A Component of the Chloroplast Protein Import Apparatus Functions in Bacteria*

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(Received for publication, May 29, 1997, and in revised form, August 4, 1997)

Tox36 is a family of 44-kDa envelope polypeptides previously identified as components of the chloroplast protein import apparatus by virtue of their close physical proximity to translocating proteins. An indication of their function thus remains at large. A heterologous in vivo approach for studying the function of Tox36 was developed in this study by introducing a member of Tox36 into E. coli to assess its effect on bacterial protein translocation. The presence of Tox36 enhances the translocation of two bacterial periplasmic proteins in a manner resembling the chloroplast system. Translocation of the two bacterial periplasmic proteins was less sensitive to sodium azide, resembling more the azide-insensitive nature of the chloroplast protein import process. Mutated Tox36 proteins were not capable of causing the same effect as that observed for unaltered Tox36. Tox36 was also capable of complementing bacterial strains with temperature-sensitive secA mutations that affected protein translocation. The combined results provide evidence that Tox36 plays a central role in the chloroplast protein translocation process.

Nuclear-encoded chloroplast proteins synthesized outside the organelle are imported into the compartment via a complex process. The import process involves a host of components such as energy, transit signals, proteinaceous envelope membrane factors, processing peptidases and chaperones (1–3). These components facilitate various steps of the translocation process such as unfolding, binding to surface receptors, translocation across envelope membranes, and maturation (1–3). The multi-subunit protein translocation machinery of the chloroplast envelope membrane plays a central role in this process (2). The implication of components with a role in the chloroplast envelope protein import apparatus have so far relied heavily on physical approaches that show spatial proximity to a translocating protein. Therefore, approaches must be developed to provide a much needed insight into the actual function of each component. A number of other components of the plastid import pathway share mechanistic features in common with factors involved in the transport of proteins across bacterial membranes and have indeed been demonstrated to be interchangeable to a certain extent. The organelar-targeting transit signals of chloroplast precursor proteins can be recognized by the bacterial protein export machinery and transported into the periplasm with correct processing (4). The processing site of many chloroplast transit sequences follows the bacterial cleavage site and are readily cleaved by E. coli signal peptidase (5). In addition to transit signals, thylakoid lumen proteins contain prokaryotic-like intra-organelar sorting domains (6, 7). Some lumen proteins even traverse the thylakoid via an azide-sensitive pathway resembling bacterial azide-sensitive protein transport (6, 7). Counterparts of the bacterial components such as GroEL, DnaK, SecA, and SecY are present in plastid (3, 8–11) and, in cases concerning GroEL and SecA counterparts, have been demonstrated to function in a similar manner (3, 8, 12, 13). The parallelism of the chloroplast and bacterial protein translocation systems suggests that other plastid components are likely to be functionally interchangeable to some degree in a bacterial environment. Therefore, adaption of the bacterial system for studying the chloroplast envelope protein import apparatus may open up valuable opportunities and features afforded by the bacterial system that can be of assistance for elucidating functional aspects. This potential was realized by expressing a 44-kDa Tox36 component of the chloroplast envelope protein import apparatus in bacteria (14, 15). (The 44-kDa components formerly designated Com44/Cim44 have been renamed in accordance with a newly implemented universal nomenclature).

EXPERIMENTAL PROCEDURES

Construction of Plasmids and Bacterial Strains—DNA fragments encoding Bce44B and Oee1Dhfr were subcloned into pGEM11Z (Promega) to facilitate T7 RNA polymerase-directed expression (15, 16). Truncated Bce44B were generated by exonuclease III/S1 digestion and joined to the DNA sequence for the first four residues of the transit peptide for the pea small subunit of ribulose-1,5-bisphosphate carboxylase (17). Bce44B-N1 lacked 42 residues from the NH2 terminus, and the sequence of the fusion site was MASMISSLSVPQQ. Bce44B-N2 was made by joining the DNA sequence for the first 23 amino acids of Bce44B to the N1 deletion. The EcoRI-HindIII DNA fragment encoding the NH2-terminal 23 residues and the 5′-untranslated region was inserted into pGEM4 via EcoRI and Smal (HindII was converted to a blunt end) and was used for the construction of Bce44B-N2. The Bce44B-N2 fusion was completed by joining to an Aap-718-HindIII DNA fragment retrieved from Bce44B-N1 via the BamHI and HindIII sites of the above vector. The Aap-718 and BamHI sites were made blunt, and the sequence of the fusion point is -GLGVPP-. Bce44B-N1 and -N2 were subsequently transferred to pGEM11Z. Procedures for the introduction of recombinant plasmids into the E. coli strain JM109(DE3), MC4100, and MM52 were as described (18).

Subfractionation and Protein Analysis Procedures—Expression and subfractionation of the indicated bacterial strains were conducted as described (18) without isopropyl-thio-β-D-galactosidase induction. Inner and outer membranes, right-side-out and inside-out vesicles were prepared and assessed as described (19–24). Samples were analyzed via standard immunoblotting procedures using IgGs against Bce44B (Tox36) (14) and β-lactamase (5′-3′).

Induction of Alkaline Phosphatase—Cells were grown in LB-ampicillin.

* This work was supported by grants from the Natural Sciences and Engineering Research Council (Canada) (to K. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: Tox, protein translocation component of the chloroplast outer envelope; Oee1Dhfr, a fusion between the transit peptide of the 33-kDa protein of the photosystem II oxygen-evolving complex and mouse cytosolic dihydrofolate reductase; IgG, immunoglobulin G.

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Expression and Localization of Bce44B in Bacteria—Expression of bce44B (a member of the Toc36 envelope proteins) in bacteria gave rise to a multiple-protein pattern (Fig. 1A). The two largest bands correspond to full-length Bce44B (44 kDa) and a smaller 42-kDa product. The expressed proteins were predominantly in the cytosol. Other smaller proteins that fractionate with the cytosol and inclusion bodies are most likely derived from a combination of post-translational degradation and translation-related events, e.g. internal translation initiation or premature translation termination. A portion of the larger-sized Bce44Bs (44 and 42 kDa) co-fractionated with cytoplasmic membranes (both outer and inner) and crude inclusion bodies (Fig. 1A) and were removable from these fractions by detergent-containing washes (Fig. 1A). These bands were absent in the control Oee1Dhfr cells (Fig. 1A). A portion of the Bce44Bs associated with membrane vesicles (right-side-out and inside-out) were resistant to postfractionation thermolysin treatments, becoming sensitive only in the presence of Triton X-100 (Fig. 1A). Mock protease treatments did not exhibit any degradation. The protease-resistant membrane-associated Bce44Bs are most likely protected by the bacterial membranes and appear to be associated in a manner similar to its natural habitat, the chloroplast envelope (15). These results indicate that the bacterial protein translocation machinery recognizes Bce44B, assembling Bce44B with membranes. Interestingly, the distribution of Bce44B in the bacterial membranes was also dual in nature, resembling the situation found in the chloroplast envelope, with the exception of being opposite to the directionality of the envelope distribution pattern. Higher amounts of Bce44B were instead found associated with the outer membrane rather than the inner membrane. The directionality of the distribution of Bce44B may reflect the direction of protein translocation, namely outward in bacteria and inward in chloroplasts.

Bce44B Enhances Protein Translocation in Bacteria—The behavior displayed by Bce44B in bacterial membranes raises the possibility that Bce44B may be functional as a foreign component of the bacterial protein transport apparatus. This prediction was assessed by monitoring changes to the translocation of two periplasmic enzymes. Alkaline phosphatase is a single copy gene and is transported into the periplasm upon induction by phosphate starvation. Bce44B-containing bacteria (Bce44B cells) displayed a growth profile distinct from the control Oee1Dhfr strain (Oee1Dhfr cells) expressing a chimeric chloroplast precursor protein. Bce44B cells displayed a healthier growth rate with two periods of growth in low phosphate, whereas Oee1Dhfr cells exhibited only one weak period and very little overall growth. Bce44B cells harvested from the two growth periods contain higher levels of processed alkaline phosphatase relative to the Oee1Dhfr cells at the same sampling time (Fig. 1B). The differences were apparent even under phosphate-abundant conditions. The amount of transported alkaline phosphatase detected reflects changes in protein translocation activity. Even though phosphate is vital for the

RESULTS

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survival of a bacterium and there is a variety of mechanisms operating for survival under phosphate limitation, the data suggest that the presence of Bce44B contributes to the enhanced levels of periplasmic alkaline phosphatase, allowing cells to grow further by releasing more phosphates from limited sources.

The same effect was observed with β-lactamase, a plasmid-borne multi-copy gene (25). β-lactamase levels are higher than induced alkaline phosphatase, representing a more sensitive monitor of protein translocation changes without complications posed by low phosphate. The level of antibiotic tolerance conferred thus reflects changes to protein transport activity. Bce44B cells formed colonies with ampicillin concentrations as high as 3 mg/ml, whereas Oee1Dhfr cells cannot form colonies beyond 1 mg/ml (Fig. 1C). The plasmid copy number was determined to be comparable in both strains (approximately 700–800) and was not expected to have an impact on β-lactamase expression. The higher level of ampicillin tolerance displayed by the Bce44B cells was mirrored in the enhanced level of transported β-lactamase (Fig. 1C). The level of processed β-lactamase is higher on a per cell basis in Bce44B cells than in Oee1Dhfr cells at all ampicillin concentrations tested. These findings parallel the results derived from chemical cross-linking experiments indicating the close physical proximity of Bce44B to β-lactamase during or shortly after translocation, implicating the involvement of Bce44B (data not shown).

**Effects of Sodium Azide on Protein Translocation—**Sodium azide is a potent inhibitor of bacterial protein translocation activity acting via the SecA protein (26, 27). Both alkaline phosphatase and β-lactamase utilize the SecA-dependent pathway and thus can be inhibited by azide, accumulating as precursor forms. In contrast, transport across the chloroplast envelope is azide-tolerant, even though the translocation of some proteins across the internal thylakoid system is azide-sensitive (6, 7). The Bce44B cells were therefore tested for changes in azide sensitivity to assess whether protein translocation in these cells has been altered by Bce44B to resemble the chloroplast protein transport system. The level of sodium azide (0.5 mM) used partially inhibits SecA activity and the effects on transport were monitored with anti-β-lactamase IgGs (Fig. 2).

In the absence of azide, Bce44B cells displayed higher levels of exported/processed β-lactamase than Oee1Dhfr cells at all the time points sampled. In the presence of azide, the Oee1Dhfr cells displayed increasing amounts of precursor β-lactamase and a concomitant decrease in the levels of mature transported form. The pattern was different in the Bce44B cells, where both precursor and mature forms of β-lactamase increased, suggesting that Bce44B is capable of sustaining the transport of β-lactamase at an elevated level even though SecA was partially hampered by azide. These results suggest that Bce44B can partially compensate for the azide-sensitive SecA-dependent protein transport pathway and, to an extent, function in a manner resembling its chloroplastic nature.

**Compensation of a Temperature-sensitive secA Mutant Bacteria Strain by Bce44B—**The compensatory capabilities of Bce44B were further investigated using the temperature-sensitive secA bacteria mutant, MM52, and its parental wild-type strain, MC4100 (28). MM52 displays mild protein-translocation deficiencies at 30 °C and is exacerbated at 37 °C. At 30 °C, Bce44B had the same effect in both strains as described above (Fig. 3A). Plasmid copy numbers of these strains were comparable. One observable difference was the rate of colony growth, the MM52 strains required 16 h for the MC4100 16 h versus the 16 h for the MC4100 strains.

The compensatory effects of Bce44B on protein translocation were active even under nonpermissive conditions. Bce44B-containing MM52 cells (MM52-Bce44B) displayed increasing levels of mature β-lactamase over the 2-h study period at both permissive and nonpermissive temperatures, similar to the pattern exhibited by the MC4100 counterparts (Fig. 3B). In contrast, Oee1Dhfr-containing MM52 cells (MM52-Oee1Dhfr) displayed deficiencies at both 30 °C and 37 °C, manifesting in the accumulation of precursors. The compensatory effect of Bce44B was also evident in the presence of azide. MM52-Oee1Dhfr cells exhibited the expected deficiency, manifesting in the accumulation of β-lactamase precursors at a relatively low azide concentration (0.125 mM) compared with the MM52-Bce44B cells, which behaved like the MC4100 counterparts (Fig. 3C). MC4100 strains began displaying precursor accumulation only when azide was elevated above 0.37 mM. MC4100-Oee1Dhfr cells began displaying the precursor form at 0.25 mM azide.

**The Effect of Bce44B Can Be Abolished by Truncations—**The positive effect Bce44B exerts on protein transport is most likely due to its membrane-associated interaction with the bacterial protein translocation apparatus since mutations affecting membrane association and/or complexing mechanisms of Bce44B abolish its capabilities. Bce44B-N1 and Bce44B-N2 did not display any of the observed effects, behaving more similarly to the control cell lines (Fig. 4A-C). The truncations appear to affect outer and inner membrane distribution, more evenly in the case of Bce44B-N1 and only to the inner membrane in the case of Bce44B-N2. Other structural features affected by the truncations were not investigated and will be the subject of an upcoming study. Bce44B-N1 cells were not able to form colonies above 500 µg/ml of ampicillin, and Bce44B-N2 cells formed colonies only up to 250 µg/ml. Bce44B-N1 and Bce44B-N2 cells were relatively more azide-sensitive with higher amounts of precursor accumulation at a lower azide level, which corroborates the higher sensitivity to ampicillin and the much lower protein transport activity observed.

**DISCUSSION**

Bce44B is a member of the Toc36 chloroplast envelope proteins (formerly designated Com44/Cim44) previously identified to be proximal to a partially translocated chimeric chloroplast

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**Fig. 2.** Time course study of the effect of Bce44B on the azide-sensitive nature of β-lactamase protein transport.

| Cell line    | Bce44B cells | Oee1Dhfr cells |
|-------------|--------------|---------------|
| Time (hrs)  | 0  | 0.5 | 1.0 | 1.5 | 2.0 |
| No azide    | ![Graph](image1.png) | ![Graph](image2.png) |
| Azide       | ![Graph](image3.png) | ![Graph](image4.png) |
precursor protein and by virtue of this criterion was ascribed a role in protein translocation (14, 15). The current study provides much needed functional evidence that Bce44B is a central component of the chloroplast protein import apparatus. The evidence suggests that Bce44B is clearly involved in protein translocation whether it is in bacterial membranes or in the chloroplast envelope. Both systems recognize and assemble Bce44B in a similar manner into both the outer and inner cytoplasmic membranes of the bacterium. Interestingly, the distribution pattern of Bce44B between the outer and inner cytoplasmic membranes was similar to the chloroplast envelope relative to the direction of protein translocation. Bacterial membrane-associated Bce44B affects the SecA-dependent protein transport of periplasmic enzymes by enhancing translocation via a more azide-tolerant mode of action and can be found proximal to translocating or newly translocated proteins (chemical cross-linking data not shown). The compensatory capabilities of Bce44B in the protein transport mutant bacterial strain, MM52, provide further evidence that Bce44B is actively involved in the facilitation of protein transport. This capability occurs even during the impairment of secA at nonpermissive temperatures, providing strong evidence that the observed enhancement of periplasmic protein transport is not due to the up-regulation of the endogenous bacterial protein translocation system.

FIG. 3. Compensation of the secA temperature-sensitive mutation in bacterial strain MM52 by Bce44B. A, formation of colonies on nutrient agar plates containing increasing concentrations of ampicillin. B, time course study of the effect of Bce44B on the azide sensitivity of ß-lactamase transport in MM52 and the control strain MC4100 at 30 °C (permissive temperature) and 37 °C (nonpermissive temperature). The identity of the precursor and mature forms is marked with p and m, respectively. C, effects of increasing sodium azide concentration on the transport of ß-lactamase in MM52- and MC4100-derived strains. Precursor and mature forms are marked as in panel B.
that the effects are due to intact membrane-associated Bce44Bs and are not attributed to the mere presence of Bce44B in the cell, causing the effects via up-regulation of the bacterial protein transport system. The truncated Bce44B proteins in fact perturbed bacterial protein transport, which further suggests the process that Bce44B effects is protein translocation.

The overall outcome of this study clearly indicates that it may be very useful to explore heterologous in vivo systems for studying the functional aspects of other protein translocation components of the chloroplast envelope. The outcome of this study also adds more evidence to the hypothesis that different protein translocation systems share common mechanistic features and, in our case, protein transport across the chloroplast envelope and the bacterial cytoplasmic membranes.

Acknowledgments—The authors thank Dr. J. Beckwith for providing the strains MM52 and MC4100, Dr. K. Poole for critical reading of the manuscript, and Z. W. Ko for technical assistance.

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