Cell Envelope Signaling in Escherichia coli

LIGAND BINDING TO THE FERRICHROME-IRON RECEPTOR FhuA PROMOTES INTERACTION WITH THE ENERGY-TRANSDUCING PROTEIN TonB*

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The ferrichrome-iron receptor of Escherichia coli is FhuA, an outer membrane protein that is dependent upon the energy-coupling protein TonB to enable active transport of specific hydroxamate siderophores, interaction by certain phages, and cell killing by the protein antibiotics colicin M and microcin 25. In vivo cross-linking studies were performed to establish at the biochemical level the interaction between FhuA and TonB. In an E. coli strain in which both proteins were expressed from the chromosome, a high molecular mass complex was detected when the ferrichrome homologue ferricrocin was added immediately prior to addition of cross-linker. The complex included both proteins; it was absent from strains of E. coli that were devoid of both FhuA or TonB, and it was detected with anti-FhuA and anti-TonB monoclonal antibodies. These results indicate that, in vivo, the binding of ferricrocin to FhuA enhances complex formation between the receptor and TonB. An in vitro system was established with which to examine the FhuA-TonB interaction. Incubation of TonB with histidine-tagged FhuA followed by addition of Ni²⁺-nitritriacetate-agarose led to the specific recovery of both TonB and FhuA. Addition of ferricrocin or colicin M to FhuA in this system greatly increased the coupling between FhuA and TonB. Conversely, a monoclonal antibody that binds near the N terminus of FhuA reduced the retention of TonB by histidine-tagged FhuA. These studies demonstrate the significance of ligand binding at the external surface of the cell to mediate signal transduction across the outer membrane.

High affinity iron uptake in Gram-negative bacteria such as Escherichia coli is a stepwise process that involves recognition of a ferric iron chelator (siderophore) by a receptor within the outer membrane, translocation of the siderophore-Fe(III) complex into the periplasm, and internalization of the iron by a cytoplasmic membrane permease in a periplasmic binding pro-tein-dependent manner (reviewed in Refs. 1–4). Dissection of the energy requirements for high affinity uptake of iron (5) and of vitamin B₁₂ (6–9), transport of which shares common elements with siderophore-Fe(III) transport mechanisms, identified the importance of the TonB and ExbB/D proteins of the cytoplasmic membrane to couple proton-motive force with active transport at the outer membrane (Ref. 10; reviewed in Refs. 2 and 11). TonB homologues have been identified in many Gram-negative bacteria, including Salmonella enterica serovar Typhimurium (12), Yersinia enterocolitica (13), Haemophilus influenzae (14), and Pseudomonas aeruginosa (15). In addition, complexes between TonB or its homologues and other proteins (ExbB, ExbD, and as yet unidentified proteins) were detected with anti-E. coli TonB monoclonal antibodies (mAbs1; Ref. 16). These findings demonstrate that the TonB-dependent energy transduction system is shared among many Gram-negative bacteria and suggest that high affinity energy-dependent iron uptake in Gram-negative aerobes is accomplished using TonB and its accessory proteins (16).

Physical association between TonB and a TonB-dependent outer membrane receptor was first demonstrated biochemically by in vivo cross-linking experiments in which TonB was coupled to the E. coli enterobactin (also known as enterochelin) receptor, FepA (17). This finding substantiated genetic analyses that suggested interactions between TonB and other outer membrane transporters, namely the ferrichrome-iron receptor FhuA (18, 19) and the vitamin B₁₂ receptor BtuB (20–22). However, formation of the FepA-TonB complex appeared to be independent of the presence of ferric enterobactin, since the strains used carried mutations in the enterobactin biosynthetic genes and were negative on chrome azurol S plates used for detecting siderophore excretion (23). Paradoxically, TonB did not appear to form cross-links with any of the other outer membrane receptors. BtuB and FhuA have subsequently been shown to compete for a limiting amount of TonB function; ferrichrome decreased the rate of vitamin B₁₂ transport, and, conversely, vitamin B₁₂ inhibited ferrichrome uptake if BtuB were overexpressed (24). These results imply that ligand-bound outer membrane receptors have a means of signaling to TonB that they are occupied. Indeed, outer membrane receptors have been demonstrated to change conformations following addition of cognate ligands (25–27). The binding of ferrichrome-iron to purified FhuA led to a conformational change in the receptor detectable both as a reduction in reactivity of certain anti-FhuA mAbs and as a gain in resistance to trypsinolysis at lysine 67 but not at lysine 5 of FhuA (26). We proposed that

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1 The abbreviations used are: mAb, monoclonal antibody; PVDF, polyvinylidene difluoride; NTA, nitrilotriacetate; LDAO, N,N-dimethyldodecylamine N-oxide; PAGE, polyacrylamide gel electrophoresis; TLN, Tris-HCl/LDAO/NaCl.
such a conformational change in FhuA may act as a signal to TonB in vivo, indicating that the receptor is loaded with ligand.

To understand the functional relevance of the ligand-induced conformational change in FhuA and to investigate whether receptors other than FepA can form complexes with TonB, we initiated studies of FhuA-TonB interactions. We now report that FhuA can be cross-linked to TonB and that the cross-linking between FhuA and TonB is significantly enhanced by the presence of the ligand ferricrocin. Additionally, we have established an in vitro system with which to evaluate the coupling between FhuA and TonB and the effect of normally membrane-impermeant macromolecules upon the interaction. Complementary results from in vivo and in vitro experiments confirm that ligand binding to FhuA enhanced its physical association with TonB.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents—**Goat anti-mouse immunoglobulin (IgG (heavy plus light chain)-horseradish peroxidase conjugate and goat anti-mouse IgG1-horseradish peroxidase conjugate antibodies were from Southern Biotechnology Associates (Birmingham, AL). The enhanced chemiluminescence Western blotting assay (ECL) kit was obtained from Amersham Corp. Immobilon-P polyvinylidene difluoride (PVDF) membrane was from Millipore Corp. Ni²⁺-nitrilotriacetate (NTA)-agarose resin was from Qiagen, imidazole from ICN Biomedicals, Inc., and N,N-dimethylformamide (DMF) from ultracentrifugation, enriched for cytoplasmic membrane vesicles (36), was solubilized with stirring for 45 min at room temperature in 50 mM Tris-HCl, pH 7.8, 2% DDAO, and 0.5 mM phenylmethylsulfonyl fluoride to give a total soluble protein concentration of approximately 10 mg/ml. Extracts were stored at −20 °C. Immediately prior to column experiments the samples were thawed, diluted 10-fold with 50 mM Tris-HCl, pH 7.8, 0.1% LDAO, 100 mM NaCl (TLN) plus 5 mM imidazole, and filtered over 0.45-μm pore size cellulose acetate filters (Millipore Corp.).

**Construction of a FhuA Column and Its Use to Capture Solubilized TonB—**Hexahistidine-tagged FhuA (FhuA.H₆) from the outer membrane of the E. coli K-12 strain AW740/pXH405 was purified to apparent homogeneity over Ni²⁺-NTA-agarose (26). To construct the FhuA.H₆-Ni²⁺-NTA-agarose column, a 2-mg aliquot of concentrated FhuA.H₆ was diluted 1:10 in TLN and applied over 20 column volumes. A major peak was eluted at an imidazole concentration of approximately 80 mM. Samples (8 μl) of column fractions (1 ml) were mixed with an equal volume of 4 × concentrated SDS-PAGE sample buffer, boiled for 1 min, and then resolved on 9% polyacrylamide gels. Proteins were transferred to PVDF membranes and probed with anti-FhuA and anti-TonB mAbs. The primary antibodies were detected either with an anti-mouse secondary-specific rhodamine-phosphatase-conjugated secondary antibody (187, 1; Ref. 41) followed by visualization using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium or with a goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibody followed by visualization by enhanced chemiluminescence.

In a parallel experiment, a duplicate 2-mg aliquot of concentrated FhuA.H₆ was applied over 10 column volumes, mixed with a 20-fold molar excess of ferricrocin, and loaded onto a bed of 1 ml of Ni²⁺-NTA-agarose in an HR 5/5 column (Pharmacia Biotech Inc.). A flow rate of 0.5 ml/min was used for all column chromatography.

**Construction of FhuA-TonB Interaction—**To investigate the influence of FhuA-specific ligands upon the protein-protein interaction, an in vitro experiment of smaller scale was devised. Aliquots (5 μl) of either FhuA.H₆ or FhuA.A021–128.H₆ were added to 10 column volumes of 50 mM Tris-HCl, pH 7.8, and elution with imidazole was performed exactly as described above for the FhuA.H₆ sample. To eliminate the possibility that the presence of TonB₆₆–100 in the eluate was due to some affinity of TonB to FhuA, the experiment was repeated without application of FhuA.H₆ to the resin.

**In Vitro Analysis of the Effect of Ligands upon the FhuA-TonB Interaction—**To investigate the influence of FhuA-specific ligands upon the protein-protein interaction, an in vitro experiment of smaller scale was devised. Aliquots (5 μl) of either FhuA.H₆ or FhuA.A021–128.H₆ in TLN were incubated in solution with a 20-fold molar excess of ferricrocin, with 50 μl of undiluted RK4691 cell lysate containing colicin M (Ref. 34; 2°-fold dilution of stock gave a clear zone of lysis on the indicator strain MC4100), with 50 μl of undiluted AY261 cell lysate containing microcin J23, or with 10° plaque-forming units of the FhuA-specific-phase TS5 or Δ68 for 10 min. All incubations were at 25 °C. For some experiments, hybridoma supernatants (specific Ig concentration, 5–10 μg/ml) containing anti-FhuA mAbs, FhuA-derived mAbs (16), and anti-TonB mAbs (16) were added to an additional 10 column volumes. For one of these mAbs bind determinants located between amino acids 1–20, 21–59, 381–417, and 417–550 of FhuA, respectively. For both of these mAbs, FhuA.A021–128.H₆ was added at 10 column volumes, and the protein-protein interaction was detected either with an anti-mouse secondary-specific rhodamine-phosphatase-conjugated secondary antibody (187, 1; Ref. 41) followed by visualization using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium or with a goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibody followed by visualization by enhanced chemiluminescence.

In a parallel experiment, a duplicate 2-mg aliquot of concentrated FhuA.A021–128/H₆ was applied over 10 column volumes, mixed with a 20-fold molar excess of ferricrocin, and loaded onto a bed of 1 ml of Ni²⁺-NTA-agarose in an HR 5/5 column (Pharmacia Biotech Inc.). A flow rate of 0.5 ml/min was used for all column chromatography.
lysate from K1086/huA cells was mixed with 1 μl of a 1 m stock of either peptide 1 (corresponding to amino acids 1–18 of FhuA) or peptide 2 (corresponding to amino acids 636–651 of FhuA) for 10 min prior to the addition of the TonB-containing sample to FhuA.H6. Before use, the pH of the peptide solutions was adjusted to neutrality with 100 mM Tris-HCl, pH 7.4. After a 15-min incubation, 2.5 μl of TLN-equilibrated Ni2+-NTA-agarose resin was added and mixed for 10 min. The resin was pelleted by centrifugation and washed twice with TLN containing 5 mM imidazole, and bound proteins were eluted by addition of 30 μl of TLN containing 50 mM EDTA. Aliquots (5 μl) of the eluate were mixed with electrophoresis sample buffer, boiled for 1 min, and loaded onto 9% polyacrylamide gels. Resolved proteins were transferred to PVDF membranes and probed with anti-FhuA mAb Phu8.4 and anti-TonB mAb 4H4 detected by goat anti-mouse IgG1-horseradish peroxidase-conjugated antibodies and chemiluminescence. Standard proteins for relative mobility were 0.1 μg of purified FhuA.H6 (lane 1) and prestained markers with apparent molecular masses as indicated between panels. Cell extracts, having been subjected to cross-linking, were collected from the following E. coli strains: SG303 (lanes 2 and 3), SG303/huA (lanes 4 and 5), SG303/huA(pGC01) (lanes 6 and 7), and GSM01(pGC01) (lanes 8 and 9). An equal number of cells were cross-linked in each sample. C, extended exposure of lanes 1–5 of the immunoblot shown in B. fc, ferricrocin.

**FIG. 1. Identification of TonB and FhuA proteins by immunoblot analysis of formaldehyde-cross-linked samples.** Duplicate panels of SDS-soluble proteins were resolved by SDS-PAGE, transferred to PVDF membranes and probed with anti-TonB mAb 4H4 (A) or with anti-FhuA mAb Phu8.3 (B). Primary antibodies were detected by horseradish peroxidase-conjugated secondary antibodies and chemiluminescence. Standard proteins for relative mobility were 0.1 μg of purified FhuA.H6 (lane 1) and prestained markers with apparent molecular masses as indicated between panels. Cell extracts, having been subjected to cross-linking, were collected from the following E. coli strains: SG303 (lanes 2 and 3), SG303/huA (lanes 4 and 5), SG303/huA(pGC01) (lanes 6 and 7), and GSM01(pGC01) (lanes 8 and 9). An equal number of cells were cross-linked in each sample. C, extended exposure of lanes 1–5 of the immunoblot shown in B. fc, ferricrocin.

**RESULTS**

**FhuA Can Be Cross-linked in Vivo to TonB**—Previous results demonstrated the existence of a high molecular mass complex containing both PepA and TonB (17). We posited that a complex between FhuA and TonB could be identified by using a similar in vivo formaldehyde cross-linking protocol. The anti-TonB mAb 4H4 detected prominent bands with apparent molecular masses of 57–59 kDa and ~175 kDa (Fig. 1A). Anti-TonB mAb-reactive bands with relative mobilities greater than the band at 69 kDa are not displayed because they have been characterized previously (10, 16, 17, 36). It should be noted that unequivocal assignments of molecular masses of bands based on their mobility in SDS-PAGE was not possible (as noted in Refs. 16 and 17). Since formaldehyde cross-links are heat-labile, protein samples were not completely denatured by boiling prior to application to the polyacrylamide gel.

The prominent band of ~175 kDa was present in cells in which FhuA was expressed at chromosomal levels (E. coli strain SG303) but absent from SG303/huA cells. This band was detected when FhuA was expressed from the multicopy plasmid pGC01 in SG303/huA cells but was lost in the isogenic tonB::kan strain GSM01 (pGC01). This band therefore represented a candidate FhuA-TonB complex.

**Ferricrocin Increases the Abundance of the FhuA-TonB Complex**—To establish the specificity of the candidate FhuA-TonB complex, the effect of adding the FhuA-specific siderophore ferricrocin immediately prior to formaldehyde cross-linking was examined. Ferricrocin induced the appearance of the band migrating to ~175 kDa in strain SG303 and increased the abundance of the complex in SG303/huA(pGC01). Addition of ferricrocin did not significantly change the SDS-soluble protein profile of either SG303/huA or GSM01(pGC01).

To verify that the ~175-kDa complex contained FhuA, an identical panel of cross-linked proteins was probed by immunoblotting with the anti-FhuA mAb Phu8.3 (Fig. 1B). In the SDS-soluble cross-linked proteins from strain SG303, the most prominent band corresponded to monomeric wild-type FhuA of ~80 kDa. Less intensely mAb-reactive bands of ~90, 100, and 110 kDa were also identified. Upon longer exposures of the x-ray film (Fig. 1C), a band migrating with an apparent molecular mass of ~175 kDa was detected in the SDS-soluble extract from SG303 cells that had been preincubated with ferricrocin. As was apparent upon probing with the anti-TonB mAb, no band migrating to ~175 kDa was present when FhuA was expressed from the chromosome in the absence of exogenously added ferricrocin. The formation of this ~175-kDa complex was dependent upon both FhuA and TonB since in strains SG303/huA and GSM01(pGC01) the complex was absent. Fur-
thermore, the complex that was detected by the anti-FhuA mAb displayed an identical pattern of abundance as influenced by addition of ferricrocin. These data therefore demonstrated that a complex between FhuA and TonB was formed in vivo and that its amount increased by preincubation with ferricrocin.

When FhuA was expressed from the high copy number plasmid pGC01, a family of FhuA-containing, formaldehyde-cross-linked complexes appeared above the FhuA monomer in the range of 90–130 kDa (Fig. 1B). These bands had mobilities identical to the less prominent bands that were seen above the FhuA monomer when FhuA was expressed from the chromosome in strain SG303 (particularly in longer exposures; see Fig. 1C) and were independent of the presence of TonB since their profile was unchanged between SG303(fhuA(pGC01) and GSM01(pGC01). At present, we have not established the identity of these FhuA-containing complexes. A similar set of anti-FhuA mAb-reactive bands was disclosed in vivo cross-linking with E. coli strain SG303/fhuA(pGC405) (data not shown). In these experiments, the FhuA-containing complexes migrated slower than the FhuA66–100 H6 monomer, which had a relative mobility of ~72 kDa. This observation indicates that amino acids 21–128 of FhuA are apparently not required for the formation of these higher molecular mass complexes.

**TonB Binds Specifically to FhuA in Vitro**—We developed an in vitro system with which to examine the interaction between FhuA and TonB that would allow for further assessment of its specificity. Purified FhuA H6 protein was applied to a Ni2+-NTA-agarose column. Detergent-solubilized samples containing TonB were introduced into the column, the resin was washed and bound proteins were eluted with imidazole. Initial experiments showed that trace amounts of wild-type TonB were retained by the Ni2+-NTA-agarose resin even in the absence of FhuA H6. Two modifications were employed to circumvent this limitation. We postulated that the proline-rich region of TonB might be responsible for the interaction between TonB and the resin. The TonB derivative TonB66–100, which lacks the majority of the proline-rich region and which confers wild-type TonB phenotype upon cells (36), was therefore used for in vitro column experiments. Incubation of 5 μM imidazole in the binding and wash buffers eliminated nonspecific retention of proteins by the Ni2+-NTA resin.

Elution of bound FhuA H6 also led to the recovery of TonB66–100 (Fig. 2, middle blots). In the absence of FhuA H6 on the resin and with an equivalent amount of TonB66–100 applied in a control experiment, no TonB66–100 was detected in the eluate (Fig. 2, top blots). Retention of TonB66–100 by the Ni2+-NTA-agarose resin therefore required prior application of FhuA H6. Examination of the eluate fractions for FhuA by total protein staining (data not shown) indicated that the fractions containing the FhuA peak corresponded to those containing the TonB66–100 peak, as determined by immunoblotting with an anti-TonB mAb. The co-elution of TonB66–100 with FhuA H6 implies that the two proteins interact in this assay since TonB66–100 displayed no affinity for Ni2+-NTA-agarose in the absence of bound FhuA H6.

To evaluate whether ferricrocin enhanced complex formation between FhuA and TonB as it did in vivo, purified FhuA H6 was incubated with a 20-fold molar excess of ferricrocin immediately prior to application onto the resin. When an amount of TonB66–100-containing sample that was equivalent to that used previously (Fig. 2, top and middle blots) was applied onto the FhuA H6-ferricrocin column, an increased amount of TonB66–100 was eluted (Fig. 2, bottom blots). Immunoblotting of column fractions with anti-FhuA mAbs (data not shown) indicated that the same amount of FhuA H6 was eluted from the column regardless of the absence or presence of ferricrocin. The observed increase in TonB66–100 retained by the FhuA H6-ferricrocin complex further demonstrates the specificity of interaction between the two proteins since ferricrocin is specific for FhuA.

The in vitro results were extended to examine the effects of other FhuA-specific ligands, such as colicins and phage, upon the FhuA-TonB interaction. This necessitated development of a microscale in vitro experimental protocol due to the relative scarcity of these ligands, to maintain ligand excess relative to FhuA. Using this protocol, complexes between FhuA and TonB that were formed in solution were captured by Ni2+-NTA-agarose resin. Cell extracts containing wild-type TonB instead of TonB66–100 were used to verify that the native TonB protein interacted with FhuA H6 in vivo as it did in vitro. Immunoblotting of resolved Ni2+-NTA-agarose eluates with anti-FhuA and anti-TonB mAbs identified qualitative differences in TonB binding to FhuA H6 (Fig. 3). Quantitation of the amounts of TonB and FhuA H6 on the immunoblot established reproducible and significant alterations in the amount of TonB retained in the presence of TonB66–100. The addition of ferricrocin to FhuA H6 induced a substantial increase in the subsequent recovery of TonB (Fig. 3, compare lanes 3 and 4). Relative to the amount of TonB bound to the FhuA H6-ferricrocin complex, only the addition of colicin M led to a similar large amount of TonB, which co-eluted with FhuA H6 from the resin (Fig. 3, lane 9). Phage φ80 induced a 3-fold increase in the amount of TonB, which bound to FhuA H6 and which was subsequently captured by the Ni2+-NTA-agarose resin (Fig. 3, lane 10). Addition of phage T5 or microcin 25 (Fig. 3, lanes 11 and 12) in place of ferricrocin led to the recovery of amounts of TonB that were similar to that obtained after no ligand addition (Fig. 3, lane 3).

Our library of anti-FhuA mAbs (35) has been used previously to probe FhuA topology, to investigate interactions between receptor and its ligands, and to identify ligand-induced conformational changes (26). We investigated whether binding of mAbs to the FhuA H6-ferricrocin complex could alter the association between FhuA and TonB. Since the determinants that are recognized by each mAb have been mapped (35) to regions of the primary sequence of FhuA, data on antibody-induced alteration of the FhuA-TonB interaction would provide insight into regions of the receptor that are critical for the interaction.
Ligand Binding Promotes FhuA-TonB Interaction

Figu 3. Flu A-specific ligand after the interaction between FhuA and TonB. Equal amounts of purified FhuA.H6 protein were incubated with FhuA-specific ligands (fc, ferricrocin; CM, colicin M; 660, phase $\phi 80$; 75, phage TX1; m25, microcin 25), with anti-FhuA mAbs, and then with TLN-solubilized cell extract containing TonB, which in some cases was preincubated with peptide 1 corresponding to amino acids 1–18 or with peptide 2 corresponding to amino acids 636–651 of the mature FhuA sequence. Complexes were captured with Ni2+NTA-agarose, resolved by SDS-PAGE, and identified by immunoblotting with both anti-FhuA mAb FhuA.8.4 and anti-TonB mAb 4H4. Primary antibodies were detected by horseradish peroxidase-conjugated secondary antibodies and chemiluminescence. Molecular masses are as indicated (in kDa); the various additions for samples and corresponding controls are indicated in the grid beneath the blot. "quant" identifies the results from quantitation (performed in triplicate as described under "Experimental Procedures") as the percentage of TonB recovered after each treatment relative to the amount of TonB in lane 4. The results of quantitation of the amount of TonB in each lane (quant%) relative to the amount of TonB in lane 4 as described under "Experimental Procedures" are shown as means from three replicate experiments; standard deviations (std dev) are listed in the final row.

Incubation of mAb FhuA.3 with the FhuA.H6-ferricrocin complex prior to addition of TonB led to a substantial reduction in the amount of TonB that was subsequently eluted (Fig. 3, lane 5). Since the determinant of mAb FhuA.3 is located in a region between amino acids 1 and 20 of mature FhuA, this result suggests that sequences within this region are important for physical association between FhuA and TonB. The possibility that this inhibitory effect was due to nonspecific steric hindrance by the antibody (~160 kDa) to prevent TonB from interacting with FhuA was unlikely since the binding of mAbs FhuA.5.1 (~160 kDa) and FhuA.3 (M heavy chain isotype; ~800 kDa) to their determinants did not significantly reduce the association of TonB with FhuA (Fig. 3, lanes 6 and 7). Incubation of the surface-reactive mAb FhuA.8.1 with the FhuA.H6-ferricrocin complex modestly reduced the subsequent retention of TonB relative to that without mAb addition.

A synthetic pentapeptide corresponding to the TonB box of the E. coli ferric coprogen receptor FhuE was reported (43) to inhibit TonB-dependent receptor activity in growing cells. Could a peptide that includes the TonB box of FhuA inhibit physical association between FhuA and TonB in vitro? To address this question, a synthetic peptide (peptide 1) corresponding to amino acids 1–18 of mature FhuA was used to prevent TonB from interacting with FhuA, as was another peptide (peptide 2) corresponding to amino acids 636–651 of FhuA. Reduced the amount of TonB bound to the FhuA.H6-ferricrocin complex (Fig. 3, lanes 13 and 14). Our assay for in vitro interaction also demonstrated that TonB bound specifically to the hexahistidine-tagged FhuA derivative FhuA.A021–128.H6, as well as it did to FhuA.H6 (data not shown). Furthermore, ferricrocin and colicin M binding to FhuA.A021–128.H6 increased the amount of TonB, which was subsequently co-eluted with FhuA.A021–128.H6 from the Ni2+-NTA-agarose resin.

DISCUSSION

Our identification and characterization of a FhuA-TonB complex indicates that FepA is not the sole high affinity receptor with which TonB interacts, thus broadening the anecdotal observations (17) and increasing the likelihood that all TonB-dependent receptors directly contact TonB. The finding that the ferrichrome homologue ferricrocin enhanced the interaction between FhuA and TonB extends our earlier results of ligand-induced conformational changes in FhuA (26). Localization of the conformational changes to periplasmically exposed regions of FhuA led to our hypothesis that FhuA signals TonB via such a structural alteration, thereby indicating its requirement for energy to translocate bound ligand across the outer membrane. The ferricrocin-induced enhancement of FhuA-TonB coupling that was established by in vivo formaldehyde cross-linking experiments and by in vitro interaction assays supports the ligand-induced signaling hypothesis. An excess of FhuA over TonB, achieved by overexpression of FhuA from a multicopy plasmid or by application of milligram amounts of purified FhuA.H6 to the Ni2+-NTA-agarose resin, was sufficient to overcome the apparent ferricrocin-dependence of complex formation. This observation can be explained if a fraction of the receptors modulates their conformation independently of the presence of ferricrocin. However, in the more physiologically relevant expression of chromosomally encoded FhuA, even prolonged exposure to x-ray film of immunoblots with anti-FhuA mAbs did not reveal the FhuA-TonB complex in the absence of ferricrocin. If the complex were present without added ligand, it was beneath the detection limit of the enhanced chemiluminescence system. Formation of detectable amounts of the FhuA-TonB complex therefore appears to require ferricrocin under physiological circumstances, but is not absolutely dependent upon the presence of bound ligand when the receptor is overexpressed.

The prominent band of ~175 kDa migrated slightly faster than the previously characterized FepA-TonB complex of 195 kDa (17). That this latter complex was not evident on immunoblots probed with anti-TonB mAbs was probably due to the araB mutations in our strains, leading to a block in enterobactin synthesis. Recent results (2) indicate that enterobactin enhances complex formation between FepA and TonB. Thus, the earlier results of Skare et al. (17), taken together with those presented here, suggest that the FepA-TonB complex was visualized in ent+ strains due to synthesis of enterobactin intermediates, which, although undetectable by the chrome azurol S plate assay, were nonetheless able to cause the requisite conformational change in FepA that allowed cross-linking to TonB.

Particularly intriguing is the set of anti-FhuA mAb-reactive bands with relative mobilities lower than that of monomeric FhuA, species that were disclosed by immunoblotting of SDS-soluble cross-linked proteins. What is the identity of proteins with which TonB interacts, thus broadening the anecdotal observations? They did not contain TonB since they were present in a strain in which the chromosomal tonB gene was disrupted by insertion of a kanamycin resistance cassette. One or some of the bands with relative mobilities lower than that of monomeric FhuA may represent multimeric FhuA complexes. The enterobactin receptor FepA apparently forms trimers in vivo since nonadenurating lithium dodecyl sulfate-gel electrophoresis of outer membrane fractions containing FepA revealed an anti-FepA mAb-reactive band, the relative mobility of which was approximately 3-fold greater than that of the FepA monomer (44). Cross-linking in vivo with formaldehyde.

2 K. Postle, unpublished results.
followed by SDS-PAGE and immunoblotting with an anti-FepA mAb also revealed high molecular mass bands that were proposed to represent FepA dimers and trimers (17). In contrast, FhuA was identified to be monomeric on the basis of sedimentation equilibrium and velocity measurements (45). Since the purified FhuA that was used in those biophysical experiments retained biological activity, it was concluded that the functional state of FhuA was probably monomeric. However, these findings are also consistent with an alternate interpretation; FhuA may be organized as a trimer of independently functional monomers within the outer membrane. Our preliminary experiments indicate that FhuA trimers exist in samples of purified protein, whether or not subjected to formaldehyde cross-linking (data not shown). The multimeric organization of FhuA \textit{in vivo} and its significance in receptor and transport activity thus remain to be demonstrated. The identities of the molecules that associate with FhuA and that give rise to the higher molecular mass complexes (90–130 kDa) remain enigmatic, but they may include other cell envelope proteins that influence FhuA multimerization or FhuA activity.

That TonB-dependent receptors physically interact with TonB was originally inferred by recognizing that siderophore receptors, the vitamin B\textsubscript{12} receptor, and group B colicins all share a short stretch of amino acid homology near their N termini (Refs. 11, 46, and 47; for a recent inventory of 36 TonB-dependent receptors for which the sequences are known, see Refs. 48). This consensus amino acid sequence, termed the “TonB box,” is thought to interact with the TonB protein, thereby enabling ligand translocation across the outer membrane. Which sequences of FhuA and TonB are required for interaction between the two proteins? The TonB box is a likely candidate, although sequences outside of this region are also thought to play a role in the interaction between TonB and TonB-dependent receptors. Only mAb FhuA.8.3 greatly reduced the retention of TonB by the FhuA.H\textsubscript{6}-ferricrocin complex. Such inhibition was probably due to specific steric hindrance of the FhuA-TonB interaction at or near the determinant which is bound by FhuA.8.3. This finding underscores the value of mAbs as highly specific probes of FhuA in the investigation of protein-protein interactions. To identify N-terminal sequences of FhuA that are critical for association with TonB, we are currently attempting to delineate precisely the epitope which is bound by mAb FhuA.8.3. Lynase 5 of FhuA plays a role in the binding of FhuA.8.3 since trypsinolysis of purified FhuA.H\textsubscript{6} at this residue abolished mAb reactivity (26).

Loss-of-function mutations within the TonB box of FepA, BtuB, and FhuA have in all cases included the substitution by a turn-promoting residue, either Gly or Pro (11). Recent examination of one such mutation (I14P) involving the TonB box of the enterobactin receptor FepA revealed that such alterations result in the loss of specific \textit{in vivo} formaldehyde-mediated cross-linking to TonB. The mutation did not involve a formaldehyde-reactive residue, yet it altered the conformation of FepA at several distally located protease-sensitive sites (49). These observations, coupled with the finding that seemingly invariant residues within the consensus TonB box sequence could be mutated without significant loss of receptor functions (11, 18, 21, 50), lead to the suggestion that the TonB box is not directly involved in interactions with TonB, but affects the conformation of distal sites through which this interaction occurs (49).

That a synthetic peptide carrying the TonB box of FhuA did not reduce the physical association between FhuA and TonB \textit{in vitro} lends support to the role of TonB box conformation as opposed to primary sequence. Thorough characterization of FhuA peptides which may inhibit FhuA-TonB coupling is now possible using our \textit{in vitro} interaction assay. Such analyses should reveal the sequence specificity of the FhuA-TonB interaction if indeed it is uniquely dependent upon regions of FhuA primary sequence, as inferred from the results of Tuckman and Osborne (43).

Experiments \textit{in vitro} allowed us to reproduce a hallmark of the FhuA-TonB interaction \textit{in vivo}: that ferricrocin binding enhanced complex formation. The ability of colicin M and, to a lesser extent, phage \textit{ø80} also to promote the physical association between FhuA and TonB extends our \textit{in vitro} approach to other receptor-specific ligands. Furthermore, this observation supports the conclusion that independently of an energy source, physiologically relevant conformational changes occur for FhuA to signal its requirement for TonB energy coupling (26). Our findings therefore provide novel insights into the early steps of TonB-dependent transport activity.

The inability of phage T5 and of the protein antibiotic microcin 25 significantly to increase FhuA-TonB coupling may be explained if these FhuA-specific ligands induce conformational changes in the receptor that are different from those induced by ferricrocin and colicin M. Indeed, our preliminary results suggest that different subsets of anti-FhuA mAbs identify conformational changes induced by select ligands of FhuA (data not shown). Phage T5 binding to FhuA induced a change in FhuA conformation that resulted in ion conductance across lipid membranes (51) and both phage DNA transfer into and ferrichrome efflux from proteoliposomes (52, 53). However, one would not predict that this same conformational change would increase FhuA-TonB association since T5 is a TonB-independent ligand. Alternatively, the large size of the phage particles relative to the other ligands may in some manner preclude the enhancement of FhuA-TonB interaction as detected \textit{in vitro}. The identification and isolation of tail fiber proteins that constitute the minimal requirement for phage to participate in receptor recognition (54, 55) will assist in the analysis of TonB coupling with TonB-dependent receptors. Future studies need to focus upon the ligand-induced conformational dynamics of FhuA which govern its physical association with TonB and upon those regions of FhuA which are required for the interaction with TonB. Accordingly, our finding that the hexahistidine-tagged FhuA derivative FhuA\textsubscript{Δ21–128.H\textsubscript{6}} participated in FhuA-TonB interactions as well as did FhuA.H\textsubscript{6} lays groundwork for our continuing investigations into the nature of the FhuA-TonB association.

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