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Protein secretion in Gram-negative bacteria: assembly of the three components of ABC protein-mediated exporters is ordered and promoted by substrate binding

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One of the strategies used by Gram-negative bacteria to secrete proteins across the two membranes which delimit the cells, is *sec* independent and dedicated to proteins lacking an N-terminal signal peptide. It depends on ABC protein-mediated exporters, which consist of three cell envelope proteins, two inner membrane proteins, an ATPase (the ABC protein), a membrane fusion protein (MFP) and an outer membrane polypeptide. *Erwinia chrysanthemi* metalloproteases B and C and *Serratia marcescens* hemoprotein HasA are secreted by such homologous pathways and interact with the ABC protein. Using as protein substrates HasA and GST–PrtC, a chimeric protein which has a glutathione *S*-transferase moiety fused to a large C-terminal domain of protease C, we developed a simple system to identify proteins bound to the substrate based on substrate affinity-chromatography using heme– or glutathione–agarose. We show an ordered association between the protein substrates and the three exporter components: the substrate recognizes the ABC protein which interacts with the MFP which in turn binds the outer membrane component. Substrate binding is required for assembly of the three components.

Keywords: ABC exporter/affinity chromatography/membrane ATPase/multiprotein complex/protein secretion

Introduction

Protein translocation across biological membranes is a fundamental process in cell biology. Many exported proteins, in both prokaryotes and eukaryotes, are transported across a single membrane as precursors with an N-terminal signal peptide. There are however, a number of proteins which cross two membranes in a single step. Most mitochondrial proteins, for example, are encoded by nuclear genes and imported into the mitochondrial matrix passing directly through the mitochondrial outer and inner membranes at translocation contact sites between them (Glick *et al.*, 1991). Similarly, several extracellular proteins in Gram-negative bacteria lack signal peptides and cross the two membranes of the cell envelope in one step without periplasmic intermediates. One such independent pathway involves specific ATP-driven protein translocators of the ATP-binding cassette (ABC) super family. This system has been highly conserved during evolution and mediates *trans*-membrane transport of a wide variety of substrates in both prokaryotes and eukaryotes (Higgins, 1992).

ABC proteins most often consist of two membrane-embedded hydrophobic and two conserved hydrophylic ATP-binding domains. These domains can be either parts of a single polypeptide, as for example the mammalian multidrug resistance transporter (MDR) (Gottesman and Pastan, 1993) and the cystic fibrosis chloride channel (CFTR) (Riordan *et al.*, 1989), or separate polypeptides as in many prokaryotic transport systems. In several cases, the four domains of the ABC transporter, whether a single or several polypeptides, appear to be sufficient to mediate *trans*-membrane translocation of solutes as demonstrated by *in vitro* ATP-dependent transport by the purified components reconstituted into proteoliposomes. Nevertheless, most of the ABC protein-mediated transport systems from Gram-negative bacteria involve accessory proteins. For example, uptake systems require a soluble periplasmic substrate-binding protein. Similarily, ABC protein-mediated exporters involved in protein secretion invariably require two accessory envelope proteins. One is an inner membrane protein with a periplasmic domain, the C-terminus of which may interact with the outer membrane. Because of their membrane topology, these proteins are classified into the Membrane Fusion Protein family (MFP) (Dinh *et al.*, 1994). The second accessory component is an outer membrane protein (Wandersman and Delepelaire, 1990). Consequently, the whole apparatus required for protein secretion, consisting of the ABC transporter and the two accessory proteins, will herein be referred to as the ABC protein-mediated exporter.

These Gram-negative bacterial protein exporters are dedicated to the secretion of one or several closely related proteins such as toxins, proteases and lipases. The genes encoding the three secretion proteins and the exoproteins are usually all linked, consistent with the specificity of the systems. For example, the four highly homologous metalloproteases of *Erwinia chrysanthemi*, PrtA, B, C and G, are encoded by genes clustered with the three genes encoding their ABC exporter, in order the genes for PrtD, the ABC protein, PrtE, the MFP, and PrtF, the outer membrane component (Létoffé *et al.*, 1990). The *Escherichia coli* α hemolysin gene *hlyA* is adjacent to the *hlyB* and *hlyD* genes which encode the ABC protein and the MFP respectively (Mackman *et al.*, 1986). Similarily, *hasA*, the *Serratia marcescens* structural gene for an extracellular hemoprotein required for heme acquisition (Létoffé *et al.*, 1994a) is linked to *hasD* and *hasE* which encode the ABC protein and the MFP respectively. In these two last cases, the genes encoding the outer membrane components are unlinked.

Most of the exoproteins that are substrates for these exporters do not have an N-terminal signal sequence but have an uncleavable secretion sequence located in the

50 C-terminal amino acids (Mackman *et al.*, 1986; Delepelaire and Wandersman 1990; Ghigo and Wandersman 1994). These secretion sequences are specific for each system and have in some cases been shown to interact directly with the ABC protein. Thus, purified PrtD and the purified HlyB cytoplasmic domain display ATPase activity (Koronakis *et al.*, 1993) regulated by binding of the polypeptide substrate or of its C-terminal signal sequence (Delepelaire, 1994). Interaction between the ABC protein and its substrate has also been evidenced by genetic studies on hemolysin transporter (Zhang *et al.*, 1993) and on protease and HasA hybrid transporters obtained by combining components from each system (Létoffé *et al.*, 1994b; Binet and Wandersman 1995). These results emphasized the role of the ABC protein in protein secretion without clarifying the functions of the other two components.

We report a simple system based on affinity chromatography for studying the association between substrates and various exporters. The substrates used were HasA, a heme-binding protein which is retained on hemin–agarose, and GST–PrtC, a chimeric protein consisting of the glutathione *S*-transferase moiety fused to a large fragment of protease C still carrying the C-terminal secretion signal. We show that the two types of affinity resin (hemin–and glutathione–agarose) also bind the three secretion proteins via the substrates. It was thus possible to demonstrate an ordered association of the three components of ABC protein-mediated exporters, from both inner and outer membranes, and to establish that substrate binding was required for the formation of this multiprotein complex.

Results

GST–PrtC and HasA form complexes with exporter proteins (PrtD, PrtE and PrtF) during abortive secretion

The GST–PrtC fusion protein and HasA carry intact C-terminal secretion signal. Yet they were not secreted by the PrtD, PrtE and PrtF exporter and, when co-expressed with protease, they both inhibited protease secretion (Figure 1). For HasA, it has been shown that the block affects PrtD (Binet *et al.*, 1995). Probably, HasA and GST–PrtC remain attached to the membrane via PrtD, and stuck in the exporter. We therefore tried to extract them from membrane preparations and to test whether they are associated with the exporter proteins. Crude membrane pellets were prepared and solubilized in detergent from bacteria harboring plasmids expressing the *prtD*, *prtE*, *prtF* and *hasA* or *gst-prtC* genes. The HasA-producing preparation was incubated with hemin–agarose gel beads and the GST–PrtC preparation with glutathione–agarose. Bound proteins were eluted from the beads by heating in SDS sample buffer. For each chromatography, both bound and unbound proteins were separated by SDS–PAGE. Coomassie blue staining of the bound proteins revealed several proteins which were not further characterized (data not shown). PrtD, PrtE, PrtF, HasA and GST–PrtC in the fractions were each immunodetected with specific antibodies. Both HasA and GST–PrtC were retained on the corresponding affinity agarose, showing that the membrane-bound forms did not lose their biological activity after solubilization in detergent (Figure 2A and B). In both cases, the three secretion proteins were retained with the substrates on the corresponding affinity agarose. The secretion proteins were only retained in the presence of the substrates and, for HasA, could be competitively eliminated by the addition of free heme. In both the GST–PrtC and the HasA preparations, a proportion of each secretion protein did not bind to the corresponding affinity agarose though both protein substrates were in stoichiometric excess, present at concentrations sufficiently high to block completely protease secretion *in vivo*. The unbound secretion proteins could result from dissociation during solubilization. It is also possible that there was a limiting concentration of a cellular component in the assay which has not yet been identified and that PrtD was in excess due to plasmid expression as compared with this limiting component.

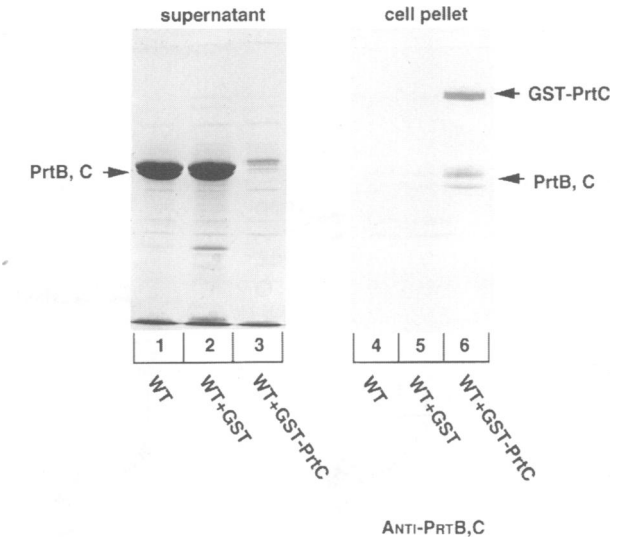

Fig. 1. Inhibition of protease secretion by the GST–PrtC chimeric fusion protein. Left: Coomassie blue stained gel of supernatants from strains carrying various plasmids; the equivalent of 1 $OD_{600\ nm}$ unit of supernatant of a late exponential growth phase culture was loaded in each lane. Lane 1, C600 (pRUW6); lane 2, C600 (pRUW6 + pGEX1); lane 3, C600 (pRUW6 + p*gst-prtC*). Right: immunoblot of cell pellets of the same cultures revealed with anti-PrtB, C antibodies. The equivalent of 0.2 $OD_{600\ nm}$ unit was loaded in each lane.

These results suggest that the entire exporter complex was extracted with detergent and remained associated with the substrates during the chromatography procedure. Furthermore, during interaction with the secretion proteins, the conformation of HasA and GST–PrtC remained compatible with binding to their respective substrate: heme or glutathione. Neither GST–PrtC nor HasA is secreted by the PrtD, PrtE and PrtF transporter to which they remained attached. Thus this experimental system allows obtention of multiprotein complexes corresponding to a sort of frozen exporter.

We further tested whether this stable association of the three secretion proteins with the substrates occurred only in the case of abortive secretion. HasA is efficiently secreted by the hybrid transporters HasD, PrtE, PrtF or HasD, HasE, TolC. We therefore tested whether these secretion proteins co-purified with HasA on hemin–agarose.

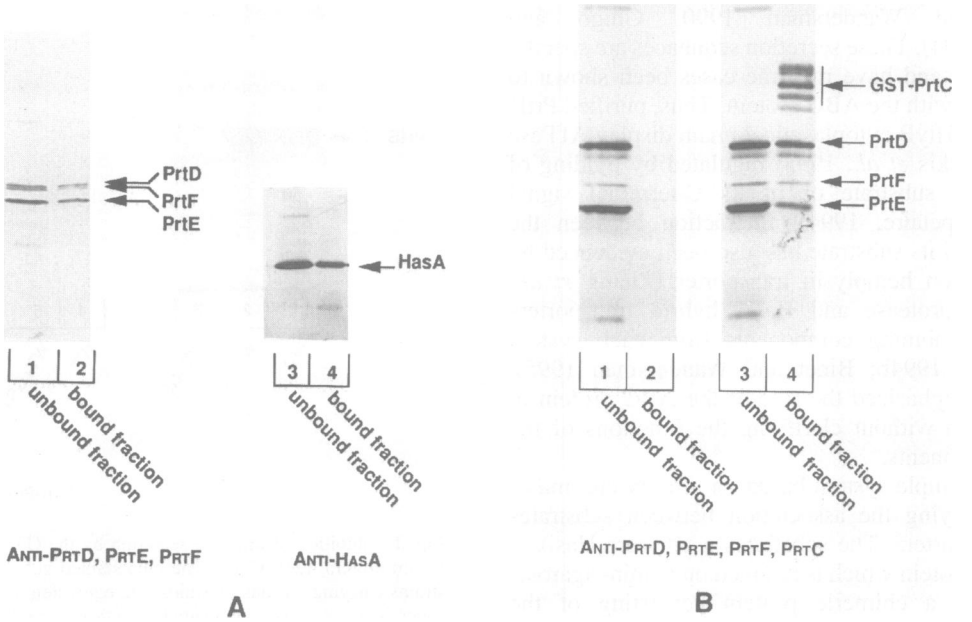

Fig. 2. Co-precipitation of transporter proteins with both HasA (A) and GST–PrtC (B) substrates on affinity resins. (**A**) Immunodetection of PrtD, PrtE, PrtF and HasA in the unbound and bound fractions after treatment of the solubilized membranes with hemin–agarose (see Materials and methods). The solubilized membranes were obtained from strain C600 (pRUW4 *-inh1*, *prtD*, *prtE*, *prtF-* + pSYC134-*hasA-*) producing PrtD, PrtE, PrtF and HasA. Fifty percent of the fraction retained on the hemin–agarose (lane 2) and 15% of the unbound fraction (lane 1) were loaded on 10% SDS–polyacrylamide gel and probed with a mixture of anti-PrtD, anti-PrtE and anti-PrtF antibodies used at 1/1000, 1/3000 and 1/2000 dilutions respectively. The same amounts (lanes 3 and 4) were loaded on to a 15% SDS–polyacrylamide gel and probed with anti-HasA antibodies used at a 1/2000 dilution. (**B**) Immunodetection of PrtD, E and F and of the GST–PrtC fusion protein in the solubilized and bound fractions after treatment of the solubilized membranes with glutathione–agarose (see Materials and methods); lanes 1 and 2, the solubilized membranes were obtained from strain C600 (pRUW4-*inh1*, *prtD*, *prtE*, *prtF-* + pGEX1-*gst-* -) producing PrtD, E and F and the GST. Lane 1, solubilized fraction; lane 2, bound fraction. Lanes 3 and 4, the solubilized membranes were obtained from strain C600 (pRUW4 + p*gst-prtC*) producing PrtD, E and F and GST–PrtC fusion protein; lane 3: solubilized fraction ; lane 4, bound fraction; in both cases the bound fraction was concentrated 15 times as compared with the solubilized fraction, loaded on to a 10% SDS–polyacrylamide gel and probed as above.

HasA forms a complex with exporter proteins (HasD, PrtE, PrtF) during secretion

Membrane proteins from bacteria harboring plasmids expressing the *hasD*, *prtE*, *prtF* and *hasA* genes were prepared as above and subjected to hemin–agarose chromatography. Immunodetection, performed with anti-HasA, anti-HasD, PrtE and PrtF antibodies, showed that the three secretion proteins were retained with HasA on the hemin–agarose (Figure 3). The same experiment was performed with membranes of cells expressing HasD, HasE, TolC and HasA; only anti-HasD and anti-TolC antibodies were used as anti-HasE antibodies were not available. Both secretion proteins were retained with HasA on hemin–agarose (Figure 3). Thus, the secretion proteins of both systems associated with the substrate during secretion.

The various combinations of secretion proteins tested were all functional, able to secrete protease and/or HasA. To ascertain whether the co-precipitation of secretion proteins with substrates reflects a working association, we tested whether non-functional exporters were able to form secretion protein complexes.

HasA forms a complex with PrtD and PrtE but not with TolC in the presence of the inactive exporter PrtD, PrtE, TolC

The hybrid exporter PrtD, PrtE, TolC is unable to secrete either protease or HasA whereas the hybrid exporter PrtD, HasE, TolC can secrete protease (Létoffé *et al.*, 1994a). Thus, the outer membrane protein TolC may interact with HasE but not with PrtE. Membrane proteins from bacteria harboring plasmids expressing the *prtD*, *prtE*, *tolC* and *hasA* genes were prepared as above and subjected to hemin–agarose chromatography. PrtD and PrtE, but not TolC, bound to HasA on hemin–agarose (Figure 4). TolC was present in the membrane preparation and recovered in the non-adsorbed fraction and was therefore not associated with the two other secretion proteins (Figure 4). Similarly, hemin–agarose chromatography of membrane proteins extracted from cells containing PrtD, HasE and TolC (a functional exporter) together with HasA led to the retention of HasA, PrtD and TolC. The involvement of TolC in the complex was therefore dependent on the nature of the MFP: HasE but not PrtE led to TolC inclusion. The MFP appears to interact directly with the outer membrane protein. The possible association of PrtD and PrtE with HasA in the absence of the outer membrane component suggests that the substrate can interact with an exporter complex lacking one component. We investigated whether the substrate could also interact with each of the secretion proteins separately or whether assembly is ordered.

Association of PrtD, PrtE and PrtF with HasA is ordered

Membrane proteins were prepared as above from bacteria harboring plasmids producing HasA together with either PrtD alone, PrtE alone, PrtF alone or the following combinations: PrtD + PrtE, PrtD + PrtF or PrtE + PrtF.

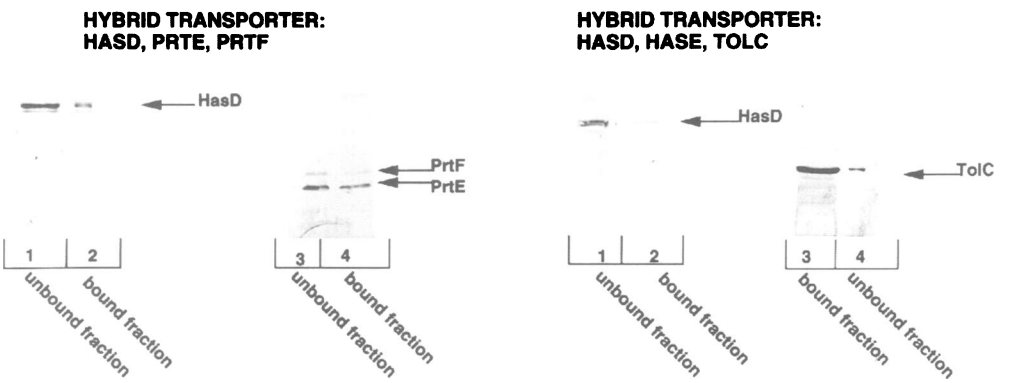

Fig. 3. Co-precipitation of transporter proteins with HasA during secretion. Left: Immunodetection of HasD, PrtE and PrtF in the unbound and bound fractions prepared as described for Figure 2A. The solubilized membranes were obtained from strain C600 (pSYC13-*hasA*, *hasD* + p*PrtEF)* producing HasA, HasD, PrtE and PrtF proteins. Immunodetection of HasA in the unbound and bound fractions (not shown) gave the same results as in Figure 2A. Fifty percent of the bound and 15% of the unbound fractions were resolved by 10% SDS–PAGE and probed with anti-HasD, PrtE and PrtF antibodies used as described for Figure 2A (anti-HasD antibodies were used at a 1/1000 dilution). Right: Immunodetection of HasD and TolC in the unbound and bound fractions prepared as in Figure 2A. The solubilized membranes were obtained from strain C600 *tolC::*Tn*5* (pSYC34-*hasA*, *hasD*, *hasE*- + p*tolC* 238) producing HasA, HasD, HasE and TolC proteins. Immunodetection of HasA in the unbound and bound fractions (not shown) was as in Figure 2A and is not shown again. Fifty percent of the bound and 15% of the unbound fractions were resolved by 10% SDS–PAGE and probed with a mixture of anti-HasD and anti-TolC antibodies used as described in Figure 2A (anti-TolC antibodies were used at a 1/1000 dilution).

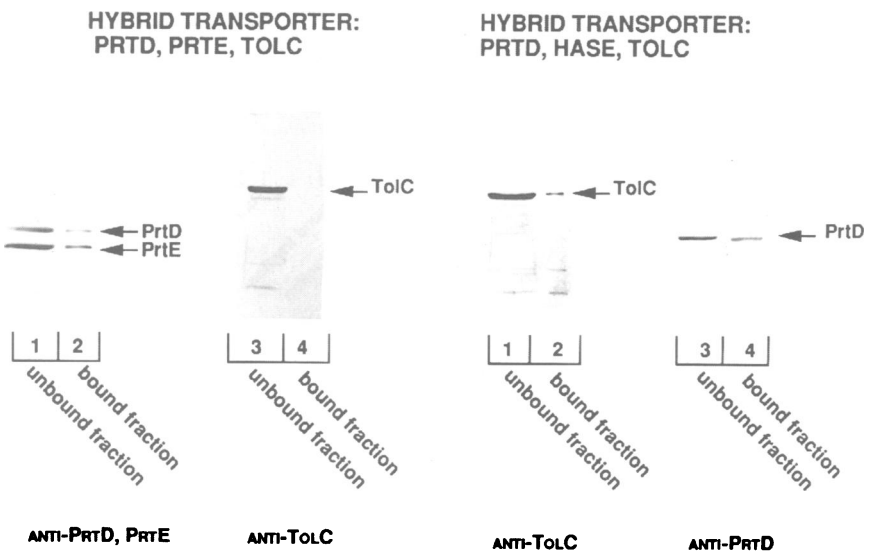

Fig. 4. Co-precipitation of various hybrid transporter proteins with HasA. The protein compositions of the two hybrid transporters are indicated above the figure. Immunodetection of HasD, PrtE and TolC in the unbound and bound fractions prepared as in Figure 2A. Left: the solubilized membranes were prepared from strain C600 *tolC::Tn5* (p*prtDE* + pSYC134 -*hasA*- + p*tolC*238) producing HasA, PrtD, PrtE and TolC. Right: the solubilized membranes were obtained from strain C600 *tolC::Tn5* (p*prtD* + pSYC21 -*hasA*, *hasE*-- + p*tolC*238). HasA in the unbound and bound fractions was as in Figure 2A. Fifty percent of the bound and 15% of the unbound fractions were resolved by 10% SDS–PAGE and probed with a mixture of anti-PrtD, PrtE and anti-TolC antibodies (left) or with anti-PrtD and anti-TolC antibodies (right).

Isolated PrtD bound HasA, but neither PrtE nor PrtF formed a complex with HasA either independently or together (Figure 5). However, in the presence of PrtD, PrtE but not PrtF, interacted with HasA. In all the experiments, the unbound proteins were recovered in the non-adsorbed fraction, excluding the possibility that the observed results were due to differences of degradation in different strains. Comparable results were obtained with the GST–PrtC fusion (not shown). These experiments show that the substrate recognizes the ABC protein first. The secretion complex formation could be either spontaneous or mediated by the binding of the substrate to the ABC protein. To discriminate between these two possibilities, we tested whether the formation of exporter protein complexes was dependent on the presence of substrate.

Association of the exporter proteins PrtD, PrtE and PrtF is promoted by the presence of the transporter substrate

To test whether the secretion proteins were associated in the absence of a substrate for secretion, membrane proteins were prepared from cells harboring a plasmid expressing the *prtD*, *prtE* and *prtF* genes. The preparation was incubated with HasA protein, purified from culture supernatants and the mixture subjected to hemin–agarose chromatography. PrtD, but not PrtE or PrtF, was retained with HasA on the affinity agarose (Figure 6). PrtE and

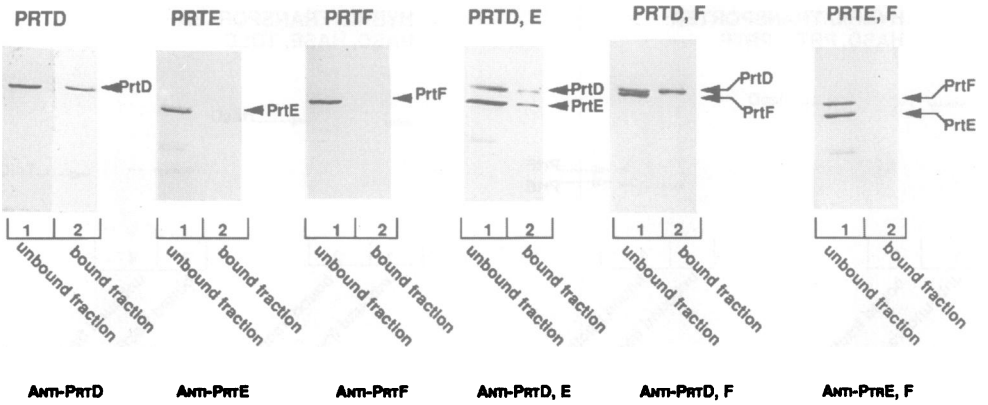

Fig. 5. Co-precipitation of incomplete transporter proteins with HasA. The protein compositions of the incomplete transporters are indicated above the figure. The solubilized membrane proteins were obtained from the following strains: PRTD, C600 (p*prtD* + pSYC134 -*hasA*-); PRTE, C600 (p*prtE* + pSYC 134 -*hasA*-); PRTF, C600 (p*prtF* + pSYC134 -*hasA*-); PRTD, E, C600 (p*prtDE* + pSYC134 -*hasA*-); PRTD, F, C600 (p*prtDF* + pSYC134 -*hasA*-); PRTE, F, C600 (p*prtEF* + pSYC134 -*hasA*-). Immunodetection of HasA in the unbound and bound fractions was as shown in Figure 2A. Fifty percent of the bound and 15% of the unbound fractions were resolved by 10% SDS–PAGE and proteins immunodetected with anti-PrtD, PrtE and PrtF antibodies or with the corresponding mixtures used as described above.

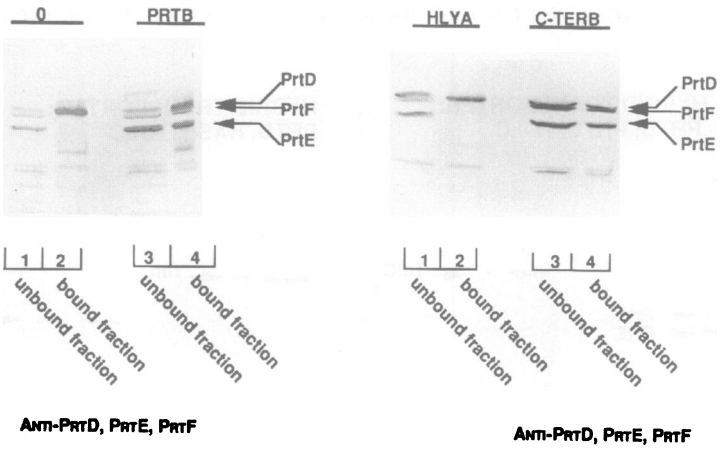

Fig. 6. Co-precipitation of transporter proteins in the presence of various transporter substrates. Transporter proteins were PrtD, PrtE and PrtF in all the experiments. Transporter substrates produced concomitantly with the transporter are indicated above the figure. The solubilized membrane proteins were obtained from the following strains: 0, C600 (pRUW4 -*inh1*, *prtD*, *prtE*, *prtF*-); PRTB, C600 (pRUW4 -*inh1*, *prtD*, *prtE*, *prtF*-) + pRUW500- *prtB*-); HLYA, C600 (pRUW4 + pHYCA-*hlyC*, *hlyA*-), C-TERB, C600 (pRUW4 *inh1*, *prtD*, *prtE*, *prtF*- + PRUW37-*lacZ-Cter-prtB*-). HasA in the unbound and bound fractions was as shown in Figure 2A and is not shown again. Fifty percent of the bound and 15% of the unbound fractions were resolved by 10% SDS–PAGE and proteins immunodetected with anti-PrtD, PrtE and PrtF antibodies or with the corresponding mixtures used as above.

PrtF were present in the membrane preparation and were recovered in the unbound fraction (Figure 6). The absence of PrtE and PrtF from the HasA–PrtD complex could have been due either to a different conformation of HasA prepared from a culture supernatant unable to promote formation of the three-protein complex, or to such a complex not forming in membranes of cells in the absence of the exporter substrates. Membrane proteins from cells harboring plasmids expressing *prtD*, *prtE* and *prtF* together with *prtB*, the structural gene for metalloprotease B, were prepared and incubated with HasA protein and passed through the hemin–agarose column. In this experiment, all three secretion proteins (PrtD, PrtE and PrtF) precipitated with HasA (Figure 6). This result shows that HasA prepared from a culture supernatant has a conformation competent for the interaction with the whole exporter. Membrane proteins from cells expressing only the exporter genes were mixed with membrane proteins of cells expressing only *hasA*. Only the ABC protein co-precipitated with HasA, confirming that the failure of the complex to form does not arise from a change in HasA properties. Similar experiments were performed with membrane proteins from cells producing PrtD, PrtE and PrtF without PrtB, incubated with membrane proteins of cells producing only GST–PrtC. Only PrtD bound to the glutathione resin (data not shown). These experiments indicate that the secretion proteins are assembled prior to incubation with HasA or GST–PrtC and that assembly in the cell envelope is promoted or interactions strengthened by the presence of the substrate, in this case, PrtB. A similar result was obtained with cells producing the C-terminal peptide of protease B instead of the complete substrate, indicating that association is driven by the secretion sequence (Figure 6). HlyA uses a different ABC transporter, is not secreted by the protease transporter and does not inhibit protease secretion. Presumably, therefore, it does not interact with the protease transporter. Membrane proteins from cells harboring one plasmid expressing *prtD*, *prtE* and *prtF* and a second expressing *hlyC* and *hlyA* were prepared and incubated as above with HasA protein. The presence of

HlyA did not cause secretion protein association (Figure 6).

Discussion

In Gram-negative bacteria, at present, three secretion mechanisms have been identified (Wandersman, 1996). One of them is a two-step process initiated by translocation across the inner membrane by a signal peptide-dependent general export pathway. Periplasmic intermediate translocation across the outer membrane requires specific transporters comprising 1–14 accessory proteins depending on the type of transporter (Pugsley, 1993). Among the two signal peptide-independent pathways which bypass the periplasm, one involves a transporter consisting of >20 secretion proteins and the second, the ABC protein-mediated exporter, involves three proteins located in both membranes. The lack of periplasmic intermediate has led to the assumption that the secretion proteins can form a multiprotein complex carrying the substrate from the cytoplasm to the extracellular medium. Previous experimental evidence for interaction between proteins belonging to the two Gram-negative membranes has proceeded from genetic and *in vivo* crosslinking experiments on the TonB system (Skare *et al.*, 1993).

Using HasA and GST–PrtC (a chimeric protein which has a glutathione *S*-transferase moiety fused to a large C-terminal domain of protease C) as protein substrates, we developed a simple system based on substrate affinity-chromatography using heme– or glutathione–agarose to show an ordered association between the protein substrates and the three exporter components. The three secretion proteins only bound to the resins in the presence of the corresponding substrate, indicating the binding specificity. This is the first biochemical evidence on affinity between proteins forming an ABC protein-mediated exporter. Several types of natural or hybrid transporters are available which are active for both HasA and protease, for protease only or for neither of these substrates. Using an exporter able to secrete HasA, such (HasD, PrtE and PrtF) multiprotein complexes were demonstrated, showing that the association between HasA and the exporter is both stable enough and has a long enough half-life to be isolated. Hybrid transporters unable to promote HasA or protease secretion were considered as non-functional although the defective step is unknown. One such hybrid, PrtD, PrtE, TolC, did not form the three-component complex. Instead, only the two inner membrane components co-precipitated with HasA. TolC remained in the unbound fraction. This was not due to some inherent property of TolC since it could be co-precipitated with HasA in other transporters such as HasD, HasE and TolC or PrtD, HasE and TolC. These experiments clearly establish that the exporter–protein association is specific and whether an outer membrane protein, TolC in this case, is retained on the resin depends on the nature of the MFP component. A direct interaction between the transporter elements is also suggested by experiments showing that secretion protein interaction is ordered: the substrate recognizes the ABC protein, which interacts with the MFP, which in turn binds the outer membrane component. This does not rule out the possibility that the substrate interacts with these two proteins, either transiently or after successive modifications mediated by the ABC protein and the MFP. It is also possible that secondary, direct interactions with MFP and OMP occur only during secretion.

We also show that the substrate plays a critical role in the secretion protein association. HasA protein purified from extracellular medium, added to solubilized membrane proteins from cells containing the exporter, but no substrate, was co-retained on hemin–agarose only with the ABC protein. In a similar experiment performed with solubilized membrane proteins from cells producing the secretion proteins together with one of the substrates, such as protease B or the C-terminal peptide of protease B, the three secretion proteins all bound to the resin. The presence of both substrate and exporter is thus necessary for the formation of a complex prior to solubilization. Induction of multiprotein complex formation by the substrate is very likely initiated by substrate binding to the ABC protein, which may cause either a chemical or a conformational change of the ABC protein, allowing it to bind to the MFP, or may stabilize a pre-existing loose association between the secretion proteins or prevent their dissociation. We have previously observed that cells producing the three secretion proteins of various ABC protein-mediated exporters without concomittent protein secretion display a higher drug sensitivity, suggesting that the secretion proteins may interact even in the absence of substrate (Wandersman and Létoffé, 1993). Such a role of the peptide ligand has been observed for the SDS stabilization of MHC class II molecules and also for the assembly of class I heavy chains with β2 microglobulin (Townsend *et al.*, 1989; Denzin *et al.*, 1995). In conclusion, we report biochemical evidence for interaction between proteins belonging to the two bacterial Gram-negative membranes and the involvement of substrates in this association.

Materials and methods

Bacterial strains, plasmids, growth conditions

Escherichia coli strain C600 (F⁻ *thr leu fhuA lacY thi supE*) and TG1 [*thi supE* Δ (*lac-proAB*)/F′*traD36 proAB lacI*^{q} *ZΔM15*] are from our laboratory collection. The parental *tolC*::Tn*5* was strain GC7442 from the collection of T.Ogura (Kumamoto University Medical School, Kumamoto, Japan) and C600 *tolC*::Tn*5* was made by phage P1 transduction from GC7442 into C600 recipient strain. The nature of the vector, the antibiotic resistance and the foreign genes inserted in the various vectors are indicated in brackets. Plasmids: pRUW4 (pACYC184, CAM, *-prtD, prtE, prtF, inh1-*); pRUW6 (pACYC184, SPC, *-inh, prtD, prtE, prtF, prtB, prtC-*); pSYC34 (pUC18, AMP, *-hasA, hasD, hasE-*); pSYC134 (pUC18, AMP, *-hasA-*) pSYC13 (pUC18, AMP, *-hasA, hasD-*); pSYC21 (pUC18, AMP, *-hasA, hasE-*), pRUW500 (pUC18, AMP, *-prtB-*); pRUW37 (pUC18, AMP, *-lacZ-Cter-prtB-*); pSF4000 (pACYC184, CAM, *-hlyC, hlyA, hlyB, hlyD-*). The plasmids p*prtD*; p*prtE*, p*prtF*, p*prtDE*, p*prtEF*, p*prtDF*, are all derivatives of pACYC184 and were described in Binet *et al.* (1995). pAX629 (pACYC184, CAM, *tolC*) was described previously (Wandersman *et al.*, 1990). A 1.5 kb DNA fragment carrying *tolC* from pAX629 was inserted into pAM238 to give p*tolC*238. A 6.5 kb DNA fragment from pSF4000 carrying the *hlyC* and *hlyA* genes was inserted into pBGS18 to give pHYCA. Plasmid p*gst-prtC* was constructed as follows: a 1.35 kb *Sma*I–*Eco*RI DNA fragment including the 410 3′ codons of PrtC was ligated into pGEX-1 digested with *Sma*I and *Eco*RI, yielding a chimeric *gst-prtC* gene encoding a chimeric protein of 70 kDa. In the absence of IPTG (minimal expression), the presence within the same cell of this chimeric protein together with a plasmid (pRUW6) allowing the synthesis and secretion of the proteases B and C led to 95% inhibition of protease B and C secretion to the supernatant as compared with the control. With increasing concentrations of IPTG, the inhibition became stronger, up to 99.5% at 50 μM IPTG; conversely, both the PrtB and C and the GST–PrtC fusion protein were found in the cell pellet (Figure 1).

For the GST–HasD fusion protein, a 573 bp *Eco*RV fragment from pSYC34 carrying the last 181 codons of HasD was ligated with pGEX3X digested with *Sma*I, yielding a chimeric *GST-HasD* gene coding for a chimeric GST–HasD protein of 45 kDa.

All media and antibiotics were used as described previously (Miller, 1992).

Extraction, manipulation of plasmids and in vitro cloning

Isolation of plasmids, transformation of *E.coli*, ligation with T4 DNA ligase, agarose electrophoresis of DNA and DNA fragment purification were as described in Sambrook *et al.* (1989).

Isolation of total membranes and membrane protein solubilization

One hundred millilitre cell cultures of *E.coli* bearing various plasmids were grown at 37°C in LB medium and were harvested at an $OD_{600\ nm}$ of 0.4–0.6. The samples were centrifuged for 15 min at 5000 *g* at 4°C and the cell pellet was washed once with 10 ml of 100 mM Tris–HCl pH 8, EDTA 1 mM (TE) and resuspended in 20 ml of the same buffer. Cells were broken in a French press operated at 10 000 p.s.i. A crude membrane pellet was collected by centrifugation (60 min, 50 000 *g* at 4°C). The pellet was washed once in TE and resuspended in TE at ~250 OD units/ml. Membrane proteins were solubilized by incubation for 1 h at 4°C in 60 mM Tris–HCl, pH 8.0, 0.6 mM EDTA, 20% glycerol, 5 mM $MgCl_2$, 100 mM NaCl, 1% lauryl maltoside. Insoluble material was removed by centrifugation in an Eppendorf centrifuge for 1 h. The supernatant, containing the solubilized membrane proteins, was kept frozen at –80°C until use. All plasmids carrying the various combinations of genes encoding PrtD, PrtE and PrtF were derivatives of pACYC184 and they all produced similar amounts of the secretion proteins as determined by immunodetection with anti-PrtD, anti-PrtE and anti-PrtF antibodies (data not shown). The *hasA* gene was on a pUC derivative under the *plac* promoter. Protease B and the C-terminal signal of protease B (C-terB) were also encoded by pUC derivatives. The genes encoding the transporter substrates HasA, PrtB, C-terB and HlyA were on multi-copy plasmids and were therefore fully induced even in the absence of IPTG in strain C600. The *gst-prtC* gene was on a plasmid also carrying $lacI^q$ and was not induced by IPTG for affinity purification of the transporter proteins since almost complete inhibition of secretion was already observed in the absence of IPTG. All experiments involving TolC as outer membrane component were performed in strain C600 *tolC*::Tn*5* with a pAM238 derivative plasmid carrying *tolC* under the *plac* promoter to give large amounts of intracellular TolC, comparable with the amounts of PrtF produced from pRUW4. In experiments involving HasD alone or HasD and HasE, the proteins were encoded by the plasmid encoding HasA, a pUC derivative, under the *plac* promoter. The amounts of HasD produced from the various constructs were similar as determined by immunodetection with anti-HasD antibodies. Amounts of HasE produced could not easily be tested since the anti-HasE antibodies are not available. Hemolysin was encoded by a pBGS derivative under the *plac* promoter, also carrying the gene encoding HlyC, which is required for hemolysin fatty acylation (Hughes *et al.*, 1992).

Membrane protein affinity chromatography on hemin–agarose and glutathione–agarose

Hemin insolubilized on cross-linked 4% beaded agarose, containing 7.7 µmol of ligand per ml, was washed three times in 2 M NaCl, 50 mM Tris–HCl pH 8 and was then resuspended in buffer A (50 mM Tris–HCl pH 8, 0.5 mM EDTA, 5 mM $MgCl_2$, 20% glycerol, 0.1% lauryl maltoside). Washed beads were first serially diluted to determine the hemin–agarose concentration which does not allow a non-specific PrtD fixation in the absence of HasA. Mixtures containing 200 µl of solubilized membrane preparation and 100 µl of the appropriate hemin–agarose diluted preparation were incubated for 1 h at 4°C on a wheel and then centrifuged for 5 min at 5000 *g*. The supernatants corresponding to the unbound fraction were collected and proteins resolved by SDS–PAGE. The pellets were washed five times in 1 ml of buffer A and then resuspended in 50 µl of SDS sample buffer in the presence of protease inhibitors (1 mM EDTA, 1 mM PMSF) and boiled for 5 min. The agarose beads were pelleted at 5000 *g* for 5 min and the eluted proteins were resolved by SDS–PAGE. The same protocol was used for glutathione–agarose chromatography, except that the solubilized membranes were used directly for affinity purification and protease inhibitors were omitted from the last step.

HasA preparation from culture supernatant

HasA protein produced in *E.coli* was prepared from 1 l of an overnight culture of TG1 (pSYC34) grown in LB medium. The culture supernatant was precipitated with ammonium sulfate (80% saturation). This preparation contained almost pure HasA protein at a concentration of ~2 mg per ml estimated by Coomassie Blue staining after SDS–PAGE.

Incubation of HasA protein with membrane protein preparation followed by hemin affinity chromatography

Two hundred microliters of membrane protein prepared as above were mixed with 20 µg of HasA protein diluted in 20% glycerol, 1% lauryl maltoside and incubated at 4°C for 1 h. The mixture was then added to 100 µl of hemin–agarose prepared as above. Subsequent steps were as described above.

Anti-HasD antibodies preparation

The GST–HasD chimeric fusion protein, which was strongly induced by IPTG (100 µM), was insoluble and formed inclusion bodies which were easily harvested by low speed centrifugation. The chimeric protein was then purified by two cycles of preparative gel electrophoresis, electroeluted and injected into rabbits to raise antibodies. The antibodies were used in Western blots at a 1/1000 dilution.

Protein analysis and immunodetection technique

SDS–PAGE was carried out as described by Laemmli (1970). Immunodetection was performed as previously described (Delepelaire *et al.*, 1991).

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