Substrate-induced assembly of a contiguous channel for protein export from *E.coli*: reversible bridging of an inner-membrane translocase to an outer membrane exit pore

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The toxin HlyA is exported from *Escherichia coli*, without a periplasmic intermediate, by a type I system comprising an energized inner-membrane (IM) translocase of two proteins, HlyD and the traffic ATPase HlyB, and the outer-membrane (OM) porin-like TolC. These and the toxin substrate were expressed separately to reconstitute export and, via affinity tags on the IM proteins, cross-linked *in vivo* complexes were isolated before and after substrate engagement. HlyD and HlyB assembled a stable IM complex in the absence of TolC and substrate. Both engaged HlyA, inducing the IM complex to contact TolC, concomitant with conformational change in all three exporter components. The IM–OM bridge was formed primarily by HlyD, which assembled to stable IM trimers, corresponding to the OM trimers of TolC. The bridge was transient, components reverting to IM and OM states after translocation. Mutant HlyB that bound, but did not hydrolyse ATP, supported IM complex assembly, substrate recruitment and bridging, but HlyA stalled in the channel. A similar picture was evident when the HlyD C-terminus was masked. Export thus occurs via a contiguous channel which is formed, without traffic ATPase ATP hydrolysis, by substrate-induced, reversible bridging of the IM translocase to the OM export pore.

Keywords: bacterial secretion mechanism/mitochondrial import/*in vivo* protein translocation/TolC membrane pore/traffic ATPase

Introduction

Proteins exported by Gram-negative bacteria such as *Escherichia coli* must cross two membranes. The most direct method of transferring a protein substrate from the cytoplasm across the inner and outer membranes (IM and OM, respectively) would be by ‘all-or nothing’ interaction between specific protein components of the two membranes, bypassing the intervening periplasmic space. This simplest approach is found as the conserved type I signal-peptide-independent mechanism, which is exemplified by the export of the 110 kDa HlyA protein toxin from *E.coli* (Koronakis and Hughes, 1994) and is effected without requiring a periplasmic intermediate generated by the Sec complex (Koronakis *et al.*, 1989). Export is mediated by

a specific translocator comprising an IM translocase of two proteins (Wagner *et al.*, 1983), one of which is a traffic ATPase which provides essential energy from ATP hydrolysis (in toxin export, HlyB) (Gentschev and Goebel, 1992; Koronakis and Hughes, 1993; Koronakis *et al.*, 1993), and a single protein in the OM, the trimeric exit pore TolC (Wandersman and Delepelaire, 1990; Koronakis *et al.*, 1997). These three proteins are recognized as the core, and probably only protein components of the translocator, and several lines of evidence support the view that direct interaction between the energized IM and OM mediated by them is central to the mechanism. Substrate HlyA proteins with defective export signals (located at the extreme C-terminus) are located in the cytoplasm or membrane fractions, not in the periplasm (Koronakis *et al.*, 1989; Stanley *et al.*, 1991). Similarly, in the absence of the OM exit pore, the IM translocase does not accumulate substrate in the periplasm, nor does it allow secretion of substrate from spheroplasts, i.e. the OM component is an integral part of the active translocator (Koronakis *et al.*, 1997). HlyA export shares with conventional Sec-dependent IM secretion an early requirement for the total proton-motive force (ΔP), but subsequent translocation does not require ΔP or its components (Koronakis *et al.*, 1991). A translocation intermediate in this late stage was not located in the cytoplasm, periplasm or on either periplasmic face, prompting the suggestion that it was blocked in an energetically primed membrane fusion channel or complex.

It is likely that the supposed interaction between the two bacterial membranes would be mediated principally between OM TolC and the IM HlyD. Whereas the HlyB traffic ATPase is an integral IM protein characterized by the large cytoplasmic ATPase domain (Koronakis *et al.*, 1993, 1995), sequence and topological analyses of HlyD protein fusions suggest that it has a single transmembrane domain, a short cytoplasmic N-terminal domain and a large C-terminal periplasmic domain (Schulein *et al.*, 1992). While traffic ATPases are widespread throughout prokaryotic and eukaryotic cells (Higgins *et al.*, 1986), HlyD and related proteins are found uniquely in Gram-negative bacteria, as part of type I protein export and also systems involving drug resistance transporters of the major facilitator superfamily and oligosaccharide exporters of the heavy metal resistance/nodulation/cell division family. They have indeed been pre-emptively entitled ‘membrane fusion proteins’ (Dinh *et al.*, 1994), forming a loose family having similar sizes and hydropathy profiles, all probably embedded at their N-termini in the IM which they span. Topological studies using reporter fusions have substantiated the sequence prediction that the bulk of the protein is located in the periplasmic space, and the C-terminal region has been confirmed to be essential for HlyA translocation (Schulein *et al.*, 1994). Analysis of two-

Fig. 1. Reconstitution of the HlyA export system *in vivo*, tagged with His(HlyD). Exported HlyA in the culture supernatant of *E.coli* BL21(DE3) carrying the wild-type chromosomal *tolC* gene and either the three compatible plasmids pACT7HlyB, pT7HlyD and pLGHlyA separately expressing, respectively, *hlyB*, *hlyD* and *hlyA* (left lane), or with pACT7HlyB, pLGHlyA and pT7HisHlyD containing a 5' extension, thereby creating a His-tag fusion at the HlyD N-terminus (right-hand lane). After precipitation of 1 ml cell-free supernatant with 10% TCA, HlyA was resolved on a 10% SDS–polyacrylamide gel and probed with anti-HlyA polyclonal antiserum.

dimensional crystals suggest that TolC also has a periplasmic domain, extending from the OM trimeric porin-like structure formed by the TolC monomers, possibly spanning most of the way to the IM (Koronakis *et al.*, 1997). The prediction of interactions has been supported by experiments in which translocating substrate of a related type I system co-precipitated both IM and OM exporter component proteins from detergent-solubilized cell extracts (Létoffé *et al.*, 1996).

To describe the *in vivo* sequence of events in this export process, we wanted to establish in particular whether part or all of the exporter complex is pre-formed in the absence of substrate, whether engagement of the substrate induces the bridging contact between the inner and outer membranes, and, if so, whether the bridge remains in place or is reversible. To do this, we have reconstituted the HlyA toxin export system *in vivo* by expressing the components on separate compatible replicons to allow expression in different combinations. We used the integral IM proteins HlyD and HlyB as His-tagged bait so that we could isolate, by *in vivo* chemical cross-linking and affinity chromatography, intermediate and complete export complexes formed during the engagement and translocation of the substrate protein. By substituting a characterized mutant HlyB traffic ATPase into the *in vivo* system, we were also able to assess the role of ATP hydrolysis in export complex formation.

Results

Reconstitution of the HlyA export system to isolate membrane complexes before and during *in vivo* toxin translocation

To establish the *in vivo* interactions between components of the resting and active export machinery, the export system was reconstituted such that the individual components could be expressed in the cell in different combinations. The IM proteins HlyB and HlyD, and also the translocation substrate HlyA, were expressed from compatible plasmids in the TolC⁺ *E.coli* BL21(DE3) or its isogenic TolC[−] derivative BL923(DE3). To isolate potential membrane complexes, six histidine residues were fused to the N-terminus of HlyD. The reconstituted system incorporating (His)HlyD was able to export the toxin HlyA into the *E.coli* culture supernatant at a similar level to that of the wild-type system (Figure 1).

In vivo cross-linking has been used extensively to define specific interactions in the membrane translocation machineries of bacteria and mitochondria, and in this study its combination with affinity isolation via the non-translocating IM components allows comparative analysis of the core protein interactions in the intact actively translocating complex and (sub)complexes 'frozen' as they occur *in vivo*. To stabilize protein complexes in the type I HlyA export machinery, intact *E.coli* cells were treated with the chemical cross-linker Dithiobis (succinimidyl propionate) (DSP), which has a fixed spacer arm of 12 Å and a disulfide bond that can be cleaved with reducing agents. Cells were then lysed and the target complexes were affinity-purified from the extract via the His-tag of HlyD, which, as the integral IM component most likely to be at the physical core of the complex(es) and bridge to TolC, was chosen to be the primary probe throughout the experiments. In experiments to compare the degree of bridging to TolC, the other integral IM component, HlyB, was His-tagged instead. Isolated complexes containing (His)HlyD [or (His)HlyB] were treated with DTT to cleave the DSP cross-linker, and the protein components of the complex were resolved by SDS–PAGE and identified by immunoblotting with polyclonal antibodies raised individually against each of the proteins HlyA, HlyB, HlyD and TolC. Assay by immunoblotting excludes identification of any other proteins associating with the complexes, but in the specific type I membrane machinery the three proteins HlyB, HlyD and TolC are recognized as the core, and probably only components.

HlyD and the traffic ATPase HlyB form an IM complex in the absence of translocation substrate and the OM exit pore TolC

Without *in vivo* cross-linking, none of the HlyA, HlyB or TolC was co-isolated with the affinity-purified (His)HlyD (Figure 2, lane 1), while with DSP cross-linking, all four proteins were clearly isolated on the Ni-agarose (lane 2). As predicted, the three exporter components act together in a complex during export. The immediate question arising from this is whether the export proteins form a pre-formed active 'all-or-nothing' complex, or whether sequential complexes are formed at the two membranes, possibly influenced by the substrate.

A parallel analysis was therefore performed on *E.coli* cells in which HlyB and (His)HlyD were expressed in the absence of the substrate HlyA. The results (Figure 2, lane 3) show that the two IM proteins were cross-linked to each other to the same degree as they were in the presence of substrate. Furthermore, although the OM component TolC was present in these *E.coli* cells, it did not co-purify with the substrate-independent HlyB/HlyD IM complex, and the possibility that TolC nevertheless facilitated formation of the IM complex was ruled out by the undiminished detection of the complex when HlyB and (His)HlyD were co-expressed and cross-linked in the isogenic TolC[−] strain, with or without substrate (Figure 2, lanes 6 and 7). Thus HlyD and HlyB exist in a pre-formed complex in the IM, independent of the translocating substrate and the OM pore TolC.

Both components of the IM complex interact with the translocation substrate HlyA

The (His)HlyD cross-linking experiments also revealed that HlyD can interact independently with the toxin

Fig. 2. *In vivo* interaction between IM and OM components of the HlyA translocation machinery. Export machinery components contacting (His)HlyD in TolC⁺ and TolC⁻ *E. coli* cultures expressing combinations of HlyB and HlyA, as indicated. Following *in vivo* crosslinking with DSP, cross-linked complexes containing (His)HlyD were purified from the membrane fractions of lysed cells using Ni-NTA beads. Cross-linked proteins were released by cleavage of the DSP molecule with DTT, resolved on 10% SDS-polyacrylamide gels and identified by immunodetection with anti-HlyD, anti-HlyB, anti-HlyA and anti-TolC antisera.

substrate, as not only did HlyA cross-link with HlyD in the complete export machinery (Figure 2, lane 2), but also in the absence of HlyB (Figure 2, lane 4), of TolC (Figure 2, lane 6), and of both HlyB and TolC (Figure 2, lane 8). The HlyA signal in lane 8 appears low. However, considering the reproducibly lower expression of all the assay proteins in the TolC⁻ strain, the signal ratio of HlyA to HlyD agrees very well with that evident in the corresponding TolC⁺ strain, shown in Figure 2, lane 4. On the other hand, when the second IM component HlyB was also present (Figure 2, lane 6), we consistently found a relatively stronger HlyA signal, supporting the view that both HlyB and HlyD contribute to substrate binding. HlyD

Fig. 3. *In vivo* engagement of HlyA by HlyB. Recruitment of HlyA into the IM complex by (His)HlyB in the presence and absence of HlyD. Following *in vivo* crosslinking with DSP, cross-linked complexes containing (His)HlyB were purified from the membrane fractions of lysed cells using Ni-NTA beads. Cross-linked proteins were released by cleavage of the DSP molecule with DTT, resolved on 10% SDS-polyacrylamide gels and identified by immunodetection with anti-HlyB and anti-HlyA antisera.

can thus interact independently with IM HlyB and with the substrate HlyA, without requiring or recruiting TolC.

In a parallel experiment the HlyB protein was His-tagged in place of HlyD, and this revealed that in the same way (His)HlyB also cross-linked to HlyA substrate in both the complete system and also when the IM partner HlyD was omitted (Figure 3). This shows that both the HlyD and HlyB components of the IM complex can bind the translocating substrate, and that the binding is not dependent upon assembly of the IM HlyB-HlyD complex.

Engagement of the translocating substrate HlyA induces the IM complex to make a bridge, via HlyD, to the OM exit pore TolC

The data so far show that the export proteins HlyB and HlyD form a pre-formed complex in the IM, independently of OM TolC. The export proteins do not associate in an 'all-or-nothing' complex, but rather comprise two separable components at the two membranes. When TolC is absent, the substrate HlyA is still engaged by the preformed IM complex (His)HlyD/HlyB (Figure 2, lane 6), illustrating that the total absence of HlyA export by TolC⁻ bacteria is entirely due to the absence of the OM pore, as loss of TolC does not hinder earlier events at the IM. As no periplasmic intermediate exists, the data indicate that a contiguous translocation channel must be induced connecting the IM complex to the TolC OM exit pore. This is shown indeed to be the case by the behaviour of the three export proteins in the presence and absence of substrate (Figure 2, lanes 2 and 3). In these two states the HlyB/HlyD IM complex assembles equally well, but only when HlyA is engaged does the IM HlyB/HlyD complex recruit TolC, so that TolC is co-isolated via cross-linked (His)HlyD (lane 2). Without HlyA substrate, TolC is not recruited (lane 3).

It is HlyD, rather than the traffic ATPase HlyB, that is likely to be the membrane-bridging component of the HlyB-HlyD IM translocator, and the experiments show that (His)HlyD does cross-link strongly to TolC in the IM-OM bridged complex. It is nevertheless possible that

Fig. 4. *In vivo* substrate-induced interaction between IM and OM exporter components. (A) Exported HlyA in the culture supernatant of *E. coli* expressing the complete reconstituted export machinery including either (His)HlyB or (His)HlyD. (B) Substrate-induced bridging of the IM complex to OM TolC, isolated by (His)HlyB. Following *in vivo* DSP crosslinking, cross-linked complexes containing (His)HlyB were purified from membrane fractions of lysed cells using Ni-NTA beads. Cross-linked proteins were released by cleavage of DSP with DTT, resolved on 10% SDS-polyacrylamide gels and identified by immunodetection with anti-HlyD, anti-HlyB, anti-HlyA and anti-TolC antisera. The TolC blot shown was exposed for 10 times longer than in the equivalent (His)HlyD experiment shown in Figure 2.

this interaction could occur via HlyB, and so to attempt to illustrate which IM protein mediates the TolC connection, substrate-dependent bridging was assayed with His-tagged HlyB in place of (His)HlyD. Switching the tag did not affect export of HlyA (Figure 4A), and the *in vivo* cross-linking (Figure 4B) confirmed the results obtained using (His)HlyD in Figure 2, i.e. HlyB and HlyD are present in a preformed complex that in the presence of substrate HlyA, bridges to OM TolC. However, to visualize the TolC protein in Figure 4, lane 1, to a degree similar to that of Figure 2, lane 2, exposure of the TolC immunoblot required 10-fold longer (our comparison is based on the equal amount of the IM HlyB-HlyD complex in the two experiments). The observation that His-tagged HlyB is substantially less efficient than (His)HlyD in cross-linking to TolC under the same conditions provides strong support for the view that HlyD is the critical IM bridging component.

Fig. 5. Reversibility of HlyA-induced TolC-HlyD membrane bridging. (A) Cells expressing HlyA, HlyB, TolC and (His)HlyD, as in Figure 2, were harvested at mid-exponential phase, and one half was cross-linked immediately with DSP (t_0), the other half was first incubated for 30 min in minimal medium containing spectinomycin (t_{30}). Cross-linked proteins were isolated, released, resolved (SDS-10% PAGE) and immunoblotted as before. (B) Upper panel: export of HlyA substrate engaged in the membrane complex into the minimal medium after 30 min. After precipitation of 1 ml cell-free supernatant with 10% TCA, HlyA was resolved on a 10% SDS-polyacrylamide gel and probed with anti-HlyA polyclonal antiserum. Lower panel: stability of TolC (10 ml cell culture) during the 30 min assay. TolC was resolved on a 10% SDS-polyacrylamide gel and probed with anti-TolC polyclonal antiserum.

The specific export bridge between the IM and OM is transient, and is reversed after translocation of the substrate

The substrate HlyA induces bridging of the IM and OM exporter components, creating a contiguous translocation apparatus, presumably a channel, to the external medium. The question arises of whether this is a permanent structure, or whether it can revert to its distinct IM and OM components once the substrate has passed through. To answer this, the presence of the bridge was compared at two time points, as HlyA was engaged in the export machinery (i.e. as before in Figures 2 and 4), and then after the substrate had all been exported. To do this, synthesis of the substrate had to be induced for a short time in cells expressing the export components. A culture of *E. coli* TolC⁺ cells expressing (His)HlyD, HlyB and HlyA was harvested and split into two aliquots; one was resuspended in buffer and cross-linked immediately, as before, with DSP (0 min in Figure 5), while prior to identical cross-linking the other aliquot was first resuspended in minimal medium and incubated for 30 min at 37°C in the presence of the protein synthesis inhibitor spectinomycin, in order that a pre-formed HlyA substrate pool could be exported. After this time, HlyA was not detected in the cells (not shown). Pulse-labelling experiments have shown that HlyA appears in the supernatant seconds after synthesis, with most of the HlyA pool being extracellular after 5 min (Felmlee and Welch, 1988). The HlyB/(His)HlyD IM complex was evident in both cases (Figure 5A), but while immediate ('substrate engaged') cross-linking showed, as before, the complex to contain OM TolC, there was no TolC cross-linked to (His)HlyD when the HlyA pool had been exported and it was no longer present in the cell (Figure 5A). That all the substrate HlyA molecules engaged in the secretion machinery at

Fig. 6. HlyA substrate-induced changes in the protease sensitivity of the export machinery components. Proteinase K and trypsin accessibility of (His)HlyD, HlyB and TolC in spheroplasts of *E. coli* with or without HlyA. Cultures (5 ml) were converted to spheroplasts, and treated with protease at 100, 200 or 400 µg/ml for 30 min on ice. Protease treatment was terminated with 20% TCA, and proteins were resolved and probed with anti-HlyD, anti-HlyB or anti-TolC polyclonal antisera.

0 min had indeed been exported was confirmed by immunoblotting HlyA in the supernatant (Figure 5B, upper panel), and furthermore, there was no difference in the level of TolC in total cell lysates from the two aliquots (Figure 5B, lower panel), showing that the result was not due to TolC turnover and disappearance during the 30 min HlyA export.

Thus, engagement of the substrate HlyA induces the bridging of IM HlyB/HlyD to OM TolC, and once this engaged substrate has been exported, the bridge is uncoupled and the IM HlyD/HlyB and OM TolC components revert to their separate pre-induction state.

Translocating HlyA induces conformational change in each of the IM and OM exporter components

As the substrate HlyA interacts with HlyB/HlyD and triggers bridging to TolC, it is possible that its engagement induces conformational change in the export machinery components. This was addressed by assaying, in *E. coli* spheroplasts, the protease accessibility of membrane components HlyD, HlyB and TolC in the presence and absence of HlyA. After limited proteolysis by either proteinase K or trypsin for 30 min on ice, immunoblotting showed that in the presence of substrate HlyA, the HlyD, HlyB and TolC proteins were each more susceptible to protease (Figure 6). Substrate-induced assembly of the functional IM-OM exporter complex is thus concomitant with conformational change in all three of the protein components.

HlyD, the IM bridging component to trimeric OM TolC, also oligomerizes to a trimer

The trans-periplasmic structure connecting the cytoplasm to the external medium appears from predictions of protein topology and the data presented to be effected primarily by TolC-HlyD interaction. As TolC has been demonstrated by two-dimensional crystallization in phospholipid bilayers to be a trimeric structure (Koronakis *et al.*, 1997), we assessed the *in vivo* oligomerization state of its putative partner HlyD in the membrane. To do this, *in vivo* cross-linking was performed on cells expressing (His)HlyD, but

Fig. 7. *In vivo* oligomerization of HlyD. Cultures expressing (His)HlyD and either all or none of HlyA/HlyB/TolC were treated with cross-linker DSG, and cross-linked proteins were isolated as before and probed with anti-HlyD antiserum. The locations of size markers are shown in kDa.

with disuccinimidyl glutarate (DSG) rather than DSP, a non-cleavable chemical cross-linker with a shorter fixed spacer arm of 7.7 Å, such that it cross-links only to peptides or proteins that are very close. After cross-linking, (His)HlyD was affinity-purified and immunoblotted with anti-HlyD antisera, revealing that in addition to the monomeric form of HlyD migrating at below 60 kDa, a high-molecular-weight form of HlyD, migrating at ~180 kDa, was identified in all recombinant strains tested, e.g. when HlyD was expressed alone or in the complete export system (Figure 7). HlyD thus forms a trimer in the IM of *E. coli*, even in the absence of the OM TolC, the translocation substrate HlyA or the IM traffic ATPase HlyB, which suggests that the induced double-membrane export complex, perhaps a channel, could comprise a trimeric HlyD-TolC structure.

ATP hydrolysis by the traffic ATPase HlyB is essential for export, but not for the stages of exporter assembly

HlyB forms a pre-formed IM complex with HlyD, in which substrate is engaged, and this activated IM complex undergoes substrate-induced bridging to TolC via HlyD. Is ATP hydrolysis by HlyB, which is essential for HlyA export (Koronakis *et al.*, 1995), required for any of these critical assembly steps? In an investigation carried out to answer this question, the wild-type HlyB was replaced with the mutant HlyB^{G609D}, in which a single substitution of an ATP-binding pocket glycine residue (by aspartate) has completely disabled hydrolysis but not binding of ATP, which caused a complete loss of HlyA export (Koronakis *et al.*, 1995).

The mutant HlyB^{G609D} was confirmed as unable to support export of the HlyA toxin when substituted into the reconstituted system in *E. coli* (Figure 8A), but *in vivo* DSP cross-linking of the (His)HlyD-complexed components (Figure 8B) showed that the mutant HlyB^{G609D} interacted in a way indistinguishable from that of the wild-type HlyB. Without the substrate HlyA, HlyB^{G609D}

Fig. 8. Assembly and function of an export complex containing mutant HlyB^{G609D}. (A) HlyA exported by complexes comprising TolC, (His)HlyD and either wild-type HlyB or HlyB^{G609D}. (B) TolC–(His)HlyD bridging in the export complex containing mutant HlyB^{G609D}, cross-linked in the presence and absence of HlyA. Proteins cross-linked to (His)HlyD were purified and immunoblotted as before. (C) HlyD oligomers (DSG cross-linked) in the complex containing mutant HlyB^{G609D} in the presence and absence of HlyA [i.e. corresponding to lanes 2 and 3 in (B)].

formed an IM complex with HlyD (Figure 8B, lane 3), and the substrate was engaged and triggered the IM HlyD/HlyB^{G609D} bridge to the OM TolC (Figure 8B, lane 2). Furthermore, the substitution with HlyB^{G609D} did not compromise trimerization of HlyD, as assessed by *in vivo* cross-linking with DSG (Figure 8C), nor did it preclude the conformation changes induced in HlyD, HlyB^{G609D} and TolC by the substrate HlyA (confirmed by protease accessibility as before; not shown). The HlyA substrate was therefore engaged but not translocating, and there was accumulation of HlyA in the HlyB^{G609D} export

complex. This lends strong support for the view that the specific ATP hydrolysis is required for movement of the substrate through the assembled export apparatus.

Masking the HlyD C-terminus also blocks translocation without inhibiting substrate engagement or assembly of the exporter complex

The C-terminal domain of the IM HlyD is predicted from topology studies to be in the periplasm, and is essential for HlyA translocation (Schulein *et al.*, 1994). We were now able to assess the importance of the HlyD C-terminus in sequential steps of export complex assembly, and to do so we created a variant in which the (His)HlyD C-terminus was masked by the in-frame fusion to a 10-amino-acid c-Myc epitope. This variant, (His)HlyD^{myc}, was unable to support export of HlyA when substituted for (His)HlyD in the reconstituted export complex (Figure 9A). However, *in-vivo* cross-linking showed that HlyD^{myc} oligomerized, as did the wild-type HlyD (Figure 9B), that it did not alter the HlyA-induced changes in exporter protein protease sensitivity (not shown), nor prevent pre-assembly of the IM HlyB–HlyD complex (Figure 9C, lane 3), engagement of substrate HlyA or substrate-induced bridging to OM TolC (Figure 9C, lane 2). These data suggest that the (His)HlyD^{myc} secretion block does not occur in recruitment or assembly of the export complex; this is supported by the apparent accumulation of substrate HlyA apparent in the complete system compared with that when HlyB was omitted (Figure 9C, compare lanes 2 and 4). The blockage appears to apply at a late stage of HlyA export, i.e. the function of the contiguous channel in releasing the engaged HlyA substrate.

Discussion

We have established the protein interactions underlying the direct type I double-membrane export of the 110 kDa HlyA toxin by Gram-negative bacteria. By reconstituting the export process in *E. coli* with separately expressed components and translocation substrate, cross-linked membrane complexes formed *in vivo* were isolated before and after substrate engagement. The two IM exporter proteins HlyD and the traffic ATPase HlyB, plus the substrate HlyA, were expressed on distinct compatible plasmids, either in the wild-type strain chromosomally encoding the OM exit pore TolC, or in a null mutant lacking TolC. Complexes formed during export were isolated by affinity chromatography facilitated by attaching N-terminal His tags (which did not impair export) to HlyD, the probable IM component contacting the OM TolC and sometimes HlyB. Our findings from this series of experiments were as follows. (i) The traffic ATPase HlyB formed an IM precomplex with HlyD, independent of TolC and without the substrate HlyA. (ii) Both of the IM proteins HlyB and HlyD could bind the substrate independently. (iii) None of the bilateral IM interactions (HlyD–HlyB, HlyD–HlyA or HlyB–HlyA) triggered bridging to the OM TolC. The TolC exit pore was recruited to the export complex only when the substrate HlyA was engaged with the IM HlyD/HlyB complex, i.e. IM substrate engagement induces bridging of the pre-formed IM complex to the OM. (iv) When a pool of HlyA substrate was allowed to complete its export without *de novo* protein synthesis, IM–OM

Fig. 9. Assembly and function of an export complex containing HlyD^{myc}. (A) HlyA exported by complexes comprising TolC, HlyB and either wild-type (His)HlyD or mutant (His)HlyD^{myc}. (B) HlyD oligomers (DSG cross-linked) in a complex containing mutant HlyD^{myc} in the presence and absence of HlyA substrate. (C) TolC–(His)HlyD bridging in an export complex containing the mutant (His)HlyD^{myc}, cross-linked in the presence and absence of HlyA. Proteins cross-linked to (His)HlyD^{myc} were purified and immunoblotted as before.

bridging was reversed and the components of the exporter separated to their pre-engagement states, i.e. the substrate-dependent bridging is dynamic. (v) Translocating HlyA induced conformational changes in each of the three exporter proteins HlyD, HlyB and TolC, concomitant with assembly of a *trans*-periplasmic channel. (vi) Cross-linking of TolC to the IM complex via His-tagged HlyB was 10- to 20-fold less effective than when assayed via His(HlyD), substantiating the view of HlyD as the IM bridging component. (vii) IM HlyD oligomerized in the membrane, apparently to a trimeric structure that would mirror its

OM partner TolC, which we have shown by electron microscopy to be a trimeric pore. (viii) Substitution with a mutant traffic ATPase, HlyB^{G609D}, which binds but does not hydrolyse ATP, blocked HlyA export but did not impair HlyD oligomerization, pre-assembly of the IM HlyB–HlyD complex, substrate recruitment or substrate-induced bridging to OM TolC. (ix) In the absence of ATP hydrolysis, the HlyA substrate was trapped within the assembled export complex and appeared to accumulate in a non-stoichiometric manner. (x) Masking the HlyD C-terminus also blocked HlyA export, and likewise did not inhibit HlyD trimerization, substrate engagement or assembly of the IM precomplex or the complete IM–OM channel, suggesting inhibition of translocation channel operation.

Our data depict direct protein transfer from the cytosol across both membranes out of the Gram-negative bacterial cell by a contiguous *trans*-periplasmic export channel formed by cooperative association of IM and OM components (Figure 10). The frozen (sub)complexes identified provide a series of ‘snapshots’ that allow a sequence of events to be suggested, and this dynamic view is emphasized by the engagement and disengagement of the channel observed when a pool of substrate is allowed to translocate out of the cell (Figure 5). In depicting the interactions between the core membrane components of the type I export machinery, our data support and open further questions, for example the possible role of ATP binding by the traffic ATPase (rather than hydrolysis) and total proton-motive force (Koronakis *et al.*, 1991, 1993) in the stages of channel assembly, and the nature of the bilateral and trilateral interactions between multimeric HlyB and HlyD, and TolC. Our *in vivo* data show that the HlyB traffic ATPase and HlyD bridging protein, interacting in a preformed IM complex, both contribute to substrate recruitment. Such bilateral interaction between the type I bridging protein (‘membrane fusion protein’) and protein substrate was not observed in detergent-solubilized EDTA-washed membranes containing a heterologous type I transporter (Létoffé *et al.*, 1996), but this could reflect a lower affinity of hybrid non-cognate components, which were used to arrest the substrate protein in a non-functional transporter, or the detergent sensitivity of complexes that are not stabilized by crosslinking. Distinct interaction between the bridging protein and traffic ATPase in the absence of substrate was not assayed in these earlier experiments (Létoffé *et al.*, 1996). It is widely assumed that HlyB interacts directly with substrate protein, and this is lent support by genetic experiments (Zhang *et al.*, 1993). One simple explanation which accommodates these data is that substrate binding by each of the two IM proteins is not mutually exclusive, and that they bind to different domains of the large HlyA protein, either simultaneously or sequentially. Such possibilities are indeed explicitly left open by Létoffé and colleagues (1996).

In addition to sharing principles with the general bacterial Sec system of the cytoplasmic membrane (Wickner and Lodish, 1985; Duong *et al.*, 1997), and the type II and III protein export systems of Gram-negative bacteria (which require the action of 12 or more exporter proteins; Pugsley, 1993), the direct type I export appears to have particular similarities with protein import into the mito-

Fig. 10. Proposed stages in type I export of HlyA toxin.

chondrial matrix (Schatz and Dobberstein, 1996). The import process is also mediated by cooperative association between two complexes in the outer and inner membranes, and does not involve intermediates in the intermembrane space (Kubrich *et al.*, 1995). Matrix-targeted proteins can be arrested during transport, and translocation intermediates span both membranes and can be cross-linked to subunits of both translocation complexes. The two systems disengage in the absence of precursor, i.e. assembly of the contiguous import channel is reversible (Horst *et al.*, 1995). The early stage of passage across the mitochondrial IM is dependent on the membrane potential, promoting assembly of the IM import machinery such that it engages substrate, which in turn triggers opening

Fig. 11. Possible shuttle mode of substrate transfer.

of the IM channel and insertion of the substrate protein (Hwang *et al.*, 1989). This process of opening and closing of the channel would be followed by 'ratcheted' translocation into the matrix driven by ATP hydrolysis (Voos *et al.*, 1996). It has been suggested that mitochondrial import machines might have evolved from transporters of Gram-negative prokaryotic ancestors: in addition to the above similarities, the protein channel subunit TOM40p of the mitochondrial OM has a predicted β -sheet structure that is typical of bacterial OM pore-forming proteins like TolC (Schatz and Dobberstein, 1996).

As in the mitochondrial mechanism, the assembly of the type I channel is substrate-induced, and the IM and OM components revert to their independent state after export of the substrate. However, the accumulation of substrate in the assembled HlyB/HlyD/TolC channel seen in the absence of HlyB ATP hydrolysis suggests a modified model of export in which ATP binding and substrate engagement promote the opening of the IM entrance to the export channel, enabling the substrate to enter and trigger formation of the IM-OM bridge. Without hydrolysis of bound ATP, the (IM) entrance to the channel stays open and allows accumulation of HlyA, which is apparently unable to exit the channel on the other (OM) side. It is possible that ATP hydrolysis closes the IM entrance, so that rounds of ATP binding and hydrolysis control ('gate') opening and closing of the channel entrance. It is still further feasible that oscillation between the open and closed states at the entrance determines inversely the states of the channel exit, so that the channel would open at one end or the other, but not both, and that 'rocking' between the two states would repeatedly engage and expel substrate (Figure 11). Such a contiguous gated channel able to accumulate bulk substrate seems an attractive alternative to ATP hydrolysis supporting a ratchet mechanism in which substrate translocates domain by domain. Such a view would also be in agreement with electrophysiological experiments on the HlyB-related traffic ATPase CFTR that forms a single-membrane ion channel (Gadsby and Nairn, 1994; Gunderson and Kopito, 1994, 1995). These show that ATP binding to nucleotide-binding fold NBF2 opens the primed, closed channel, while ATP hydrolysis closes it. Whereas gating of ion channels such as CFTR results in the bulk flow of ions across a membrane, the toxin export channel of *E.coli* would in this scenario open selectively to the engaged

substrate HlyA. Once the substrate has been exported, the HlyD–TolC bridge would disengage and the IM and OM components revert to their resting state. The type I mechanism could thus be described as a substrate-responsive ‘shuttle’ transfer.

Materials and methods

Bacterial strains and recombinant plasmids

All manipulations were performed in *E. coli* BL21(DE3) (F[−] *ompT*, r_B[−]; Studier and Moffatt, 1986). BL923 is a *tolC*[−] derivative of BL21(DE3) constructed by P1 phage transduction of the mutant *tolC* gene from *E. coli* MC1061, generated by mutagenesis with *N*-methyl, *N*'-nitro, *N*-nitrosoguanidine (MNNG, Sigma). The *tolC*[−] mutant genotype of the transductant was confirmed by sensitivity to 0.05% sodium deoxycholate, loss of hemolysin export, complementation with recombinant *tolC* (Hiraga *et al.*, 1989) and TolC immunoblotting.

Construction of recombinant clones

Plasmid pACT7HlyB is the vector pACYC184 containing a *Clal*–*Sall* fragment from pARHlyB (Koronakis *et al.*, 1993) that has *hlyB* 3' of the T7 promoter. Plasmid pACT7HlyB^{G609D} has substituted a 1.1 kb *PstI*–*EcoRI* fragment of pACT7HlyB from pEK50^(G609D) (Koronakis *et al.*, 1995), the mutant pACT7HlyB^{G609D} is unable to complement the export defect of pEK50^(G609D). Plasmid pT7HisHlyD contains (His)HlyD, HlyD with an N-terminal fusion of six histidine residues and a linker sequence spanning a thrombin cleavage site, created by mutating the start codon of *hlyD* to an *NdeI* site and cloning this into the *NdeI* site of the expression vector pET15b (Novagen). Plasmid pACT7HisHlyB was created by substituting the pET15b T7 expression region located between the *Bgl*II and *Hind*III sites of plasmid pACT7HlyB. The *hlyB* coding sequence of pARHlyB was subcloned using the *NdeI* and *Bam*HI sites, resulting in the same His tag as in (His)HlyD. In pT7HisHlyD^{myc}, insertion of a 35 bp linker encoding a 10-amino-acid c-Myc epitope 9E10 (EQKLISEEDL), followed mutation of the *hlyD* stop codon to a *HpaI* restriction site. Plasmid pT7HlyD was created by converting the *hlyD* start codon into an *NdeI* recognition site by oligo-directed mutagenesis and subsequent ligation of *hlyD* on a 2.4 kb *NdeI*–*Bgl*III fragment into the T7 expression vector pAR3039 (Studier and Moffatt, 1986). Plasmid pT7HisDT7B has the genes encoding (His)HlyD and HlyB transcribed in opposite directions by two T7 promoters, achieved by ligating *Bam*HI-cut pT7HisHlyD with a 3 kb *Bgl*II–*Bam*HI fragment containing the *hlyB* and T7 promoter of pACT7HlyB. Plasmid pLGHlyA carries the *hlyA* sequence from pEK50 on a 3.3 kb *Bam*HI fragment inserted in the single *Bam*HI site of pLG339, a plasmid with a pSC101 replicon that is maintained together with pBR322- and pACYC184-based plasmids. Plasmid pACARA is pACYC184 containing the 1.3 kb *Clal*–*Sall* fragment of pBAD18 (Guzman *et al.*, 1995) spanning the multiple cloning site (mcs), the pBAD promoter and repressor gene *araC*. HlyA was ligated into the mcs to give pACARAHlyA.

In vivo protein cross-linking

E. coli BL21(DE3) cells carrying defined plasmid combinations were grown in 2× TY to A₆₀₀ 0.7, washed with cross-linking buffer (150 mM NaCl, 20 mM NaPO₄ pH 7.2), and concentrated 10-fold in the same buffer for cross-linking with 0.2 mM DSP, and 20-fold for 0.135 mM DSG. Cross-linking was carried out for 30 min, with DSP at 37°C, with DSG on ice, and was quenched with 20 mM Tris. Cells were harvested, resuspended in freeze–thaw lysis buffer (10 mM Tris–HCl pH 7.4, 1 mM EDTA) with 1 mM PMSF, and lysed by repeated freezing and thawing.

Purification of His-tagged protein complexes

Cell membranes were isolated after addition of DNase (100 µg/ml) and MgSO₄ (10 mM) by 10 min centrifugation at 16 000 g, were solubilized in 8 M urea, 10 mM Tris–HCl, 100 mM NaH₂PO₄, 1% Triton X-100, 0.2% Sarkosyl, adjusted to pH 8.0, mixed with 50 µl 50% Ni-NTA resin for each 10 ml cell, and incubated for 1 h with mixing. The resin was washed three times for 10 min, first with 8 M urea, 10 mM Tris, 100 mM NaH₂PO₄, 1% Triton X-100, pH 6.3, and then with 0.5 M NaCl, 20 mM Tris, 5 mM imidazole, 0.1% SDS, pH 8.0. Bound proteins were eluted with 8 M urea, 50 mM Tris, 2% SDS, 0.4 M imidazole, pH 6.8, and the cross-linkers were reduced with 50 mM DTT for 30 min at 37°C.

Identification of components in the export complexes

Samples were resolved on an SDS 10% polyacrylamide gel transferred to a polyvinylidene difluoride membrane (Problot, Applied Biosystems).

PAGE-separated proteins were probed with polyclonal antisera raised in rabbits immunized with (i) denatured extracellular HlyA (Koronakis *et al.*, 1989), (ii) purified TolC protein (Scottish Antibody Production Unit, Carlisle), (iii) a HlyD polypeptide comprising amino acids 88–478 expressed from a T7 plasmid and purified from inclusion bodies, and (iv) a synthetic peptide corresponding to the C-terminal 10 amino acids of HlyB. Goat-anti-rabbit antibodies conjugated to horse-radish peroxidase (Pierce) and a chemoluminescence kit (Supersignal, Pierce) were used for immunodecoration.

Protease accessibility of HlyB, HlyD and TolC

Escherichia coli BL21(DE3) cells carrying pT7HisDT7B plus or minus pACARAHlyA were grown to A₆₀₀ 0.5 and induced with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) and 0.2% L-arabinose for 30 min. Cells (5 ml) were harvested and resuspended in 0.25 ml 0.1 M Tris–acetate, pH 8.0, containing 0.5 M sucrose and 5 mM EDTA. Lysozyme was added (20 µl of 2 mg/ml), followed by 0.25 ml ice water. After incubation for 5 min on ice, MgSO₄ was added to a final concentration of 18 mM and the cells were collected by centrifugation for 45 s in a microcentrifuge. The spheroplasts were resuspended in 0.5 ml 50 mM Tris–acetate, pH 8.0, containing 0.25 M sucrose and 10 mM MgSO₄, and aliquots (50 µl) were incubated on ice for 30 min with proteinase K or trypsin. Protease treatment was terminated with an equal volume of 20% TCA solution, and the precipitate was collected by centrifugation, washed once with acetone and resuspended in protein sample buffer (8 M urea, 50 mM Tris, 2% SDS, pH 6.8). Proteins were resolved by SDS–PAGE and assayed by immunoblotting as above.

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