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Selective extracellular release of cholera toxin B subunit by *Escherichia coli*: dissection of *Neisseria* Iga_β-mediated outer membrane transport

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The C-terminal domain (Iga_β) of the *Neisseria* IgA protease precursor is involved in the transport of covalently attached proteins across the outer membrane of Gram-negative bacteria. We investigated outer membrane transport in *Escherichia coli* using fusion proteins consisting of an N-terminal signal sequence for inner membrane transport, the *Vibrio cholerae* toxin B subunit (CtxB) as a passenger and Iga_β. The process probably involves two distinct steps: (i) integration of Iga_β into the outer membrane and (ii) translocation of the passenger across the membrane. The outer membrane integrated part of Iga_β is the C-terminal 30 kDa core, which serves as a translocator for both the passenger and the linking region situated between the passenger and Iga_β core. The completeness of the translocation is demonstrated by the extracellular release of the passenger protein owing to the action of the *E.coli* outer membrane OmpT protease. Translocation of the CtxB moiety occurs efficiently under conditions preventing intramolecular disulphide bond formation. In contrast, if disulphide bond formation in the periplasm proceeds, then translocation halts after the export of the linking region. In this situation transmembrane intermediates are generated which give rise to characteristic fragments resulting from rapid proteolytic degradation of the periplasmically trapped portion. Based on the identification of translocation intermediates we propose that the polypeptide chain of the passenger passes in a linear fashion across the bacterial outer membrane.

Key words: Gram-negative/protein transport/secretion/translocation intermediates

Introduction

Secretion of proteins into the extracellular environment of Gram-negative bacteria requires passage across a cell envelope consisting of two membranes, the inner and the outer membranes, separated by the periplasmic space (Kellenberger, 1990). The *Neisseria* and *Haemophilus* IgA proteases have N-terminal signal sequences (Pohlner *et al.*, 1987; Poulsen *et al.*, 1989) that specify their transport across the cytoplasmic membrane, probably using the Sec-dependent pathway (for review see Schatz and Beckwith, 1990). Outer membrane translocation of the IgA proteases is achieved by a transport function contained in the C-terminal Iga_β domain previously called 'helper' (Pohlner *et al.*, 1987). Iga_β integrates into the outer membrane and is assumed to form a translocation pore for covalently attached protein domains.

The IgA protease of *Neisseria gonorrhoeae* MS11 has been extensively studied (Halter *et al.*, 1984, 1989; Pohlner *et al.*, 1987). The precursor protein encoded by the *iga* gene of this strain has a molecular mass of 169 kDa and is composed of several distinct domains, including the signal sequence, the protease domain (Iga_P), the α-domain (Iga_α) and the C-terminal 45 kDa comprising Iga_β. During inner membrane transport the signal sequence is removed and Iga_β subsequently assembles into the outer membrane to direct extracellular transport of the attached domains. The release and maturation of the exported polypeptide chain is accomplished by sequential autoproteolytic cleavage at three internal cleavage sites, a, b and c, generating several extracellular products. Iga_β remains in the outer membrane as the β^{45} protein.

Extracellular transport, autoproteolytic release and maturation of IgA protease and the accompanying products also occur in recombinant *Escherichia coli* and *Salmonella* species. Thus additional secretion functions do not appear to be required for Iga_β-mediated outer membrane translocation (Meyer *et al.*, 1987). This unique feature of the IgA protease secretion system contrasts with the export requirements of other exoproteins of Gram-negative bacteria, which need additional transport proteins (Howard and Buckley, 1983; d'Enfert *et al.*, 1987; Hirst and Holmgren, 1987; Wandersman and Delepelaire, 1990).

Previously we reported that translational fusion with Iga_β of strain *N.gonorrhoeae* MS11 is sufficient to mediate extracellular transport of cholera toxin B subunit (CtxB) to the cell surface of *Salmonella typhimurium* (Klauser *et al.*, 1990), whereas CtxB by itself is restricted to the periplasm in enterobacteria (Pearson and Mekalanos, 1982). Due to the lack of IgA protease activity in the respective CtxB – Iga_β hybrids, the recombinant bacteria displayed the CtxB moiety on the cell surface. Furthermore, it was shown that transport to the cell surface of the covalently linked CtxB was influenced by the conformational state of the polypeptide. Transport of CtxB across the outer membrane was blocked by intramolecular disulphide bridges and appeared to proceed only under reducing conditions, which favour an unfolded state of CtxB. This requirement for 'export competence' is consistent with other currently investigated preprotein translocation models (Randall and Hardy, 1986; Eilers and Schatz, 1986; Sanz and Meyer, 1988).

Here we present studies in *E.coli* on the Iga_β-mediated extracellular transport of covalently attached CtxB showing that the integration of Iga_β into the outer membrane is independent of the translocation of the passenger polypeptide across the membrane. A major tool facilitating this analysis was the specific proteolytic activity of the outer membrane protease OmpT, which releases the CtxB moiety into the exterior of the cell by specific cleavage within Iga_β. Our

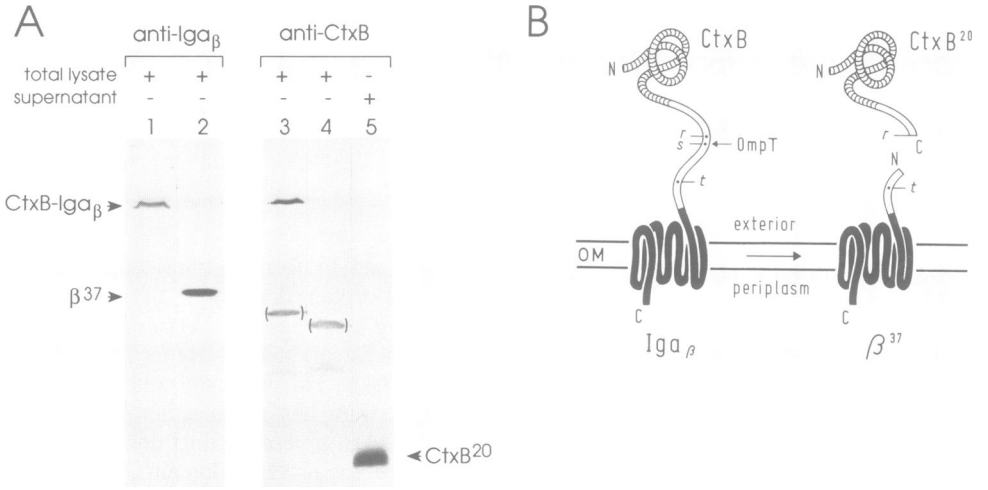

Fig. 1. OmpT-dependent processing of CtxB−Iga$_\beta$ hybrids and extracellular release of CtxB. (**A**) Total cellular proteins and culture supernatants were probed by immunoblot analysis with anti-Iga$_\beta$ monoclonal antibody HAL-2 and anti-CtxB serum AK55. Lanes 1 and 3, lysates of *S.typhimurium* G30(pTK49); lanes 2 and 4, lysates of *E.coli* GC1(pTK49); lane 5, culture supernatant of GC1(pTK49). The arrows denote the CtxB−Iga$_\beta$ protein B49 and the OmpT proteolysis products β^{37} and CtxB^{20}. Samples originated from liquid cultures supplemented with 5 mM 2-mercaptoethanol. Proteins that are present in the non-recombinant host strains and that cross-react with serum anti-Fp42 are in parentheses. (**B**) Schematic representation of OmpT-dependent extracellular release of CtxB^{20}. The diagram on the left illustrates the presumed topology of a CtxB−Iga$_\beta$ hybrid molecule in the outer membrane (OM) after extracellular transport of the CtxB moiety. The surface-displayed CtxB (dashed) is connected to the membrane-integral portion (black) via the linking region (white) of Iga$_\beta$, which contains the OmpT cleavage sites r, s and t. The membrane-integral portion is likely to form a 'β-barrel' in the outer membrane, consisting of anti-parallel, amphiphilic β-strands. The diagram on the right illustrates the situation after cleavage by OmpT at site s. The Iga$_\beta$-derived cleavage product β^{37} remains integrated in the outer membrane while the 20 kDa conjugate, CtxB^{20}, consisting of the CtxB moiety and the N-terminal 8 kDa of Iga$_\beta$, is released into the culture supernatant.

data strongly suggest that the second step (translocation) is a folding-sensitive linear process: conditions permitting only limited translocation of the polypeptide chain lead to the formation of transmembrane intermediates. Iga$_\beta$ represents a one-component protein translocator with ideal features for investigations on the mechanism and the requirements of polypeptide chain translocation across biological membranes.

Results

OmpT-directed extracellular release of CtxB

We previously engineered hybrid proteins consisting of an N-terminal signal sequence, the CtxB moiety and the *Neisseria* Iga$_\beta$ domain necessary for outer membrane translocation (Klauser *et al.*, 1990). Other than the removal of the N-terminal signal peptide these CtxB−Iga$_\beta$ fusions maintain full size when produced in *S.typhimurium* G30 (Figure 1A, lanes 1 and 3). In contrast, expression in *E.coli* cells results in additional processing of CtxB−Iga$_\beta$ fusions yielding a cell-associated cleavage product termed β^{37}. This cleavage product corresponds to the C-terminal 37 kDa portion of Iga$_\beta$ specifically recognized by the monoclonal antibody HAL-2 (lane 2). A 20 kDa protein (CtxB^{20}), corresponding to the N-terminal CtxB polypeptide attached to a small N-terminal portion of Iga$_\beta$ (Figure 1B), appears in the culture medium (Figure 1A, lane 5) but is not detectable in total lysates (lane 4).

The β^{37} and CtxB^{20} cleavage products arise from the proteolytic activity of the *E.coli* outer membrane protease OmpT (Grodberg and Dunn, 1988). As demonstrated by the immunoblot analysis of total lysates of the *ompT* mutant strains UT4400(pTK62) and UT5600(pTK62), the CtxB−Iga$_\beta$ hybrid protein B62 (Figure 4) remains stable and no OmpT-dependent processing products appear (Figure 2A, lanes 3 and 4). In contrast, the *ompT*$^+$ control strains GC1(pTK61) and UT2300(pTK62) produce the protein β^{37} (lanes 1 and 2). This clearly demonstrates that proteolysis of CtxB−Iga$_\beta$ hybrids was mediated by OmpT causing the release of the CtxB conjugate (CtxB^{20}) into the medium (see Figure 1B).

According to the results obtained with recombinant *S.typhimurium* G30 (Klauser *et al.*, 1990) the CtxB moiety attached to Iga$_\beta$ should be displayed on the surface of the *E.coli ompT* cells. Whole cells and culture supernatants of strains UT5600(pTK62) and UT2300(pTK62) were tested for surface exposure of CtxB and the presence of soluble CtxB^{20} in the supernatant. In a whole-cell ELISA (Van der Ley *et al.*, 1985) the *ompT* strain UT5600(pTK62) gave a strong signal, suggesting that the CtxB moiety was exposed at the cell surface (Figure 2B). The signal obtained from the supernatant was no higher than the background level, suggesting that no CtxB^{20} was released from the cells. In contrast, the *ompT*$^+$ strain UT2300(pTK62) gave only a weak cell-associated signal but elicited a strong extracellular signal (Figure 2B).

To determine the proteolytic site, which was apparently located within Iga$_\beta$, the β^{37} cleavage product derived from the hybrid protein B61 was recovered from *E.coli* GC1(pTK61) cells and subjected to N-terminal sequencing. We found that the β^{37} protein band actually consisted of two protein species of slightly different size, which under certain experimental conditions migrated as distinct protein bands in SDS−PAGE (β^{37} and β^{37*}; Figure 2A, lane 1). Analysis of the N-terminus of the lower band (β^{37}) revealed the sequence Arg-Ala-Ile-Ser-Ser-Glu, demonstrating that Iga$_\beta$ is cleaved at the peptide bond Arg−Arg1161 (see Pohlner *et al.*, 1987), termed site s (Figure 1B). These data confirmed that the OmpT protease of *E.coli*, which is

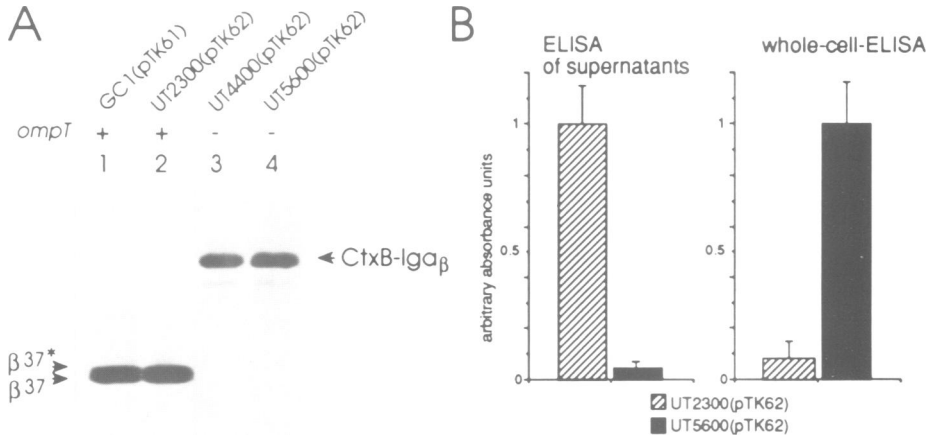

Fig. 2. Effect of the *E.coli ompT* genotype. (**A**) Total cellular proteins of recombinant *E.coli ompT*$^+$ and *ompT* cells producing the CtxB−Iga_β fusions B61 and B62 were reacted by immunoblot analysis with the Iga_β-specific monoclonal antibody HAL-2. The proteins CtxB−Iga_β, β^{37} and β^{37*} are marked with arrowheads. β^{37} evolves from cleavage of Iga_β between Arg−Arg1161 (site s) and β^{37*} presumably from cleavage between Arg−Arg1148 (site r). (**B**) Extracellular CtxB was detected in an ELISA using culture supernatants as a source of antigen. Cell surface located CtxB was detected by whole-cell ELISA. In both cases anti-CtxB serum AK55 was used. Results are presented as arbitrary absorbance units derived by setting the highest mean value for strain UT2300(pTK62) in the ELISA of supernatants and UT5600(pTK62) in the whole-cell ELISA to 1 U. The other mean values are presented as proportions of one arbitrary unit. Standard deviations reflect the variations in several independent experiments. Extracellular and cell-associated signals cannot be compared directly.

reported to cleave between paired basic amino acid residues in proteins (Sugimura and Nishihara, 1988), causes the cleavage of Iga_β. The formation of the slightly larger protein (β^{37*} cleaved at site r) can probably be attributed to cleavage between residues Arg−Arg1148 of Iga_β. However, since β^{37*} was not sequenced, proteolysis by OmpT between the adjacent paired residues Lys−Ser1157 or Arg−Asn1159 cannot be ruled out.

Topological features of Iga_β

The immunoblot analysis of GC1(pTK61) lysates using the polyclonal serum anti-Fp42 revealed a further Iga_β-specific protein band with a molecular mass of 32 kDa (Figure 3A), which is not recognized by antibody HAL-2 (Figure 1A, lane 2). This cleavage product, termed β^{32}, is absent in *ompT* strains (Figure 7A, lanes 1 and 3), showing that OmpT cleaves Iga_β at a third site (t) as well as at sites r and s. N-terminal sequence analysis of the β^{32} protein, isolated from a membrane preparation of UT2300(pTK61) revealed the sequence Ala-Ala-Gln-Pro-Arg. Site t is thus defined by residues Arg−Ala1203. Proteolysis by OmpT between Lys−Gln and Lys−Ala pairs has been described (Teo, 1987). Site t must be utilized less efficiently than sites r and s, otherwise β^{37} would be quickly converted into β^{32} and would therefore be undetectable. Conversion seems to progress slowly during bacterial growth, as β^{32} is more abundant in late exponential cultures (data not shown).

The epitope for the monoclonal antibody HAL-2 resides between amino acid residues Arg1161 and Arg1202 of Iga_β (sites s and t; Figures 1 and 3). Immunofluorescence analysis with HAL-2 antibody of *ompT* cells expressing the B49 fusion suggests that the 5 kDa region between sites s and t is exposed at the cell surface (data not shown). This is confirmed by the accessibility of this region to externally added trypsin in physiologically intact GC1(pTK61) cells. Protein β^{37} is entirely converted into the tryptic product β^{32} (Figure 3A, lane 2). Since there is no visible size difference between the β^{32} proteins present in untreated and trypsinized bacteria (lanes 1 and 2), OmpT and trypsin

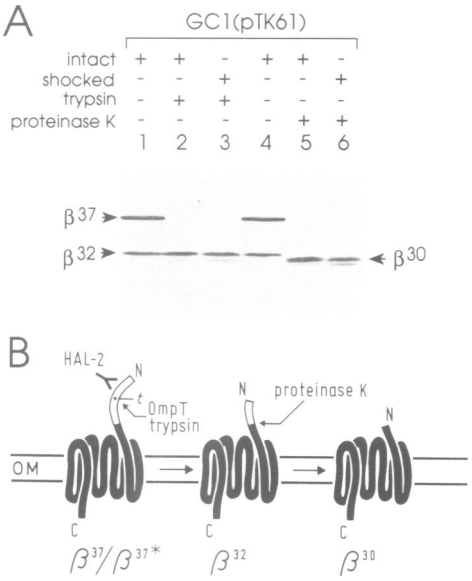

Fig. 3. Accessibility of Iga_β to externally added proteases. (**A**) Total cellular proteins from protease-treated *E.coli* GC1(pTK61) cells were reacted by immunoblot analysis with the Iga_β-specific serum anti-Fp42. The proteolytic products of Iga_β, β^{37}, β^{32} and β^{30} are marked with arrowheads. (**B**) Schematic representation of Iga_β-derived cleavage products. The diagram on the left specifies the 37 kDa proteins β^{37*} and β^{37}, generated by OmpT proteolysis at cleavage sites r and s, respectively. β^{37*} and β^{37} carry the epitope for the monoclonal antibody HAL-2. The diagram in the middle shows the β^{32} protein, generated by OmpT proteolysis at site t (Arg−Ala1203). As indicated, β^{32} is also evolved by cleavage with externally added trypsin. The diagram on the right shows the β^{30} protein, which evolves by incubation of the bacteria with proteinase K. The resistance to proteases of broad specificity like proteinase K indicates that β^{30} is the membrane-integral portion of Iga_β.

probably both utilize cleavage site t. The fact that cleavage site t is fully accessible to trypsin under conditions that allow the protease to gain access to the cell surface but not to the periplasmic space (Klauser *et al.*, 1990) is consistent with

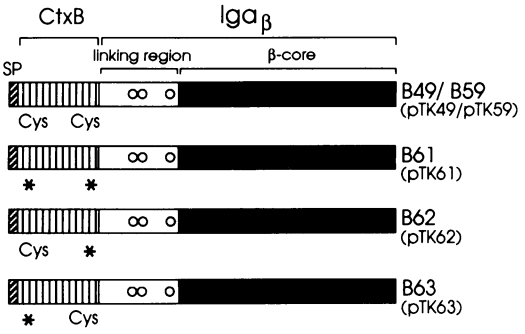

Fig. 4. Schematic representation of CtxB−Iga$_\beta$ hybrid proteins. The hybrids consist of a 21 amino acid signal peptide (SP), the CtxB domain (Lockman and Kaper, 1983) and the 45 kDa Iga$_\beta$ domain (Val1097−Phe1505) of the IgA protease precursor of *N.gonorrhoeae* MS11. Iga$_\beta$ is further dissected into linking region and β-core. The positions of the OmpT cleavage sites r (Arg−Arg1148), s (Arg−Arg1161) and t (Arg−Ala1203) are indicated (open circles). The hybrids differ with respect to residues Cys9, Ala10, Cys86 and Trp88 of the CtxB moiety: proteins B49 and B59 still contain these residues; protein B61 carries the exchanges Cys9Leu, Ala10Glu, Cys86Gly and Trp88Gly; B62 carries the exchanges Cys86Gly and Trp88Gly; and B63 carries the exchanges Cys9Leu and Ala10Glu. The short joining region between CtxB and Iga$_\beta$ (Klauser *et al.*, 1990) is the same for the protein pairs B61 and B62 and B59 and B63. Amino acid exchanges were introduced by PCR directed, site-specific mutagenesis. Plasmids encoding the respective hybrids are in parentheses.

the idea that *E.coli* OmpT protease activity is surface oriented (Grodberg and Dunn, 1988).

The β^{32} tryptic cleavage product was almost completely resistant to further degradation by trypsin in shocked cells (Figure 3A, lane 3). However, treatment of both intact and shocked cells with proteinase K produced a group of slightly smaller cleavage products (β^{30}) with a molecular mass of ~30 kDa (lanes 4 and 5), indicating that β^{30} is the portion of Iga$_\beta$ that lies within the membrane. The further degradation to β^{30} is explained by the broader specificity of proteinase K, which removes additional amino acids from the exposed N-terminus (Figure 3B), whereas trypsin is restricted to cleavage at basic amino acids.

In conclusion, the 45 kDa Iga$_\beta$ domain, which is defined as the autoproteolytic cleavage product detectable in the outer membrane of its authentic host (Pohlner *et al.*, 1987), can be divided into two domains, the protease-resistant, membrane-integral 'core' specified by β^{30} and an N-terminal, protease-sensitive, cell surface-exposed portion, the 'linking region', which joins the passenger protein with the membrane-embedded β^{30} core.

Membrane assembly of Iga$_\beta$ is independent of CtxB translocation

From previous studies it appeared that the formation of intramolecular disulphide bonds between Cys9 and Cys86 in the periplasm produced a CtxB conformation that could not be transported. To assess whether this conformational constraint similarly affects the extracellular release of CtxB^{20} from recombinant *E.coli* GC1 cells, the secretion of fusion proteins B49, B59, B61, B62 and B63, which differ with respect to the number of cysteines present in the CtxB moieties (see legend to Figure 4 and Klauser *et al.*, 1990), was studied in more detail. *E.coli* GC1 recombinants, each producing one of these proteins, were grown in liquid culture under non-reducing and reducing conditions. The culture

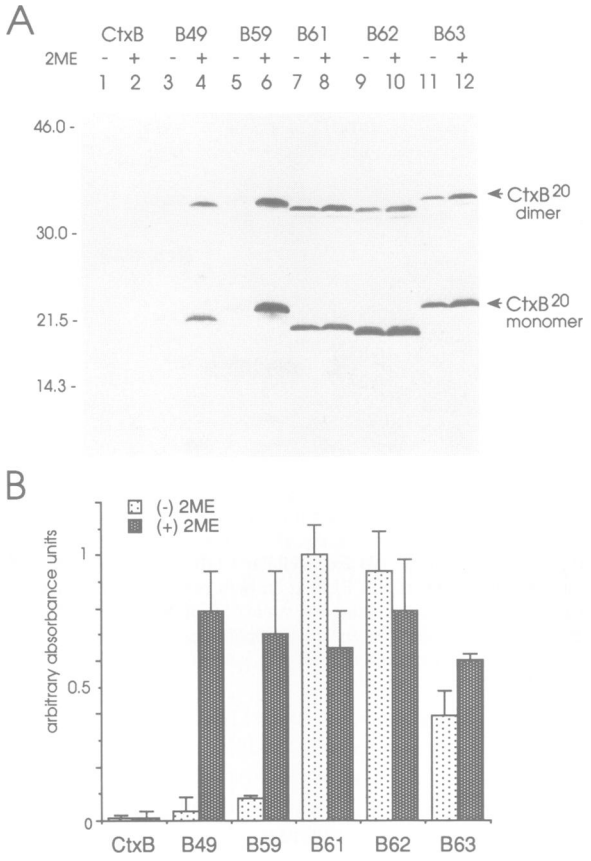

Fig. 5. Influence of export-competence on extracellular secretion of CtxB^{20}. (**A**) Culture supernatants were probed by immunoblot analysis with anti-CtxB serum AK55. Supernatants were prepared from *E.coli* GC1 cells producing the proteins CtxB (encoded by plasmid pTK2; Klauser *et al.*, 1990), B49, B59, B61, B62 and B63 grown in the absence (−) or presence (+) of 5 mM 2-mercaptoethanol (2ME). Extracellular CtxB^{20} tends to form oligomers even after heating the samples in reducing SDS sample buffer, as indicated by the presence of dimers. Monomers and dimers are indicated with arrowheads. The size variations of the monomers and dimers probably correspond to variations in the length of the joining region between CtxB and Iga$_\beta$ (Klauser *et al.*, 1990). (**B**) Relative quantification of extracellular CtxB products by ELISA of culture supernatants using anti-CtxB serum AK55. Recombinant *E.coli* GC1 cells, producing the proteins as described in panel A, were used. Results are presented as arbitrary absorbance units. The arbitrary scale was derived by setting the highest mean value of strain GC1(pTK61), grown without 2ME, to 1 U. The other mean values are presented as proportions of one arbitrary unit. Standard deviations reflect variations in the results of several independent experiments.

supernatants were prepared and analysed by immunoblotting (Figure 5A) and ELISA (Figure 5B) using CtxB-specific serum. CtxB^{20} proteins derived from fusions B49 and B59 were released into the culture medium only under reducing conditions [Figure 5A (lanes 4 and 6) and B] and were not released under non-reducing growth conditions [Figure 5A (lanes 3 and 5) and B]. This dependence on 2-mercapto-ethanol for CtxB^{20} extracellular secretion was not observed for proteins B61, B62 and B63 (lanes 7−12). Since these proteins lack either one or both cysteine residues, they are incapable of forming intramolecular disulphide bonds, so translocation of the CtxB moiety to the cell surface proceeds independently of the culture conditions. The authentic CtxB protein produced by strain GC1(pTK2) was secreted into the periplasm (data not shown) but did not appear in the

extracellular medium under either reducing or non-reducing growth conditions [Figure 5A (lanes 1 and 2) and B].

To test whether cleavage of Iga$_\beta$ by OmpT also occurs when the CtxB moiety is retained at the periplasmic side of the outer membrane, the total lysates of *E.coli* GC1 recombinants were analysed by immunoblotting. Regardless of whether or not CtxB was competent for translocation across the outer membrane, CtxB−Iga$_\beta$ was cleaved by OmpT as indicated by the appearance of β^{37} (Figure 6A), demonstrating that it was correctly assembled in the outer membrane. This result suggests that the assembly of Iga$_\beta$ into the outer membrane is a separate process and that it is distinct from the translocation of the attached CtxB polypeptide chain (Figure 6B).

Folding-induced translocation arrest of CtxB is released by intrinsic periplasmic proteases

The translocation of the protein attached to the Iga$_\beta$ moiety probably occurs by linear movement of the polypeptide chain across the membrane. Such a continuous process is indicated by the fact that translocation is conformation-dependent and that the assembly of Iga$_\beta$ into the membrane and the appearance of CtxB at the exterior of the cell can be uncoupled. If the mechanism of translocation is indeed a linear movement one would expect that transport proceeds until the whole protein is exported or until a transport-incompatible structure within the translocating chain blocks the process. In the latter case, membrane spanning translocation intermediates should be formed that are partially extracellular and partially intracellular.

Since CtxB forms intramolecular disulphide bonds that appear to arrest translocation, one would expect to recover full-length translocation intermediates under *ompT* conditions. However, non-translocated CtxB fused to Iga$_\beta$ appeared to be unstable (Figure 6, lanes 3 and 5), indicating that non-translocated portions of CtxB are rapidly degraded by periplasmic proteases. The resulting degradation products would be remnants of translocation intermediates with a size equivalent to the membrane-integral β^{30} region plus the linking region to the site of the translocation block, i.e. the disulphide bond between Cys9 and Cys86 that prevents almost the complete CtxB from translocation. In the case of the B59 fusion (Figure 4) this would be a predicted size of ~45 kDa.

The experiment suggesting linear translocation was performed using *E.coli* and *S.typhimurium* strains producing proteins B59 and B63 (Figure 4). The strains were cultured under non-reducing conditions and total lysates of the cells were prepared and analysed by immunoblotting. As Figure 7A illustrates, there is substantially less full-length B59 (lane 3) than control protein B63 (lane 1). The residual amount of full-length B59 could be explained if a proportion of molecules had been translocated before disulphide bonds were formed. In contrast to B59, the control protein B63 was efficiently translocated, presumably due to its inability to form intramolecular disulphide bridges (Figure 4). More importantly, as postulated above, a cluster of Iga$_\beta$-specific proteolytic cleavage products of a narrow molecular weight range is seen in cells producing B59 (Figure 7A, lane 3, asterisk), but not in cells producing B63 (lane 1). These products migrate at ~46−50 kDa and thus lack most of the CtxB moiety (Figure 7B, diagram on the right). To characterize the degradation products and to confirm that

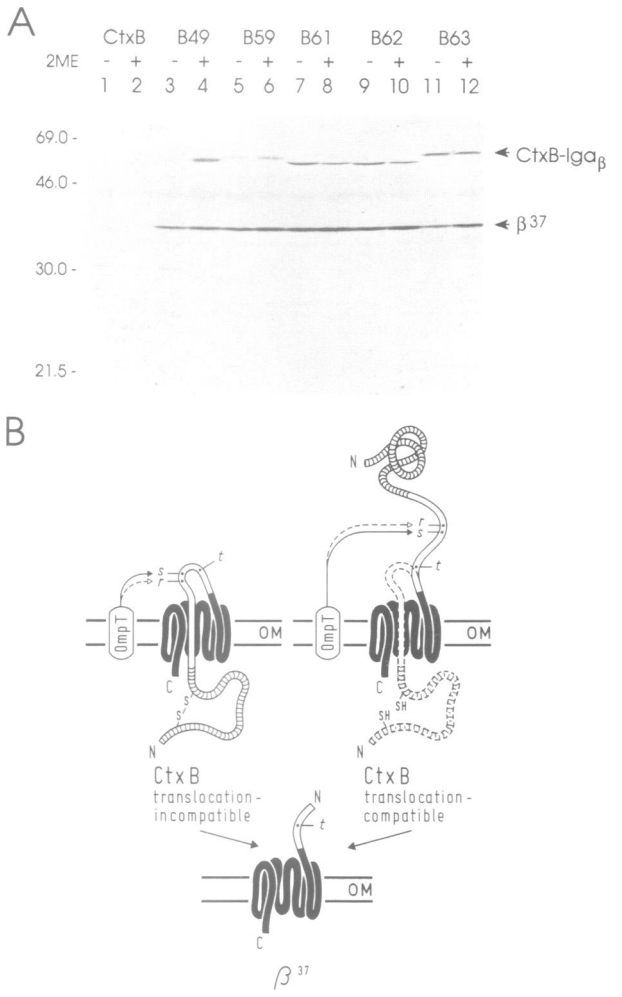

Fig. 6. Dissection of Iga$_\beta$-mediated extracellular transport into two steps. (**A**) Total cellular proteins from recombinant *E.coli* were probed by immunoblot analysis reacted with Iga$_\beta$-specific monoclonal antibody HAL-2. The lysates were prepared from liquid cultures of *E.coli* GC1 grown under non-reducing (−2ME) and reducing conditions (+2ME). The GC1 recombinants (*ompT*$^+$) produce the proteins CtxB, B49, B59, B61, B62 and B63. Note that the CtxB−Iga$_\beta$ hybrid bands are visible in addition to the cleavage product β^{37}, a fact that can be attributed to incomplete proteolysis by OmpT. The hybrid bands are less abundant in cells derived from non-reducing cultures: this only concerns the proteins B49 and B59, which are capable of forming intramolecular disulphide bonds (lanes 3 and 5). (**B**) The diagram illustrates that the production of β^{37} (bottom) by OmpT proteolysis occurs irrespective of whether or not CtxB is competent for translocation across the outer membrane (top). Left diagram (top): CtxB is retained at the periplasmic face of the outer membrane by the presence of an intramolecular disulphide bond. The linking region containing the OmpT cleavage sites r, s and t is competent for transport to the cell surface, where it is accessible to OmpT activity. Right diagram (top): OmpT proteolysis also occurs when the CtxB is competent for translocation. Since β^{37} forms irrespective of CtxB translocation it is concluded that Iga$_\beta$-meditated extracellular transport is a two-step process. Step I is the integration into the outer membrane. Step II is the translocation of the passenger.

the linking region and a small C-terminal portion of CtxB have escaped periplasmic degradation by translocation, B59-producing cells were incubated with IgA protease: as shown in Figure 7A the remaining CtxB portions of the translocation intermediates are specifically cleaved from Iga$_\beta$ at the engineered IgA protease cleavage site (Klauser *et al.*, 1990) to leave $\beta^{45\#}$.

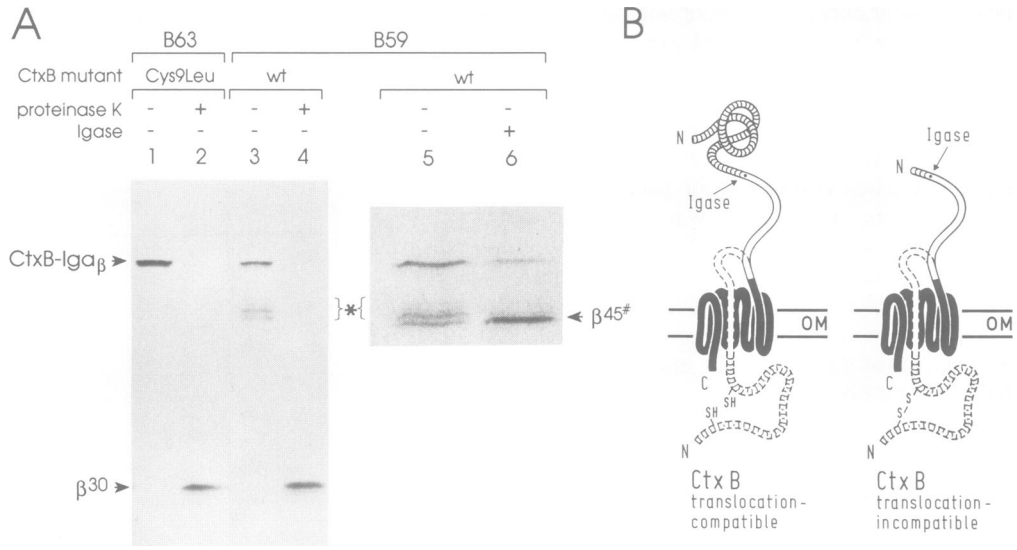

Fig. 7. Detection of translocation intermediates. **(A)** Total cellular proteins of recombinant *E.coli* and *S.typhimurium* were probed by immunoblot analysis using serum anti-Fp42. Lysates were prepared from B59- and B63-producing cells of strains UT5600 (lanes 1–4) and G30 (lanes 5 and 6) grown in liquid culture at 37°C under non-reducing conditions (without 2-mercaptoethanol). Protein B59 harbours wild-type CtxB (wt) and B63 carries the amino acid exchange Cys9Leu within CtxB. As indicated, the cells were treated with proteinase K (lanes 2 and 4) or purified IgA protease (Igase, lane 6) prior to preparation of the lysates. The protein bands, CtxB–Iga$_\beta$, β^{30} and $\beta^{45\#}$ (= Igase proteolysis product including Iga$_\beta$ from Val1097–Phe1505 plus seven amino acids at the N-terminus, Klauser *et al.*, 1990), are marked with arrowheads. The protein bands (asterisked) specify remnants of translocation intermediates, which result from degradation of the non-translocated CtxB moiety by periplasmic proteases. **(B)** Schematic representation of the Iga$_\beta$-mediated linear polypeptide chain translocation. The diagram on the left illustrates that CtxB that is in a translocation-compatible form (i.e. has its cysteine residues reduced) escapes periplasmic degradation by being translocated to the protease-free cell surface. This leads to the formation of the full-length CtxB–Iga$_\beta$ hybrid. The diagram on the right shows that CtxB that is translocation-incompatible because of an intramolecular disulphide bond is retained at the periplasmic face of the outer membrane and is susceptible to degradation by proteases. Periplasmic degradation forms cleavage products containing the complete Iga$_\beta$ (β-core plus linking region) and short portions of the CtxB C-terminus. These can be removed by incubation with IgA protease (Igase). The positition of the artificially introduced Igase cleavage site is indicated.

To establish that the degradation products were correctly inserted into the outer membrane (Figure 7B), physiologically intact cells were treated with proteinase K and analysed for the production of β^{30}. Proteinase K treatment of either B59- or B63-producing cells led to the formation of an equal amount of β^{30}, showing that not only the full-length hybrids but also the degradation products of B59 were correctly inserted in the membrane (Figure 7A, lanes 2 and 4). Furthermore, IgA protease and proteinase K treatment of physiologically intact cells, which gave rise to the formation of proteins $\beta^{45\#}$ and β^{30} respectively, demonstrated that the linking region was exposed on the cell surface.

Discussion

We have begun to characterize the mechanism by which the Iga$_\beta$ domain of the *Neisseria* IgA protease precursor transports covalently attached proteins across the outer membrane of a Gram-negative bacterium. In the *in vivo* model system we employed CtxB–Iga$_\beta$ fusion proteins with *E.coli* as host. Our data indicate that the transport of the CtxB passenger across the outer membrane is a two-step event consisting of (i) membrane assembly of the 'translocator' domain and (ii) translocation of the passenger. The studies revealed translocation intermediates, which led us to assume a linear movement of the unfolded polypeptide chain of the passenger protein across the membrane.

In contrast to other prokaryotic and eukaryotic protein translocation systems presently under investigation, the Iga$_\beta$-dependent transport system seems to represent a simple version of a membrane-bound translocator consisting of a single protein. No accessory secretion function for Iga$_\beta$ has been found. The transport system is not restricted to IgA protease, and several other heterologous polypeptides besides CtxB have been successfully transported on to the *E.coli* cell surface (our unpublished results). Iga$_\beta$ fusion proteins require an N-terminal leader peptide to ensure their transport across the inner bacterial membrane probably via the Sec-dependent pathway. Subsequent translocation of the passenger across the outer membrane is directed by the 30 kDa C-terminal core region, which becomes the membrane-integral part of Iga$_\beta$ in the outer membrane. The translocation process may result in (i) the exposition of the passenger protein on the bacterial surface, as observed in *S.typhimurium* (Klauser *et al.*, 1990) and *E.coli ompT* strains, or (ii) the release of the passenger into the medium. Extracellular release may either be due to autoproteolysis as used by the authentic IgA protease system (Pohlner *et al.*, 1987), or catalysed *in trans* by other proteases, such as OmpT located in the outer membrane (Figures 1 and 6). With regard to the latter alternative, host-dependent processing has also been shown to be involved in the extracellular release of the *Serratia marcescens* serine protease by recombinant *E.coli* (Miyazaki *et al.*, 1989).

Principally two different mechanisms can be postulated for the transport of protease or heterologous passenger proteins across the outer membrane: a two-step process, as initially proposed by Pohlner *et al.* (1987), whereby membrane assembly of Iga$_\beta$ precedes the translocation of the attached polypeptide chain, or a one-step transport process intrinsically coupled to the assembly of Iga$_\beta$ into the

outer membrane. Our data clearly support the first alternative because stable integration of Iga_β into the outer membrane occurs irrespective of the translocation-competence of the passenger, as evidenced by β^{37} formation due to intrinsic OmpT protease activity (Figure 6) and protease resistance of the β-core region (Figure 3). Furthermore, a single-step process would inevitably require large passenger proteins to be in a compact form when they cross the membrane since translocation of a long unfolded polypeptide chain otherwise could not be an immediate event. This implication, however, contrasts with the observed dependence of Iga_β-directed translocation on an unfolded passenger conformation: the folding state of the CtxB passenger is stabilized by an intramolecular disulphide bridge between the two cysteine residues and leads to a blockage in translocation (Figures 5 and 7).

The requirement for an unfolded state appears to be a common feature of translocation systems involving preproteins. Stabilization of a folded structure has been shown to arrest the import of preproteins into mitochondria (Schleyer and Neupert, 1985; Eilers and Schatz, 1986; Rassow *et al.*, 1989) and preprotein transfer across the cytoplasmic membrane of *E.coli* (Randall and Hardy, 1986; Tani *et al.*, 1989). However, some degree of folding seems to be compatible with translocation. Thus a preprotein evidently containing secondary and tertiary structure (Lecker *et al.*, 1990), a branched polypeptide (Vestweber and Schatz, 1988) and a preprotein containing a disulphide-constrained loop of 13 amino acids (Tani *et al.*, 1990) were competent for translocation across their respective model membranes. In this context it is interesting that the secreted portion (P121) of the authentic Iga precursor consists of ~1000 amino acids of which only two are cysteine residues. The cysteines are 11 amino acids apart (Pohlner *et al.*, 1987) and thus probably incapable of stabilizing a bulky tertiary structure that might interfere with translocation. It would be interesting to determine systematically the maximal size of a cysteine bond-induced loop that is tolerated for Iga_β-mediated outer membrane transport.

Mechanistic parallels may be drawn between Iga_β-mediated translocation and the general transport pathway of proteins across the bacterial inner membrane. *In vitro* studies of proOmpA import into inner membrane vesicles containing the *sec* gene products revealed that preprotein translocation proceeded through a series of intermediates. The process was controlled by the presence of ATP and the electrochemical potential. These intermediates were detected as OmpA cleavage fragments in the lumen of the vesicles, which were protected from degradation by externally added proteases (Schiebel *et al.*, 1991). Consistent with this finding we were able to demonstrate the existence of outer membrane translocation intermediates. Intramolecular disulphide bond formation in the passenger domain of CtxB fusions caused blockage of outer membrane transport and the generation of translocation intermediates. The periplasmic segments of these intermediates were, however, prone to degradation by periplasmic proteases. As a result, stable fragments of the CtxB−Iga_β fusions of the predicted size were formed in OmpT negative strains (Figure 7A). This observation strongly supports the idea of a linear movement of the unfolded passenger.

It appears that Iga_β encompasses several distinct functions required for the translocation of preproteins across biological membranes, and usually contributed by separate protein entities. Recent studies on the import of preproteins into the lumen of the endoplasmic reticulum and the mitochondrial matrix, and export into the periplasm of *E.coli* demonstrated the requirement for soluble as well as peripheral and membrane-integral receptors. These are involved in (i) the recognition of transport signals (Bernstein *et al.*, 1989; Lill *et al.*, 1990), (ii) the targeting to the membrane (Hartl *et al.*, 1990; Connolly and Gilmore, 1989), (iii) the initial integration into the membrane (Stuart *et al.*, 1990; Schiebel *et al.*, 1991) and (iv) the translocation across the membrane (Krieg *et al.*, 1989; Bieker and Silhavy, 1990; Vestweber *et al.*, 1989). For the Iga_β-mediated outer membrane transport, the signal recognition function of the passenger protein is substituted by the covalent linkage to Iga_β. Iga_β itself, which in structural terms resembles a typical outer membrane protein (Vogel and Jähnig, 1986; Struyvé *et al.*, 1991), targets the whole fusion to the outer membrane. The initial membrane integration of the passenger can be attributed to the configuration of Iga_β in the outer membrane: because the N-terminal end of the membrane-integral β-core is located at the surface (Figure 3), this must also be true of the adjacent passenger (and linking) segment (Figure 7). With respect to these three functions, Iga_β can be considered as a specialized form of a 'general translocation system'. While the general transport functions are reusable for multiple translocations, Iga_β, due to its specialization, only serves for a single translocation event.

An open question concerns the energy source serving to translocate the Iga_β-fused protein across the outer membrane. For preprotein translocation across eukaryotic membranes and the inner bacterial membrane, both ATP and an electrochemical potential are probably required (for review see Eilers and Schatz, 1988). Since no ATP consuming process has ever been reported to occur in the periplasm, ATP hydrolysis cannot be considered as a driving force for Iga_β-mediated outer membrane translocation. Recent data on proOmpA import into *E.coli* inner membrane vesicles suggest that the movement of the polypeptide chain could be driven at a later stage by the electrochemical potential across the membrane without ATP hydrolysis (Schiebel *et al.*, 1991). Analysis of extracellular aerolysin secretion in *Aeromonas salmonicida* indicated that an electrochemical gradient across the outer membrane exists that may drive translocation (Wong and Buckley, 1987). It is, however, questionable whether such gradients could be maintained in the outer membrane of *E.coli*, which is known to harbour hole-forming porins (Sen *et al.*, 1988). Instead one might assume that the energy for the Iga_β-mediated outer membrane translocation results from the hydration and proper folding of the passenger polypeptide as soon as it reaches the bacterial cell surface. Both the low concentration of free water in the periplasm (Brass *et al.*, 1986) and the proposed linear movement of Iga_β-mediated translocation are consistent with this theory.

Another open question is whether Iga_β forms a true pore for the translocation of the peptide chain. Secondary structure predictions show that the Iga_β core might form a β-barrel through which a peptide could be transported (T.Klauser, J.Pohlner, J.Krämer, K.Otzelberger, F.Jahnig and T.F.Meyer, in preparation). Alternatively, the fused passenger protein may cross the outer membrane at the interphase between Iga_β and the lipid bilayer. Whatever route is taken by the passenger, the Iga_β-directed outer membrane transport constitutes an attractive one-component

system, which provides insight into the mechanism of protein translocation across biological membranes.

Materials and methods

Bacterial strains, media and growth conditions

E.coli strain GC1 ($K12_{r-m+}$ MDU; Meyer *et al.*, 1982) was from our laboratory collection. *E.coli* strains UT2300, UT4400 and UT5600 (Earhart *et al.*, 1979) were kindly provided by J.Grodberg and J.J.Dunn. *S.typhimurium* G30A (Osborn *et al.*, 1964) was from the collection of P.A.Manning. Cells were cultured in LB at 37°C. Reducing growth conditions were achieved by supplementing the medium with 5 mM 2-mercaptoethanol (Klauser *et al.*, 1990).

Plasmid construction

The construction of plasmids pTK2, pTK49, pTK59 and pTK61 has been described previously by Klauser *et al.* (1990). Plasmid pTK2 encodes CtxB including the signal sequence (Lockman and Kaper, 1983) and plasmids pTK49, pTK59 and pTK61 encode the CtxB−Iga_β hybrids B49, B59 and B61 respectively (Figure 4 and Klauser *et al.*, 1990). Plasmid pTK62, encoding B62, was constructed by ligating the *NdeI*−*SacII* vector fragment of plasmid pTK59 to a mutated *ctxB* gene contained on a 0.31 kb *NdeI*−*SacII* fragment. This fragment was synthesized by the polymerase chain reaction (Saiki *et al.*, 1988) using plasmid pTK2 as the template and oligonucleotides TK017 (5'-TATCTTCAGCATATGCACAT-3') and TK016 (Klauser *et al.*, 1990) as primers. Plasmid pTK63, encoding B63, was constructed from the 0.49 kb *NdeI*−*EcoRI* PCR fragment, introduced into the *NdeI*−*EcoRI* vector fragment of plasmid pTK59. For amplification, plasmid pTK59 served as the template and oligonucleotides JO024 (5'-TCCAGCGCATCCAAGGGG-3') and TK015 (Klauser *et al.*, 1990) were used as primers.

Preparation of bacterial lysates and culture supernatants

Cells from 1 ml liquid culture (A_{600} = 1.0−1.2) were suspended in 0.9 ml PBS (140 mM NaCl, 20 mM potassium phosphate pH 7.5) and precipitated with 100 μl 100% TCA (trichloroacetic acid) for 30 min at 0°C. The precipitate was collected by centrifugation, washed with 80% ethanol, dried and resuspended in 100 μl SDS sample buffer. After boiling, samples corresponding to 50 μl liquid culture were analysed by SDS−PAGE and immunoblotting. Supernatants were prepared from liquid cultures by centrifugation, and filtered through 0.45 μm filter cartridges (Schleicher & Schuell, Inc.). Extracellular proteins were precipitated with TCA (10% final concentration, 0°C, 1 h). Precipitates were washed with 80% ethanol, dried and dissolved in SDS sample buffer. After boiling, samples corresponding to 2 ml of culture supernatant were subjected to SDS−PAGE and immunoblotting.

ELISA techniques

The whole-cell ELISA was performed as described by Van der Ley *et al.* (1985) with modifications (Klauser *et al.*, 1990). For ELISA of culture supernatants, plates (Nunc Immuno F) were coated with culture supernatant preparations either undiluted or in serial dilutions in PBS at 4°C overnight. Wells were blocked with 3% BSA in PBS for 1 h at 37°C and, after washing, anti-CtxB serum AK55 (1:200) was added and incubated for 1 h at 37°C. Incubation (30 min at 37°C) with alkaline phosphatase-conjugated protein A (1:2000) and the substrate *p*-nitrophenyl-phosphate (1 mg/ml), dissolved in 0.1% ethanolamine pH 10.3, followed. Absorbance was measured at 405 nm using an ELISA reader (SLT-Lab instruments). Washing steps in between the incubations were performed with PBS-Tween (PBS supplemented with 0.05% Tween-20). Antibody dilutions were made in PBS without detergent.

Immunoblotting

The procedure was performed as described previously (Klauser *et al.*, 1990) except that transfer buffer I (200 mM glycine, 25 mM Tris−HCl pH 8.3, 20% methanol, 0.1% SDS) was used for electroblotting. The anti-Iga_β antibody HAL-2 was used in a 1:1000 dilution and the polyclonal rabbit antisera anti-Fp42 (Halter *et al.*, 1989) and the anti-CtxB serum AK55 were used in 1:200 dilutions. Alkaline phosphatase-conjugated goat anti-mouse IgG or protein A were used in 2000-fold dilutions.

Preparation of proteins β^{37} and β^{32} for N-terminal sequencing

Total cell membranes were prepared from an overnight culture of GC1(pTK61) grown in 1 l LB at 37°C. After harvesting and washing in 0.9% NaCl, the cells were lysed by three freeze−thaw steps in liquid nitrogen with sonification steps in between. Undisrupted cells were removed by low-speed centrifugation. Membranes were collected by ultracentrifugation (Beckman TL.100), washed and resuspended in water. Aliquots were mixed with SDS sample buffer, seperated by SDS−PAGE and electroblotted onto a polyvinylidene difluoride filter (Matsudeira, 1987) using transfer buffer II (20 mM Tris−HCl pH 8.3, 20% methanol, 0.1% SDS). The filter was subsequently stained with solution I (0.275% Coomassie Brilliant Blue R250, 50% methanol, 10% acetate) and destained with solution II (50% methanol, 10% acetate). The bands corresponding to the proteins β^{37} and β^{32} were excised and the filter pieces rinsed in water. The dried filters were subjected to automated N-terminal sequencing (120A Analyzer, Applied Biosystems).

Protease treatment of cells

The procedure was performed as described previously (Klauser *et al.*, 1990) with the following modifications. PBS was used as the incubation buffer. Trypsin, proteinase K and IgA protease were used at final concentrations of 0.5 mg/ml, 0.1 mg/ml and 5 μg/ml respectively. Proteolysis was terminated using TCA precipitation as described above (preparation of bacterial lysates and culture supernatants) and 1 mM phenylmethylsulphonyl fluoride was added to inhibit further proteolysis.

Materials

DNA modifying enzymes were from Boehringer, Pharmacia and Biolabs. *Taq* polymerase was from Perkin Elmer. All other chemicals or reagents were from Serva, Sigma, Roth and Merck.

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