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TRANSFERRIN-IRON UPTAKE BY GRAM-NEGATIVE BACTERIA

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1. ABSTRACT

Members of the families Neisseriaceae, Pasteurellaceae and Moraxellaceae are capable of transferrin-iron acquisition in the absence of siderophore production. They do so via expression of a bi-partite receptor composed of two dissimilar proteins, TbpA and TbpB. Both proteins are surface exposed, iron-regulated and capable of binding transferrin. However, other physiochemical, antigenic, and immunogenic characteristics of the proteins are quite distinct. TbpB is a lipoprotein, which like the mammalian transferrin receptor is capable if discriminating between apo- and holo-transferrin. Expression of TbpB is not essential for transferrin-iron uptake, and in rare situations, the gene that encodes this protein is not linked to the gene encoding the second component. TbpA is a member of a family of TonB-dependent transporters, others of which accomplish ferric-siderophore and vitamin B12 uptake at the expense of a proton gradient across the cytoplasmic membrane. However, unlike the other TonB-dependent receptors where vitamins or ferric-siderophores are wholly internalized, the bacterial transferrin receptor must remove iron from transferrin at the cell surface. This review focuses on the structure-function relationships in the transferrin-binding proteins, their sequence and antigenic diversity, and the mechanisms by which they accomplish transferrin-iron uptake. The contribution of these proteins to pathogenesis and vaccine development based on TbpA and TbpB are also discussed.

2. INTRODUCTION

Iron is required by most living organisms, where it participates in redox reactions, oxygen transport, and iron detoxification processes (for review see reference 1). Because iron is an essential macronutrient for most organisms, efficient strategies have evolved for its acquisition. The ability to sequester iron is recognized as a virulence factor since the environment in a mammalian host is believed to be iron limited. Mechanisms for iron acquisition fall into two general categories: synthesis of siderophores and cognate receptors, or receptor-mediated acquisition of iron bound to host proteins (1). While these two mechanisms are not mutually exclusive, many of the enteric bacteria rely on the former strategy, while several mucosal pathogens rely on the latter. Members of the families Neisseriaceae, Pasteurellaceae, and Moraxellaceae are capable of utilizing host iron-binding proteins as iron sources via a receptor mediated process, without synthesis of a siderophore intermediate. While the pathogenic *Neisseria* species express receptors that recognize siderophores made by other microbes (2, 3), they do not excrete any detectable siderophores themselves (4). In addition to scavenging heterologous siderophores, the pathogenic *Neisseria* are capable of utilizing the human iron-binding proteins, transferrin, lactoferrin, and hemoglobin as iron sources (5, 6). Likewise, other mucosal pathogens, including *Moraxella catarrhalis* (7), *Actinobacillus pleuropneumoniae* (8), *Haemophilus influenzae* (9), and *Pasteurella multocida* (10) express a specific receptor for acquisition of transferrin-bound iron.

Transferrin is a monomeric, serum glycoprotein of approximately 80,000 Da whose function is to transport iron to tissues. Primarily synthesized in the liver, transferrin expression has also been detected in extrahepatic tissues including T-lymphocytes, brain and Sertoli cells (11). The transferrin concentration in serum is approximately 25 micromolar (12) and the protein is only about 30%

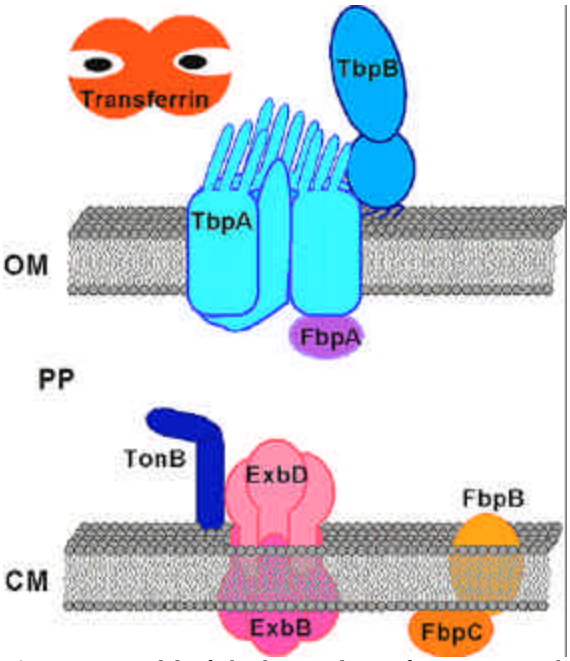

Figure 1. Model of the bacterial transferrin-iron uptake system. The glycoprotein transferrin consists of two similar lobes, each of which are capable of binding a single atom of ferric iron (black ellipses). The transferrin-binding proteins TbpA and TbpB (blue) are located in the outer membrane (OM), while the ferric binding protein (FbpA, lavender) is believed to be periplasmically-located (PP). Other members of the periplasmic-protein dependent uptake system include FbpB and FbpC (orange), which are thought to be an iron permease and an ATPase, respectively. TonB (indigo) is tethered to the cytoplasmic membrane (CM) via its amino-terminal region. ExbB and ExbD (pink) are integral, cytoplasmic proteins that harness the proton-motive force, with which TonB effects conformational changes and iron uptake. After transferrin binds to the Tbp complex, iron is extracted and passed to FbpA, which ferries it across the periplasm. The iron then traverses the cytoplasmic membrane via the FbpB and FbpC proteins.

saturated with iron, leading to a situation where the apo form (without iron bound) is typically in excess of the holo form (with iron bound) (13). The transferrin molecule can be divided into two similar lobes (N and C), each of which are capable of binding a single atom of ferric iron (Fe3+) in coordination with the anion carbonate. Iron uptake in mammals is accomplished via a dimeric protein receptor, which preferentially binds the ferrated or holo form of transferrin. The receptor is a transmembrane, lipid modified protein which is endocytosed by the cell in the process of iron uptake. As the endocytosed vesicle becomes acidified, protonation of the anion is believed to facilitate iron release from transferrin (14). The deferrated transferrin is then recycled to the cell surface where it is released into the extracellular fluid for another cycle of iron loading (14). The purpose of this review is to describe the mechanism by which Gram-negative bacterial pathogens have usurped this process of mammalian iron transport by adapting strategies very similar to those used by other bacteria to acquire siderophore-bound iron.

3. COMPONENTS OF THE BACTERIAL TRANSFERRIN-IRON UPTAKE SYSTEM

3.1. The receptor proteins, TbpA and TbpB

Two iron-repressed, transferrin-binding proteins (Tbps) were originally isolated by transferrin-affinity chromatography from *Neisseria meningitidis* (15). Due to their relative sizes, these proteins were referred to as Tbp1, the larger protein, and Tbp2, the smaller protein. Using a similar technique, the homologous gonococcal proteins were isolated and used to generate a polyclonal antiserum against Tbp1. Gonococcal expression libraries were screened with this antiserum for clones that expressed portions of Tbp1. Simultaneously, clones were isolated that repaired a defect in transferrin receptor function (trf). These clones represented two ends of the gene that encoded Tbp1. By chromosome walking, the rest of the gene was cloned, sequenced and named *tbpA* (16). At this time, it became clear that the product of the *tbpA* gene, subsequently re-named TbpA, was a member of the TonB-dependent family of bacterial outer membrane transporters. With the homologous proteins as templates, we proposed that TbpA traversed the outer membrane via at least 22 transmembrane beta-strands, connected extracellularly by variously-sized loops that served as ligand-binding domains (figure 1). Subsequently when the crystal structures of two TonB-dependent transporters were solved, it was clear that this family of proteins shared a globular domain, referred to as a "plug" or "cork", which served to occlude the inner orifice of the beta-barrel formed by the 22 transmembrane beta-strands. TbpA has a similarly-sized and positioned domain, which putatively serves a similar function.

TbpA has been insertionally inactivated in *N. meningitidis* (17), *N. gonorrhoeae* (16), *M. catarrhalis* (18), and *H. influenzae* (19). The lack of expression of TbpA results in mutants that are incapable of growth on medium containing transferrin as a sole iron source. This observation is consistent with the proposal that TbpA serves as the portal of entry for iron through the Gram-negative outer membrane.

The *tbpB* gene was identified in the pathogenic *Neisseria*, primarily based on its proximity to the *tbpA* gene (17, 20). *tbpB* in both *N. meningitidis* and *N. gonorrhoeae* is located immediately upstream of *tbpA*, the genes being separated by an intergenic region of 86 base pairs. The predicted protein contains a putative signal II cleavage site, suggesting that TbpB is a lipoprotein. This was confirmed in the case of gonococcal TbpB by intrinsic labeling with ^{14}C-palmitate (20). The TbpB protein expressed by *A. pleuropneumoniae* has likewise been shown to be lipidated (8, 21). It has been proposed that TbpB is surface exposed and loosely-tethered to the outer leaflet of the outer membrane (figure 1); however, it is possible that there are as yet unidentified transmembrane domains that allow this protein to span the outer membrane. Sequencing of *tbpB* revealed no outstanding homologues, leaving the structure of the TbpB protein a matter of continued speculation. An

interesting characteristic shared by TbpBs but not by TbpAs is their ability to discriminate between apo and holo-transferrin (22-25). Thus, this bacterial lipoprotein, like its eukaryotic counterpart, preferentially binds the ferrated form of transferrin suggesting that it recognizes the "closed", iron-bound conformation of transferrin (14). This specificity for ferrated transferrin could explain the observation that mutants lacking TbpB are capable of transferrin-iron acquisition, albeit at a lower rate. Gonococcal mutants lacking TbpB were capable of 50% of wild-type levels of iron uptake from transferrin, after adjusting for the amount of receptor proteins made by the respective isogenic mutants (reference 20 and unpublished observations). Thus, while TbpB was not absolutely required for transferrin-iron uptake, its presence made the process more efficient. The ability of this protein to preferentially recognize the ferrated ligand might be important in both initial recognition of the iron-laden ligand and also in release of the "used" transferrin, once iron has been removed.

3.2. The periplasmic-binding protein dependent transport system

The *fbp* (ferric binding protein) gene cluster, located at a locus distant from the *tbp* gene cluster in the pathogenic *Neisseria*, encodes proteins that bear similarity to the periplasmic-binding protein dependent cytoplasmic permease systems (PBP) (figure 1) such as those for maltose uptake and histidine import (26). FbpA, the protein encoded by the first gene in the putative operon, was originally described as a major, iron-regulated, outer membrane protein (27). Subsequently it was shown that FbpA received ^{55}Fe that had been delivered to the cell by transferrin, lactoferrin or citrate, implicating this protein in the periplasmic transport of ferric iron (28). Interestingly, the FbpA protein, which has been crystallized, bears a striking resemblance to a single lobe of transferrin (29), leading to speculation that this protein is the "primordial" transferrin, existing prior to the putative gene duplication event from which the current bi-lobed transferrin arose. FbpA binds ferric iron with an affinity near that of transferrin, and the iron is coordinated with similar residues and an anion (30). These observations make it feasible for FbpA to serve as the periplasmic transporter of ferric iron after it has been stripped from transferrin and passed into the periplasm through the pore in TbpA (figure 1).

The genes located downstream of *fbpA* (*fbpB* and *fbpC*), encode proteins that resemble cytoplasmic permeases and ATPases, respectively (31). The FbpB gene product (figure 1) resembles MalF and HisQ, both members of a well-characterized class of cytoplasmic permeases (32). The FbpC gene product (figure 1), like its homologues, contains a highly-conserved Walker box (32) consistent with ATP binding capability by this component. It is proposed that the FbpC protein provides the energy, in the form of ATP hydrolysis, to drive uptake through the FbpB protein, which serves as the permease (31). It should be noted that these steps are directly analogous to those known to occur in the process of ferric-siderophore and vitamin B12 uptake in Gram negative bacteria (33). While there is some controversy as to whether all of the members of this putative operon are co-expressed (34-36), it seems that those pathogens capable of transferrin-iron uptake, also encode genes for PBP-dependent internalization of ferric iron. For some time, it was believed that FbpA was an essential gene, as all attempts to inactivate it failed. However, insertional inactivation mutants were successfully generated in a strain that expressed a functional hemoglobin receptor (37-39), consistent with the idea that FbpA is required for ferric iron acquisition, but not for import of heme-bound iron. Presumably, there is a heme-specific PBP dependent uptake system in these mucosal pathogens which has not yet been identified.

3.3. The energy transducing system

Those pathogens that can utilize transferrin-bound iron also express homologues of the TonB-ExbB-ExbD energy transduction system (figure 1), which has been well-characterized in enteric bacteria (for review see reference 40). Although functionally conserved, the sequences of these genes among the *Neisseriae* and *Pasteurellae* are highly divergent, sharing only approximately 35-45 % similarity with those in *Escherichia coli* (41-43). While not the focus of this review, this group of interacting proteins is thought to harness the proton-motive force, generated across the cytoplasmic membrane, and pass it off to TonB-dependent receptors in the outer membrane (for review see reference 44). ExbB and ExbD have been proposed to sense the proton gradient, and to transduce this potential energy to TonB. TonB has been shown to physically interact with TonB-dependent siderophore and vitamin transporters (45, 46), which is believed to be the event that causes a conformational change in the transporter eliciting a cycle of substrate internalization through the beta-barrel.

While the energy transduction process is believed to be similar in the enterics and mucosal pathogens, TonB-dependent internalization of transferrin-bound iron is notably different from ferric siderophore and vitamin transport in several respects. Transferrin-bound iron must be removed at the surface prior to transport through TbpA. In contrast, the entire ferric-siderophore is internalized without an iron removal event at the outer membrane. It seems likely that iron extraction from transferrin requires TonB-derived energy, however this issue has not been experimentally addressed. In the gonococcal system, it has been demonstrated that TonB mutants are inhibited in transferrin release from TbpA (47); however, it is unclear if TonB is utilized for iron removal first, which facilitates release, or if TonB is required for the release event itself.

Like the situation in the enterics, a sequence near the amino-terminus of TbpA, termed the TonB-box, is required for energization of the receptor (47). Conversion of Ile_{16} to Pro resulted in a de-energized phenotype; that is, the mutant receptor was competent for transferrin binding, but unable to accomplish transferrin-iron uptake. This mutant, like those defective in the *tonB-exbB-exbD* genes (43), was unable to efficiently release transferrin from the cell surface, a phenomenon which might have contributed to the extraordinarily high apparent binding affinity attributable to mutant TbpA (47). We have been able to co-

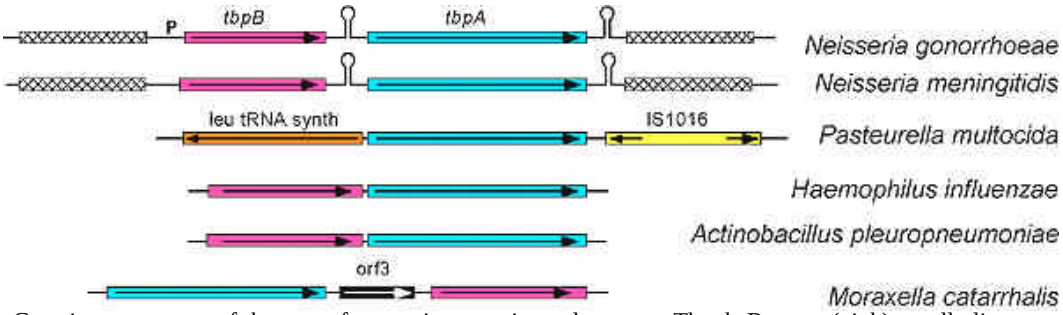

Figure 2. Genetic arrangement of *tbp* genes from various species and genera. The *tbp*B genes (pink) usually lie upstream of the *tbp*A genes (blue), except in the case of the loci in *M. catarrhalis* (50) and *P. multocida* (10). In the case of *M. catarrhalis*, the gene order is reversed and the two are separated by an open reading frame (orf3) of unknown function. In the case of *P. multocida*, only a *tbp*A gene has been identified and characterized, and it is flanked by a gene that encodes a leucine tRNA synthetase (orange) and an IS element (yellow). The *tbp* genes in the pathogenic *Neisseria* are separated by a potential stem-loop structure; the hairpin loop that lies downstream of *tbp*A is likely a rho-independent transcription termination signal. The cross-hatched boxes flanking the *tbp* loci in the pathogenic *Neisseria* represent extended regions of repeats, which encode no detectable open reading frames. Long black arrows represent the direction of transcription for each gene, while the short black arrows (at either end of IS1016) represent the terminal repeats. P represents the putative promoter region that lies directly upstream of gonococcal *tbp*B. Genes are not drawn to scale.

purify TonB with TbpA in affinity purification experiments (48). The TonB-box mutant, however, did not facilitate this physical association, consistent with the idea that a wild-type TonB box sequence is necessary for interaction between TonB and the transporter (48). While this is similar to the situation in E. coli, we have also detected an interaction between TbpA and TonB without the addition of ligand, that is, in immunoprecipitation experiments using an antibody specific for recombinant TbpA (48). Another intriguing difference between E. coli and neisserial TonBs is that the latter appears to be more profoundly regulated by internal iron concentrations (48). A rationale to explain both of these findings is that iron uptake from transferrin might be regulated by iron stress more so than by receptor occupation. Thus, in E. coli, TonB is believed to charge the transporter for uptake only when ligand is bound, thereby making efficient use of limited TonB pools (49). In the case of the Neisseriae, having ligand bound could be advantageous as a molecular shield, while charging the receptor for iron removal and iron uptake would only be useful if diminished iron pools warranted uptake.

4. GENETIC ORGANIZATION AND DIVERSITY OF THE TRANSFERRIN-IRON UPTAKE SYSTEM

4.1. Genetic arrangement and regulation

The genes encoding the Tbps are linked in the chromosome, typically with the TbpB gene preceding the TbpA gene; however, this arrangement is not universal (figure 2). In *M. catarrhalis*, the *tbpA* gene precedes the *tbpB* gene, and the two are separated by an open reading frame of unknown function (50). The *tbpA* gene of *Pasteurella multocida* is located downstream of a gene that encodes a leucine tRNA synthetase, and is flanked 3' by an IS element (10) (figure 2). Surprisingly, there is no linked *tbpB* gene in this organism (10), which is similar to the recently described transferrin receptor of *Histophilis ovis* (51). Both observations are consistent with the idea that TbpB is not absolutely required for transferrin-iron uptake, but makes the process more efficient when it is present.

The neisserial *tbp* gene locus is the only one described to date in which the *tbp* genes are separated by any significant distance. The gonococcal *tbpB* gene is separated from *tbpA* by 86 base pairs (figure 2), although the two are co-transcribed in a single bicistronic operon (52). Within the intergenic region is a region of potential secondary structure consisting of 35 nucleotides, with a calculated free energy of dissociation of –23.6 kcal (16). The presence of a secondary structure in the bicistronic operon or in a *tbpB*-specific monocistronic message could be important for maintaining the optimum stoichiometry of *tbp*-specific mRNA species, and could translate to effects on the stoichiometry of the functional Tbp complex. Given that this genetic arrangement of the *tbp*s is unique to the pathogenic *Neisseria*, it follows that the intergenic region and its impact on Tbp expression, could be particularly important to the biology of these organisms. Similarly unique to these organisms is the presence of flanking silent regions extending more than 2 kb in both directions (53, 54). The significance of these repeat-rich regions and their impact on Tbp expression remain to be explored.

The mechanism by which the Tbps are expressed as a function of iron stress has been studied in *N. meningitidis* and in *N. gonorrhoeae* (55, 56). Both operons are preceded by putative promoters, which contain sequences with significant similarity to Fur-binding sites. The Fur (ferric uptake regulator) protein, is a global regulator that represses gene transcription in the presence of high internal pools of ferrous iron (57). The neisserial *fur* genes have been characterized, and a point mutation in gonococcal *fur* resulted in decreased iron-dependent repression of the Tbps (58). While the Fur binding site has not been genetically characterized in the *tbp* locus, it is clear that Fur mediates repression of these genes. To date, iron is the only well-characterized mechanism that influences expression of the transferrin receptor components. Decreased external pH appears to negatively impact expression of the Tbps, however the mechanism by

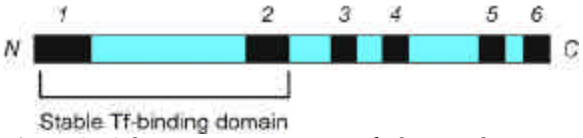

Figure 3. Schematic representation of TbpB. The protein is shown from amino- (N) to carboxy-terminus (C). Numbers above the gene correspond to 6 regions (black boxes) of sequence similarity identified among the TbpB proteins characterized to date. The amino-terminal half of TbpB (bracketed region) contains a stable transferrin-binding domain that resists heating and SDS.

which it does so is unclear. Addition of ligand has no effect on Tbp expression (52). This is in contrast to the situation with some siderophore transporters, whose expression is increased in the presence of substrate (59). Unlike many other genes in the chromosomes of *Neisseria* spp. and *Haemophilus influenzae*, there is no evidence that the *tbp* genes are "contingency loci" (60), that is loci that are preceded by homopolymeric stretches of nucleotides capable of undergoing slipped-strand mispairing (61).

4.2. Sequence and antigenic diversity in the transferrin-iron uptake system

Neisserial TbpA sequences are quite well-conserved, sharing between 80 and 99% similarity (62). The lowest degree of conservation is detected when comparing TbpAs from *N. gonorrhoeae* and *N. meningitidis* strain B16B6. This meningococcal strain expresses the low-molecular weight isotype of TbpB, and represents approximately 20% of meningococcal strains surveyed (63). On the other hand, those strains that express a high-molecular weight TbpB (represented by strain M982) express a TbpA protein that is very similar (>94% similarity) to gonococcal TbpAs (62). When the analysis is extended to other genera, significant sequence similarity is retained. Pairwise comparisons between gonococcal TbpAs and the distantly-related TbpA from *Pasteurella haemolytica* still result in similarity scores between 51 and 53%. Thus, the structure-function relationships in TbpAs are constrained to a large extent, necessitating that sequences be preserved across general.

In contrast, TbpBs are very diverse, sharing only 57 to 75% similarity among the pathogenic Neisseria spp. (64). When extended to the other genera, this analysis yields a range of from 42 to 47% similarity. Six regions of sequence conservation can be identified in alignments of TbpBs (figure 3). Originally, this analysis was applied to the nine TbpBs that had been characterized from the pathogenic Neisseria spp. (64). When this analysis is extended to those TbpB sequences currently available in GenBank, the same patterns of sequence conservation emerge. The first region of conservation corresponds to the putative signal peptide and the contiguous ca. 50 amino acids. The second region of homology is confined to a small stretch of amino acids near the middle of the protein. Four shorter regions of conservation are located in the carboxy-terminal half of the protein (figure 3). Region 3 contains two tandem cysteine residues, which are present in all except the TbpBs of M. catarrhalis. The significance of this conserved sequence and its contribution to the function of TbpB remain unclear.

The antigenic variability of TbpB is also greater than that described for TbpA (63, 65-67), suggesting that the latter could prove to be the better, and more conserved, vaccine target. We have assessed antigenic variability using antisera generated against short peptides of gonococcal TbpA (62). In general, the antigenic variability tracks with observed sequence diversity, as expected, and extending the length of the antigenic peptide beyond ca. 20 amino acids generates antibodies that are more cross-reactive strain to strain (reference 68 and unpublished data). In contrast, antibodies against meningococcal high-molecular weight TbpBs cross react with TbpBs within that size class, but not with proteins of the low molecular weight isotype (63). Taken together these results suggest that TbpBs are more variable than TbpAs and thus antibody responses generated against the former are less cross-reactive. On the other hand, while antigenic variation does exist among TbpAs, by extending the length of the immunizing epitope, we can generate a cross-reactive immune response. It remains to be seen if this immune response can protect against infection.

5. STRUCTURE-FUNCTION RELATIONSHIPS IN TBPA

Using computer prediction methods, sequence diversity, and similarity with other TonB-dependent transporters, we have developed a two-dimensional topology model for gonococcal TbpA (figure 4 and reference 69). After the crystal structure of the *E. coli* ferric-enterobactin receptor (FepA) was solved (70), we utilized this information to identify those regions of TbpA that were homologous to the observed beta-strands of FepA. This approach therefore mandates that our TbpA topology model has 22 transmembrane beta-strands, as this is the case for FepA. If there are more than 22 transmembrane strands in TbpA, as one might expect given that TbpA is ca. 20 kDa larger than FepA, these strands are likely to be near the carboxy-terminus of the protein. In the carboxy-terminal third of the protein, more amphipathic beta-strands are predicted and there is very little sequence variability, consistent with structural constraints and/or limited surface accessibility.

We began testing the topology model by creating TbpA loop deletions in *N. gonorrhoeae* (69). Deletion of loops 4 and 5 (figure 4) resulted in gonococcal mutants that were incapable of binding transferrin or utilizing this protein as a sole iron source. Deletion of loop 8 resulted in a mutant that bound transferrin, but with diminished affinity but wild-type capacity (i.e., copy number). This mutant was, however, incapable of growth on transferrin-bound iron, regardless of the presence or absence of TbpB. These results implicate loops 4 and 5 as critical transferrin binding epitopes and further indicate that tight interactions between TbpA and transferrin are required for iron extraction and subsequent growth. We have also cloned and over-expressed gene fragments that encode various loops of gonococcal TbpA (68). We tested the binding capabilities of each of the loop fusions and found, quite surprisingly, that loop 5 and a larger fusion consisting of

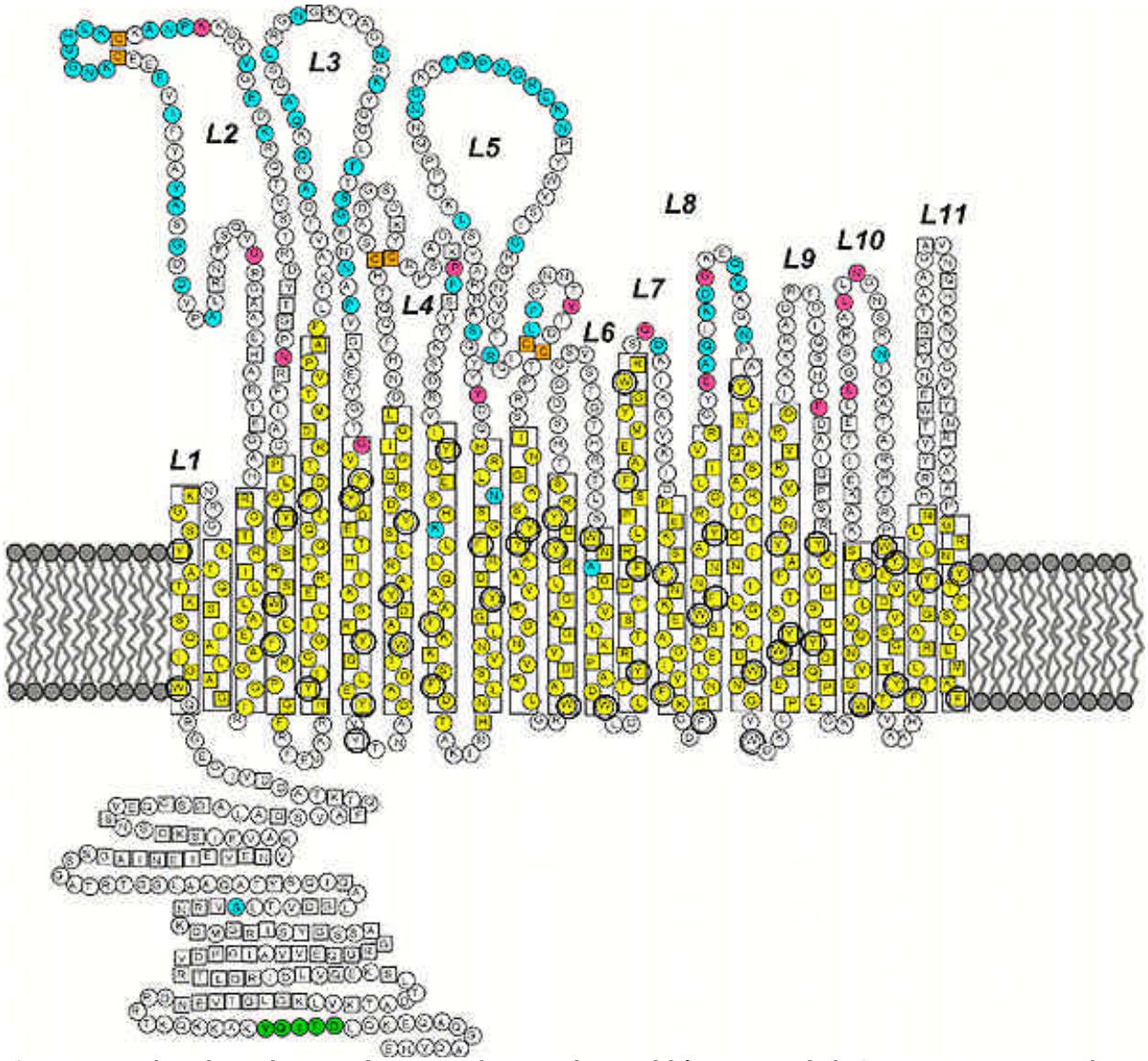

Figure 4. Hypothetical, two-dimensional, outer membrane topology model for gonococcal TbpA. Twenty-two transmembrane, amphipathic beta-strands (vertical rectangles) are predicted from an alignment between gonococcal TbpA and *E. coli* FepA. These 22 transmembrane strands are connected by 11 extracellular loops (numbered L1-L11) and short periplasmic turns. The protein contains 6 conserved Cys residues (orange), which are suggested to form disulfide bridges in three extracellular loops. The characterized TonB-box is shown in green. Regions that are well conserved among TbpA proteins (62) are shown in yellow. These coincide precisely with the predicted beta-strands. Positions of sequence diversity in an alignment of gonococcal TbpAs (62) are shown in blue. Residues highlighted in pink are those that are conserved among the TbpAs that bind human transferrin but distinct in TbpAs that bind other animal species of transferrin. These residues could reflect positions at which species specificity is imposed by the receptor. Residues that are circled are the aromatic amino acids, W, Y or F. Residues shown in squares are those that are completely conserved among the TbpA proteins, regardless of the species of transferrin that is bound. The TbpA sequence shown is that of gonococcal strain FA19.

loops 4 and 5 bound transferrin specifically. These results coincide with those of the deletion analysis and identify at least one ligand binding domain within loop 5. Interestingly, this loop is one of the most variable in terms of sequence identity and length (reference 62 and figure 4), leading to the hypothesis that vulnerable binding epitopes are camouflaged with and surrounded by antigenic diversity (68).

We have also generated loop-specific antisera, with which we have confirmed the surface exposure of loops 2 and 5 (71). As another test of surface exposure, we have utilized an epitope insertion approach to verify the surface exposure of loops 2, 3, 5, 7, and 10 (72). Insertion into these loops variously impacts ligand binding (72), suggesting that binding epitopes are distributed among various loops and that perhaps high affinity binding of

transferrin is mediated by the convergence of multiple binding domains located on physically-disjointed, surface-exposed loops.

6. STRUCTURE-FUNCTION RELATIONSHIPS IN TBPB

Because no TbpB homologues have been identified for which the crystal structure is known, no topology models have been developed for these proteins. The amino-terminal half of TbpB is required to form a stable transferrin binding domain (figure 3), which withstands heating and treatment with SDS (64, 73). A lower-affinity binding domain was identified in the less diverse carboxy-terminal half of meningococcal TbpB (74), and may be characteristic of TbpBs in general. The observation that meningococcal TbpB contains two transferrin binding domains (74, 75) and internal sequence redundancy (76) has led to the concept that, like the ligand it binds, TbpB is a bi-lobed protein (figure 1).

We have proposed previously that the structure of TbpB is particularly sensitive to drying, as the relative amount of transferrin bound to TbpA vs. TbpB was different depending on whether the cells were presented with ligand in a solid-phase (dot blot) assay or in a liquid-phase assay (25). However, since the ligands used as probes in these two assays are different, it seems prudent to entertain an adjunct or alternative hypothesis for this finding. For the solid-phase assay, HRP-labeled transferrin is employed, whereas in the liquid-phase binding assay, ^{125}I-labeled transferrin is the ligand. The larger issue is that the commercially-available HRP-labeled protein is only partially saturated with iron while the ^{125}I-labeled ligand is completely saturated. Thus, is seems likely that at least one reason for the under-estimation of TbpB-specific binding in the solid-phase assay is that the ligand in this assay is poorly recognized, given that TbpB preferentially binds the ferrated form of transferrin. Thus, this discrepancy could be a direct reflection of the selectivity of TbpB for holo-transferrin.

7. MECHANISM OF UPTAKE

7.1. What's known

Several observations suggest that TbpA and TbpB function together to bind transferrin and relieve it of its iron. First, both proteins bind transferrin on the cell surface, but they do so differently. Their respective interactions with transferrin are differentially sensitive to ionic strength conditions, and their ligand specificities differ. In spite of these differences, the proteins expressed individually on the surface of the gonococcus bind to transferrin with similarly-high affinities, in the nanomolar range. However, when the two proteins were expressed simultaneously, new binding phenomena emerged, none of which were similar to those observed with the individual proteins (25). These observations suggest that TbpA and TbpB function together on the cell surface, to elicit binding characteristics that are unique to the complex. Second, the protease accessibility of TbpB is dramatically influenced by the presence and TonB-dependent energization of TbpA (47). These results indicate that the two proteins are at least in close proximity to one another in the outer membrane, and suggest that they form a complex. Finally, transferrin affinity isolation experiments yield TbpA and TbpB when TbpA is expressed, but neither protein when TbpA is absent (under high ionic strength conditions) (17, 25). Likewise, the *in vitro* characteristics of purified meningococcal Tbps suggest that the two proteins form a stable complex (77). Thus, all of the available evidence leads to the conclusion that TbpA and TbpB, while capable of independent, distinct interactions with ligand, actually operate as one unit in the outer membrane to facilitate ligand binding and possibly iron extraction.

Mutants defective in TonB-dependent energization of TbpA released transferrin very slowly and inefficiently (47). This implies that energy is required for ligand release, but it is unclear if iron extraction is a necessary prerequisite for release and is the energy-requiring step that is prevented in these mutants. There is also a rapid release of ligand that occurs within the first 5 minutes after addition of cold competitor (47). This release is energy independent and may be due to the presence of a low affinity binding site on TbpB. This result may also suggest that some population of TbpB is free, i.e., not complexed with energized TbpA and therefore not influenced by TonB-derived energy.

Very little is known about the precise molecular steps involved in the uptake of transferrin-bound iron. It is clear that TbpB makes the process of iron uptake from transferrin more efficient (20), perhaps by virtue of its specificity for holo-transferrin. Iron uptake is also completely dependent upon the presence of TbpA and TonB-derived energy (16, 47). Also apparent is the fact that radiolabeled iron donated by transferrin is ultimately captured by FbpA in the process of internalization (28). However, the method of iron extraction from transferrin, the point at which TonB-derived energy is expended in the transport process, TbpB's precise role in uptake, and whether or not iron is transiently bound to TbpA during transport, are all currently unanswered questions.

7.2. Speculation

Based on what is known about the eukaryotic transferrin receptor and its function in iron uptake, we can speculate about how iron might be extracted from this glycoprotein. Iron release from transferrin is potentiated by the presence of the eukaryotic receptor and an acceptor molecule, citrate (14). It is believed that protonation of the coordinating anion facilitates iron removal, at which point the iron is released and subsequently bound by the acceptor. Perhaps the same is true for the bacterial transferrin receptor, in which case the source of the protonating species must be determined. Could TonB, the protein that harnesses the proton motive force, be the source of the protons that are ultimately used to effect the iron extraction event? The presence of TbpB could provide the selectivity of the ferrated ligand to the receptor complex, in terms of initial recognition, and in addition provide an efficient release mechanism. Given that the bacterial receptor binds transferrin with a K_d in the

nanomolar range, release of the ligand could hamper rapid turnover and iron uptake efficiency. However, since TbpB binds apo-transferrin only very poorly, the deferrated ligand could be efficiently released, after iron has been extracted. After the iron has been removed from transferrin, where does it go? It seems unlikely that iron could "float" through the barrel formed by TbpA to associate with FbpA situated below (figure 1). Perhaps there are transient iron binding sites located within the globular "plug" domain, which then serves to usher the released iron through the pore, and to subsequently hand it off to the periplasmic binding protein. In this scenario, the "plug" functions not only to guard the periplasm from entry of unwanted species, but also as a chaperone for that which gets through the entry gate, set up by the extracellular loops. Thus, the overall process might proceed as follows. TonB is charged at the cytoplasmic membrane and then associates with TbpA, facilitated or interfered with by the sequence of the TonB box. In this system, the frequency with which this interaction takes place is influenced more by iron stress levels than by the presence of bound ligand. TonB influences the conformation and/or protonation of TbpA, a signal that is transduced to transferrin. Transferrin is then compelled to release its bound iron, perhaps by protonation of the coordinating anion. The iron is then transiently bound to the "plug" domain, which in this system serves as the acceptor molecule. The plug changes conformation in the process of transport, which allows periplasmically-located FbpA to get access to the transported iron. From this point, FbpA binds the ferric ion in a manner similar to that of a single lobe of transferrin using phosphate as the coordinating anion, and shuttles the ion across the periplasm to the cytoplasmic membrane permease system, composed of FbpB and FbpC. At this level, reduction might occur, which would facilitate iron release from the PBP-dependent system and subsequent assimilation into the cytoplasm.

8. PATHOGENESIS AND VACCINE IMPLICATIONS

It is difficult to discuss the transferrin receptor without regard to its impact on pathogenesis and its utility as a potential vaccine component. The fact that this receptor is not subject to high-frequency phase or antigenic variation, and that it is expressed by all gonococci and meningococci tested to date indicate that the function of this receptor *in vivo* is critical to the survival of these organisms. Gonococcal mutants that lack this receptor are incapable of eliciting signs or symptoms of urethritis in human male volunteers (78). This transferrin receptor deficient strain was constructed in the background of a naturally-occurring strain that does not express the lactoferrin receptor. This wild-type strain, unlike the isogenic transferrin receptor mutant, does cause experimental urethritis in human males. It is possible that the lactoferrin receptor could substitute for the transferrin receptor in terms of creating a viable, infectious gonococcus, but the fact remains that in nature, nearly 50% of gonococcal strains do not express a functional lactoferrin receptor (6). These observations suggest that expression of the transferrin receptor, not the lactoferrin receptor, provides a selective advantage to the gonococcus as it infects its human host. In an estradiol-treated mouse model (79), transferrin, lactoferrin and hemoglobin receptors were not required for colonization of the female genital tract (80). While perhaps not surprising, given the specificity of the gonococcal transferrin and lactoferrin receptors for the human iron-binding proteins, these experiments suggest the presence of other possible iron sources available to gonococci in the female genital tract. While the preceding results pertain specifically to the gonococcal Tbps and their roles as virulence factors and vaccine antigens, similar experiments have been conducted with the Tbps from *N. meningitidis* and *A. pleuropneumoniae*. Meningococcal Tbps have been shown to elicit bactericidal antibodies and protect against challenge in animal models (81, 82). Similarly, Tbps from *A. pleuropneumoniae* elicit a protective immune response (83) and are necessary to cause disease experimental animals (84).

Since the components of transferrin receptor are expressed and necessary *in vivo*, it stands to reason that they might be suitable targets for immunoprophylaxis. However, antigenic variation, and in the case of TbpA, strategically-located antigenic variation, might hide vulnerable epitopes from surveillance. In addition, there is accumulating evidence (85-87) that the gonococcus modulates downward the immune response against self-epitopes, and perhaps other non-self antigens as well. This will require that epitopes be identified that will elicit a functional, protective immune response and that these epitopes be presented to the immune system absent highly immunogenic, antigenically-variable sequences. In addition, these epitopes will have to be presented to the immune system with an appropriate mucosal adjuvant so that a functional, cross-protective immune response can be elicited, even if native infections do not elicit such a response.

9. PERSPECTIVES

While we have learned a great deal about the structure, function, antigenicity and variability of the proteins that comprise the bacterial transferrin receptor, there is still a much that we do not clearly understand. The following is a list of outstanding questions that, when answered, would significantly contribute to a complete understanding of the bacterial transferrin-iron uptake process. 1. How is iron removal accomplished at the outer membrane and at the cytoplasmic membrane? Both transferrin and FbpA bind ferric iron with extremely high affinity and use similar coordination strategies, thus the iron removal process at both locations might be similar. 2. How does TonB contribute to the iron removal process at the outer membrane? Is this protein involved in iron extraction from transferrin, in release of the deferrated ligand, or in both? Does the latter process depend upon the successful completion of the former? 3. What is the structure of TbpB? Is the protein entirely extracellular or are there as yet undefined transmembrane domains? Is TbpB important in both initial recognition of holo-transferrin and in release of deferrated-transferrin? Does TbpB serve another function? 4. How are TbpA and TbpB

transported to the cell surface? Do they form a stable complex in the outer membrane and if so, how do they find one another? Does their association with each other depend on external factors such as energy or ligand? Answers to these questions will aid in our understanding of the basic mechanism that underlies this transport process and will focus future vaccine development efforts.

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