JOURNAL OF BACTERIOLOGY, Feb. 1997, p. 1400–1403 Vol. 179, No. 4
0021-9193/97/$04.00+0

NOTES

Evidence that KpsT, the ATP-Binding Component of an ATP-Binding Cassette Transporter, Is Exposed to the Periplasm and Associates with Polymer during Translocation of the Polysialic Acid Capsule of *Escherichia coli* K1

JOSEPH M. BLISS AND RICHARD P. SILVER*

Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, New York 14642

Received 26 July 1996/Accepted 24 November 1996

KpsT utilizes ATP to effect translocation of the polysialic acid capsule of *Escherichia coli* K1. We have previously proposed a mechanistic model for the action of this protein. Here, we provide evidence to support two predictions of the model: that KpsT associates with polymer and that KpsT is accessible from the periplasmic surface of the inner membrane.

The polysialic acid (polySia) capsule of *Escherichia coli* K1 is recognized as a key virulence determinant in invasive infections (15). Capsule expression is mediated by the 14 gene products of the 17-kb *kps* cluster (4, 19, 23). This cluster is divided into three functional regions with the central region 2 responsible for synthesis, activation, and polymerization of the capsular polymer and the flanking regions 1 and 3 involved with translocation of the polymer from its cytoplasmic site of synthesis to the cell surface (4, 19, 23). We believe translocation to be mediated by a multicomponent complex of proteins, effecting translocation in a single step at sites of membrane fusion (for a review, see reference 4). The details of this translocation process have been the focus of recent study in our laboratory.

Translocation of polySia at the cytoplasmic membrane is mediated by an ATP-binding cassette (ABC) transporter, KpsMT, encoded by region 3 (16, 17, 21). ABC transporters have been described in organisms ranging in diversity from bacteria to humans and are involved in transport of a varied array of molecules in both directions across cell membranes (2, 9). Despite their widely varied functional roles, members of this superfamily share a common structural organization and are believed to function through similar mechanisms to transport their substrates (2, 9). ABC transporters are comprised of an integral inner membrane protein (the membrane component, e.g., KpsM) and a hydrophilic protein that binds ATP (the ATP-binding component, e.g., KpsT). The membrane components commonly have six membrane-spanning segments and are believed to form a dimer in the membrane to provide a porelike structure for substrate transport. Although strongly conserved in structure, the membrane components bear little sequence homology throughout the superfamily. In contrast, the ATP-binding components are well conserved, with the highest homology in the nucleotide binding domain. This domain contains Walker motifs A and B (24) characteristic of proteins that bind ATP. Several ATP-binding components have now been shown to bind ATP, and ATP hydrolysis is known to provide energization for transport (for reviews, see references 1 and 8).

ATP-binding components of ABC transporters have traditionally been considered peripheral inner membrane proteins, localized to the cytoplasmic surface. More recently, however, the accessibility of the ATP-binding component of both the histidine and maltose permeases, HisP and MalK, respectively, from the periplasmic surface has been reported (3, 20). Although not an ABC transporter, SecA, the ATP-binding protein of the protein secretion system, also exhibits this property (13). These proteins are all thought to undergo ATP-dependent conformational changes that result in insertion into the membrane at sites defined by the membrane components of each system (3, 6). Consistent with this view, we have proposed a model for the mechanism of action of KpsT that involves association with polySia, an ATP-binding-induced conformational change, insertion into the membrane through interaction with KpsM, and subsequent ATP hydrolysis that returns KpsT to its original conformation and releases polymer to the extracytoplasmic compartment (4). This model was developed on the basis of our characterization of a dominant negative *kpsT* mutation that disrupts polymer translocation in an otherwise wild-type K1 strain, EV36 (5).

The dominant negative mutation that we characterized is a glutamic acid-to-glycine substitution at position 150 of KpsT (E150G). This residue is adjacent to Walker motif B and therefore is likely to be part of the protein's nucleotide binding domain, but the E150G protein is not deficient in ATP binding. We predict, therefore, that the defect in E150G is in ATP hydrolysis (5). Furthermore, we showed that certain additional mutations in *kpsT* could provide intragenic suppression of the dominant negative phenotype of the protein (5). These suppressors included mutations that disrupt ATP binding, alter the "helical domain" believed to be involved in interaction with KpsM, or alter the "linker peptide" believed to function in signaling between the ATP-binding domain and the helical domain. According to our model, the E150G protein has a dominant negative effect because it binds ATP and polymer

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Rochester Medical Center, 601 Elmwood Ave., Box 672, Rochester, New York 14642. Phone: (716) 275-0680. Fax: (716) 473-9573. E-mail: silver@medinfo.rochester.edu.

and inserts into the membrane but is unable to hydrolyze the bound ATP to return to its original conformation, resulting in a "locked" translocation complex. All translocation sites become occupied by these complexes so that the wild-type protein no longer has access to translocation sites, and the cells thus become unencapsulated. The suppressors exert their effect by interfering with steps necessary to progress to this locked complex, i.e., ATP binding, ATP-induced intramolecular signaling, or interaction with KpsM. Consistent with this general model are recent studies of SecA that have more fully elucidated the role of ATP binding and hydrolysis in promoting insertion of SecA into the membrane and deinsertion. A mutation in D209 (analogous to D149 in motif B of KpsT) preserves ATP binding by SecA, albeit at a depressed level, but eliminates the protein's translocation ATPase activity (14). This protein, defective in protein translocation, was shown to be stably inserted into the membrane but defective in deinsertion (7). These findings substantiate the notion that mutations in this conserved region of ATP binding proteins may affect ATP hydrolysis while preserving binding and, further, that ATP hydrolysis in KpsT may be essential for deinsertion.

We have conducted experiments to test two predictions based on this model. As described above, HisP, MalK, and SecA have all been shown to exhibit some accessibility from the periplasm as each shows sensitivity to protease digestion from the periplasmic surface. In similar experiments we have been unable to demonstrate such sensitivity for KpsT. Since the E150G protein may be stably membrane inserted due to an inability to hydrolyze ATP, while the wild type may cyclically insert and deinsert, we hypothesized that the E150G mutation may provide a tool by which susceptibility to digestion from the periplasmic space could be demonstrated. The second prediction from our model is that KpsT associates with polySia during the translocation process. This notion is based in part on the finding that wild-type cells overexpressing KpsT, despite being fully encapsulated, accumulate polymer in the cytoplasm (5). The observation that the genes encoding KpsM and KpsT overlap (16) suggests that these proteins are normally present in similar amounts to form transport complexes. Since increasing the amount of KpsT results in polymer accumulation, such accumulation may result from an interaction between these molecules as they await access to KpsM and translocation. In this communication we describe experiments that support both of these predictions.

Protease accessibility of E150G from the periplasmic surface. To assess their exposure to the periplasmic surface, the accessibility of wild-type KpsT and E150G KpsT to proteolysis in right-side-out membrane vesicles (ROV) was investigated. ROV are prepared by lysis of spheroplasts in large volumes of hypotonic buffer. Under these conditions they lose their cytoplasmic components and reseal, maintaining their original orientation (10). ROV were prepared according to the method of Prossnitz et al. (18) from EV36 expressing wild-type KpsT (pSR340) or E150G KpsT (pSR577). Inside-out membrane vesicles (IOV) from the same strains were prepared by lysis in a French press as described previously (12). Oriented membrane vesicles were treated with trypsin essentially as described previously (11). ROV were treated with 5 mg of trypsin per ml for 0, 2.5, 5, and 10 h, after which time the proteins were precipitated and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and KpsT was visualized by immunoblotting (Fig. 1A). After an initial drop in the KpsT signal intensity in both strains, extended incubation resulted in additional degradation of the E150G protein in EV36 vesicles (Fig. 1A, lanes 6 to 8) whereas the wild-type protein remained stable (Fig. 1A, lanes 2 to 4). The initial

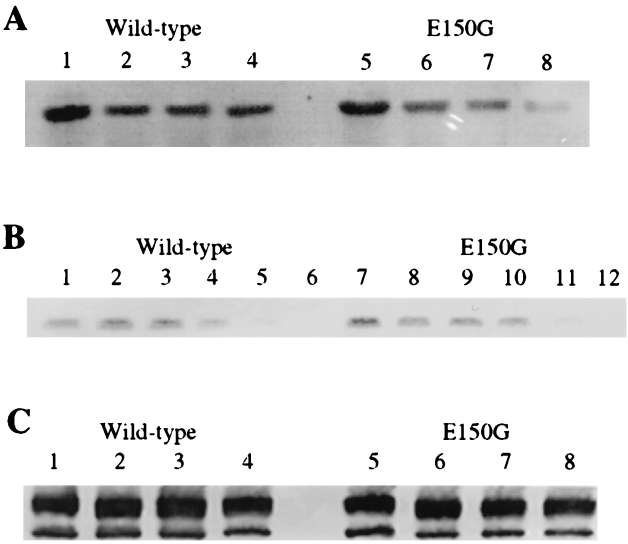

FIG. 1. Trypsinization of oriented membrane vesicles. (A) Immunoblot of KpsT following treatment of ROV prepared from EV36/pSR340 (lanes 1 to 4) or EV36/pSR577 (lanes 5 to 8) with 5 mg of trypsin per ml for 0 (lanes 1 and 5), 2.5 (lanes 2 and 6), 5 (lanes 3 and 7), and 10 h (lanes 4 and 8). (B) Immunoblot of KpsT following a 2-min incubation of IOV prepared from EV36/pSR340 (lanes 1 to 6) or EV36/pSR577 (lanes 7 to 12) with trypsin at the following concentrations: 0 (lanes 1 and 7), 0.01 (lanes 2 and 8), 0.1 (lanes 3 and 9), 1 (lanes 4 and 10), 10 (lanes 5 and 11), and 100 μg/ml (lanes 6 and 12). (C) Immunoblot of F_1 protein following treatment of ROV prepared from EV36/pSR340 (lanes 1 to 4) or EV36/pSR577 (lanes 5 to 8) with 5 mg of trypsin per ml for 0 (lanes 1 and 5), 2.5 (lanes 2 and 6), 5 (lanes 3 and 7), and 10 h (lanes 4 and 8).

decrease in signal was consistently observed regardless of trypsin concentration, length of incubation, or vesicle preparation used and is believed to represent a population of KpsT that is improperly localized during the preparative procedure. The increased sensitivity to trypsinization of E150G in EV36 vesicles compared with the sensitivity of wild-type KpsT suggests that the mutant protein has greater accessibility from the periplasmic space than the wild-type protein, as predicted by our model.

A possible alternative explanation for the increased trypsin sensitivity of E150G over that of the wild type is that the two proteins are equally exposed but that the mutant protein is inherently more sensitive to the protease and therefore preferentially degraded. To confirm that the difference in trypsin sensitivity was due to a difference in accessibility to the protease in ROV and not a difference in sensitivity, the wild-type and E150G proteins were trypsinized in IOV (Fig. 1B). Both proteins were exquisitely sensitive, being undetectable after a 2-min incubation in the presence of trypsin at a concentration of 100 μg/ml (Fig. 1B, lanes 6 and 12). This concentration was 50-fold less than that employed in the ROV incubations. By reducing the trypsin concentration further in serial 1:10 dilutions and repeating the 2-min incubations, the two proteins were shown to have an equivalent sensitivity to degradation, arguing against an inherent difference in sensitivity between E150G and wild-type proteins (Fig. 1B, compare lanes 1 to 6 and 7 to 12).

An important control in protease experiments involving ROV is to show that vesicle integrity was not compromised by trypsinization. The finding that wild-type and E150G KpsT proteins exhibit a difference in sensitivity to trypsin in ROV despite showing marked and equal sensitivity in IOV is itself a control that suggests that the protease did not gain access to the vesicle interior. As an additional control, the F_1 subunit of

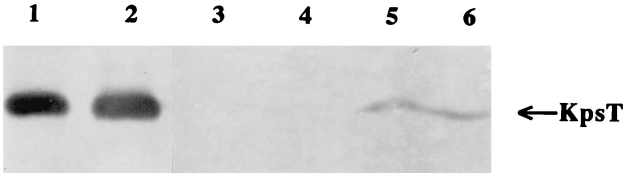

FIG. 2. Anti-KpsT immunoblot of cell lysates and polySia immunoprecipitates. Lane 1, HB101/pSR340 cell lysate; lane 2, EV36/pSR340 cell lysate; lanes 3 and 4, H.46 immunoprecipitate from HB101/pSR340 lysate plus added polySia before (lane 3) and after (lane 4) boiling in the presence of β-ME; lanes 5 and 6, H.46 immunoprecipitate from EV36/pSR340 lysate before (lane 5) and after (lane 6) boiling in the presence of β-ME.

the H^+ ATPase, a known cytoplasmically localized protein, was also visualized by immunoblotting over the same time course of trypsin exposure. This protein remained stable throughout the incubation, indicating that vesicle integrity was not compromised (Fig. 1C).

Association between KpsT and polySia. To assess a potential interaction between KpsT and polySia, lysates prepared from EV36 harboring pSR340, previously shown to accumulate intracellular polySia (5), were immunoprecipitated with horse antiserum directed against polySia (H.46). Cells for immunoprecipitation experiments were grown in 50-ml L-broth cultures to logarithmic phase and harvested by centrifugation. Cells were resuspended in 1 ml of 50 mM NaP_i (pH 8.0) containing 1 mg of lysozyme per ml and incubated on ice for 30 min. Cells were then lysed by brief sonication on ice. Following centrifugation for 15 min at 10,000 × *g* to remove debris, 0.9 ml of lysate was extracted with 0.1 ml of 20% Triton X-100 (per volume, in 50 mM NaP_i [pH 8.0]) for 20 min at 37°C. The detergent extracts were centrifuged at 28,000 × *g* for 50 min. To immunoprecipitate polySia, 0.5 ml of the resulting supernatant was mixed with 0.5 ml of H.46 immune globulin. A clearly visible flocculent precipitate formed after 30 min on ice. Samples were microcentrifuged at 1,000 rpm in an Eppendorf 5415C centrifuge for 5 min to pellet the precipitate, and precipitates were washed three times in 1 ml of ice-cold 50 mM NaP_i (pH 8.0). Immunoprecipitates were resuspended in 100 μl of Laemmli SDS-PAGE loading buffer without β-mercaptoethanol (β-ME). Portions (40 μl) of each sample were loaded twice on a 12% polyacrylamide SDS-PAGE gel. One of the two samples was combined with β-ME at a final concentration of 2% (vol/vol) and placed in a boiling water bath for 10 min prior to loading. The presence or absence of KpsT in the precipitates was determined by immunoblotting with KpsT-specific antiserum. Lysates prepared in the same way from an unencapsulated *E. coli* K-12 strain, HB101, harboring pSR340 were used as a control and produced no visible precipitate after incubation with H.46 globulin. To form an immunoprecipitate from these lysates for control purposes, 75 μl of purified polySia (1 mg/ml in 50 mM NaP_i [pH 8.0]) was added to HB101 lysates prior to immunoprecipitation with H.46 globulin. KpsT was found to coprecipitate with polySia in EV36/pSR340 lysates but not in HB101/pSR340 lysates with exogenous polySia added (Fig. 2 [compare lanes 5 and 6 with lanes 3 and 4]). The HB101/pSR340 lysate, however, did contain amounts of KpsT protein similar to those of the EV36/pSR340 lysate (Fig. 2 [compare lanes 1 and 2]) and produced a comparable amount of immunoprecipitate from the added polySia (data not shown). This finding not only demonstrates that KpsT was not simply entrained by the immune complexes in EV36/pSR340 but also argues that KpsT lacks an inherent affinity for polySia and must be presented with it in the context of the capsule synthesis/expression machinery for the association to be detected. In an effort to detect possible linkages between polySia and KpsT, the precipitated samples were run both in the absence of β-ME, without boiling (Fig. 2, lane 5), and with boiling in the presence of β-ME (Fig. 2, lane 6). No differences in the migration of coprecipitated KpsT were detected under these conditions. This finding implies that the linkage connecting KpsT with polySia is either indirect, e.g., involving another protein, or labile in nature such that reducing agents are not required for dissociation.

To determine if any other *kps* components were important for the coprecipitation of KpsT with polySia, pSR340 was transformed into a variety of chromosomal *kps* mutant strains including chromosomal deletions in *kpsM*, *kpsD*, and *kpsE*, as well as an insertion mutation in *kpsC* (4, 19, 23). Extracts from each of these strains were immunoprecipitated and analyzed as described above. KpsT was detected in the precipitates of all of these strains (data not shown). Likewise, to determine if any available *kpsT* mutations would alter the immunoprecipitation, plasmids encoding the following *kpsT* mutations were transformed into EV36: K44E, G84D, S126F, E150G, G155R, C163Y, and H181Y (17). Immunoprecipitates from each were analyzed as described above. Again, each of these mutated KpsT proteins was detected in the immunoprecipitate (data not shown), arguing that none of these residues is crucial for the interaction with polySia.

An interaction between polySia and protein was first demonstrated by Troy and McCloskey (22). Additionally, Weisgerber and Troy (25) described experiments in which immunoprecipitated polySia made from labeled K1 membranes was found to be associated with a protein of 20 kDa. This protein underwent a marked shift in mobility after boiling, a treatment that releases bound polymer (25). Using the same antiserum, we have shown that KpsT, a 25-kDa protein, coprecipitates with polySia but only in a K1 background. In contrast to the results of Weisgerber and Troy (25), however, there was no significant difference in the mobility of immunoprecipitated KpsT before and after boiling. In their experiments, Weisgerber and Troy used purified membranes containing radiolabeled proteins for the in vitro synthesis of polySia, followed by immunoprecipitation. Our experiments utilized polySia synthesized in vivo as a source for immunoprecipitation. The lack of a difference in mobility before and after boiling in our experiments may reflect a technical difference in the detection limits of immunoblotting versus autoradiography or inefficient transfer of the polySia-linked KpsT to the filter in the immunoblotting procedure. Alternatively, our results may indicate that the true nature of the linkage between KpsT and polySia is a labile one that is easily disrupted in the ionic environment of Laemmli buffer and that a more stabile linkage is obtained in vitro. One further possibility is that the protein detected by the experiments of Weisgerber and Troy is a protein distinct from KpsT, perhaps involved in the polymerization reaction. In any event, our results are consistent with a mechanism in which KpsT associates with the transported substrate and inserts into the membrane to initiate translocation of polymer.

We are grateful to Alan Senior for providing F_1 antiserum, Willie Vann for providing purified polySia and H.46 immune globulin, and Ronald Pigeon for critically reviewing the manuscript.

This work was supported by grant AI39615 from the National Institutes of Health. J.B. is a student in the Medical Scientist Training Program funded by NIH grant 2T32GM07356 and was also supported by an Interdepartmental Training Grant in Genetics and Regulation (5T32GM07102) from the Public Health Service.

REFERENCES

1. **Ames, G. F.-L., and A. K. Joshi.** 1990. Energy coupling in bacterial periplasmic permeases. J. Bacteriol. **172:**4133–4137.
2. **Ames, G. F.-L., C. S. Mimura, S. R. Holbrook, and V. Shyamala.** 1992. Traffic ATPases: a superfamily of transport proteins operating from *Escherichia coli* to humans. Adv. Enzymol. **65:**1–47.
3. **Baichwal, V., D. Liu, and G. F.-L. Ames.** 1993. The ATP-binding component of a prokaryotic traffic ATPase is exposed to the periplasmic (external) surface. Proc. Natl. Acad. Sci. USA **90:**620–624.
4. **Bliss, J. M., and R. P. Silver.** 1996. Coating the surface: a model for expression of capsular polysialic acid in *Escherichia coli* K1. Mol. Microbiol. **21:** 221–231.
5. **Bliss, J. M., C. F. Garon, and R. P. Silver.** 1996. Polysialic acid export in *Escherichia coli* K1: the role of KpsT, the ATP-binding component of an ABC transporter, in chain translocation. Glycobiology **6:**445–452.
6. **Economou, A., and W. Wickner.** 1994. SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion. Cell **78:**835–843.
7. **Economou, A., J. A. Pogliano, J. Beckwith, D. B. Oliver, and W. Wickner.** 1995. SecA membrane cycling at SecYEG is driven by distinct ATP binding and hydrolysis events and is regulated by SecD and SecF. Cell **83:**1171–1181.
8. **Higgins, C. F.** 1990. The role of ATP in binding-protein-dependent transport systems. Res. Microbiol. **141:**353–360.
9. **Higgins, C. F.** 1992. ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. **8:**67–113.
10. **Kaback, H. R.** 1971. Bacterial membranes. Methods Enzymol. **22:**99–120.
11. **Kerppola, R. E., and G. F.-L. Ames.** 1992. Topology of the hydrophobic membrane-bound components of the histidine periplasmic permease: comparison with other members of the family. J. Biol. Chem. **267:**2329–2336.
12. **Kerppola, R. E., V. K. Shyamala, P. Klebba, and G. F.-L. Ames.** 1991. The membrane-bound proteins of periplasmic permeases form a complex: identification of the histidine permease HisQMP complex. J. Biol. Chem. **266:** 9857–9865.
13. **Kim, Y. J., T. Rajapandi, and D. Oliver.** 1994. SecA protein is exposed to the periplasmic surface of the *E. coli* inner membrane in its active state. Cell **78:**845–853.
14. **Mitchell, C., and D. Oliver.** 1993. Two distinct ATP-binding domains are needed to promote protein export by *Escherichia coli* SecA ATPase. Mol. Microbiol. **10:**483–497.
15. **Moxon, E. R., and J. S. Kroll.** 1990. The role of bacterial polysaccharide capsules as virulence factors. Curr. Top. Microbiol. Immunol. **150:**65–85.
16. **Pavelka, M. S., Jr., L. F. Wright, and R. P. Silver.** 1991. Identification of two genes, *kpsM* and *kpsT*, in region 3 of the polysialic acid gene cluster of *Escherichia coli* K1. J. Bacteriol. **173:**4603–4610.
17. **Pavelka, M. S., Jr., S. F. Hayes, and R. P. Silver.** 1994. Characterization of KpsT, the ATP-binding component of the ABC-transporter involved with the export of capsular polysialic acid in *Escherichia coli* K1. J. Biol. Chem. **269:**20149–20158.
18. **Prossnitz, E., A. Gee, and G. F.-L. Ames.** 1989. Reconstitution of the histidine periplasmic transport system in membrane vesicles: energy coupling and interaction between the binding protein and the membrane complex. J. Biol. Chem. **264:**5006–5014.
19. **Roberts, I. S.** 1995. Bacterial polysaccharides in sickness and in health. Microbiology **141:**2023–2031.
20. **Schneider, E., S. Hunke, and S. Tebbe.** 1995. The MalK protein of the ATP-binding cassette transporter for maltose of *Escherichia coli* is accessible to protease digestion from the periplasmic side of the membrane. J. Bacteriol. **177:**5364–5367.
21. **Smith, A. N., G. J. Boulnois, and I. S. Roberts.** 1990. Molecular analysis of the *Escherichia coli* K5 *kps* locus: identification and characterization of an inner-membrane capsular polysaccharide transport system. Mol. Microbiol. **4:**1863–1869.
22. **Troy, F. A., and M. A. McCloskey.** 1979. Role of a membranous sialyltransferase complex in the synthesis of surface polymers containing polysialic acid in *Escherichia coli*. J. Biol. Chem. **254:**7377–7387.
23. **Vimr, E., S. Steenbergen, and M. Cieslewicz.** 1995. Biosynthesis of the polysialic acid capsule in *Escherichia coli* K1. J. Industr. Microbiol. **15:**352–360.
24. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the α- and β- subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **8:**945–951.
25. **Weisgerber, C., and F. A. Troy.** 1990. Biosynthesis of the polysialic acid capsule in *Escherichia coli* K1: the endogenous acceptor of polysialic acid is a membrane protein of 20 kDa. J. Biol. Chem. **265:**1578–1587.