respectively, and those for FA6819 (Tbp1⁺ Tbp2⁻) (C) were 2.3 nM and 1.8×10^9 molecules per μg , respectively. The lines in panels B and C do not intersect all of the points since they represent the best-fit, single-site binding parameters estimated by the nonlinear algorithm of Lundon-1 and do not represent the least-squares, linear relationship between the points. B/F, bound/free ratio.

The Scatchard-Rosenthal plots of binding data from transferrin binding to FA6747 (Tbp1⁻ Tbp2⁺) and FA6819 (Tbp1⁺ Tbp2⁻) are shown in Fig. 3B and C, respectively. Lundon-1 analysis indicated that Tbp2 (Fig. 3B) expressed in the absence

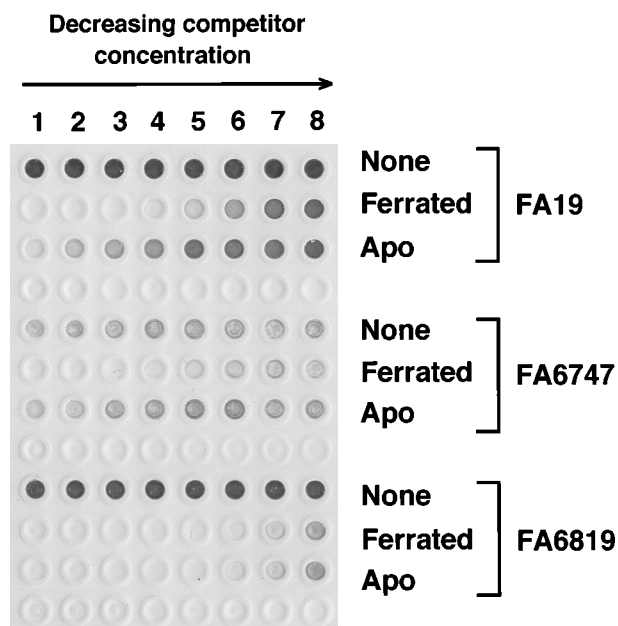


FIG. 4. Solid-phase, transferrin-binding assay. Rows of dots contain iron-stressed whole cells of FA19 (wild type), FA6747 ($Tbp1^- Tbp2^+$), and FA6819 ($Tbp1^+ Tbp2^-$), as indicated at right. Probes for rows: None, 0.33 μ g of HRP-labeled transferrin (2.8 nM) per ml without any competitor added; Ferrated, 0.33 μ g of HRP-labeled transferrin (2.8 nM) per ml mixed with twofold dilutions of saturated transferrin as competitor; Apo, 0.33 μ g of HRP-labeled transferrin per ml mixed with twofold dilutions of apotransferrin as competitor. Competitor concentrations for columns (micromolar): 1, 6.4; 2, 3.2; 3, 1.6; 4, 0.8; 5, 0.4; 6, 0.2; 7, 0.1; 8, 0.05. All HRP-labeled transferrin solutions included 0.8 μ M deferoxamine mesylate. The blot was scanned with a Relisys 2412 scanner, and the image was annotated with Adobe Photoshop software.

of Tbp1 bound transferrin with an apparent K_d of 7.4 nM, and the Tbp2 copy number was approximately 9.5×10^9 molecules per μ g of total cellular protein in this strain. Analysis of binding data from FA6819 (Fig. 3C) indicated that Tbp1, expressed in the absence of Tbp2, bound transferrin with a K_d of 2.3 nM and a copy number of approximately 1.8×10^9 molecules per μ g of total cellular protein. The lines in Fig. 3B and C do not intersect all of the points since they represent the best-fit, single-site binding parameters estimated by the nonlinear algorithm of Lundon-1 and do not represent the least-squares, linear relationship between the points.

Competitive transferrin binding in solid phase. The gonococcal transferrin receptor as expressed in FA19 was previously shown to discriminate slightly (4- to 16-fold) between apo- and ferrated forms of transferrin in a solid-phase, transferrin-binding assay (9). We assessed the ability of the $Tbp1^-$ and $Tbp2^-$ mutants to discriminate between the different forms of transferrin in the same assay format. Deferoxamine mesylate was also added during the assay whose results are depicted in Fig. 4 so that the competitor apotransferrin could not scavenge contaminating iron during the course of the experiment. As shown in Fig. 4, both the wild-type strain and FA6747 ($Tbp1^- Tbp2^+$) were capable of discriminating between apo- and ferrated forms of transferrin, as evidenced by the inability of excess apotransferrin to block binding of ferrated, HRP-labeled transferrin. FA6819 ($Tbp1^+ Tbp2^-$), however, did not appear to discriminate between ferrated transferrin and apotransferrin, since both forms competed equally well with the ferrated, HRP-labeled transferrin for binding. These results indicated that Tbp2 was capable of discerning between apo- and ferrated forms of transferrin and suggested

that Tbp2, rather than Tbp1, might be responsible for the discriminatory phenotype of the receptor in FA19. We also noted that FA6747 ($Tbp1^- Tbp2^+$) in this assay format bound much less transferrin relative to the wild-type strain than it did in the liquid-phase format (compare FA6747 and FA19 curves in Fig. 2). Tbp2 expressed in the absence of Tbp1 may be more susceptible to the drying process used in the solid-phase format or the washing procedures of the two formats may differ sufficiently such that transferrin binding to Tbp2 in solid-phase experiments was underestimated.

Competitive transferrin binding in liquid phase. The ability of Tbp1 and Tbp2 to discriminate between ferrated transferrin and apotransferrin was also assessed in a liquid-phase binding assay format. As shown in Fig. 5A, FA19 showed a modest but statistically significant ability to discriminate between ferrated transferrin and apotransferrin at the two highest concentrations of competitor tested (P value of 0.02 at 500 nM transferrin; P value of 9.5×10^{-5} at 1,500 nM transferrin). In contrast, FA6747 ($Tbp1^- Tbp2^+$) showed a striking preference for ferrated transferrin in that apotransferrin was hardly recognized at all by this strain (Fig. 5B). FA6819 ($Tbp1^+ Tbp2^-$) did not distinguish between the ferrated and apo-forms of transferrin (Fig. 5C).

Effect of sialylation on liquid-phase transferrin binding. Since sialylation of gonococcal lipooligosaccharide has been shown to hinder access of antibody to outer membrane porins (19), we wanted to determine if this modification affected the accessibility of the transferrin receptor. We tested the effect of growth with CMP-NANA on liquid-phase transferrin binding to the panel of gonococcal strains. Sialylation of the lipooligosaccharide of FA19 did not change the ability of the transferrin receptor to bind its ligand (data not shown). Sialylated strains FA6747 ($Tbp1^- Tbp2^+$) and FA6819 ($Tbp1^+ Tbp2^-$) were similarly unaffected in transferrin binding (data not shown). Strains from each of the three assays were effectively sialylated under the growth conditions used since cultures grown in the presence of CMP-NANA lost the ability to bind the 3F11 (3) monoclonal antibody (data not shown).

Affinity purification of Tbps from gonococcal mutants. Irwin et al. (23) demonstrated that meningococcal Tbp2 could not be affinity purified from a mutant lacking Tbp1 and concluded from this that Tbp1 and Tbp2 interact with each other in the wild-type strain. The results presented in Fig. 6 indicated that, like its meningococcal homolog, gonococcal Tbp2 could not be purified from FA6747 ($Tbp1^- Tbp2^+$) in the presence of Sarkosyl (Fig. 6B, lanes 7 and 8). However, Tbp2 could be affinity purified from FA6747 ($Tbp1^- Tbp2^+$) in the presence of the nonionic detergent Triton X-100 (Fig. 6B, lane 9). This binding was specific and not due to incomplete solubilization, since no Tbp2 was purified in the mock experiment to which no biotinylated transferrin was added (Fig. 6B, lane 10). The absence of Tbp2 did not affect the ability to purify Tbp1 from FA6819 ($Tbp1^+ Tbp2^-$) (Fig. 6A, lanes 11 to 14). Increasing the salt concentration in the wash solutions modestly increased the yield of Tbp1 and Tbp2 from FA19 (Fig. 6A, lanes 1, 2, and 3).

Protease accessibility of Tbp1 and Tbp2 in gonococcal mutants. We assessed surface exposure of the individual transferrin-binding proteins by exposing the iron-stressed wild-type and mutant strains to trypsin. The results presented in Fig. 7 showed that both Tbp1 and Tbp2 were accessible in the wild-type strain to low concentrations of trypsin, indicating that both proteins were surface exposed. Data shown in Fig. 7A indicated that Tbp1 was more susceptible to proteolytic attack in the absence of Tbp2 (Fig. 7A, compare lanes 3 to 5 and lanes 13 to 15). Virtually all of the Tbp2 expressed in the absence of Tbp1 was readily accessible to trypsin, and transferrin-binding

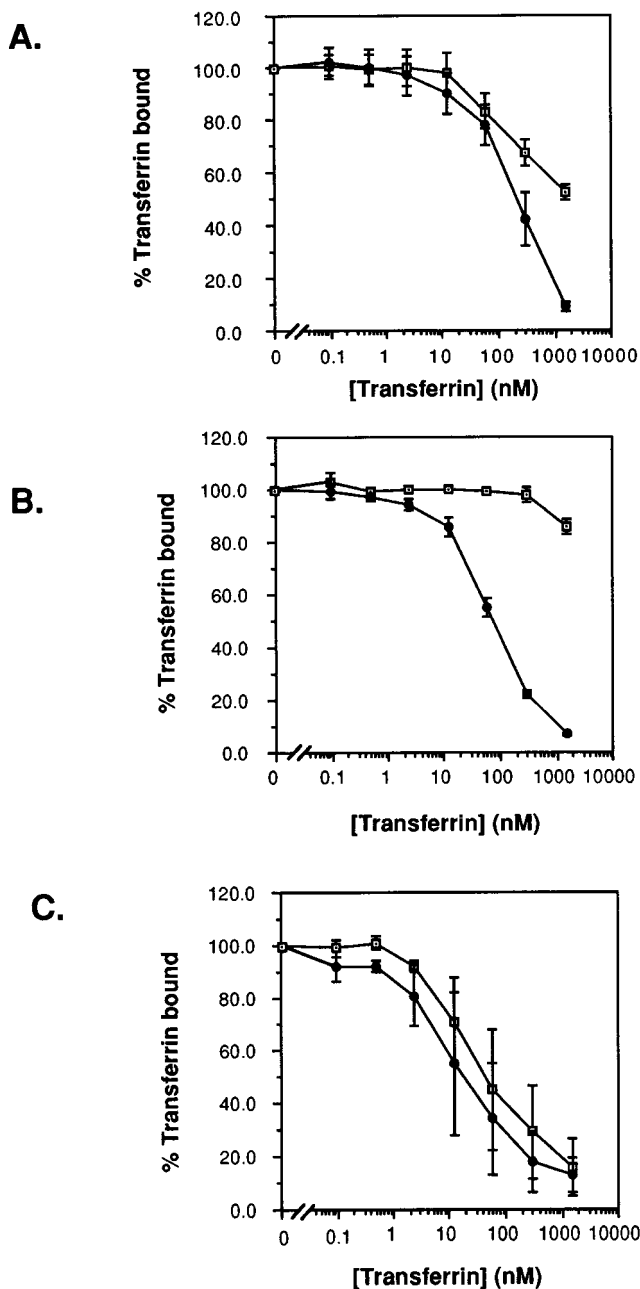


FIG. 5. Competitive liquid-phase transferrin binding to mutants. Percentages of transferrin specifically bound to FA19 (wild type) (A), FA6747 (Tbp1⁻ Tbp2⁺) (B), and FA6819 (Tbp1⁺ Tbp2⁻) (C) as functions of competitor concentration are shown. Open squares represent binding in the presence of apo-transferrin, and closed circles represent binding in the presence of ferrated transferrin. Datum points represent means of three individual experiments. Error bars indicate standard deviations within each triplicate set.

activity was completely abolished by 10 min. For the wild-type strain, a fraction of the Tbp2 expressed was accessible to trypsin but a population remained resistant to cleavage for at least 30 min (Fig. 7B). This resistant population was recalcitrant to degradation even when trypsin concentrations were increased 10-fold (data not shown). Since Fbp, a basic, periplasmic protein (8) was not affected by trypsin exposure in these experiments (data not shown), we conclude that gross lysis, which

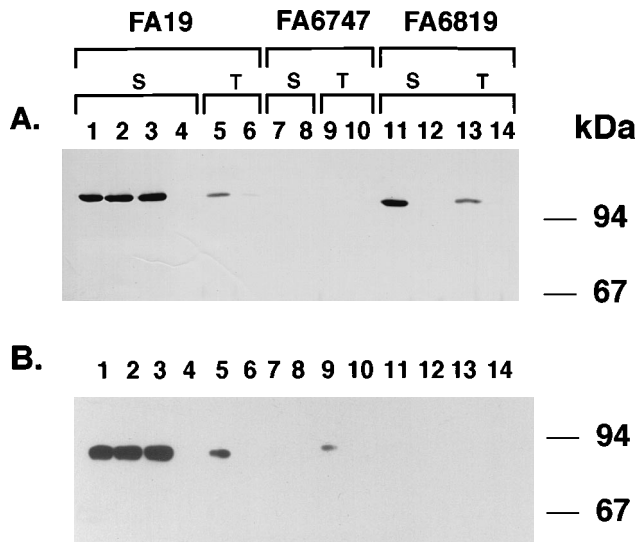


FIG. 6. Affinity-purified, transferrin-binding proteins. Western blots probed with anti-Tbp1 antiserum (A) and with HRP-labeled transferrin to visualize Tbp2 (B). Lanes 1 to 6, affinity-purified proteins purified from FA19 (wild type); lanes 7 to 10, FA6747 (Tbp1⁻ Tbp2⁺); lanes 11 to 14, FA6819 (Tbp1⁺ Tbp2⁻). S and T, proteins isolated in the presence of Sarkosyl and Triton X-100, respectively. Lanes 1, 2, and 3 contain proteins purified from FA19 in Sarkosyl in the presence of 100 mM, 500 mM, and 1 M NaCl, respectively. Even-numbered lanes from 4 to 14 contain proteins isolated in control experiments to which no transferrin was added. Approximate positions of molecular mass standards are indicated at right. Blots were scanned with a Relisys 2412 scanner, and images were annotated with Adobe Photoshop software.

could expose proteins that otherwise were not surface exposed, was not occurring.

Protease accessibility of Tbp1 and Tbp2 in the presence of transferrin. We added saturating concentrations of transferrin before exposing the cells to trypsin to determine whether ligand binding effected exposure of trypsin recognition sites. Under the experimental conditions used here, transferrin was not detectably digested by trypsin (data not shown). Tbp1 was protected from trypsin in the presence of transferrin (compare Fig. 7A and 8A), most profoundly so in the wild-type strain that also expressed Tbp2 (lanes 1 to 5 of Fig. 7A and 8A). After 10 min of trypsin exposure, full-length Tbp1 was noticeably diminished in the absence of Tbp2 (compare lanes 12 and 13). The protease digest pattern of Tbp2 was not dramatically altered in the presence of transferrin, although comparison of Fig. 7B and 8B indicates that Tbp2 might be more protected from trypsin in the absence of transferrin. Tbp2 detection in these experiments required binding of peroxidase-conjugated transferrin; thus, the 58-kDa trypsin-digest product seen in lanes 5 and 6 of Fig. 8B remained capable of binding transferrin.

DISCUSSION

Although we know that both Tbp1 and Tbp2 bind transferrin and somehow contribute to the ability of the gonococci to utilize transferrin as an iron source (2, 15), we do not know the precise roles that each of these proteins play in transferrin binding and growth on transferrin-derived iron. This study using specific gonococcal mutants lacking either Tbp1 or Tbp2 was designed to begin to answer these questions. The results presented here indicate that Tbp1 and Tbp2 expressed independently in the mutants bound transferrin with relatively high affinity in the liquid-phase binding experiments. The apparent affinities of each binding protein and of the wild-type receptor

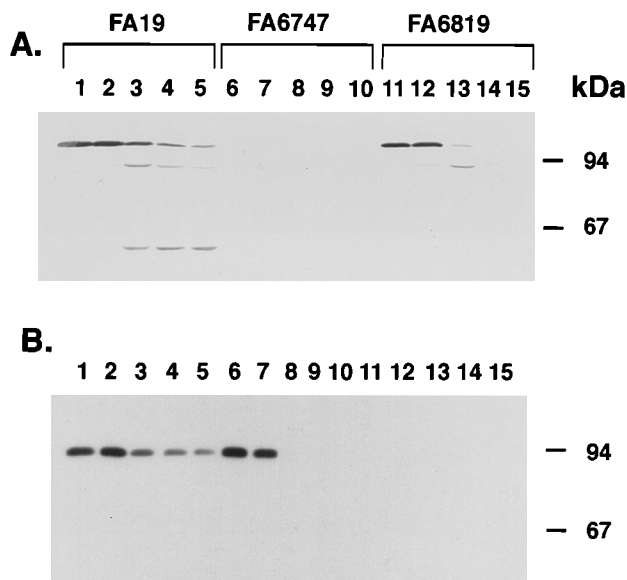


FIG. 7. Protease accessibility of transferrin-binding proteins. Western blots probed with anti-Tbp1 antiserum (A) and HRP-labeled transferrin to visualize Tbp2 (B). Lanes 2 to 5, trypsin-treated, whole-cell lysates of FA19 (wild type); lanes 7 to 10, FA6747 ($Tbp1^- Tbp2^+$); lanes 12 to 15, FA6819 ($Tbp1^+ Tbp2^-$). Lanes 1, 6, and 11 contain untreated whole-cell lysates from FA19, FA6747, and FA6819, respectively. Whole cells were treated with trypsin for 0 min (lanes 2, 7, and 12), 10 min (lanes 3, 8, and 13), 20 min (lanes 4, 9, and 14), and 30 min (lanes 5, 10, and 15). Approximate positions of molecular mass standards are indicated at right. Blots were scanned with a Relisys 2412 scanner, and images were annotated with Adobe Photoshop software.

were considerably higher than that reported previously for the transferrin receptor in the same gonococcal strain, FA19 (9). Previous estimates and those presented here for the gonococcal transferrin receptor may differ for several technical reasons.

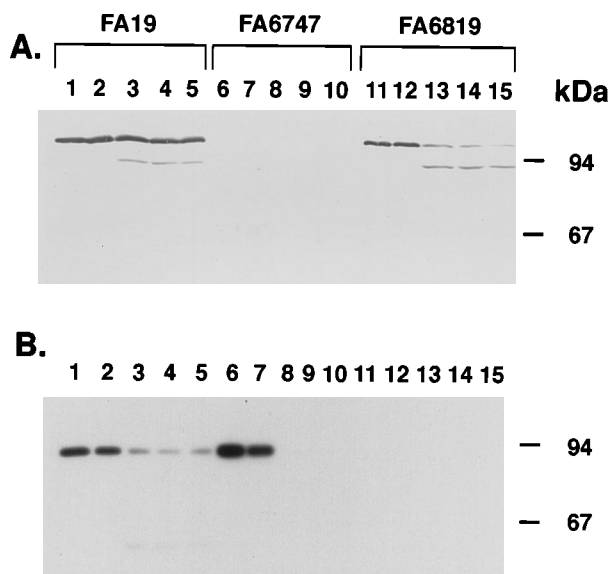


FIG. 8. Protease accessibility of Tbp1 and Tbp2 in the presence of transferrin. Panels and lanes are as described in the legend to Fig. 7. Cultures were mixed with 100 nM transferrin prior to trypsin exposure. Approximate positions of molecular mass standards are indicated at right. Blots were scanned with a Relisys 2412 scanner, and images were annotated with Adobe Photoshop software.

Blanton et al. (9) indicated that their calculated affinity may have been underestimated because of the slow centrifugation procedure used to separate bound from unbound ligand. The data presented in this work were generated by using a rapid filtration step that allowed unbound ligand to be separated from cells and bound ligand in approximately 15 s. In equilibrium-phase binding studies of receptors with affinities in the nanomolar range, the allowable separation time is approximately 1.7 min (7), which is considerably less time than was required to complete the centrifugation-based washes used in the previous study. Additionally, K_d is influenced by the receptor number, with apparent K_d increasing with receptor concentration (7). Thus, binding assays ideally should be conducted with the lowest possible receptor number; the binding studies presented here were conducted with 20- to 100-fold fewer cells per assay than those of previous studies. A filtration-based separation might also retain blebs and membrane fragments that could otherwise be lost by centrifugation, increasing the yield of total bound counts.

One interpretation of the observation of two transferrin-binding sites in the wild-type gonococcus is that these two binding sites represented the two characterized binding proteins. The copy numbers of the individual binding components in the wild-type strain suggested that the low-affinity site was sixfold more prevalent in cultures grown under these conditions. The estimates of K_d indicated that the wild-type binding sites differed in affinity by about 20-fold. This interpretation, however, is based upon the assumption that the binding proteins are noninteracting; that is, binding of transferrin to the first protein had no effect, sterically or conformationally, on binding to the second protein. We do not know this to be true, and in fact, other evidence indicated that the proteins did interact with one another (see below). Thus the two binding sites in the wild-type strain might be interpreted as two conformational states of a complex receptor, composed of both Tbp1 and Tbp2. Note that since the binding assays were conducted in growth media containing glucose and at room temperature, the cells were energized and capable of iron uptake from transferrin, a process which changes the ligand. Thus, both the ligand and the conformation of the receptor were potentially changing during the course of the assay. Analysis of the mutants indicated that neither protein expressed individually bound transferrin with binding parameters that approximated those of the wild-type strain. This suggested that the wild-type binding phenomena resulted from the combination of Tbp1 and Tbp2 at the cell surface. Determination of the precise stoichiometry of Tbp1 and Tbp2 in the wild-type strain will require further experimentation. Extrapolation from binding data obtained with the mutants to wild-type stoichiometry estimates could lead to erroneous conclusions. However, the mutant data indicated that the transferrin-binding site composed of Tbp1 only was about threefold higher in affinity than that composed of Tbp2 only. There were also ca. fivefold more Tbp2-specific transferrin-binding sites than Tbp1-specific binding sites as indicated by analysis of the mutants expressing either of these proteins individually.

Copy number estimates are reported here as numbers of sites per microgram of total cellular protein rather than the more traditional numbers of sites per CFU. All receptors in the culture remained competent to bind transferrin, even if they resided on cell fragments rather than viable cells. Conversion to CFU from micrograms of whole-cell protein is further complicated by the observation that the $Tbp1^-$ mutant was the least viable of the tested strains; that is, there were fewer CFU per microgram of whole-cell protein in cultures of this strain (data not shown). However, we could estimate that in the

wild-type strain grown under these conditions, there were approximately 750 high-affinity and 4,700 low-affinity binding sites per CFU.

Overall, the affinities of the transferrin receptor in the wild-type strain and of Tbp1 and Tbp2 individually are near the estimates made for the mammalian receptor, K_d , i.e., 5 to 20 nM (55). Thus, the bacterial receptor could effectively compete with its mammalian counterpart *in vivo*, even at low transferrin concentrations such as those probably existing on mucosal surfaces (42), and in other nonhematogenous gonococcal niches (39, 49). In fact, this analysis indicated that the high-affinity binding site on the wild-type gonococcus was capable of binding transferrin with even higher affinity than its mammalian counterpart. Binding transferrin with such a high affinity might necessitate a mechanism by which the receptor could recognize the deferrated ligand and, in a distinct step, release it from the receptor.

The mutant that expressed only Tbp2 showed a striking ability to discriminate between ferrated transferrin and apotransferrin. Tbp2 had a 100-fold higher affinity for ferrated transferrin than for apotransferrin, which is similar to the figure quoted for the mammalian transferrin receptor (55). Since transferrin exists *in vivo* in a partially saturated state (56), the ability of Tbp2 to differentiate between ferrated transferrin and apotransferrin might allow the gonococcus to identify ferrated transferrin molecules rapidly and bind them preferentially. The ability of Tbp2 to discriminate between these two forms of transferrin also could be important in release of transferrin from the cell surface after the iron has been removed. Blanton et al. (9) reported that the gonococcus, like the meningococcus (48), could discriminate slightly between ferrated and apotransferrin in the solid-phase, transferrin-binding assay but that they were unable to detect differential binding to the wild-type strain in liquid-phase experiments (9). In this study, we were able to detect discrimination between ferrated transferrin and apotransferrin by the wild-type strain in both liquid-phase and solid-phase experiments in the presence of deferoxamine mesylate. The extent of binding and discrimination by Tbp2 was underestimated in the solid-phase binding experiments.

Tbp1 and Tbp2 appear to interact with one another in wild-type gonococci, as evidenced by the altered properties exhibited by the separated proteins in the affinity purification assay. Sarkosyl-solubilized Tbp2 could be affinity purified in the presence but not in the absence of Tbp1, suggesting that Tbp2 might have bound to transferrin through Tbp1 under these conditions. Alternatively, Tbp2 in the absence of Tbp1 may have adopted a different conformation so that it no longer bound to transferrin in Sarkosyl. We found that Tbp2 without Tbp1 did not bind to transferrin in Sarkosyl, but binding was observed in the nonionic detergent Triton X-100. Since both proteins were capable of binding independently to transferrin in the affinity purification assay, given the appropriate detergent solubilization conditions, both proteins obviously were capable of independent transferrin binding and were not simultaneously required to reconstitute a binding site. More Tbp1 was purified as the salt concentration of the Sarkosyl-containing wash solution was increased, possibly because high-ionic-strength conditions enhanced hydrophobic interactions between Tbp1 and transferrin.

Protease accessibility experiments suggested that the conformation or exposure of Tbp2 was dependent upon the presence of Tbp1, consistent with the hypothesis that the two proteins interact. When Tbp1 was absent, virtually all of the expressed Tbp2 was readily accessible to trypsin. When Tbp1 was present, as in the wild-type strain, a portion of the expressed

Tbp2 remained resistant to trypsin exposure for at least 30 min while a fraction was trypsin accessible. It is possible that the apparent effect of Tbp1 on the conformation of Tbp2 was indirect. The absence of Tbp1 in the membrane may have altered the conformation and/or protease accessibility of Tbp2. Membranes lacking Tbp1 might be more prone to lysis or bleb formation, which may expose Tbp2 abnormally, although Tbp2 was not accessible to trypsin in these cultures. The exquisite sensitivity of Tbp1 to relatively low trypsin concentrations was in contrast to observations for outer membrane porins (46, 52), which were found to be relatively protease resistant. Tbp1 expressed without Tbp2 was more susceptible to trypsin than it was in the wild-type strain. In the presence of both transferrin and Tbp2, Tbp1 was quite resistant to trypsin exposure. On the other hand, Tbp2 appeared more susceptible to cleavage by trypsin when both Tbp1 and transferrin were present. This may suggest that when both Tbp1 and Tbp2 are present, ligand binding causes Tbp2 to adopt a more open conformation, allowing more access by trypsin. The trypsin accessibilities of both Tbp1 and Tbp2 in whole cells argue strongly that the proteins interact at the cell surface.

The results presented here suggest a model in which both Tbp1 and Tbp2 are surface-exposed, transferrin-binding proteins that together constitute the functional transferrin receptor, although both proteins can bind transferrin independently. Tbp1, by its homology with the TonB-dependent outer membrane receptors, is postulated to form the channel through which the iron from transferrin traverses the outer membrane (16). The affinity purification assays and protease accessibility experiments suggest that Tbp1 and Tbp2 interact, since the presence of Tbp1 influences the binding properties and conformation or exposure of Tbp2. Similarly, Tbp2 effects the binding and exposure properties of Tbp1. Tbp2, although not essential for growth on transferrin as an iron source *in vitro* (2), may increase the specificity of the functional receptor for ferrated transferrin and/or may be employed in release of the deferrated transferrin from the functional receptor once the iron has been removed.

The protease accessibility experiments suggested that Tbp2 exists in at least two conformations in the wild-type strain. In one form, Tbp2 is in an open, protease-accessible conformation, and in another form, Tbp2 is in a more closed conformation or is less accessible. Perhaps when Tbp2 is expressed without Tbp1 it exists in essentially one protease-sensitive form. Tbp2 in this protease-sensitive state is able to distinguish between ferrated transferrin and apotransferrin. These observations suggest that some aspect of Tbp1 conformation, function, or energization might alter the conformation of Tbp2 in the wild-type strain, thereby interconverting Tbp2 between protease-sensitive and protease-resistant states. In this model, the two transferrin-binding sites predicted from the binding data might be interpreted as two conformational states of the transferrin receptor, consisting of both Tbp1 and Tbp2. These binding conformations might then correspond to the open and closed forms expressed by Tbp2. Support for this speculative model of transferrin receptor structure and function will require further experimentation.

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