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MECHANISMS OF IRON ACQUISITION BY THE HUMAN PATHOGENS *NEISSERIA MENINGITIDIS* AND *NEISSERIA GONORRHOEAE*

Kyle H. Rohde and David W. Dyer

Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Biomedical Research Center Room 366, 975 NE 10th Street, Oklahoma City, Oklahoma 73104

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1. ABSTRACT

It is well established that bacterial pathogenesis is dependent on the ability to acquire iron within the host. The success of the highly adapted obligate human pathogens *Neisseria meningitidis* (NM) and *Neisseria gonorrhoeae* (NG) can be attributed in part to the efficient utilization of multiple host iron (Fe) sources, allowing replication on mucosal surfaces, in the bloodstream, and intracellularly. Most Gram-negative bacterial strategies for scavenging iron from the human host rely on the TonB protein to energize active iron transport across the outer membrane. Pathogenic *Neisseria* express multiple high-affinity iron transporters including a family of two-component TonB-dependent receptors as well as multiple single-component TonB-dependent Fe transporters. This review describes our current understanding of the mechanisms *Neisseria* have evolved to utilize various iron sources encountered during infection of the human host. Recent studies have provided insight into the interaction of neisserial outer membrane receptors with host iron carrier proteins. Emerging structural information on neisserial iron transporters will be compared with the crystal structures and biochemical data available for homologous *Escherichia coli* TonB-dependent Fe-siderophore receptors. In the process, we will highlight the aspects of the iron transport process that are unique and those that remain to be experimentally demonstrated in *Neisseria*. These include receptor structure/function, the mechanism of iron removal from protein ligands, the fate of Fe and heme-Fe after traversing the outer membrane, and the role of TonB-associated energy in receptor functions. Finally, we will discuss regulatory mechanisms that control the expression of iron scavenging systems. The investigation of iron metabolism in NM and NG is important for understanding the biochemistry of this virulence factor, the development

of vaccines targeted at outer membrane iron receptors, and therapeutic interventions exploiting these transporters as high affinity drug delivery systems.

2. INTRODUCTION

2.1. Pathogenesis of meningococcal and gonococcal disease

NM and NG, two closely related Gram-negative human pathogens, cause very different diseases yet are adapted to occupy similar niches within the host. Several comprehensive reviews discuss neisserial pathogenesis and host cell interactions (1-4). NM is a leading cause of bacterial meningitis and sepsis in the United States and is responsible for epidemics in the "meningitis belt" of sub-Saharan Africa (5-9). NG, however, is a sexually transmitted disease (STD) agent associated with mucosal inflammatory disease (urethritis and cervicitis), salpingitis, and pelvic inflammatory disease with sequelae including infertility and ectopic pregnancies (3). The first step of both neisserial infections is the colonization of a mucosal surface mediated by adhesins such as pili, Opa, and Opc proteins. NM typically attaches to the nasopharyngeal mucosa whereas NG adheres to male urethral epithelia or the female uterine cervix (1, 3). Engagement of host epithelial receptors (CD66 and heparan sulfate proteoglycan receptors) triggers the engulfment of bacteria into vacuoles, with subsequent intracellular multiplication. Some intracellular NM traverse the mucosal epithelium,escape into the subepithelial stromal tissue, and gain access to the bloodstream. Though less frequently, NG also can penetrate the epithelial barrier to produce bacteremia and disseminated gonococcal infection (DGI) involving the skin and synovial tissue of joints. DGI is typically a chronic infection that is not life-threatening, although the sequelae can be significant and life-long. In contrast, invasive meningococci multiply rapidly in the bloodstream (reaching 10^8 CFU/ml), leading to the development of fulminant meningococcal sepsis. Other pathophysiological signs of advanced meningococcal disease include petecchial hemorrhaging, shock, penetration of the blood-brain barrier and meningitis, disseminated intravascular coagulation (DIC), consumption coagulopathy, multiple organ failure, and death. Despite the considerably different clinical syndromes that these pathogens cause, the Fe transport mechanisms that they possess are highly conserved between the two organisms.

2.2. Iron acquisition as a virulence factor

Iron (Fe) is essential for the growth of almost all living cells due to its role in cellular functions such as oxygen transport and storage, electron transport, the reduction of ribonucleotides and dinitrogen, and the decomposition of peroxides (10-12). In these processes, Fe serves as a cofactor or prosthetic group for numerous essential proteins including hemoglobin, myoglobin, cytochromes, ribonucleotide reductase, nitrogenase, peroxidase, catalase, and succinic dehydrogenase. Thus, eukaryotic cells and microorganisms share the need for Fe in order to carry out basic metabolic tasks. However, the chemical properties of iron present two problems for the human host physiology. Ferric iron (Fe^{3+}) is extremely insoluble ($K_{sp}=10^{-38}$M) at physiological pH in an aerobic, aqueous environment (13). In addition, Fe^{3+} is highly cytotoxic due to its ability to induce free radical formation via the Fenton reaction, leading to lipid peroxidation, DNA strand breakage, and protein denaturation (14, 15). To suppress these problems, human physiology has evolved carrier proteins to sequester Fe intracellularly, complexed with ferritin and hemoproteins, and extracellularly, bound to transferrin (Tf) and lactoferrin (Lf). As a result, the concentration of free Fe in serum is limited to only 10^{-18}M (12, 16-19).

However, most microorganisms require ~10^{-6}M Fe to support growth, approximately 10^{12}-fold more than is available in human tissues and body fluids (17-19). Thus, many nonpathogens are incapable of causing disease because they lack the capacity to obtain essential Fe for growth during infection. The term "nutritional immunity" was coined to describe this nonspecific host defense against infection (20). Further, human pathogens must contend with the host hypoferremic response. This cytokine-mediated acute-phase response to septicemic infection further reduces Fe availability by increasing serum Lf and Tf levels (reducing Tf Fe saturation), suppressing intestinal assimilation of Fe, and increasing intracellular Fe storage in the liver (17, 18, 20, 21). A key to the success of NM and NG is the redundant mechanisms these microbes have evolved to overcome this "iron blockade". *In vitro* and *in vivo* experiments have shown that pathogenic *Neisseria* exploit numerous host iron sources including ferric and ferrous salts, heme (Hm), siderophores, Tf, Lf, hemoglobin (Hb), and hemoglobin complexed to haptoglobin (Hp) (22-28). Animal disease models and human challenge experiments have clearly demonstrated that iron assimilation supports meningococcal and gonococcal disease. Holbein *et al.* demonstrated that intraperitoneal injection of Fe-dextran enhanced NM infection in mice and this was reversible by Fe chelation (29, 30). Similarly, this model showed that the administration of human Tf, Lf, or Hb dramatically increased mortality in infected mice (31, 32). Fe-starved meningococci grown at low pH (6.6) gained a 1200-fold increase in relative virulence (33). The impaired virulence of NM and NG mutants defective in specific Fe transporters tested in the mouse and human challenge infection models, respectively, directly confirmed the need for active Fe uptake to cause disease (34-36). Recent studies have shown that signature-tagged meningococcal mutants in *tonB*, *exbB*, and *exbD*, which have a pleiotropic defect in Fe-uptake, were unable to cause systemic infection in an infant rat model (37).

2.3. Bacterial mechanisms of iron assimilation

Bacteria use diverse means to obtain Fe *in vivo.* Bacterial pathogens such as *Escherichia coli* and *Vibrio cholerae* secrete iron-regulated cytolysins to increase the availability of Fe complexed to intracellular Hm and Hb (38-41). *Streptococcus mutans* (42) and *Listeria monocytogenes* (43) express either membrane-bound or secreted Fe reductase enzymes, respectively, to release Fe from the host carrier proteins Tf and Lf. The marine pathogen *Vibrio vulnificus* relies on a heme-inducible secreted protease to release heme-Fe from HbHp

complexes (44). However, the best characterized strategy of bacterial Fe acquisition has been studied in enteric bacteria that secrete low-molecular-weight high-affinity Fe chelators called siderophores. Siderophores can solubilize Fe from mineral complexes in the environment or can compete Fe from Tf and Lf (13, 45). Once complexed to Fe, the ferri-siderophore is transported into the cell through specific outer membrane receptors. For organisms that occupy both human and environmental niches, this strategy is effective for scavenging Fe from diverse Fe sources. However, siderophore-dependent Fe uptake is energetically expensive due to the need to synthesize and metabolize these Fe-chelating molecules (46).

Despite early assertions (47-49), it has been conclusively established by numerous investigators that *Neisseria* do not produce and secrete siderophores (25, 50, 51). NM and NG elaborate a set of iron-repressible proteins (FeRPs) in response to Fe deprivation encountered either *in vitro* or within the human body (51, 52). Further study of neisserial FeRPs has revealed a group of TonB-dependent receptors that acquire Fe by binding directly to and removing Fe from Tf, Lf, Hb, and HbHp complexes (23-27, 53-57). This strategy would seem more favorable energetically than siderophore-mediated Fe uptake since it does not require the synthesis and secretion of an Fe chelator by the bacterium. A discussion of the molecular mechanisms of Fe assimilation in *Neisseria*, with particular emphasis on the biochemistry of the distinct steps of the transport process, is presented in the following sections. First, we will discuss well-characterized siderophore-dependent systems in *E. coli* as a backdrop against which neisserial Fe transport mechanisms can be viewed.

3. TONB-DEPENDENT IRON TRANSPORT – THE *E. COLI* PROTOTYPE

Iron acquisition is an example of a physiological problem Gram-negative bacteria face – the active transport of ligands across the semi-permeable, de-energized outer membrane (OM). The presence of non-specific porin channels in the OM that exclude solutes of >600 Da prevents passive diffusion of larger Fe-containing complexes and precludes the establishment of a potential energy gradient that could be used to drive such a transport process. A large family of high-affinity OM transporters has been characterized in various Gram-negative bacteria that solve this "Gram-negative dilemma" through a common interaction with the TonB energy transduction complex (58-64). Receptors for ligands as diverse as vitamin B_{12}, phage T1, cytotoxic colicins, Fe-siderophores, and Fe carrier proteins interact with TonB to facilitate the energy-dependent internalization of their respective ligands. The *in vivo* relevance of TonB-mediated processes to the pathogenesis of several disease agents, including *Haemophilus influenzae, Vibrio cholerae, Shigella dysenteriae,* and NM has been established using animal models of infection (37, 59, 61, 63). For extensive discussions of TonB-dependent transport systems, readers are referred to several excellent reviews on the subject (62, 65-68).

The key components of TonB-dependent Fe transport systems include: 1) a high affinity OM receptor that functions as a gated porin, 2) TonB, 3) the potential energy provided by the PMF across the inner membrane, 4) a periplasmic Fe binding protein, and 5) a cytoplasmic membrane ABC transporter to shuttle the Fe into the cytosol. The molecular characterization of neisserial Fe acquisition systems is at a stage of relative infancy. However, extensive study of several related *E. coli* TonB-dependent siderophore receptors (BtuB, FhuA, FepA, and FecA) has yielded novel structural and mechanistic models of Fe transport that appear remarkably conserved among different members of this protein family.

3.1. Structure-function of the TonB-ExbB-ExbD energy transduction complex

The iron-regulated *tonB* gene encodes a 26-kDa protein with an unusual primary structure (69, 70). The hydrophobic amino-terminal portion of TonB contains an uncleaved signal sequence that anchors the protein in the inner membrane (IM) (69, 71). Several lines of evidence revealed that the N-terminus is also essential for TonB activity and interaction with auxiliary proteins (see below). Recent studies have identified the sequence motif SXXXH (where X is any amino acid) in the transmembrane alpha-helix as the minimal structural requirement for a functional interaction of TonB with the PMF (72). The central region of TonB is comprised of 17% prolyl residues and includes two highly charged domains: a negatively charged Glu-Pro repeat followed by a positively charged Lys-Pro repeat (69). This core domain was predicted to adopt an extended, rigid conformation extending up to 10 nm, leading to speculation that this region may allow TonB to span the periplasmic space and contact OM receptors directly (73, 74). The structural rigidity of this region was thought to contribute to the ability of TonB to physically transfer PMF-induced conformational changes to OM receptors. However, neither the tandem duplication (75) nor complete deletion (76) of this proline-rich region prevented TonB-dependent transport functions. In the deletion mutant, impaired contact between TonB and the FhuA receptor was suggested by reduced phage ϕ80 adsorption in a high osmolarity medium that expands the periplasmic volume. Thus, the role of this highly conserved proline-rich domain in TonB energization of receptors remains unclear.

A topology model depicting TonB anchored in the cytoplasmic membrane by its N-terminal domain with the largely, hydrophilic central region extending into the periplasm has been empirically confirmed by analysis of TonB-PhoA fusion proteins (77). Notably, fusions of alkaline phosphatase or beta-lactamase to the extreme C-terminus of TonB remain enzymatically active, suggesting that this region is periplasmically localized despite its moderate hydrophobicity. By analyzing truncated TonB constructs, Anton and Heller demonstrated that deletion of the distal 15 C-terminal residues, which includes a hydrophobic domain, rendered TonB inactive (78). The recent determination of the crystal structure of the C-terminal domain of TonB by Chang *et al.* (79) revealed that this region of TonB (residues 164-239) dimerizes to form a novel structural fold. Chang *et al.* have modified existing models of TonB-driven transport processes to account for

this new data regarding the apparent dimeric structure of TonB (79). Consistent with these observations, Higgs *et al.* identified putative dimers and trimers of TonB in crosslinking experiments (80).

Two additional accessory proteins, ExbB and ExbD, are essential components of the TonB energy transduction complex. The 26-kDa ExbB is anchored in the IM by three transmembrane domains with the majority of the protein in the cytoplasm – only the amino terminus protrudes into the periplasm (81). In contrast, topological mapping of the 17.8-kDa ExbD protein using beta-lactamase fusion constructs indicated that it has a single hydrophobic membrane-spanning region and is predominantly periplasmic (82). The proposed physical interaction of TonB, ExbB, and ExbD to form a tripartite energy-transducing complex is supported by *in vivo* crosslinking studies and *in vitro* detection of binding of His6ExbB to both ExbD and TonB (83-85). Additionally, TonB fusion proteins anchored in the membrane by heterologous signal sequences derived from either penicillin-binding protein 3 or TetA were inactive and could no longer be crosslinked to ExbB (86, 87). A point mutation in valine-17 in the transmembrane domain of TonB also abolished TonB function and crosslinking to ExbB (88). Thus, the uncleaved signal sequence of TonB is involved in interacting with ExbB and is crucial to the energy transduction by TonB. Although recent studies suggest a ratio of 1 TonB: 2 ExbD: 7 ExbB in the complex, the actual architecture and stoichiometry of the TonB-ExbB-ExbD complex remains to be determined (89).

An indispensable function attributed to the ExbB and ExbD auxiliary proteins is the stabilization of TonB. Fischer *et al.* showed that degradation of unstable TonB overexpressed from a plasmid was prevented by concomitant overproduction of ExbB (90). The chemical half-life of TonB was significantly reduced in mutants lacking either ExbB or ExbD (91, 92). A chaperone-like function has also been ascribed to ExbB based on its interaction and stabilization of cytoplasmically localized TonB (93). Finally, Skare *et al.* (85) reported that when TonB is stabilized in a *ompT-exbB-* strain in which TonB is present at wild-type levels, the absence of ExbB reduces TonB-dependent vitamin B12 transport by ninety percent. This finding suggests that in addition to stabilizing TonB, ExbB plays an important role in TonB energy transduction, perhaps by coupling TonB to the PMF or by acting to recycle TonB to an active conformation (68).

TonB is thought to provide a link between the IM-associated PMF and OM receptors energizing active transport of Fe across the outer membrane. Indirect evidence for the requirement of an IM energy source came from studies showing that cells treated with uncouplers of oxidative phosphorylation (cyanide, 2,4-dinitrophenol (DNP), or carbonyl cyanide-m-chlorophenolhydrazone (CCCP)) mimicked the transport defects of mutants lacking TonB (94-96). The PMF was identified as the energy source required for vitamin B12 uptake by BtuB in studies using an *atp* mutant lacking a membrane-bound ATP synthase (94, 97). Subsequently, studies of vitamin B12 uptake by BtuB in *btuC atp* mutants (lacking IM cobalamin transport and membrane-bound ATP synthase) clearly demonstrated that the TonB complex and PMF were acting at the outer membrane step of Fe internalization. However, the mechanism by which the PMF is harnessed to drive active transport across the OM remains unclear. As mentioned previously, the N-terminal transmembrane domain of TonB and its interaction with ExbBD are required for TonB to be "recharged" by the PMF. ExbB and ExbD may themselves constitute a proton translocation system (87), or they may simply connect TonB to an unidentified proton translocator. Proposed models of the dynamic interactions between ligands, receptors, TonB, and the PMF involved in TonB-dependent Fe transport will be discussed below.

Probably the most interesting yet least understood aspect of TonB-dependent Fe transport is the mechanism by which the TonB conveys the potential energy of the IM-associated PMF to OM receptors to drive active Fe uptake. Because of the paucity of relevant data, however, hypothetical models describing the mechanism of TonB-energy transduction are necessarily vague. Conformational changes in TonB that required an intact PMF, ExbB, and the transmembrane domain of TonB were reported by Larsen *et al.*, suggesting that PMF energy may transmitted to OM receptors through structural movements in TonB (98). Chang *et al.* (79) have postulated that dimeric TonB molecules anchored to ExbBD may undergo a PMF-driven torsional motion that powers opening of the receptor channel, analogous to that observed for flagellar rotation machinery. Alternatively, based on equal partitioning of TonB between the cytoplasmic and outer membranes in sucrose gradients of lysed cell fractions, Letain and Postle (99) speculated that energy transduction is accomplished by shuttling of TonB between the two membranes. Conditions known to hinder TonB-receptor interactions (i.e. deletion of TonB C-terminus) enhanced TonB association with the cytoplasmic fraction; conversely, conditions that adversely affect the anchoring of TonB in the inner membrane (the absence of ExbB and ExbD or substitution of a TetA N-terminal anchor) favored localization of TonB to the outer membrane. There are several caveats to this interpretation of the data presented. There is no evidence for the complete disengagement of TonB from ExbBD *in vivo* and TonB was not detected in soluble fractions (98, 99). It is possible that these observations were artifacts arising from the ability of TonB to bind to both bilayers (68, 71, 80) coupled with the forcible separation of the two membranes during cell disruption. Given the limited amount of TonB that must service multiple receptor populations (100), the instability of TonB when not associated with ExbBD (90-92), and the presumed energy input required for reinsertion of TonB into the inner membrane to recharge for additional transport cycles, the physical shuttling of TonB back and forth across the periplasm does not seem to present an efficient mechanism of energy transduction. Further experiments are required to fully test these models and to provide the additional clues needed to elucidate the novel mechanism of TonB-mediated active transport of Fe.

3.2. Conserved structural features of TonB-dependent receptors

Members of the TonB-dependent receptor family have highly conserved structures and sequence motifs, reflecting common functional characteristics: OM localization, integral membrane topology, transport channel

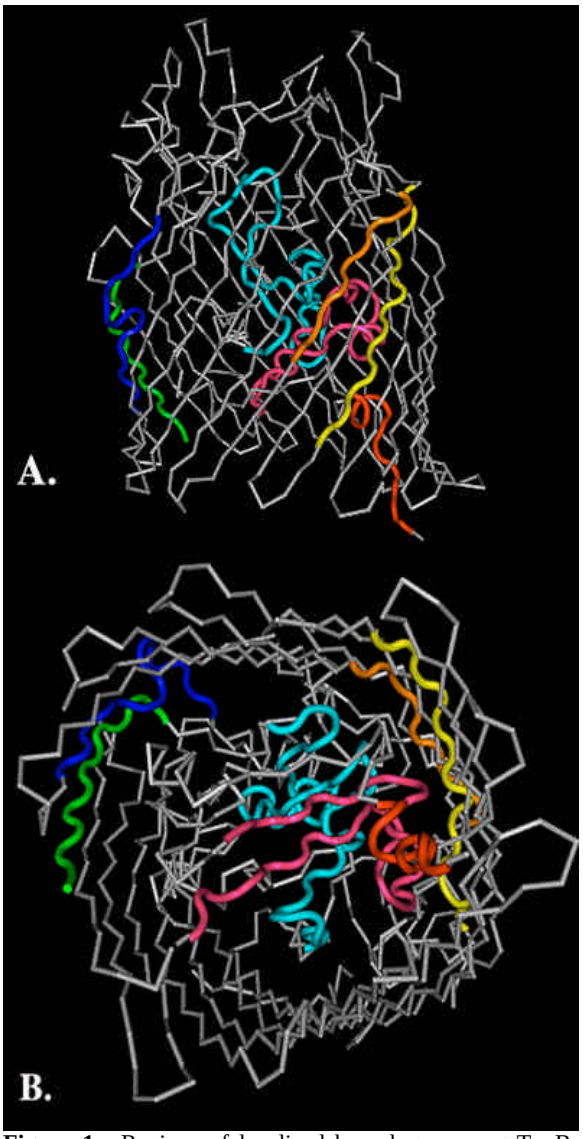

Figure 1. Regions of localized homology among TonB-dependent receptors. A. Side view of FepA. B. Bottom (from periplasmic side) view of FepA channel occluded by N-terminal plug domain. The seven consensus domains shared by TonB-dependent receptors, as described by Kadner (104) and Cornelissen *et al.* (140) were highlighted using the Cn3D program on the backbone of the FepA crystal structure as downloaded from the Molecular Modeling Database at the National Center for Biotechnology Information (MMDB#12191, PDB#1FEP). Domain 1 (red), domain 2 (light blue), domain 3 (pink), domain 4 (yellow), domain 5 (orange), domain 6 (dark blue), domain 7 (green).

formation, binding of extracellular ligands, and interaction with TonB. Like generalized porins, these receptors noticeably lack extensive hydrophobic or alpha-helical domains that might be expected in integral membrane proteins, a property that allows them to cross the cytoplasmic membrane without becoming irreversibly embedded. Membrane insertion is accomplished by the formation of porin-like "beta-barrel" channels in the OM composed of extensive anti-parallel amphipathic beta sheets (62, 67, 68, 101-103). By sequence alignment of multiple TonB-dependent receptor proteins, Kadner identified seven conserved regions of localized homology that distinguish this protein family (104). Their conservation between receptors that transport very different substrates suggest a role in central, common aspects of TonB-dependent receptor function. Figure 1 depicts the three-dimensional location of the seven domains as mapped onto the crystal structure of a representative receptor, FepA. Note that they cluster into two pairs of closely associated beta strands on one face of the barrel and three adjacent regions within the N-terminal plug domain. Domain 1 contains a conserved pentapeptide motif called the TonB box found in the amino-terminus of all known TonB-dependent OM transporters as well as TonB-dependent Group B colicins (104-106). Within domain 7, there is an invariant C-terminal phenylalanine preceded by a stretch of alternating hydrophilic and hydrophobic amino acids, a motif found in almost all outer membrane proteins (OMPs) that may be important for OM localization (107). The roles of the remaining five conserved regions defined by Kadner are unclear, but may contain sites involved in additional TonB interactions, OM localization and assembly, or interaction of subunits in multimeric receptor complexes.

Early topology models of several *E. coli* TonB-dependent receptors based on differing algorithms for identifying amphipathic membrane-spanning beta-sheets predicted the presence of 16-32 transmembrane segments (101, 108-111). Using monoclonal antibodies, deletion mutagenesis, epitope insertions, and protease accessibility, extensive surface-exposed loops involved in ligand binding were described (108-111). Siderophore receptors such as FepA and FhuA were pictured as gated porins, with large extracellular ligand-binding "gating loops" selectively restricting access to the transport channel. Evidence to support this model came from *in vivo* and black lipid bilayer studies demonstrating that deletion of putative gating loops resulted in nonspecific, passive channels that allowed TonB-independent solute diffusion (108-112).

However, the recent comparison of X-ray crystallographic structures for three TonB-dependent Fe-siderophore receptors – FhuA, FepA, and FecA – revealed a novel, highly conserved structure for these transporters (113-117). All three receptors formed hollow, 22-stranded anti-parallel beta-barrels larger than any channel formed by porins (see figure 2). The protein channels are approximately 70Å tall with an elliptical cross-section of about 35Å by 45Å, with the largest extracellular loops protruding up to 35Å above the OM surface. Consistent with earlier models, short periplasmic turns of 2-4 residues connected adjacent transmembrane strands, whereas surface loops involved in ligand binding were considerably longer (3-37 residues for FepA) (113). Most surprisingly, it was shown that the aqueous channel formed by the barrel domain was occluded by an N-terminal plug domain from the periplasmic side, not by extracellular gating loops (figure 2). The plug domain, which represents a unique

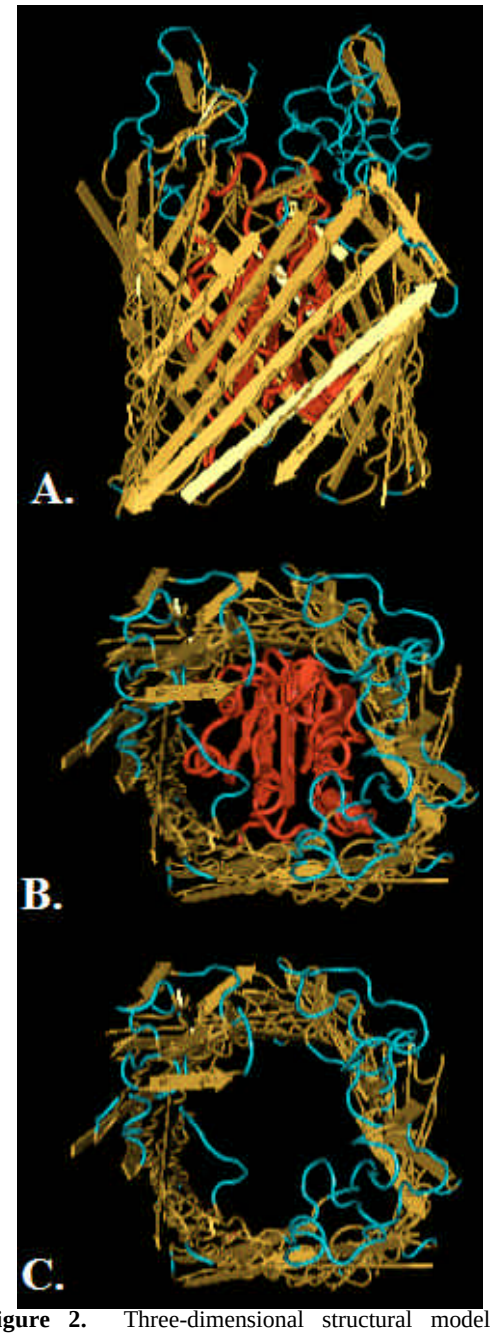

Figure 2. Three-dimensional structural model of a prototypical TonB-dependent receptor, FepA, as determined by X-ray crystallography. Panel A, Side view of intact FepA 22-stranded beta-barrel structure and extensive surface loops. Panel B, Top view of intact FepA showing transmembrane channel occluded by plug domain. Panel C, Channel formed by barrel lumen of FepA shown without the plug domain. Beta sheet regions are shown in yellow, light blue designates extracellular loops, and red denotes the N-terminal plug domain containing the TonB box (not resolved in crystal structure. Structures were downloaded from the Molecular Modeling Database at the National Center for Biotechnology Information (MMDB# 12191, PDB# 1FEP) and viewed with Cn3D 3-D structure viewer.

protein fold, is connected to the barrel by a periplasmic hinge region. The top of the plug domain is exposed to the exterior of the cell, while the surface of this region interacts extensively with the barrel lining (in the case of FhuA, 9 salt bridges and >60 hydrogen bonds) (117). Residues in both the barrel and the plug-domain could be seen contributing to the ligand binding site.

3.3. Current model of TonB-receptor-ligand interactions

This section briefly summarizes the current model(s) of the dynamic interactions involved in TonB-dependent transport based on extensive studies of *E. coli* siderophore receptors. A comprehensive analysis of this topic can be found in a review by Dr. Volkmar Braun in this issue. This will facilitate the subsequent discussion of the related yet distinct mechanisms of TonB-dependent Fe acquisition in pathogenic *Neisseria.*

3.3.1. Receptor-ligand interactions

E. coli TonB-dependent receptors bind their cognate ferri-siderophore ligands with high affinity (K_d= 2nM for FepA and 0.6nM for FhuA) and significant specificity (118, 119). Mutagenesis and X-ray crystallographic studies have identified specific extracellular loops and residues within the barrel and plug domains involved in ligand recognition (113, 115-118, 120). Several independent approaches have shown that the high-affinity binding of ligand induces conformational changes in the receptor structure. *In vivo* ligand-dependent alterations in receptor structure have been noted both indirectly through ligand-dependent changes in the stability of TonB (98) and directly by electron spin resonance (ESR) analysis of spin-labeled FepA (121, 122). Similarly, Moeck *et al.* reported that the protease accessibility and recognition of purified FhuA by monoclonal antibodies was altered in the presence of ferrichrome (123). This *in vitro* data suggests that initial ligand-induced conformational changes in the receptor protein do not require TonB.

Comparing the X-ray crystal structures of ligand-free and ligand-loaded FhuA revealed that the backbone of the barrel and position of extracellular loops were virtually unchanged (116, 117). However, relatively minor shifting (1-2Å) of two plug regions toward the ligand were propagated and amplified across the membrane, resulting in dramatic conformational changes in a periplasmic N-terminal domain termed the “switch helix”. In the ligand-free form, the periplasmic switch helix fit into a complementary hydrophobic pocket on the adjacent barrel wall. Ferrichrome binding unfolded the switch helix, which swung ~180^o to the opposite barrel wall, with some residues displaced by as much as 17Å (116, 117). Using site-directed spin labeling and electron paramagnetic resonance (EPR) spectroscopy to characterize substrate-induced changes in the TonB box of BtuB, Merianos *et al.* (124) confirmed that ligand binding induced significant structural changes in the N-terminal plug domain. It was proposed that the unwinding of the switch helix may serve to signal the ligand-loaded status of the receptor and trigger TonB-mediated active transport of the bound ligand across the outer membrane (115-117, 123, 124). Given that

multiple siderophore receptor populations must compete for a limited amount of TonB (100), the ability of TonB to specifically recognize ligand-loaded receptors would be essential for efficient TonB-dependent Fe acquisition by the large receptor population.

3.3.2. TonB-receptor interactions required for active iron transport

It has long been postulated that TonB-mediated energy transduction involves physical contact of TonB with OM receptors. Significant evidence suggests that the highly conserved TonB box motif in the N-terminus of Fe transporters physically contacts TonB. Mutations in the pentameric TonB box of various receptor proteins resulted in uptake-deficient phenotypes that mirrored those of TonB mutants (125-130). Suppressor mutations within TonB that rescued TonB box receptor mutants provided further genetic evidence for interaction of TonB with this region. These mutations were all mapped to Gln-160, implicating this residue as a potential receptor binding site (125, 128, 131). Most convincingly, Tuckman *et al*. reported the *in vivo* inhibition of several TonB-dependent processes (Fe uptake, phage infection, and colicin killing) by a synthetic TonB box consensus pentapeptide (106).

Chemical cross-linking of TonB to OM receptors such as FepA and BtuB has confirmed the physical interaction of these two components of the transport apparatus (85, 126, 129). *In vitro* "capture" binding assays using His_6-TonB as bait also have demonstrated binding of TonB to the FhuA and FepA siderophore receptors (132). Deletion of the C-terminal 48 residues of TonB abrogated crosslinking to FepA (129), while a separate study showed that truncation by as little as 15 C-terminal amino acids rendered TonB inactive (78). Thus, the highly conserved C-terminal domain of TonB, which contains a putative amphipathic helix, appeared essential for TonB-receptor interactions. A recent study by Howard *et al*. (133) employing the expression of TonB fragments lacking the IM anchor supported this conclusion. TonB fragments containing as little as 118 C-terminal amino acids inhibited TonB-dependent functions of the FepA, FhuA, and FecA receptors. Since these "periplasmic" TonB fragments, not anchored in the IM, cannot interact with the PMF, these results suggest that at least initial binding of receptors by TonB is an energy-independent process. Finally, binding of specific ligand was shown to enhance TonB-receptor interactions, as detected by either *in vivo* crosslinking or *in vitro* binding assays (126, 132, 134). This validates the proposal that ligand binding is an important conformational signal for TonB to preferentially engage loaded receptors and trigger active transport across the OM.

Recent investigations focusing on the N-terminal plug domains of FhuA and FepA have raised intriguing questions about the importance of this region in interacting with TonB. Braun *et al.* (135) constructed an FhuA mutant, FhuAΔ5-160, lacking the N-terminal plug region but stably expressing the empty beta-barrel in the outer membrane. It was predicted that this protein would form large, permanently open channels that would allow diffusion of large compounds such as antibiotics and SDS. In the absence of the TonB box, it was believed that TonB-dependent active transport of ferrichrome would be blocked. As expected, plugless FhuA did increase sensitivity to large antibiotics and the ability to grow on large maltodextrins in the absence of LamB, indicators of non-specific diffusion. Surprisingly, however, FhuAΔ5-160 supported specific ferrichrome uptake and sensitivity to phages and colicin M in a TonB- and PMF- dependent manner (135). Ferrichrome transport by this mutant receptor was reduced to 45% of wild-type, but was completely blocked in a *tonB* mutant and in cells treated with a protonophore. Thus, the plug domain (which includes the TonB box pentapeptide) appeared dispensable and sites outside of the TonB box could link FhuA to TonB and the PMF. In a separate study, unanchored C-terminal TonB fragments inhibited ferrichrome transport by FhuAΔ5-160, further indicating that TonB binds directly to the beta-barrel of Fe transporters in the absence of a TonB box or plug domain (133).

These conclusions were confirmed and extended by Scott *et al.*, who deleted and exchanged the plug domains of two siderophore receptors, FhuA and FepA (119). The empty beta-barrels and chimeric receptors still bound their cognate siderophore ligand with wild-type affinities, followed by TonB-dependent internalization. The beta barrel was shown to discriminate between ferric enterobactin and ferrichrome, despite the contribution of plug residues to the binding site. Although heterologous plug domains can partially substitute for native N-termini (119, 136), plug domains were not completely interchangeable or dispensable. The empty FepA and FhuA barrels did form leaky diffusion channels, consistent with the observations of Braun *et al.* (135). The N-terminal domains of FepA and FhuA were also required for optimal ligand binding. Deletion or exchange of this region resulted in a dramatic drop in binding capacity such that only 10% of the mutant proteins bound siderophore (119).

However, the assertion that the receptor N-terminal plug domain is not required for TonB-dependent activity was challenged recently by Vakharia and Postle (137). These authors constructed mutants of FepA containing four different deletions within the plug region. Deletion of residues 1-152 (FepAΔ1-152) removed the entire plug domain, excision of residues 7-152 (FepAΔ7–152) served to mimic the FhuA deletion (FhuAΔ5-160) studied by Braun *et al.* (135), deletion of residues 20-152 left the TonB box intact, whereas FepAΔ17-150 was identical to the construct characterized by Scott *et al.* (119). The four FepA mutant proteins could not utilize enterobactin for growth, bind colicin B, or be cross-linked to TonB. These observations do not rule out the possibility that TonB interacts with the barrel domain in a manner not detected by cross-linking. The ability to weakly co-immunoprecipitate TonB with wild-type and mutant FepA proteins containing only the barrel domain supports this proposed TonB-barrel domain interaction. How can these data be reconciled with the observations of Scott *et al*, (119) and Braun *et al.* (135) that suggested the

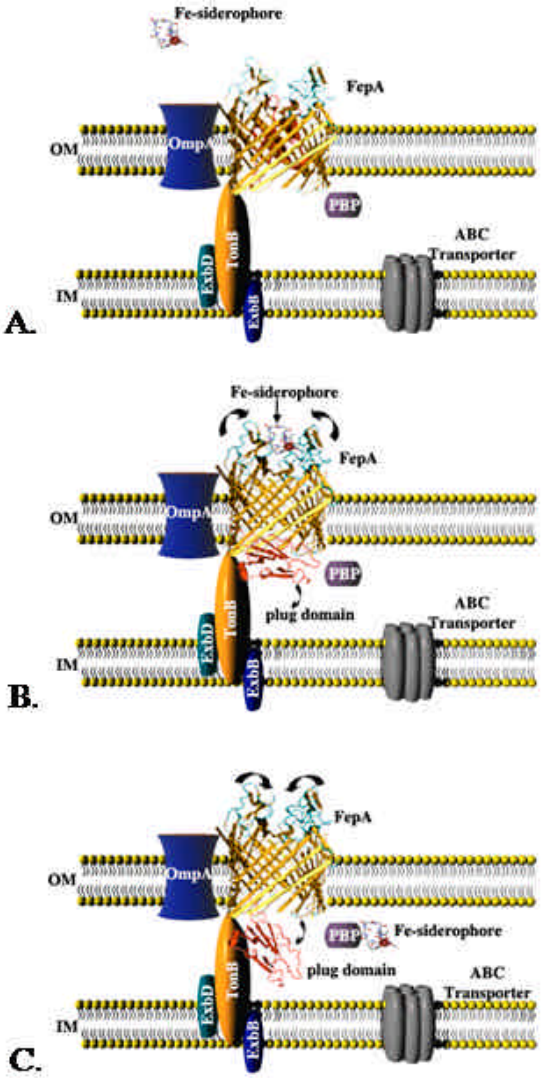

Figure 3. Model of substrate transport by prototypical TonB-dependent receptor FepA. Panel A: In the resting state, FepA surface loops are in an "open" conformation, possibly participating in interactions with outer membrane proteins (OMP) such as OmpA. TonB is docked with the OM via weak interactions with OMPs and perhaps the receptor barrel domain. Panel B: Ligand binding induces receptor conformational changes that close the outer hatch and displace the N-terminal switch helix and plug domain. This serves as a signal of receptor occupancy and triggers tight interaction with TonB. Panel C: TonB drives the active transport of substrate through the FepA channel where it is bound by a periplasmic binding protein (PBP). Panel D: The FepA-TonB complex must reset to the open, resting state to prepare for another transport cycle. An ABC transporter delivers the Fe-siderophore to the cytoplasm.

N-terminal plug domain is not required for TonB-dependent receptor functions? Vakharia and Postle demonstrated that their results stem from the different genetic backgrounds of the strains used to analyze the FepA mutant phenotypes (137). The strain in which FepA plug mutants retain TonB-dependent activity, KDF541 (a derivative of RWB18-60), contains an uncharacterized chromosomal *fepA* mutation (112). Thus, FepA expressed from the chromosome of this strain may contain the plug domain. Similarly, reportedly active FhuA barrel domains were studied in a strain that actually expressed a full length FhuA allele with 5 amino acid substitutions (136). However, FepA plug mutants expressed in the W3110 strain background, in which the chromosomal *fepA* allele was inactivated by deletion of residues 55-359 and insertion of a kanamycin resistance marker, were inactive (137). The authors suggested that FepA mutants lacking a plug domain may be rendered partially functional by interprotein complementation by plug-containing receptor proteins expressed from the chromosome. The proposed existence of FepA as a trimer (85) is consistent with this hypothesis.

Thus, currently available data suggest that the N-terminal globular plug domain is indeed important for the proper function of TonB-dependent receptors, although TonB interactions with other portions of the receptor may also be functionally significant. The most evident role for the globular plug domain within the transport channel is to preserve the integrity of the OM as a diffusion barrier. Residues in the N-terminal plug region also form hydrogen bonds with the siderophore in the ligand binding site of the receptor (115, 116). Empty beta-barrels exhibit a reduced transport rate (135), and may fail to adopt a binding-competent conformation (119). This implies that the interaction of the plug with the barrel is important for wild-type binding and transport kinetics. There is significant evidence that the direct interaction of TonB with the N-terminal receptor plug domain is crucial for ligand transport across the OM. For example, the transport defects of TonB box mutants of FhuA (131), BtuB (128), and Cir (125) argue that interaction of TonB with the beta-barrel is not sufficient to open the channel of wild-type receptors and the plug domain aids in this process. In light of recent data, the case for a putative interaction of TonB with the receptor barrel domain is somewhat weakened but warrants further investigation.

3.3.3. General model of TonB-mediated Fe uptake and energy transduction

Based on available genetic, biochemical, and structural data, the following model describes the proposed mechanism of TonB-dependent receptor-mediated siderophore uptake (figure 3).

In the "resting" state in which ligand is absent, TonB may dock transiently with the OM receptor searching for a signal of receptor engagement (figure 3A). This was suggested by the ability of TonB to bind to nonreceptor proteins such as OmpA and Lpp in the outer membrane (80) and by weak TonB-receptor interactions observed in the absence of ligand (85, 126, 132). These weak interactions would allow a limited number of TonB molecules to monitor the much larger population of receptors to identify those to which ligand is bound. Scott *et al.* demonstrated that the unoccupied receptor assumes an

"open" resting state, in which surface loops can be cross-linked to major OM proteins OmpF/C and OmpA. The proper localization of the N-terminal plug domain within the channel may be important to stabilize this 'open" conformation, since an empty beta-barrel mutant of FepA could not be cross-linked to these proteins (119). Through an induced fit mechanism (116), the binding of ligand to extracellular residues in both the plug and barrel domains causes slight conformational changes that are amplified and propagated across the membrane (figure 3B). These changes in the periplasmic switch helix and N-terminal domain allow TonB to sense and strongly engage occupied receptors, likely through interactions with both the N-terminal domain/TonB box and the barrel. TonB then releases its stored potential energy to drive conformational changes in the receptor that release the ligand from its initial high-affinity binding site to permit its transport into the periplasm (figure 3C). While the siderophore is shuttled across the IM by an ABC transporter, TonB is then somehow recharged to an energized configuration capable of powering another transport cycle through interaction with ExbBD, and driven by the PMF.

How does the ligand traverse the interior of the beta-barrel to reach the periplasm, and what transport steps require energization by TonB? Buchanan et al. proposed an "air-lock" mechanism for FepA transport of enterobactin that involves two hypothetical hatches. The first hatch, formed by extracellular loops, is envisioned to close behind the bound ligand to seal off the channel (figure 3C). This was visualized in the X-ray crystal structure of liganded FecA, which revealed extracellular loops closing the external pocket of FecA upon ferric citrate binding, (115). The conversion of active receptors to a "closed" state is also consistent with the ligand-induced loss of crosslinking of FepA surface loops to OmpF/C and OmpA (119, 138). Subsequently, TonB triggers opening of the second hatch, i.e. the plug domain, to release the ligand into the periplasm.

There is some disagreement about the structural rearrangements involved in opening the periplasmic hatch to allow solute passage. According to one model, relatively small conformational changes in the strands of the plug could form an opening large enough for transit of a small siderophore molecule. This model is viewed by some as the most energetically favorable solution, allowing minimal disruption the ~60 hydrogen bonds anchoring the plug in place (113, 115-117). A second model suggests that the entire plug domain is likely ejected from the barrel lumen during this stage of the transport process (119). The stable existence of empty beta-barrel channels has been demonstrated (119, 135) and the transport of large colicins by TonB-dependent receptors is only conceivable through a completely unobstructed pore. The reliance on TonB and the PMF may stem from the energy input needed to disrupt the extensive interactions of the N-terminal domain with the barrel. One proposed experimental test of these hypotheses would involve tethering the plug to the barrel wall via engineered disulfide bridges and determining the effect on TonB-dependent ligand transport (117).

3.3.4. Neisserial TonB-ExbB-ExbD

The existence of a TonB-ExbB-ExbD energy transduction complex in pathogenic *Neisseria* was first suggested by the identification of Fe-transporters with homology to known TonB-dependent receptors (36, 139-142). Within the past five years, homologs of *tonB, exbB,* and *exbD* have been cloned and sequenced from both *N. meningitidis* and *N. gonorrhoeae* (58, 64). Unlike *E. coli*, the three genes in *Neisseria* are linked and may be transcribed as a single operon. Insertional inactivation of *tonB* in both pathogens resulted in pleiotropic defects in Fe acquisition from Tf, Lf, Hb, and HbHp complexes (58, 64). This supported the conclusion that the TonB homologs of the pathogenic *Neisseria* also play a central role in the energy-dependent transport of Fe via OM receptors.

Currently, the mechanism of TonB function in *Neisseria* is largely inferred from the studies of *E. coli* TonB presented above. However, the low levels of homology (~25-35%) between the TonB proteins of pathogenic *Neisseria* and those of other Gram-negative bacteria suggest caution in relying heavily on extrapolation of data derived from heterologous systems for understanding the details of the neisserial TonB system (64). The functional divergence of different TonB systems was evident in complementation studies showing that the neisserial TonB system did not productively interact with the FhuA TonB-dependent receptor of *E. coli* (64). The topology of the neisserial proteins and their interaction to form a tripartite complex remain to be investigated. In addition, a role for ExbBD in the stabilization and activity of TonB, as seen in *E. coli*, has not been defined in *Neisseria*. Only two studies have addressed the role of the neisserial Exb proteins in TonB activity. The leaky phenotype of an *exbD* mutant of *N. meningitidis* interrupted by insertion of an antibiotic cassette in the 3' end of the gene suggests a role for ExbD in Fe uptake (64). Cornelissen *et al.* reported that a gonococcal *exbB* mutant exhibited ligand-binding kinetics indistinguishable from a *tonB* mutant. However, this mutant was constructed by ethyl methanesulfonate (EMS) mutagenesis and possible polar effects on *exbD* expression were not defined (130). Recently published reports of the effects of TonB and the PMF on receptor conformations and ligand binding kinetics for neisserial Tbp and Hpu receptors will be discussed in detail in section 4. Desai *et al.* recently described an alternative system that allows energy-dependent Fe uptake from Tf, Lf, and Hb in both NM and NG in the absence of a functional TonB (143). The genes and corresponding Ton-like proteins that can functionally substitute for TonB-ExbBD have not yet been isolated. Redundant TonB systems involved in Fe transport have also been identified in other Gram-negative bacteria including *Vibrio cholerae, V. parahaemolyticus, V. alginolyticus, and Pseudomonas aeruginosa* (61, 144-146).

4. TWO-COMPONENT TONB-DEPENDENT NEISSERIAL IRON TRANSPORTERS

The obligate human pathogens NM and NG have evolved multiple schemes to efficiently scavenge Fe within the human host (see table 1). However, compared to siderophore-mediated Fe uptake systems of *E. coli*, the molecular and biochemical details of these Fe assimilation mechanisms are just beginning to emerge. The best characterized family of neisserial TonB-dependent Fe transporters is a group of two-component receptors that contain an accessory lipoprotein in addition to a TonB-

Table 1. Neisserial proteins involved in iron uptake and metabolism

PROTEIN	CELLULAR LOCATION	SIZE	FUNCTION/PROPERTIES	SELECTED REFERENCES
Two-component TonB-dependent receptors				
TbpA	OM	~100 kDa	TonB-dependent Tf receptor/Fe transport channel. Forms complex with TbpB	53, 56, 140, 147, 159,167
TbpB	OM	68-85	Tf receptor accessory lipoprotein component. Specific binding of human, Fe-loaded Tf	147, 159, 166, 167
LbpA	OM	105	TonB-dependent Lf receptor/Fe transport channel	57, 139, 198, 201, 202
LbpB	OM	77	Lf receptor accessory lipoprotein. Contributes to Lf binding/use. No specific role defined	55, 199, 200, 202, 203
HpuB	OM	85	TonB-dependent receptor for Hb, Hp, and HbHp. Transports heme-Fe. Phase variable	24, 54, 141, 223, 224
HpuA	OM	35	Lipoprotein component of Hpu receptor. Required for ligand binding/utilization	141, 223, 224, 225
Single-component TonB-dependent receptors				
HmbR	OM	89	TonB-dependent Hb receptor. Phase variable	36, 224, 228, 229
FetA	OM	76	TonB-dependent enterobactin receptor. May transport other siderophore(s).	22, 231, 232, 223
TdfF	OM?	78	Homologous to TonB-dependent siderophore receptors	237
TdfG	OM?	134	Homologous to TonB-dependent Hm receptors. Only in NG, not NM	237
TdfH	OM?	101	Putative TonB-dependent receptor. Ligand unknown	237
Energy transduction apparatus				
TonB	periplasm, anchored in IM	28	Transduces PMF energy to OM receptors	58, 60, 64, 65-70, 72, 79
ExbB	IM/cytoplasm	24	Stabilizes TonB. May couple TonB to PMF	81, 83-85, 91, 92
ExbD	IM/periplasm	15	Same as ExbB	82, 83-85, 91, 92
Intracellular iron metabolism				
FbpA	periplasm	37	periplasmic Fe^{3+} binding protein	267-272
FbpB	IM?	56	? Fe3+ cytoplasmic permease, ABC transporter ?	266, 273
FbpC	IM?	38	? nucleotide binding protein, ABC transporter ?	266, 273
FetB	periplasm?	35	? Siderophore binding protein ?	22
HemO	cytoplasm	26	heme oxygenase	284-286
BfrA	cytoplasm	18	bacterioferritin subunit. Fe storage. Protection from oxidative stress	282
BfrB	cytoplasm	22	bacterioferritin subunit. Fe storage. Protection from oxidative stress	282

dependent gated channel protein. This increased level of receptor complexity may reflect their unique mode of action. In contrast to ferri-siderophore receptors, which transport the entire ligand into the periplasm, the two-component neisserial receptors extract Fe directly from large host Fe-carrier proteins. To date, two-component receptors for Tf, Lf, Hb and HbHp have been identified and characterized in both the meningococcus and gonococcus (23-27, 53-57). The mechanism by which two-component receptors remove tightly bound Fe from carrier proteins is unknown. Likewise, the precise role of the lipoprotein component that distinguishes this receptor family from well-known siderophore uptake systems remains to be fully elucidated. In the following section, we present emerging models of the mechanisms of TonB-dependent Fe acquisition by neisserial two-component receptors based on recent genetic, structural, and biochemical studies. As alluded to earlier, *E. coli* siderophore transport has served as a useful prototype upon which to base these models. It is important to note, however, that the overall sequence homology of the neisserial Fe-transporters with siderophore receptors is relatively low (i.e., meningococcal TbpA shows 19% homology with FepA) (147, 148). Investigation of the unique features exhibited by this family of two-component Fe transporters promises to provide the most insight into the novel biochemical mechanisms exploited by *Neisseria* to acquire Fe directly from host carrier proteins.

4.1. TbpAB (transferrin receptor)

Studies conducted over 20 years ago first demonstrated the conserved ability of pathogenic, but not commensal, *Neisseria* to utilize Tf as an Fe source (26). A high-affinity, energy-dependent saturable Tf receptor, TbpAB, that extracts and internalizes Fe from surface-bound Tf was subsequently isolated and characterized (53,

56, 149, 150). The observation that gonococcal mutants unable to use Tf-Fe were incapable of initiating urethritis in a human challenge model revealed that the Tf receptor is an important virulence factor and that Tf might be a more important Fe source on mucosal surfaces than previously appreciated (35). To our knowledge, similar investigations into the *in vivo* role of the Tf receptor in meningococcal pathogenesis have not been conducted. This section discusses our current understanding of the structure and function of this receptor. For a comprehensive review of bacterial strategies for Tf-Fe acquisition, please refer to the review article by Dr. Cornelissen in this issue.

The serum protein Tf is a monomeric glycoprotein of ~80-kDa with a bilobed structure that binds two ferric cations per molecule using carbonate as a counter anion (151). The N-terminal and C-terminal lobes, which have similar amino acid sequences and tertiary structures, each consist of two domains connected by a hinge region. The Fe bining site is located in the cleft between the two domains. Transferrin is the major source of Fe in the bloodstream during systemic infections by pathogens such as NM and NG (19, 40). Whereas the concentration of Tf in mucosal secretions is only 0.2-1.3 μM, there is more than sufficient Tf in serum (18-21 μM, 30-35% Fe saturated) to support robust bacterial growth (20, 148). There is also evidence to indicate that Tf is a potential source of Fe during meningococcal growth in cerebrospinal fluid (152-156).

The TbpAB receptor was the first two-component TonB-dependent Fe transporter identified in *Neisseria* and is the only Tf utilization system expressed by NM or NG. Two distinct Tf binding proteins (Tbps) have been isolated from NM and NG (140, 147). Their role as subunits of a single two-component receptor rather than distinct Tbps was suggested by the tandem genetic organization and Fe-regulated cotranscription of the corresponding genes *tbpA* and *tbpB* (140, 147). The surface exposure and *in vivo* expression of neisserial Tbps has been confirmed by serology, immunolabeling of clinical isolates, blocking of Tf use with anti-Tbp monoclonal antibodies, and protease accessibility studies (157-161). TbpA is a ~100-kDa Fe-repressible protein that can be affinity purified using human Tf (140, 147) and can confer Tf binding on *E. coli* expressing recombinant TbpA (162, 163). Its localized homology to members of the TonB-dependent receptor family, including the conserved TonB box, C-terminal phenylalanine residue, and predicted amphipathic beta-sheet structure, predict an integral outer membrane protein that forms a TonB-dependent Fe transport channel (104, 140). The second receptor component, TbpB, is a FeRP expressed by NM and NG that exhibits size heterogeneity, ranging from 68 to 85-kDa (164, 165). This novel component of a TonB-dependent receptor system is a lipoprotein, based on the presence of a signal peptidase II cleavage motif near the N-terminus of the TbpB coding sequence, consistent with (^{14}C)-palmitate labeling of the gonococcal TbpB protein (166).

Isogenic mutants of TbpA and TbpB have been constructed to determine the role of each protein in Fe acquisition from Tf (140, 166, 167). A gonococcal TbpA-B- mutant was unable to bind Tf and internalized no Fe from Tf, demonstrating that TbpAB represents the sole gonococcal Tf receptor (166). A mutant lacking TbpA could not internalize Fe from Tf or grow with a Tf Fe source, but still was able to bind Tf (140). This was consistent with the proposed role of TbpA as the Fe transport channel, and indicated that TbpB expressed alone was surface exposed and capable of binding Tf. A gonococcal TbpB- mutant, however, could utilize Tf as an Fe source, but only internalized 20% of wild-type levels of Fe from Tf (166). Thus, whereas TbpB appears to act as an accessory receptor in NG, TbpA is required for the gonococcus to grow with Tf as a sole Fe source. Analysis of defined mutants in the meningococcal TbpAB receptor confirmed that both proteins are capable of independently binding Tf like their NG counterparts. In contrast to the NG Tf receptor, however, both TbpA and TbpB of NM appear to be absolutely required for growth dependent on Tf-Fe (167). The reason for the discrepancy in the role of TbpB between these two closely related receptors is not clear.

Both NM and NG cause natural infections only in the humans. This high degree of host adaptation is reflected in the species specificity of the TbpAB receptors of NM and NG, which selectively bind human Tf (56, 168). The N-terminal domain of the TbpB lipoprotein mediates this host specific Tf recognition (169). The neisserial Tf receptors have also been shown to recognize and preferentially bind Fe-loaded Tf (56, 159, 170-172), thus limiting nonproductive binding of apo-Tf. A variety of assays have demonstrated that the preferential binding of diferric Tf over apo-Tf is attributable to the TbpB lipoprotein (159, 169, 172, 173). Equilibrium phase Tf binding analysis of a TbpA-B+ gonococcal mutant showed that TbpB had a 100-fold higher affinity for ferrated Tf than for apo-Tf (159). The ability to recognize the ferrated form of Tf is conserved among high (85-kDa) and low (68-kDa) molecular weight TbpB variants and TbpB homologs expressed by other Gram-negative pathogens (170-172). It has been proposed that the binding of TbpB to Tf may be sensitive to the Fe-dependent “closed” conformational state of Tf (172, 174). Collectively, these data indicate that the accessory lipoprotein TbpB contributes to optimal receptor activity by increasing the specificity of TbpAB for Fe-loaded human Tf.

Understanding receptor topology and architecture of TbpAB would shed considerable light on the mechanism of Tf binding and Fe transport. A model for the structure of TbpA has been recently proposed that is based largely on the recently solved crystallographic structures of the related siderophore receptors FepA (113) and FhuA (114, 116, 117). Boulton *et al.* proposed that TbpA forms an integral membrane 22-stranded beta-barrel with short periplasmic turns and 11 larger extracellular loops (175). These putative ligand-binding surface loops likely interact more extensively with the large Tf ligand than do comparable loops of FepA or FhuA with much smaller siderophore ligands, and play an additional role in the extraction of Fe from the carrier protein. The accurate prediction of three

surface loops by this model has been confirmed by the reactivity of peptide-specific antibodies with TbpA on intact cells and/or observed defects in ligand binding upon loop deletion (175). Determination of the three-dimensional crystallographic structure of a non-*E. coli* TonB-dependent transporter such as TbpA, reportedly in progess (176), will be important to extend the "barrel-and-plug" structural paradigm beyond *E. coli* siderophore transporters. Without the benefit of structurally well-defined homologs, much less is known about the topology and tertiary structure of the TbpB lipoprotein. Based on primary sequence analysis, which indicated neither amphipathic beta-sheet nor alpha-helical structures, TbpB is believed to be a peripherally associated by its N-terminal lipid with the outer leaflet of the outer membrane. The accessibility of TbpB to exogenous proteases (159) and the ability of N-terminal and C-terminal domains to bind Tf (169, 177) support a model in which the majority of the mature TbpB protrudes from the cell surface.

The closely-linked functions of TbpA and TbpB suggested that the two components physically interact to form a functional bi-partite receptor complex. This interaction was first implied by the observation that affinity purification of TbpB using human Tf required the presence of TbpA (159, 167, 178). The ability of TbpAs to facilitate affinity purification of heterologous TbpBs derived from NM, *A. pleuropneumoniae,* and *H. influenzae* revealed that the TbpA-TbpB interaction was conserved among these species (179). The mutual protection from trypsin digestion provided by each protein for the other further supports the model of a two-component receptor complex (159). Most convincingly, Boulton *et al.* have co-purified TbpA+B by gel filtration as a 300-kDa complex capable of binding Tf (170, 180).

However, the stoichiometry of the proposed two-component receptor complex is unclear. Analysis of co-purified TbpA+B complexes suggested the receptor complex consists of a TbpA dimer and one molecule of TbpB (170). Similarly, surface plasmon resonance (SPR) was used to show that human Tf bound approximately 2:1 more TbpA than TbpB (170). However, published models of the Tbp receptor complex have proposed TbpB/TbpA ratios ranging from 1:2 (180, 181) to 5:1 (159). Carefully controlled quantitative transcript analysis found a 2:1 ratio of *tbpB*- to *tbpA*- specific transcripts under Fe-deplete conditions (182). Assuming no post-transcriptional control mechanisms, these results would argue that either more TbpB than TbpA is present in functional receptor complexes or there is a significant population of TbpB not engaged in direct interaction with TbpA. Finally, Prinz *et al.* showed that the RmpM (class 4) protein of NM binds to TbpA dimers, possibly stabilizing receptor oligomers (183). Obviously, more research is needed to definitively establish the protein constituents, architecture and stoichiometry of the TbpAB Tf receptor.

What regions of Tf are bound by TbpAB? Using isolated C- and N- lobe tryptic fragments of Tf, both TbpA and TbpB were shown to bind to the C-lobe of hTf (184). In a complementary study, Retzer *et al.* took advantage of the species specificity of TbpAB and constructed chimeric bovine/human Tf's (185). The primary binding site of co-purified TbpA+B complexes was located in the C-terminal domain of human Tf. However, two recent studies identified receptor contact sites in the Tf N-lobe (177, 186). One strategy used an overlapping synthetic peptide library representing the entire human Tf sequence to map sequences that could bind TbpB (177). Both the C-terminal and N-terminal halves of TbpB bound conserved peptides shared by the two hTf lobes. Mapping the recognized peptides onto a three-dimensional molecular model of Tf revealed that TbpB likely binds a series of adjacent surface regions that wrap around each lobe (177).

Likewise, what portions of TbpAB are involved in contacts with Tf? Vonder Haar *et al.* identified a highly-structured and extremely stable N-terminal domain (~270-290 amino acids) of TbpB that retained the ability to bind Tf (187). As noted previously, solid phase and BIAcore SPR studies determined that receptor specificity for human Fe-loaded Tf was linked to this N-terminal region of TbpB (169, 171, 172). Although weaker than binding mediated by the N-terminus, the C-terminal half of TbpB also contains Tf binding sites suggesting that TbpB may have a bi-lobed structure that coordinately binds both lobes of Tf (169, 177). In contrast, Tf binding domains of TbpA have been characterized only recently. Based on a new "barrel-and-plug" model of TbpA, Boulton *et al.* identified three surface loops that were required for growth on Tf (175). They showed that deletion of loop 4 or loop 5 abrogated Tf binding, whereas deletion of loop 8 resulted in a 10-fold decrease in the receptor affinity for Tf. Subsequently, these authors demonstrated dose-dependent specific binding of Tf by recombinant affinity-tagged TbpA proteins containing only loop 5 or loops 4 and 5. No binding was detected to loop 8, indicating that perhaps conformational constraints required for its role in Tf utilization were lost upon isolated expression of this loop outside the context of the intact receptor (188).

The development of a liquid-phase equilibrium Tf binding assay by Cornelissen *et al.* has made possible the quantitative, kinetic description of TbpAB-ligand interactions and analysis of the role of TonB-derived energy in this transport process (130, 159). The binding kinetics of Tf to the wild-type receptor revealed two high-affinity binding sites (K_{d1}=0.8 nM, K_{d2}=16 nM) (159). TbpAB binds human Tf with an affinity close to the estimated 5-20 nM K_d of the mammalian Tf receptor, suggesting that TbpAB could effectively compete for Tf *in vivo* (189). TbpA and TbpB individually bind Tf with a high affinity (K_d= 2.3 nM and 7.4 nM, respectively). The different affinities of each receptor protein, distinct from the binding of the intact receptor, suggested that the TbpAB binding site results from the interaction of the two receptor proteins. Although many authors have used a solid-phase "dot-blot" assay to estimate Tf receptor binding kinetics, the observation that TbpA-B+ cells bound much less Tf relative to wild-type cells when assayed by solid-phase dot blots versus liquid phase assays suggests that the solid-phase assay should be used with caution (159). Receptor conformations critical for normal ligand interactions were

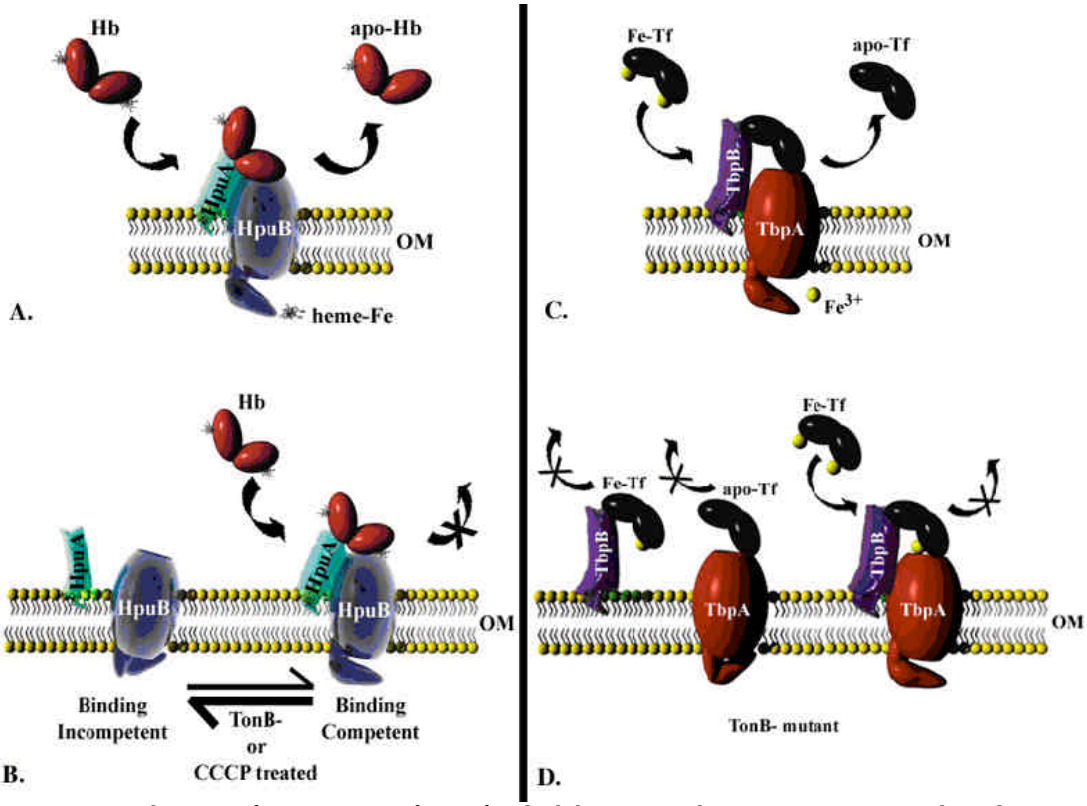

Figure 4. Distinct mechanisms of Fe acquisition from Tf and Hb by Neisserial two-component TonB-dependent receptors. A. Fe acquisition by wild-type HpuAB. Both HpuA and HpuB are required for binding and heme-Fe transport from Hb ligands. In energized cells, the majority of the HpuAB population is maintained in an 'active' conformation by TonB- and PMF- induced structural changes in receptor components. TonB is believed to facilitate the productive interaction of HpuA and HpuB to form a single high-affinity Hb binding site. The mechanism of HpuAB-mediated stripping of heme-Fe from Hb and TonB-dependent transport across the OM is unknown. B. Interaction of Hb with de-energized HpuAB. In $TonB^-$ mutants or protonophore-treated cells (referred to as de-energized), a significantly reduced Hb binding capacity suggests that most of the HpuAB population is in a binding-incompetent state. Consistent with the altered trypsin accessibility of receptor proteins in de-energized cells, HpuAB is proposed to adopt an inactive 'open' conformation. Hb binding and utilization is compromised since the close association of HpuA and HpuB is necessary to form a single high-affinity binding site. A small subset of de-energized HpuAB receptors bind Hb with approximately wild-type affinity, but heme-Fe extraction and ligand release are blocked. C. Fe acquisition by wild-type TbpBA. The bilobed Tf molecule is bound by TbpBA via two distinct binding sites before the ferric ion is transported into the periplasm. Unlike HpuB, the TbpA gated porin is capable of internalizing Fe from Tf in the absence of its partner lipoprotein. D. Interaction of Tf with de-energized TbpBA. In $TonB^-$ mutants TbpBA, like HpuAB, also assumes an altered conformation and shows impaired ligand release. However, the high binding capacity of de-energized Tf receptors, distinguishing TbpBA from HpuAB, indicates that the majority of the de-energized Tbp population is binding-competent. The ability of TbpA and TbpB individually to bind Tf with high affinity is consistent with the observed energy independence of binding capacity. The model also illustrates the ability of the TbpB lipoprotein to preferentially bind Fe-loaded Tf. No similar accessory role in determining ligand specificity has been demonstrated for the analogous HpuA lipoprotein.

apparently disrupted by the drying process. As noted earlier, Cornelissen *et al.* confirmed the ability of TbpB to preferentially bind Fe-Tf; when expressed alone, TbpB bound Fe-Tf with a 100-fold higher affinity than apo-Tf. However, the pronounced decrease in the ability of the intact TbpAB receptor to preferentially bind Fe-loaded Tf complicates understanding the *in vivo* relevance of this ligand specificity (159).

Elucidating the role of TonB in TbpAB functions also has been addressed (figure 4C and 4D). Cornelissen *et al.* showed that a TonB box mutant of gonococcal TbpA had ligand binding and protease accessibility properties indistinguishable from TonB- or ExbB- mutants (130). Consistent with several previous studies (125, 127, 128, 131), mutation of the TonB box abolished Fe uptake from Tf and growth supported by Tf. The protease accessibility of TbpB, but not TbpA, was affected by the TonB-linked energy state of TbpA. In TonB- and TonB box receptor mutants, TbpB was as sensitive to trypsin digestion as it was in the absence of TbpA. This suggested that the "tight" interaction of TbpA with TbpB that affords protease protection is TonB-dependent (130). The authors noted that the presence of ligand on TbpAB was not required for

interaction with TonB. This differs from the model of Moeck *et al.* (123) in which ligand-induced structural changes in FhuA trigger periplasmic interactions between the TonB box and TonB. As suggested above, the influence of TonB on empty TbpAB receptors may be mediated through interactions with the barrel domain of TbpA (119, 123, 135). The effects of ligand binding on TbpAB are too ill-defined to rule out interactions of TonB with the putative N-terminal plug domain of TbpA induced upon Tf binding.

In addition to energy-dependent conformational changes in the gonococcal Tf receptor, Tf binding was also profoundly influenced by mutations in TonB, ExbB, or the TonB box of TbpA. Compared with the wild-type receptor, de-energized receptor mutants had extremely high affinities (subnanomolar K_ds) for Tf that could not be accurately determined because of ligand depletion (130). The ability of bound holo- or apo- Tf to completely protect TbpA from trypsin digestion suggested that the ligand may be irreversibly bound to de-energized receptors. Measurement of the dissociation kinetics of Tf from TbpAB also suggested that TonB and an intact TonB box are required for Tf release from the receptor. A significant fraction of both ferrated and apo-Tf were irreversibly bound by de-energized TbpAB. Cornelissen *et al.* also reported the TonB-independent release of ~30% of the bound Tf within the first five minutes. The authors speculated that this population of transiently bound Tf was bound to TbpB alone, which is not known to interact directly with TonB (130).

These studies support the following model of TbpAB-Tf interactions, which reiterates and updates several previously proposed models of TbpAB function (130, 181). Extensive surface exposure, high affinity for Tf, and ability to recognize Fe-loaded human Tf makes the TbpB lipoprotein a likely candidate to serve as an initial docking site for Tf. After initial engagement by TbpB, Tf transitions to a tighter association with both TbpB and TbpA, such that Fe can be extracted but energy is now required for its dissociation. The C-lobe of human Tf, implicated as the primary binding site for TbpB (184), likely interacts first with the N-terminal domain of TbpB known to be responsible for the preferential binding of host-specific Fe-loaded Tf (169, 171, 172). The subsequent binding of the Tf C-terminal domain by TbpA (185, 186, 190) and weaker extended interaction of the C-terminal domain of TbpB with the N-lobe of Tf (177) would efficiently stabilize the initial binding event. These interactions probably involve conformational changes in both the receptor proteins and Tf that consequently position the Fe-binding cleft of Tf over the mouth of a TbpA transport channel. The complete loss of Fe uptake from Tf exhibited by a TbpA mutant lacking a single surface loop (loop 8) that retained Tf binding, albeit with a reduced affinity, implies that high-affinity ligand binding is critical to the extraction of Fe from Tf (175). Boulton *et al.* have suggested that the opposing forces exerted by TbpA and TbpB on bound Tf may be sufficient to cause conformational changes needed to induce Fe release (181). This hypothesis is supported by the inability of isolated, ferrated Tf C-lobes or N-lobes to be utilized by TbpAB as an Fe source despite their ability to bind to the receptor (191). The ability of purified TbpAB complexes to trigger Fe release from Tf and subsequent binding of Fe by FbpA or other chelator was demonstrated by Gomez *et al.* (192). However, the mechanism by which TbpAB induces the release of tightly sequestered Tf-Fe has yet to be clearly established. Alterations in the trypsin sensitivity of TbpB in the presence of Tf suggest that ligand-induced conformational changes in the receptor occur that may promote productive interactions with TonB (159). Assuming TbpA has an N-terminal plug domain, it is not known whether Tf contacts residues in the plug domain analogous to binding of siderophores to FepA and FhuA. It is likely that the extensive interaction of the large Tf ligand with TbpA surface loops could lead to propagation of structural changes to periplasmic domains of the receptor.

Other questions about TbpAB structure-function remain. Once released from Tf, does the Fe passively diffuse through the channel or are there specific Fe-binding sites in the lumen of the barrel and energy-driven structural changes that pump the Fe into the periplasm? How does TonB trigger the release of the spent ligand and reset the receptor for another cycle of Tf binding and Fe transport? The determination of the crystallographic structure of TbpA and/or TbpAB complexes would be an invaluable step toward understanding the transport mechanism, validating structural comparisons of TbpA with *E. coli* "barrel-and-plug" receptors. Detailed structural information would also allow the rational design of mutants to identify residues involved in Tf binding and to determine if the putative N-terminal plug domain of TbpA is required to interact with TonB.

4.2. LbpAB (lactoferrin receptor)

Lactoferrin (Lf) is a host Fe-carrier glycoprotein that shows ~50% amino acid homology with Tf and adopts a similar bilobed structure (40, 193, 194). The ability to retain bound Fe even under acidic conditions (pH<6.0) distinguishes Lf from Tf. Lf is commonly thought to be the primary Fe source on the mucosal epithelial surfaces where both meningococci and gonococci initially colonize (195). Whereas only trace amounts of Lf are found in serum (3-8 nM), Lf concentrations are much higher (6-13 μM) in mucosal secretions. Lf is also released by leukocytes at sites of inflammation and can cross the blood-brain barrier by receptor-mediated transcytosis, making Lf available as a potential Fe source during advanced stages of meningococcal disease (196, 197). The universal ability of meningococcal strains to utilize LF (27) and the enhanced virulence of NM in mice supplemented with human Lf (32) indicate the importance of Lf as an Fe source for the meningococcus. However, acquisition of Fe from Lf is not required for NG to colonize the urogenital mucosa or cause invasive disease. Only 50-70% of gonococcal isolates, including DGI strains, are Lf+ (27), while the naturally Lf- gonococcal strain FA1090 was shown to cause urethritis in the human male challenge model of infection (35). However, an engineered derivative of FA1090 that was Lf+Tf- was able to cause symptomatic infections in human volunteers, suggesting that the Lf receptor can support

infection and that there is sufficient Lf on the urethral mucosa to support NG growth (34). Expression of Lf utilization systems, while not essential for gonococcal disease, may contribute to differences in pathogenicity among strains.

Mickelson *et al.* first described the ability of NM, NG and commensal *Neisseria* sp. to obtain Fe from Lf (27). Iron-regulated Lf binding activity, specific for human Lf, was associated with a 105-kDa lactoferrin binding protein (Lbp) affinity purified by Schryvers and Morris (57). Subsequent cloning and characterization of the *lbpA* gene from NM (198) and NG (139), which were 94% identical, predicted a protein belonging to the TonB-dependent receptor family closely related to TbpA (46% identical, 65% similar). Bonnah *et al*. demonstrated that the Lf receptor probably contained a second protein with the affinity isolation of a second lactoferrin binding protein, LbpB (199). The N-terminal lipid modification of LbpB, predicted by the signal peptidase II cleavage motif, was confirmed by labeling with ^{3}H-palmitate (55). The accessory lipoprotein LbpB is encoded upstream of the TonB-dependent receptor LbpA in an operon transcriptionally regulated by Fe, similar to the *tbpAB* operon (55).

Isogenic mutants in the LbpAB Lf receptor have been used to evaluate the contribution of each component to receptor activity. As anticipated, mutants lacking the LbpA TonB-dependent channel protein were unable to utilize Lf-bound Fe (139, 200, 201). The loss of Lf binding by NM and NG *lbpA* mutants, as assayed by solid-phase dot blot assays, indicated that LbpB alone did not mediate Lf binding (55, 139). Liquid phase equilibrium binding assays confirmed the conclusion that LbpA is required for Lf binding (202). What, then, is the role of the LbpB lipoprotein? Mutants lacking this receptor component exhibit reduced growth with Lf, a ~50% reduction in Lf binding capacity, and ^{55}Fe uptake rates 60% of wild-type LbpAB (55, 202, 203). These results suggest an important, but non-essential, role for LbpB in the binding and internalization of Lf-Fe. Several lines of evidence support the conclusion that LbpB can bind Lf independently of LbpA (199, 200, 202, 203). It is unclear why assays of *lbpA* mutant strains expressing only LbpB failed to detect binding to Lf. Finally, Schryvers *et al.* reported that both Fe-loaded and apo-Lf competed equally with horseradish-peroxidase labeled Lf for binding to LbpAB (57). Thus, ligand binding by LbpAB does not appear to be affected by the Fe saturation of Lf, a property conferred on the Tf receptor by the TbpB lipoprotein analogous to LbpB.

The molecular architecture and functional domains of LbpAB have not been well characterized. Largely untested assumptions about the structure and function of the LbpAB receptor have been based on the close parallels with TbpAB and close similarity between the Lf and Tf ligands. LbpA is thought to form the TonB-dependent gated channel through which Lf-derived Fe is transported. Prior to the availability of the crystal structures of FepA, FhuA, and FecA, a topology model of LbpA depicting 26 transmembrane domains and 13 surface loops was proposed (198). This model has been modified by Prinz *et al.* to propose that LbpA is a 22-stranded beta-barrel with an N-terminal plug domain (204). Predicted membrane spanning regions and loop delineations are be largely unchanged from the old model, which was modified to include three previously predicted N-terminal loop regions into the plug domain. Several loops predicted by this model to be surface-exposed have been identified based on reactivity of peptide-specific antibodies with LbpA on intact cells (204).

Even less is known about the structure of the 77-kDa accessory lipoprotein LbpB. The *lbpB* from the two pathogenic *Neisseria* species are 81% identical, with 30-50% identity with the analogous TbpB lipoproteins (202). Based on primary sequence analysis, LbpB from both NM and NG contained two stretches of negatively charged residues and identical 21 amino acid C-terminal regions not found in TbpBs (200, 202). Reports that LbpB retained the ability to bind its ligand following gel electrophoresis (if sample was not boiled) (200, 203) suggested conservation of a highly structured ligand binding domain similar to that previously identified in TbpB (187). The interaction of LbpA and LbpB to form a receptor complex also is poorly understood. The existence of a heterooligomeric complex consisting of an LbpA dimer and LbpB monomer has been identified by Prinz *et al.* by two-dimensional electrophoresis using a nondenaturing first dimension (204). Although many aspects of LbpAB activity remain to be demonstrated empirically, it is believed that the essential aspects of the ligand interaction and transport process will be highly similar to the related TbpAB-Tf transport system.

4.3. HpuAB (hemoglobin and hemoglobin/haptoglobin receptor)

Hemoglobin, which sequesters nearly two-thirds of the total Fe in the human body, is yet another important Fe source for bacterial pathogens such as *Neisseria* (39, 205-207). Within erythrocytes, the 65-kDa Hb molecule exists as an $\alpha_2\beta_2$ tetramer with a heme prosthetic group bound within a hydrophobic pocket of each subunit. While intracellular hemoglobin is normally unavailable to pathogens, Hb is present at low levels in normal human serum (80-800nM) as a result of spontaneous hemolysis (208). However, Hb tetramers dissociate into $\alpha_1\beta_1$ dimers due to the rapid oxidation and dilution of Hb upon release from erythrocytes (209-211). It is this dimeric form of Hb that is encountered by pathogens during bacteremia and can be exploited as a source of Fe. The levels of Hb in the bloodstream are believed to increase in advanced meningococcal disease as a consequence of severe disseminated intravascular coagulation (DIC) and associated erythrocyte lysis (2, 212). Furthermore, the importance of Hb as an *in vivo* Fe source for NM is likely enhanced by the host's hypoferremic response to infection, which can reduce the amount of Tf-associated Fe by up to 70% (213). The availability of Hb to *Neisseria* on mucosal surfaces is not clearly understood, but Hb may serve as an important Fe source to NG in the female urogenital tract particularly during menses (214). The relevance of Hb as an Fe source during meningococcal infection was demonstrated by the decreased virulence of NM mutants

defective in Hb utilization (36) and the ability of exogenously added Hb to enhance NM lethality in mice (32). Comparable experiments addressing the role of Hb-Fe utilization during infections with NG have not been conducted.

Hemoglobin released into the bloodstream is rapidly bound by the serum glycoprotein haptoglobin (Hp) with a very high affinity (10-15M) to facilitate clearance via hepatocytes. Thus, the more physiologically relevant hemoprotein Fe source *in vivo* is probably HbHp, not Hb (215, 216). A brief summary of the unique properties of Hp is relevant to subsequent discussions about interactions of neisserial receptors with Hp. Humans carry two alleles of the Hp locus (designated 1 and 2) and any given individual is either homozygous (i.e. 1-1 and 2-2) or heterozygous (i.e. 2-1) for Hp expression. The type 1 Hp allele is shared with other primates while the type 2 Hp allele is human specific, arising from a gene duplication of an ancestral type 1 Hp locus (217). Nondenaturing starch gel electrophoresis has shown that Hp 1-1 exists as an 86-kDa monomer whereas Hp 2-2 and 2-1 polymerize to form high molecular weight multimeric complexes as large as 900-kDa (217). These dramatic structural differences may influence the interaction of Hp phenotypes with bacterial HbHp receptors. Hp serves as a natural bacteriostat because many bacteria, including *E. coli*, can not utilize Hb complexed to Hb as an Fe source (218). Not surprisingly, several successful pathogens have evolved ways to extract heme-Fe from HbHp. For example, *Vibrio vulnificus* utilizes 1-1 and 2-2 HbHp complexes but not Type 2-1 HbHp in a process that requires a heme-inducible Hb protease (44, 219). Type b *Haemophilus influenzae* encodes at least three distinct phase-variable HbHp receptors (220-222).

Both NM and NG encode a third two-component, TonB-dependent Fe transporter called HpuAB that is genetically, structurally and functionally related to TbpAB and LbpAB (24, 54). This similarity if reflected in the genetic organization of the *hpuAB* locus, which encodes the lipoprotein HpuA directly 5' to HpuB, an organization shared with the Tf and Lf receptor operons. Northern blot and RT-PCR analysis have revealed that *hpuA* and *hpuB* are encoded as an operon jointly transcribed from a single Fe-repressible promoter upstream of *hpuA* (141). In addition, HpuAB undergoes phase variation by a slipped-strand mispairing mechanism involving a poly(G) repeat motif (223, 224). HpuAB is unique among two-component TonB-dependent receptors in the ability to interact with three structurally diverse ligands. The HpuAB receptor confers on *Neisseria* the ability to bind Hb, apo-Hp, and HbHp complexes (24, 141). Data from growth assays and affinity purification experiments suggest that HbHp complexes are preferentially bound and utilized as an Fe source compared to free Hb (24). Thus, HpuAB appears to have evolved mechanisms to efficiently utilize the most physiologically abundant form of Hb. Using a *hemA* porphyrin biosynthesis mutant, Lewis *et al.* demonstrated that HpuAB extracts and internalizes the entire heme-Fe moiety from both Hb and HbHp.

The 85-kDa HpuB protein exhibited 32% identity (53% similarity) to meningococcal LbpA and contained the seven hallmark domains of TonB-dependent receptors, including 6 of 8 residues of the *E. coli* consensus TonB box motif (141). Based on homology with the TonB-dependent receptor family, HpuB is predicted to form a transport channel in the outer membrane, adopting a 22-stranded beta-barrel structure gated by an N-terminal periplasmic "plug" domain. At 35-kDa, HpuA is only half the size of TbpB and LbpB and no known homologues of HpuA currently exist in public databases. Both receptor components are surface exposed and accessible to degradation by exogenous protease treatment of intact cells (225). It is thought that the HpuA lipoprotein is peripherally associated with the outer membrane by it N-terminal cysteine-linked fatty acid (141). This model was confirmed by the observation that trypsin can cleave HpuA within 25-30 amino acids of the N-terminal anchor, seen as a ~30-kDa cleavage intermediate retaining a C-terminal epitope (225). Functional domains of HpuB likely to play a role in binding to and extracting heme-Fe from Hb, such as the TonB box, surface loops, or putative plug domain, have not been investigated. A 21-kDa domain of HpuA that is protected from trypsin digestion by HpuB, extending from approximately residue 130 to the C-terminus, has been implicated in interacting with HpuB (225). Attempts to delineate the minimal portion of HpuA required for Hb utilization by constructing C-terminal truncation mutants have been hampered by the instability of HpuA constructs truncated by as little as ten amino acids (unpublished observations).

There are several lines of evidence that indicate that HpuA and HpuB physically interact to form a functional receptor complex in wild-type cells. Chen *et al.* first reported that the absence of HpuB in an *hpuB* mutant prevented the Hb affinity purification of HpuA (223). Further indirect evidence for an HpuAB receptor complex was the alteration of the protease cleavage profile of HpuA in an *hpuB* mutant and vice versa, indicating that the presence one receptor protein influenced the conformation and exposure of the other (225). Finally, although cleavage of HpuA should release the C-terminal portion of the lipoprotein from its membrane tether, HpuA cleavage products remained associated with the cell surface, probably through interactions with HpuB (225).

In both NM and NG, isogenic mutants lacking either one or both HpuAB components have been used to investigate the role of each receptor protein in Hb and HbHp utilization. As anticipated, an *hpuAB* double knockout was unable to bind or utilize Hb or HbHp. Insertional inactivation of either *hpuA* or *hpuB* revealed that both proteins are absolutely required for growth using Hb or HbHp as sole Fe sources (24, 54, 223-225). Thus, the "accessory" HpuA lipoprotein appears to play a more integral role in ligand interactions than its TbpB and LbpB counterparts. Data suggest the contribution of HpuA and HpuB to initial ligand binding differs slightly between NM and NG. Solid-phase and liquid-phase equilibrium binding assays failed to detect ligand binding to NM expressing either Hpu protein alone (224, 225). Dot blot assays of gonococcal mutants also indicated impaired Hb binding to single *hpu* mutants. However, HpuB from NG was affinity

purified using Hb in the absence of HpuA, indicating at least a low affinity interaction between Hb and HpuB (223). Recently, Chen *et al.* identified point mutations in HpuB (HpuB*) that allowed Hb utilization even in the absence of HpuA (226). It should be noted that the growth of HpuB* variants with Hb was inhibited by human serum albumin (HSA) which binds free Hm such that *Neisseria* can no longer utilize it (23). Thus, HpuB* expressing cells were only able to utilize free Hm released from denatured Hb, not Hm from intact Hb like wild-type HpuAB. The point mutations in HpuB* proteins must have altered the receptor structure and rendered HpuB more permeable to the diffusion of free Hm (226). Further, the authors did not determine whether HpuB* mutants could utilize HbHp complexes. This data does not support the conclusion that HpuA is dispensable for the HpuAB-mediated utilization of Hb or HbHp.

Biochemical analyses of interactions between the meningococcal HpuAB receptor, Hb, and TonB have defined fundamental properties of this transporter, including features that distinguish it from other members of the two-component TonB-dependent receptor family. Liquid-phase equilibrium binding assays demonstrated specific, saturable binding of Hb to wild-type HpuAB with an affinity of ~150nM, about 10-fold lower than estimated for the TbpAB-Tf interaction (225). Unlike TbpAB, the kinetics of Hb binding indicated the presence of a single Hb binding site. This was consistent with the inability of mutants lacking either receptor protein to significantly bind Hb and the failure of recombinant MBP-HpuA to bind Hb in the absence of HpuB (unpublished observation). In contrast to the two Tf binding sites formed by TbpAB, these data suggest that the single binding site of HpuAB is formed by the extensive interaction of the two polypeptides. Competitive binding assays further revealed that the recognition of Hb by HpuAB is not species specific, whereas the neisserial Tf and Lf receptors bind only human Fe carrier proteins (225). Although HpuAB must bind Hm at some stage of the transport process, the inability of hemin to effectively compete with Hb for binding to HpuAB suggested globin, not Hm, is the primary initial contact site with Hb. In native Hb, heme is held within the globin chains surrounded by non-polar side chains, with very limited exposure of the protruding proprionate side groups of porphyrin (227). What regions of Hb interact with HpuAB and how does the binding interaction induce the release of tightly bound Hm? It is improbable that HpuAB removes heme-Fe from Hb by direct competitive binding, as the affinities of apohemoglobins for heme are very large, with dissociation constants in the 10^{-12}-10^{-15} range (227). High-affinity binding by HpuAB must induce conformational changes in Hb that reduce its affinity for the heme prosthetic group. The efficiency of HpuAB Fe acquisition would be maximized if HpuAB was able to scavenge more than one heme-Fe moiety from each bound molecule of Hb, but this aspect of transport has not been investigated. Characterization of the binding kinetics of HpuAB to the more complex ligand HbHp, which exists as three structurally diverse phenotypes, promises to provide additional insight into the function of HpuAB. Based on growth assays indicating that HbHp is preferred over Hb as an Fe source, we predict that HpuAB either has a higher affinity for HbHp and/or is able to extract heme-Fe more easily from Hp-bound Hb. It will be interesting to see how the large, multimeric form of human-specific type 2 Hp, present in type 2-2 and 2-1 HbHp complexes, affects the affinity and avidity of its interaction with HpuAB.

Energy-dependent changes in the trypsin sensitivity of HpuB revealed that TonB interacted with HpuB even in the absence of ligand and altered the conformation of the outer membrane receptor. The loss of TonB or PMF had a dramatic effect on the kinetics of HpuAB-Hb interactions. In contrast to the TbpAB receptor, the affinity of HpuAB for Hb was virtually unchanged in a TonB mutant, but the Hb binding capacity (B_{max}) of the de-energized receptor was decreased 10-fold without a noticeable reduction in receptor expression (225). Consistent with the role of TonB in transducing PMF energy to HpuAB, dissipation of the PMF with CCCP (carbonylcyanide m-chlorophenylhydrazone) closely mimicked these results. Thus, without TonB or an intact PMF, only ~10% of the de-energized HpuAB population was in a "binding competent" state (figure 4A and 4B). TonB energy transduction also was required for the dissociation of Hb from HpuAB to complete the transport cycle (225).

What is the role of TonB in HpuAB-mediated heme-Fe uptake? As noted above, the majority of the HpuAB population was in a binding-incompetent state in a TonB- mutant. These HpuAB complexes were proposed to be in an inactive "open" conformation, consistent with the increased trypsin sensitivity of receptor proteins in de-energized cells. As a result, the Hb binding site was disrupted, since the close association of HpuA and HpuB was required to form a single high-affinity binding site. Only a small subset of the HpuAB population bound Hb with wild-type affinity in a TonB mutant, but heme-Fe extraction and ligand release are blocked without a source of energy. According to this model, TonB- and PMF-induced structural changes in the receptor components appear to enhance the productive interaction of HpuA and HpuB, shifting the equilibrium of the receptor population to favor a binding- and transport- competent state. TonB-mediated energy may be required to stabilize an energetically unfavorable receptor structure (225). Thus, the influence of TonB on HpuAB function appears different from that of TbpAB (figure 4). A decrease in binding capacity was not seen for de-energized TbpAB because both receptor components bind Tf independently with a high affinity and would not require TonB to form a binding-competent structure. TonB was still required for the Tf receptor to internalize Tf-bound Fe and subsequently release the deferrated ligand (130).

5. SINGLE-COMPONENT TONB-DEPENDENT NEISSERIAL IRON TRANSPORTERS

Although much less is known about this class of neisserial receptors, several TonB-dependent Fe acquisition mechanisms have been identified that consist of only a

single protein, presumably analogous to better studied siderophore receptors like FepA and FhuA. The hemoglobin receptor HmbR and siderophore transporter FetA, which have been studied in some detail, are discussed below. Additional putative single-component TonB-dependent receptors identified from analysis of the genome sequences of NM and NG are also presented.

5.1. HmbR (hemoglobin receptor)

Stojiljkovic *et al*. reported the identification of a 89-kDa hemoglobin receptor from NM, called HmbR, that was distinguished from HpuAB by its lack of an accessory lipoprotein and its ability to utilize Hb, but not HbHp, as an Fe source (36). A survey of clinical isolates from multiple serogroups revealed that 64% of NM strains possessed both *hmbR* and *hpuAB* genes; however, the *hmbR* gene had a premature stop codon in all gonococcal strains analyzed (228). Like HpuAB, HmbR expression also undergoes phase variation (224, 228). The redundancy of two distinct systems for Hb utilization underscores the likely importance of Hb as an *in vivo* Fe source, although the advantage afforded by the expression of two Hb receptors has not been explained.

The predicted primary amino acid sequence of HmbR placed it into the TonB-dependent receptor family (36), exhibiting weak homology to TbpA (21% identical) and LbpA (22% identical). Despite their shared ability to bind and internalize intact heme-Fe from Hb, HmbR was only slightly more homologous to HpuB (28% identical, 52% similar). Mutations in *tonB* or *exbB* confirmed that HmbR heme-Fe uptake requires TonB-ExbBD energy transduction. A NM *hmbR* mutant unable to utilize Hb was attenuated in an infant rat infection model, establishing the *in vivo* relevance of HmbR and Hb use for supporting the pathogenesis of meningococcal disease (36). In solid phase dot blot assays, the binding of biotinylated Hb was specifically blocked by Hb but not heme, suggesting that, similar to HpuAB, heme does not contribute to the initial binding of Hb (229). A direct quantitative comparison of HmbR-Hb interactions with the binding kinetics of Hb to HpuAB is precluded by the lack of liquid-phase equilibrium binding data for HmbR. Although modeled after other TonB-dependent receptors, the structure of HmbR and functional role of specific domains is poorly characterized at this time. Multiple sequence alignments of bacterial Hm receptors identified two amino acid motifs, FRAP and NPNL, that are highly conserved in Hm and Hb receptors but not in TonB-dependent siderophore or vitamin B_{12} receptors (230). No functional role for these motifs has been characterized in HmbR or other heme transporters. Further analysis of the structure, ligand interactions, and TonB-dependent transport mechanism of HmbR and comparison with HpuAB will be useful in determining how two quite different receptors have evolved to accomplish similar tasks.

5.2. FetA (ferric enterobactin transporter)

Although pathogenic *Neisseria* do not produce and secrete their own siderophores (25, 50, 51), *Neisseria* are able to utilize siderophores such as aerobactin and enterobactin that are synthesized by other organisms (28, 51). Until recently, no specific siderophore receptors had been described for any neisserial species. FetA, formerly known as FrpB, is a 76-kDa protein abundantly expressed in Fe-deplete conditions by most strains of NM and NG that has homology to the TonB-dependent receptor family (231-233). FetA is closely related to CopB (71% similar), a *Moraxella catarrhalis* protein implicated in Tf and Lf utilization (234). The presence of FetA antibodies in patients infected with pathogenic *Neisseria* indicates its expression *in vivo* (52), but no specific function in Fe acquisition was attributed to FetA. Two observations led Carson *et al.* to investigate FetA as a putative ferric enterobactin receptor. First, monoclonal antibodies against the *E. coli* enterobactin receptor FepA cross-reacted with a 70-kDa neisserial FeRP corresponding closely to FetA (22). In addition, sequence analysis of the region immediately downstream of *fetA* identified coding regions homologous to components of siderophore transport systems characterized in other species.

Subsequently, the presence of both FetA and TonB was shown to be required for gonococci to utilize enterobactin as an Fe source, supporting the conclusion that FetA is a TonB-dependent siderophore receptor (22). The open reading frame downstream of *fetA*, designated *fetB*, has homology to siderophore periplasmic binding proteins. This proposed function of FetB is consistent with the loss of enterobactin utilization, but not binding, in a *fetB* mutant (22). The localization and role of FetB in siderophore transport has not been further characterized. This study reported that FetA bound ^{59}Fe-enterobactin specifically with an estimated dissociation constant of 5 μM, 250-fold lower than the affinity of FepA for enterobactin. The ability of monoclonal antibodies to FepA, mapped to an epitope implicate in ligand binding, to cross-react with FetA indicated at least partial conservation of residues in the ligand binding site. The authors concluded that the very low affinity of FetA for ferric enterobactin may indicate that FetA primarily acts as a receptor for a structurally related, but currently unknown phenolate siderophore *in vivo* (22).

5.3. Other putative single-component Fe transporters

Annotation of the completed gonococcal (FA1090 Gonococcal Genome Sequencing Project; http://www.microgen.ouhsc.edu) and meningococcal genome sequences (235, 236) revealed seven additional genes with extensive homology to conserved domains of TonB-dependent proteins (237). A preliminary study of three of these genes, designated *tdfF*, *tdfG*, and *tdfH* (TonB-dependent family), has been reported by Turner *et al.* (237) while the other four remain uncharacterized. All three gene products lacked homopolymeric or tandem DNA repeats indicative of phase-variable genes. The *tdfF* gene, present in all three sequenced neisserial genomes, is predicted to encode a 78-kDa protein. Unlike TdfG, TdfH, and other TonB-dependent receptors whose putative OM localization signals contain a C-terminal phenylalanine, TdfF terminates in a lysine residue preceeded by phenylalanine. Homology of TdfF to the FpvA pyoverdin receptor of *P. aeruginosa* (29% identity) and the FhuE coprogen receptor of *E. coli* (30% identity) suggest that

TdfF may be a siderophore transporter. The presence of an open reading frame upstream of *tdfF* with homology to a periplasmic siderophore binding protein (CeuE of *Campylobacter jejuni*) is consistent with this proposed function. However, expression of TdfF by NM or NG could not be detected by western blot (237). The gene encoding the 134-kDa TdfG protein was present in the FA1090 NG genome but was not found in either NM genome database (237). Only 17% of the gonococcal strains examined by Turner *et al.* expressed TdfG. In contrast, the *tdfH* gene was present in all three neisserial genomes, and the corresponding 101-kDa TdfH protein was expressed by 100% of the NM strains and 81% of the NG strains tested (237). Both TdfG and TdfH exhibit low level homology (11% and 16%, respectively) to the HasR hemophore receptor of *S. marcescens*. Turner *et al.* demonstrated that TdfH does not appear to be Fe-regulated, suggesting it may transport a non-Fe nutrient substrate. The four remaining uncharacterized Tdf proteins exhibit a limited degree of similarity (<30%)to various siderophore or hemin receptors. Although neisserial two-component TonB-dependent Fe transporters have received a great deal of attention, the genome sequences of NM and NG have revealed a sizable repertoire of single-component TonB-dependent receptors whose substrates and activity remain to be elucidated.

6. TONB-INDEPENDENT IRON ACQUISITION

Many bacteria including *Shigella dysenteriae* (238, 239), type B *Haemophilus influenzae* (240, 241), *Vibrio vulnificus* (242), and *Yersinia enterocolitica* (243, 244) express TonB-dependent receptors for the use of free Hm as an Fe source. The availability of Hm at the nasopharyngeal mucosa is suggested by the ability of *H. influenzae*, which requires a source of exogenous Hm, to grow on this epithelium (40). Heme is also assumed to be present on the female urogenital mucosa encountered by gonococci due to the death of epithelial cells and during menses. Currently available data indicates that all strains of NM and NG tested are able to assimilate Fe from free Hm (26, 51, 245). Unlike other neisserial mechanisms of Fe uptake, Hm assimilation is TonB-independent and does not rely on either the HpuAB or HmbR Hb receptors (58, 64). The biochemical mechanism of TonB-independent Hm transport across the outer membrane is unclear. There were several reports of hemin-binding proteins isolated from NM and NG but no specific Hm receptors have been definitively characterized (246-248). The size of Hm, which tends to aggregate due to its hydrophobicity, makes it unlikely that Hm could passively diffuse through non-specific porin channels (39, 206). However, heme has been shown to diffuse passively across *in vitro* lipid bilayers (249). In the human host, virtually all free Hm released from within cells is rapidly scavenged by the serum proteins hemopexin (Hx) and albumin (SA). The inability of pathogenic *Neisseria* to utilize Hm bound to Hx or SA (23) suggests that Hm utilization *in vivo* may not be particularly important during neisserial infections.

Biswas *et al.* have also demonstrated that utilization of ferric citrate by NG is a TonB-independent event. This is in contrast to previously characterized TonB-dependent ferric citrate transporters in other Gram-negative bacteria such as the FecA citrate receptor of *E. coli* (250, 251). The *in vivo* relevance of this Fe source is unclear.

7. Neisserial iron uptake mechanisms required for intracellular growth

Recent advances in the emerging field of cellular microbiology have revealed that NM and NG are involved in intricate interactions with host cells. Merz and So (1) have compiled a comprehensive review of the molecular interactions of pathogenic *Neisseria* with host epithelial cells. Similar to the model described for enteropathogenic *E. coli* (EPEC) (252), NM and NG colonization of mucosal surfaces begins with attachment to host cells via type IV pili (253-256). The ensuing intimate attachment, signal transduction, and cytoskeletal and membrane alterations lead to internalization and transcytosis of bacteria into subepithelial stromal tissues (1). Internalized NM and NG, which can survive and grow within epithelial cells (257, 258), are usually observed residing in vacuoles but there are reports of NG in the host cell cytosol (253, 255, 256, 258-260). How does the infection of host epithelial cells by pathogenic *Neisseria* affect Fe homeostasis? What Fe sources do these pathogens exploit to permit intracellular growth? Some answers to these questions are just becoming available.

In mammalian cells, Tf is bound at the surface by transferrin receptor (TfR) and internalized by clathrin-coated pits. Acidification of endosomes triggers the dissociation of Fe from Tf, allowing the Fe to be transported into the cytoplasm. The resulting apo-Tf remains bound to TfR and is recycled to the cell surface (261). The high affinity of neisserial TbpAB for Tf suggests that it could effectively compete with human TfR for Tf (159). Bonnah *et al.* demonstrated that this competition for Tf may alter epithelial cell Tf-Fe homeostasis (262). This study showed that infection of epithelial cells by *Neisseria* reduces the levels of TfR mRNA transcripts and cycling TfR, reducing the ability of infected cells to internalize Tf. The cellular distribution of TfR was also altered by infection, although TfR did not co-localize with intracellular *Neisseria* (262). The significance of these findings to disease pathogenesis are unclear. Reduced internalization of Tf by epithelial cells may make more Tf-Fe available to the extracellular organisms. Alternatively, the authors suggest that the alterations in Fe homeostasis may be a general response to infection aimed at depriving invasive bacteria of Fe needed to proliferate.

Several recent studies have characterized the effect of defined mutants in known neisserial Fe acquisition genes on intracellular growth and survival. Heme biosynthetic mutants, constructed by inactivation of *hemH* (ferrochelatase) or *hemA* (gamma-glutamyl tRNA reductase), require an exogenous source of Hm for growth. Gonococcal *hemH* and *hemA* mutants showed significantly reduced intracellular survival within endocervical epithelial cells *in vitro* compared with parent strains (263). This

indicated that gonococci are apparently unable to acquire heme within epithelial cells. Perhaps NG lacks receptors for non-Hb hemoproteins, which form the largest source of intracellular Hm (264), or these potential Hm sources are inaccessible due to their localization within mitochondria. Larson *et al.* were able to inhibit NM intracellular replication by chelation of Fe in cells to be infected, confirming that meningococci must also acquire Fe from the host cell to replicate (265). A meningococcal *fbpA* mutant (see section 8.1) replicated normally within cells whereas a *tonB* mutant could not multiply intracellularly unless supplemented with ferric nitrate. These findings and the observation that Hm transport is unaffected by mutation of *fbpA* (263, 266) suggest that intracellular replication of meningococci requires TonB-dependent utilization of a novel host Fe source that may be a hemoprotein.

8. THE FATE OF IRON BEYOND TRANSPORT ACROSS THE OUTER MEMBRANE

8.1. Iron transport across the periplasm and inner membrane

The transport of Fe^{3+} from Tf and Lf is the only periplasmic and inner membrane Fe uptake mechanism characterized in pathogenic *Neisseria*. Mietzner *et al.* first purified and characterized a major iron-regulated 37-kDa protein, now designated FbpA, that was expressed by all NM and NG strains tested (267, 268). The presence of a signal peptide to mediate export of FbpA across the cytoplasmic membrane (269), its localization to the periplasm in NG, and the ability of each FbpA molecule to bind a single ferric ion (270) support the model that FbpA is a periplasmic Fe-binding protein. Subsequent pulse-chase experiments showed reversible binding of Fe to FbpA and suggested that FbpA is, in fact, a transient participant in Fe acquisition (271). Biochemical analysis of the binding site and coordination of Fe by FbpA, which is approximately the size of a single Tf lobe, indicated that FbpA functions like a mono-sited analog of eukaryotic Tf (272). Two open reading frames identified downstream of *fbpA*, designated *fbpB* and *fbpC*, were predicted to encode the cytoplasmic permease and nucleotide-binding protein components of an Fe-specific ABC transporter system, respectively (273). Insertional inactivation of *fbpABC* resulted in a mutant unable to use Fe from Tf, Lf, or Fe chelates, whereas the utilization of Fe from Hm or Hb was not affected (266). Thus, FbpABC is proposed to be an ABC transporter that is a focal point for the periplasmic and inner membrane transport of Fe, but not heme, acquired from various sources. Several questions concerning FbpABC function remain to be investigated including whether FbpA interacts directly with TonB-dependent receptors, how FbpA delivers bound Fe to FbpBC, and how the putative inner membrane complex couples ATP-hydrolysis to Fe transport.

Despite the identification of periplasmic heme-binding proteins in other Gram-negative bacteria (61, 239, 244) and the availability of complete genome sequences for NM (235, 236) and NG (274), no good candidates for heme-specific PBPs or ABC transporters have been identified in these pathogens. The fate of siderophore-Fe complexes internalized by *Neisseria* is also poorly understood. West and Sparling reported the cloning of a NG gene that complements an *E. coli fhuB* mutation to allow aerobactin utilization(28). Although this gonococcal gene product has not been further characterized, *E. coli* FhuB is an integral cytoplasmic membrane protein component of an ABC transport system for hydroxymate siderophores (275-278). A homolog of periplasmic siderophore binding proteins called FetB is encoded downstream of the FetA enterobactin receptor gene (22), suggesting that genes encoding siderophore specific PBPs in *Neisseria* may be linked to the corresponding TonB-dependent receptor genes. The function of FetB as an enterobactin-specific PBP has yet to be demonstrated. The full spectrum of siderophores used and the mechanisms for their internalization by *Neisseria* have not been fully characterized.

8.2. Cytosolic iron: intracellular iron storage and heme oxygenases

Bacteria express two types of Fe-storage proteins: bacterioferritin, which have a non-heme Fe core and non-covalently bound Hm groups; and ferritin, which lacks Hm prosthetic groups (279-281). Chen *et al.* have identified and characterized a bacterioferritin (Bfr) expressed by the gonococcus (282). Bfrs usually consist of 24 identical 18 to 22-kDa subunits that form a spherical protein shell containing 600-2400 Fe atoms per molecule and 3-12 non-covalently associated protoheme IX groups (283). Purified gonococcal Bfr was ~400-kDa, composed of two similar but non-identical subunits called BfrA (18-kDa) and BfrB (22-kDa) which were both required to form a functional Bfr. A NG *bfrB* mutant exhibited reduced growth in Fe-deplete conditions, and increased sensitivity to killing by hydrogen peroxide and paraquat (282). These observations indicate that gonococcal Bfr likely plays an important role in Fe storage and minimizing Fe-induced oxidative stress.

The fate of heme following transport into the cytoplasm of *Neisseria* or other Gram-negative pathogens is not well understood. The entire heme-Fe moiety may become incorporated into Hm-containing enzymes. Alternatively, the ability of Hm to serve as an Fe source indicates that Hm is degraded by a heme oxygenase-like activity to release the Fe. The identification of *hemO* in pathogenic *Neisseria* marked the first heme oxygenase characterized in Gram-negative bacteria (284, 285). The *hemO* gene, highly conserved among commensal and pathogenic *Neisseria*, is located directly upstream of the *hmbR* Hb receptor (284). The function of HemO was initially inferred from its homology (21% identical, 44% similar) to human heme oxygenase-1, which is known to degrade heme. The phenotype of HemO knockout mutants in NM and NG included increased sensitivity to Hm toxicity and inability to utilize Hm, Hb, or HbHp as Fe sources. Recombinant NM HemO was shown to form a 1:1 complex with Hm and convert Hm into ferric-bilverdein IXα and carbon monoxide (285). The recent crystal structure determination revealed the overall fold and Hm-binding site of NM HemO was similar to mammalian heme oxygenase enzymes (286). These data confirmed the identity of HemO as a heme oxygenase (284). Without

HemO, internalized Hm accumulated to toxic levels and could not be degraded to release Fe needed for growth.

9. REGULATION OF IRON TRANSPORT

9.1. The Fur regulon

In addition to being an essential nutrient, Fe acts as a cue that regulates virulence-associated genes. The Fe-deplete environment of the human host induces the expression of Fe acquisition systems and up-regulates several bacterial toxins, including Shiga toxin of *S. dysenteriae*, diphtheria toxin of *C. diphtheria* and *P. aeruginosa* exotoxin A (12). Two putative Fe-repressible toxins belonging to the RTX family, designated FrpA and FrpC, have been identified in NM (224, 228, 287-289). However, the toxic activity of these putative RTX proteins has not been demonstrated. The molecular basis of coordinate gene regulation by Fe, widely conserved among Gram-negative bacteria, involves the Fur (ferric uptake regulator) protein. Fur was first described in *Salmonella typhimurium* (290) but has been most thoroughly characterized in *E. coli* (291-293). Fur is a ~15-kDa protein that, in the presence of an Fe cofactor, exhibits sequence-specific binding to a conserved "Fur box" motif in Fe-responsive promoters to modulate of gene expression. Although Fur has been thought to act primarily as a repressor, Fur has also been shown to activate the acid tolerance response in salmonellae (294) and upregulate expression of *sodB* in *E. coli* (295). Homologs of *fur* have been identified and cloned from NM and NG, suggesting that an analogous mechanism of Fe-responsive gene regulation is functional in these pathogens (296-298). Despite limited homology with *E. coli* Fur (50% identity), neisserial Fur proteins were able to regulate Fe-responsive promoters and complement *fur* mutants in *E. coli* (296, 298).

The direct involvement of Fur in repression of neisserial Fe uptake receptor systems has been difficult to demonstrate due to the inability to construct a *fur* null mutant (296, 298). This may be due to the Fe overload and oxidative stress resulting from constitutive expression of Fe transporter genes (299, 300). Fur-mediated regulation in *Neisseria* was inferred from the presence of putative Fur binding sites in the promoters of genes involved in Fe acquisition (55, 139-141, 231, 269, 301) and the ability of *E. coli* Fur protein to bind to the neisserial *fbpA* promoter (296). A Fur titration assay (FURTA) demonstrated that down-regulation of cloned *hmbR* gene expression in Fe-replete conditions was mediated by the Fur repressor (36). Desai *et al.* have also reported that purified gonococcal Fur binds to the *fbpA* promoter as a complex of two Fur repressor dimers (302). Thomas *et al.* used a NG missense *fur* mutant to examine the extent of the Fur regulon (299), employing two-dimensional polyacrylamide gel electrophoresis (PAGE) to monitor deregulation of Fe-controlled proteins. The derepression of at least 15 Fe-repressed proteins (Frps) and decreased expression of 17 Fe-induced proteins (Fips) suggested that Fur acts both as a repressor and activator of a large, complex regulon (299). The role of Fur as a global Fe-responsive regulator was supported by a recent study by Sebastian *et al.* examining the binding of recombinant NG Fur to putative Fur boxes identified in the gonococcal genome (303). Using electrophoretic mobility shift assays (EMSA), Fur was shown to bind promoters of genes involved in Fe uptake (*fur*, *tonB, hmbR, hemO*), secretion (*secY*), recombination (*recN*), protection against Fe-induced oxidative stress (*sodB*), metabolism (*fumC*), and all 11 genes of the *opa* surface protein family. Sebastian *et al*. also demonstrated that NG Fur recognized operator sites with high-affinities (K_d~10 nM for *tonB* and *fur*) and low affinities (K_d~100 nM for *opa*) (303). The significant variations in Fur binding affinities to different promoters within its regulon may provide a mechanism for differential Fe-responsive gene regulation. The strength of Fur-operator binding may influence the relative level of induction or repression, or may determine whether a gene is Fe-inducible or Fe-repressible. The complexity of the Fur regulon and Fur-mediated regulation is not fully understood at this time.

9.2. Phase variation and other mechanisms of regulating Fe transport

Populations of pathogenic *Neisseria* are characterized by high levels of genetic diversity. Factors contributing to this genetic heterogeneity include horizontal DNA transfer (304), antigenic variation by recombination between expressed and silent loci (305-309), and on-off switching of genes by phase variation (310-313). These mechanisms of genetic variation allow *Neisseria* to adapt to changing environmental conditions and potentially evade the immune response. A comparative genomic analysis by Snyder *et al.* identified over 100 putative phase-variable genes in the complete genomes of NM strains Z2491 and MC58 and NG strain FA1090 (314), including several neisserial TonB-dependent Fe tranporters. The HpuAB and HmbR receptors both contain poly (G) repeats within their open reading frames. These genes can be switched on or off at a rate of 10^{-3}-10^{-4} by reversible changes in the number of residues in the repeat by a slipped-strand mispairing (SSM) mechanism that alters the translational reading frame (223, 224, 228). Richardson *et al* have observed NM strain-specific differences in phase variation rates partially explained by mismatch repair defects and increased overall mutability (315). The prevalence of hypermutable strains among epidemic meningococcal isolates supports the conclusion the phase variation may play an important role in the transmission of this pathogen (316).

In addition to the absolute on-off regulation associated with typical phase variation, pathogenic *Neisseria* also employ other mechanisms to more subtly control the expression of Fe acquisition systems. For example, the level of transcription of the gonococcal siderophore receptor FetA is modulated by a reversible SSM mechanism acting at a poly-cytosine repeat within the *fetA* promoter that alters the spacing and thus the strength of the promoter (317). How do *Neisseria* regulate the expression of two-component Fe transporters encoded in a single operon to achieve proper receptor stoichiometry? In the case of *hpuAB*, which lacks any intergenic secondary structures, the two receptor components may be translationally coupled (224). In the gonococcal TbpAB Tf

receptor, the 2:1 ratio for transcripts of the upstream *tbpB* gene over *tbpA* transcripts likely results from an intergenic inverted repeat not found in the other neisserial two-component receptor operons (182). Ronpirin *et al.* proposed that this method of differential regulation of *tbpA* and *tbpB* may be due to either premature transcriptional termination at the inverted repeat or the enhanced stability of monocistronic *tbpB* mRNA products. Similar involvement of an intergenic inverted repeat in differential gene expression has been noted in the NM *fbpABC* operon (318). Finally, the two-fold higher induction of *tbpB* than *tbpA* upon Fe starvation led to the speculation that some unidentified Fe-responsive factor affected the relative abundance of the *tbp* gene products (182). The complex multilevel system involved in coordinately regulating the Fur regulon and Fe acquisition systems in the pathogenic *Neisseria* is only partly understood.

10. PERSPECTIVE

The obligate human pathogens *Neisseria gonorrhoeae* and *Neisseria meningitidis* are responsible for significant worldwide morbidity and mortality. The effective design of prophylactic and therapeutic approaches to combat these infectious diseases depends on molecular and biochemical characterization of their pathogenesis and virulence factors that are crucial for colonization and replication within the human host. As this review has highlighted, iron acquisition by pathogenic *Neisseria* is an important virulence determinant that has been the focus of much investigation. The multiple receptor-mediated Fe uptake systems expressed by NG and NM make them uniquely suited to overcome nutritional immunity and to thrive in the hostile host environment. *Neisseria* exploit siderophores produced by other organisms as well as numerous host Fe carrier proteins to fulfill their Fe requirement. In addition to the five specific Fe uptake pathways currently known, analysis of the whole genome sequences of NM and NG predict at least seven uncharacterized TonB-dependent transporters that may further expand the repertoire of Fe sources utilized by *Neisseria*. Alternatively, these TonB-dependent proteins may serve as high affinity transporters for ligands other than Fe, analogous to the *E. coli* BtuB transporter for vitamin B_{12}. The ability of NM and NG to occupy niches as diverse as mucosal surfaces, the bloodstream, cerebrospinal fluid, and intracellular compartments undoubtedly reflects the redundancy and versatility of this virulence factor. The expression of proteins involved in Fe acquisition are tightly regulated by Fe and possibly other unknown host factors to ensure stringent control of expression within the host and to permit evasion of immune responses.

A novel paradigm for TonB-dependent Fe transport that has emerged from the crystallographic and biochemical analysis of several *E. coli* siderophore uptake systems has been extrapolated to homologous neisserial TonB-dependent Fe receptors. The transport channels are envisioned to form 22-stranded "barrel-and-plug" channels that interact with TonB to drive active Fe uptake, similar to the highly conserved structures of the FepA, FhuA, and FecA siderophore receptors of *E. coli*. However, members of the best-characterized family of neisserial TonB-dependent receptors (TbpAB, LbpAB, and HpuAB) are distinguished by their ability to extract Fe from carrier proteins and the presence of an accessory lipoprotein component. More detailed characterization of receptor structure, ligand and TonB interactions, and mechanisms of Fe extraction and internalization from carrier proteins will allow for more informative comparisons between neisserial and enterobacterial strategies of Fe acquisition. In addition to providing excellent models for studying the biochemistry of Fe transport, the TonB-dependent receptors of pathogenic *Neisseria* may also serve as potential vaccine targets (158, 160, 305, 319-322) and high-affinity pumps for the delivery of "Trojan horse" therapeutics such as antibiotic-siderophore complexes and toxic heme derivatives (323, 324).

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Send correspondence to: Dr. Kyle Rohde, Biomedical Research Center Room 366, 975 N.E. 10th Street, Oklahoma City, OK 73104. Tel.: 405-2721-1201, Fax: 405-271-3117, E-mail: kyle-rohde@ouhsc.edu