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In vivo evidence for FhuA outer membrane receptor interaction with the TonB inner membrane protein of *Escherichia coli*

Karolin Günter and Volkmar Braun

Mikrobiologie II, Universität Tübingen, Auf der Morgenstelle 28, D-7400 Tübingen, FRG

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FhuA outer membrane receptor activity of *Escherichia coli* K-12 depends on the TonB inner membrane protein. The naturally occurring degradation of the TonB protein could be prevented by the FhuA receptor protein. Mutated TonB proteins could only be stabilized by mutated FhuA proteins when they functionally interacted in the uptake of ferrichrome across the outer membrane.

Escherichia coli; FhuA-TonB interaction

1. INTRODUCTION

Uptake of Fe^{3+} as ferrichrome complex and killing by colicin M requires the highly specific FhuA receptor protein in the outer membrane. After binding colicin M and ferrichrome have to be taken up into the periplasmic space and subsequently into (colicin M) or across the cytoplasmic membrane (ferrichrome). The vectorial release from the receptor across the outer membrane requires the TonB protein which is anchored in the cytoplasmic membrane but exposed to the periplasmic space [1,2]. Ferrichrome and colicin M bind to *tonB* mutants but remain on the cell surface receptors, suggesting a deficiency in the uptake across the outer membrane [2,3]. In addition, transport across the outer membrane requires energy although no energy-generating system nor energy-rich metabolites are known to reside outside the cytoplasmic membrane, leading to the concept that the TonB protein responds to the energized state of the cytoplasmic membrane, assuming an energized and an unenergized conformation, of which the energized state induces the release of the ligands at the FhuA receptor [4]. Induction of the release conformation in the FhuA receptor implies a physical interaction with the TonB protein. Indeed, point mutations in the *tonB* gene can suppress point mutations in the *fhuA* gene in that the *fhuA* mutants have regained the ability to grow on ferrichrome as sole iron source, they become sensitive to the structurally related antibiotic albomycin, and to colicin M. Depending on the *fhuA* and *tonB* mutations, restoration of FhuA activity occurs to a different extent [5]. Apparently, the altered FhuA receptor, which no longer binds to the wild-type TonB protein, interacts again with the altered TonB protein.

Correspondence address: V. Braun, Mikrobiologie II, Auf der Morgenstelle 28, D-7400 Tübingen, FRG

In this paper, we provide for the first time in vivo evidence for the direct binding of the FhuA receptor to the TonB protein. We took advantage of the physical instability of the TonB protein [1], and examined whether the naturally occurring degradation of TonB can be prevented, or delayed, by interaction with the FhuA protein. We observed that proteolytic breakdown of wild-type TonB was delayed by wild-type FhuA, and that the TonB derivatives were not, or less stabilized by FhuA, except in cases where interaction with the mutated FhuA derivatives was restored by TonB derivatives.

2. EXPERIMENTAL

2.1. *Bacterial strains and plasmids*

The activity of plasmid-encoded *fhuA* and *tonB* gene products and their mutated derivatives were studied in *E. coli* HS244 which carries a chromosomal *fhuA* mutation with little polar effect on the transcription of the downstream *fhuCDB* ferrichrome transport genes [5]. The strain is also mutated in the *tonB* and *aroB* gene. The latter mutation abolishes synthesis of enterochelin so that the iron supply depends on added ferrichrome. Growth was determined on nutrient broth agar plates supplemented with 2 μM ferrichrome, in which the available iron was limited by the addition of 0.2 mM 2,2'-dipyridyl (NBDF medium).

Plasmid pGC8 carrying a *tonB* point mutation which results in a glutamine to leucine replacement at residue 160 of the TonB polypeptide (Q160L), and plasmid pCG15 (Q160K) were previously described [2,6]. The newly isolated plasmid pCG128 (R158L) was obtained by selecting for *tonB* mutations which suppressed the *fhuA* mutation causing a FhuA 19P replacement [5].

2.2. *Recombinant DNA techniques*

The insert DNA of plasmids carrying *tonB* genes with point mutations was excised with *Bgl*II/*Hin*dII and cloned into phage M13mp19 for sequencing. Plasmid pCG128 carried the point mutation CGT/CTT. To identify the TonB proteins, the *tonB* genes were excised with *Eco*RI/*Pst*I and cloned downstream of the phage T7 gene 10 promoter of plasmid pT7-5 [8]. The *Cla*I/*Pst*I fragments of plasmid pHSC212 carrying the wild-type *fhuA* gene, or the *fhuA* derivatives *fhuA* (19P), *fhuA* (V11A) and *fhuA* (V11D) [5], were inserted into the

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Table I

Growth on ferrichrome and stabilization of TonB protein

	FhuA		FhuA(I9P)		FhuA(V11D)		FhuA(V11A)	
	Wild-type							
	Fer	Stab	Fer	Stab	Fer	Stab	Fer	Stab
TonB wild-type	+ + +	+ +	−	+	−	−	(+)	−
TonB (Q160L)	+ + +	+ +	−	−	−	−	(+)	−
TonB (Q160K)	+ + +	+	+ + +	+	−	−	(+)	−
TonB (R158L)	+ + +	+ +	+	+ +	−	−	(+)	−

+ + + strong, + weak, (+) barely detectable growth promotion by ferrichrome (Fer); and + + strong, + weaker, and − no TonB stabilization (Stab).

PstI/ClaI site of the *tonB* plasmids (Fig. 1). The 16 combinations obtained between wild-type *tonB*, wild-type *fhuA*, and their derivatives are listed in Table I.

3. RESULTS AND DISCUSSION

The FhuA (I9P) mutant expressing wild-type TonB was unable to grow on NBDF medium (Table I), as has been previously found [5]. Surprisingly, independently isolated *tonB* mutations which could suppress mutations in the TonB box of the *fhuA* gene (and of the *btuB* gene encoding the receptor for vitamin B_{12} transport) were all in the same triplet [2,5,6]. We describe here a new *tonB* mutation which is only two codons further upstream of the former. This *tonB* suppressor mutation in contrast to the other *tonB* suppressor mutations only suppressed the *fhuA* (I9P) mutation (Table I) and none of the other *fhuA* TonB box mutations, nor the *btuB415* mutation [6]. Suppression of FhuA (I9P) was weak. Cells could grow on ferrichrome as a sole iron source but slower than *fhuA* wild-type cells. They were sensitive to albomycin but a 60-fold higher concentration than in the case of the *fhuA* wild-type had to be used. TonB (R158L) conferred a 100-fold higher colicin M sensitivity to FhuA (I9P) cells than the TonB wild-type protein, which, however, was still 10-fold lower than the FhuA/TonB wild-type combination.

Growth of strain HS244 carrying on the same plasmid (Fig. 1) the three *tonB* gene mutations in all possible combinations with 3 previously isolated *fhuA* TonB box mutations [5] is summarized in Table I. The results agree with the previous findings except for the FhuA (V11A) data where we find now very weak growth in contrast to the former results where growth was better. As will be shown below this FhuA derivative is only weakly exported into the outer membrane, and probably is for this reason also unstable and displays low activity.

The *tonB* gene had to be overexpressed in order to identify the TonB protein. Therefore, the *tonB* gene and the *tonB* suppressor derivatives were cloned downstream the gene 10 promoter of phage T7 on multicopy plasmids together with either the wild-type *fhuA* gene, or the suppressible *fhuA* TonB box mutants. This system allows exclusive expression of the cloned genes when the *E. coli* RNA polymerase is inhibited by rifamycin. The amount and the stability of the plasmid-encoded TonB and FhuA proteins were studied in strain WM1576 which carried a temperature-inducible phage T7 RNA polymerase gene on plasmid pGP1-2 [8]. Cells containing the *fhuA/tonB* gene combinations listed in Table I, were pulse-labeled with [^{35}S]methionine, and then chased with a 500-fold surplus of nonradioactive methionine for 0, 15, 30, and 60 min, as has been previously outlined in detail [2]. The proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and determined by fluorography. The wild-type TonB protein was not proteolytically degraded when wild-type FhuA was expressed by the same plasmid (Fig. 2A, lanes 1–4). Wild-type TonB protein decreased during the chase period when the mutated FhuA (I9P) and FhuA (V11D) were coexpressed (Fig. 2A, lanes 5–8, and 9–12), which in combination with wild-type TonB were inactive in ferrichrome uptake. The FhuA (V11A) protein was only weakly exported (mainly the precursor form above the mature form is seen) and unstable, and the amount of the TonB protein also declined rapidly during the chase period (Fig. 2A, lanes 13–16). These data clearly indicate prevention of wild-type TonB protein degradation by wild-type FhuA, and less or no stabilization by FhuA derivatives which show weak or no activity in combination with wild-type TonB.

These experiments were repeated with the TonB derivative (Q160K). Although the interaction with wild-type FhuA supported normal growth on ferrichrome, the 60 min chase value of TonB (Q160K) was slightly reduced (Fig. 2B, lanes 1–4). Similar growth of strains carrying FhuA or FhuA (I9P) in combination with TonB (Q160K) (Table I) correlated with a similar degree of stabilization (Fig. 2B, lanes 4 and 8). No, or very weak prevention of TonB degradation was observed by FhuA (V11D) (Fig. 2B, lanes 9–12) which was also deficient in ferrichrome uptake (Table I). A few combinations of the suppressor mutants grew well on ferrichrome but TonB stabilization was less pronounced (Table I). For the assessment of these data one has to take into account that none of the suppressor combinations restored fully wild-type activity. For example, FhuA (I9P) combined with TonB (Q160K) supported growth on ferrichrome nearly as well as the wild-type

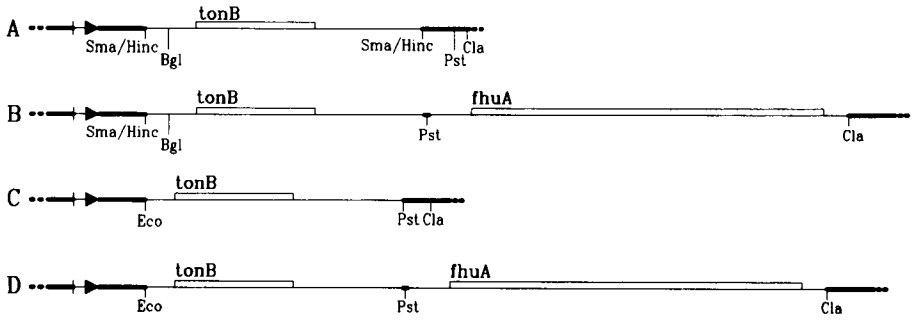

Fig. 1. Physical map of *tonB* wild-type gene (A) and of *tonB* point mutants (C) cloned into plasmid pT7-5 from phage M13mp19. The *fhuA* wild-type gene and the *fhuA* point mutants were excised from pHSC212 [5] and cloned into the pT7-5 *tonB* derivatives cleaved with *PstI-ClaI* (B,D). Vector DNA is marked by a heavy line.

FhuA/TonB combination, but sensitivity to albomycin, the structural analog of ferrichrome, was about 100-fold less. In addition, the stabilization assay is rather crude. Therefore, the partial stabilizations largely agree with the functional activities and support the major conclusion that FhuA stabilizes TonB, and that this stabilization is related to a functional interaction. The wild-type FhuA/TonB combination displays strong stabilization, FhuA (V11A), which itself is unstable, stabilizes all the TonB proteins only weakly (Fig. 2A, lanes 13–16 and 2B, 17–20), and FhuA (V11D) exhibits no interaction and no stabilization despite normal amounts of FhuA synthesized.

Irreversible adsorption of phage T7, accompanied by release of the DNA from the phage head, also requires FhuA/TonB interaction and cellular energy [4,9].

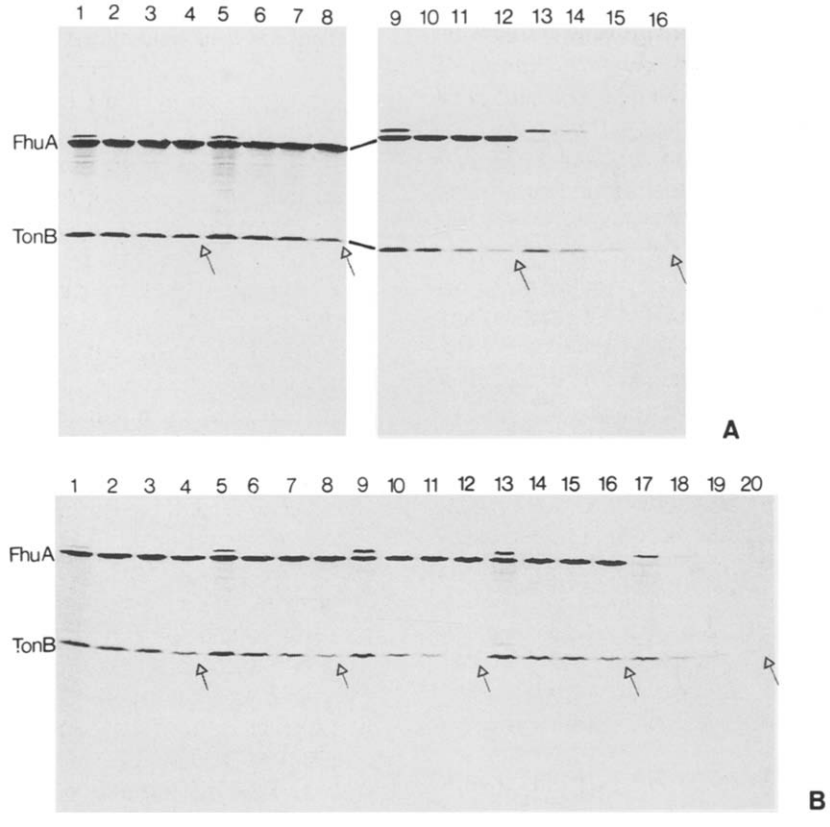

Fig. 2. Different TonB stabilities combined with various FhuA proteins. *E. coli* WM1567, transformed with pGP1-2 and plasmids containing the *tonB fhuA* genes cloned on pT7-5, was labeled for 5 min with 740 kBq with [^{35}S]methionine and then chased for 5, 15, 30, and 60 min with 6.3 mg of methionine (lanes 1–4, 5–8, 9–12, 13–16). Panel A demonstrates the stability of the TonB wild-type protein in combination with FhuA wild-type protein (lanes 1–4), with FhuA (I9P) (lanes 5–8), FhuA (V11D) (lanes 9–12), and FhuA (V11A) (lanes 13–16). Panel B shows the stability of TonB (Q160K) in combination with wild-type FhuA (lanes 1–4), FhuA (I9P) (lanes 5–8), FhuA (V11D) in the absence of ferrichrome (lanes 9–12), and in the presence of 0.2 mM ferrichrome (lanes 13–16), and with FhuA (V11A) (lanes 17–20). The arrows point to the 60 min chase values of the TonB protein.

Previously, we have shown that infection of cells by phage T1 was prevented by ferrichrome when the presumed TonB–FhuA interaction was weak. An exception were cells expressing FhuA (V11D) and TonB wild-type. They were T1 resistant and became sensitive upon addition of ferrichrome [5]. These data led us to propose that binding of ferrichrome to the FhuA (V11D) protein induced a conformation which improved coupling with the TonB protein. Therefore, stabilization of TonB by FhuA (V11D) in the presence of ferrichrome was determined. Indeed, a much lower degradation of TonB protein was observed in the presence of 0.2 mM ferrichrome (Fig. 2B, compare lanes 9–12 with 13–16).

The low amount of chromosomally encoded FhuA protein does not stabilize overexpressed plasmid-encoded TonB protein [1,2]. FhuA and its derivatives had to be synthesized in amounts similar to the TonB proteins, which indicates a direct, stoichiometric rather than catalytic, interaction between the two proteins. We take these results as an indication that the FhuA receptor protein and the TonB protein physically interact for transport of ferrichrome, albomycin and colicin M across the outer membrane, and for irreversible (productive) adsorption of phage T1 (and phage Φ80, not shown). These data further support the concept that the TonB protein in the cytoplasmic membrane induces a transport active conformation of the FhuA receptor protein (and other TonB dependent receptors and colicins) and serves as coupling factor between the cytoplasmic membrane and the outer membrane. The TonB box of the receptors and colicins, and the R158–Q160 region of the TonB protein define regions of interactions.

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