Journal of Bioenergetics and Biomembranes, Vol. 22, No. 3, 1990

MINI-REVIEW

The Mechanism of Secretion of Hemolysin and Other Polypeptides from Gram-Negative Bacteria

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Received December 6, 1989

Abstract

In the secretion of polypeptides from Gram-negative bacteria, the outer membrane constitutes a specific barrier which has to be circumvented. In the majority of systems, secretion is a two-step process, with initial export to the periplasm involving an N-terminal signal sequence. Transport across the outer membrane then involves a variable number of ancillary polypeptides including both periplasmic and outer membrane. While such ancillary proteins are probably specific for each secreted protein, the mechanism of movement across the outer membrane is unknown. In contrast to these systems, secretion of the *E. coli* hemolysin (HlyA) has several distinctive features. These include a novel targeting signal located within the last 50 or so C-terminal amino acids, the absence of any periplasmic intermediates in transfer, and a specific membrane-bound translocator, HlyB, with important mammalian homologues such as P-glycoprotein (Mdr) and the cystic fibrosis protein. In this review we discuss the nature of the HlyA targeting signal, the structure and function of HlyB, and the probability that HlyA is secreted directly to the medium through a trans-envelope complex composed of HlyB and HlyD.

Key Words: C-terminal signal sequence; hemolysin; *E. coli* secretion; HlyA, HlyB translocator; allocrites.

Introduction

In this review we shall follow the convention that the specific transport of a polypeptide to the external medium from a *Gram-negative* bacterium constitutes the process of *secretion*, while translocation of polypeptides across

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the cytoplasmic membrane to the periplasm will be termed simply *export*. In this review we shall also confine our discussion to those polypeptides which are secreted to the medium during normal growth. We shall not therefore cover the mechanism of colicin secretion, a process normally associated with changes in overall permeability of the outer membrane and frequently eventual lysis of the producing cells. For a review of the special mechanism of colicin release, the reader is referred to Pugsley (1988).

The envelope of Gram-negative bacteria has a well-defined structure: a classical protein lipid bilayer constituting the inner or cytoplasmic membrane, and an outer membrane composed of an unusual asymmetric bilayer, unique to these organisms, separated by a narrow aqueous compartment, the periplasm. Within the periplasm the peptidoglycan constitutes the shape-determining component of the cell, forming a network of individual glycan chains with an average 2-nm spacing and occasional cross bridges (Labischinski *et al.*, 1985). All available evidence indicates that the peptidoglycan layer presents no barrier to the transport of either small or large molecules, including polypeptides. As described by several contributors elsewhere in this volume, the vast majority of outer membrane and periplasmic proteins are targeted to their locations via N-terminal signal sequences through the *secA-secY* export pathway. This may be achieved by direct transport across the inner membrane followed, in the case of outer membrane proteins, by subsequent "spontaneous" assembly into the surface membrane (Baker *et al.*, 1987). Alternatively, it has been proposed (Smit and Nikaido, 1978) that outer membrane proteins may reach the surface without a periplasmic intermediate via regions of adhesion between the inner and outer membranes.

Considerable evidence now exists that such adhesion sites, or Bayer patches, exist in *E. coli*. This evidence includes electron microscopy (Bayer, 1979), the identification of "intermediate" density envelope fractions in equilibrium density centrifugation studies (Bayer *et al.*, 1982), and the enrichment of particular polypeptides in such intermediate density fractions (Bayer *et al.*, 1987; Bourdineaud *et al.*, 1989). As discussed below, such adhesion sites could also provide for direct secretion of polypeptides to the medium. We emphasize, however, that in contrast to the adhesion sites between inner and outer membranes of mitochondria, the structure and any specific role in protein transport for such sites in *E. coli* remain to be established.

Secretion of Polypeptides from Gram-Negative Bacteria Other Than *E. coli*

Some of the pathways of secretion of polypeptides from Gram-negative bacteria are illustrated in Fig. 1. In the great majority of cases proteins are

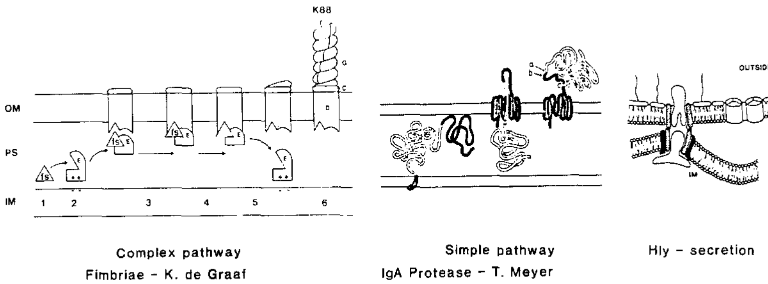

Fig. 1 Different strategies for protein secretion from Gram-negative bacteria. The majority of mechanisms involve initial transport to the periplasm utilizing an N-terminal signal sequence which is removed by signal peptidase, followed by diverse mechanisms for translocation across the outer membrane. The exception is exemplified by hemolysin which utilizes a novel C-terminal secretion signal and a specific membrane translocator which transports hemolysin directly to the medium. Other details in the text.

initially exported to the periplasm utilizing the SecA-SecY pathway and a cleavable N-terminal signal sequence. The problem in essence therefore is: What is the nature of protein transport across the outer membrane from the periplasm? As described below, this almost invariably involves the requirement for additional specific polypeptides located in the periplasm or outer membrane. Even with the assistance of accessory proteins, passage across the outer membrane, in the absence, for example, of energy-generating systems, is still difficult to envisage. A recent report, however, by Wong and Buckley (1989) suggests in fact that a protonmotive force may be involved in the transport of proaerolysin across the outer membrane of *Aeromonas hydrophila* from the periplasm. The role of accessory proteins might therefore include the direct coupling of the inner membrane protonmotive force to outer membrane translocation.

K88ab and K99 Fimbrial Proteins

As illustrated in Fig. 1, genetical and biochemical studies have identified at least two additional ancillary proteins required for transport of fimbrial subunits across the outer membrane (Mooi *et al.*, 1986). Fimbrial subunits (FaeG protein) can normally be detected in the periplasm in kinetic experiments, while release from the inner membrane during export and its subsequent stability appears to depend upon its association with the *faeE* gene product. Subsequent interaction of the FaeG-FaeE complex with an outer membrane protein, FaeD, then appears to be sufficient to achieve the

movement of the fimbrial subunits across the outer membrane onto the cell surface. At the present time the nature of this last translocation step or the means of recognition of the FaeE complex by the translocator in the outer membrane is unknown.

Cholera Toxin

The A and B subunits of the *Vibrio cholera* toxin are first exported to the periplasmic space, accompanied by the cleavage of the N-terminal signal sequence. The toxin then assembles with one A subunit and a pentameric B subunit and accumulates to very high concentrations before transport across the outer membrane occurs (Hirst and Holmgren, 1987a). Kinetic studies have indeed shown that transport to the medium from the periplasm occurs with a half-life of 13 min. This last step, remarkably, appears to involve the transport of the fully active, large multimeric structure by a completely unknown mechanism (Hirst and Holmgren 1987b). When the cholera toxin genes are expressed in *E. coli*, the active toxin accumulates in the periplasm but is not released (Hirst *et al.*, 1984). The way is therefore open for the identification of secretion-specific genes in *V. cholerae* that might be expected to promote secretion from *E. coli*.

Pullulanase

Some of the most detailed studies of protein secretion in other Gram-negative bacteria have been concerned with pullulanase produced by *Klebsiella pneumoniae*. Pullulanase, encoded by *pulA*, is initially synthesized with an N-terminal signal sequence which, unusually, is fully acylated at the cysteine residue immediately distal to the signal cleavage site for a lipoprotein signal peptidase (Pugsley *et al.*, 1986). No periplasmic intermediates have been reported in *K. pneumoniae*, and when the *pulA* gene alone is expressed in *E. coli*, the enzyme is not secreted but is found associated with both inner and outer membrane fractions rather than in the periplasm (Michaelis *et al.*, 1985; Takizawa and Murooka, 1985). Such an association may be due to the lipoprotein nature of pullulanase, A number of studies have now shown that several genes flanking the *pulA* gene itself are necessary for pullulanase transport and relatively slow release from the cell surface of both *K. pneumoniae* and *E. coli*. At least one protein involved in this process has been identified as a lipoprotein (PulS) localized to the outer membrane (d'Enfert and Pugsley, 1989), but many additional genes are apparently also required (M. Kornacker and A. P. Pugsley, personal communication).

Pullulanase is apparently finally released from cells in the stationary phase of growth as large aggregates, probably as micelles formed by the fatty acyl chains (Pugsley *et al.*, 1986). However, in a variant form of *K. pneumoniae* pullulanase, monomers, nonacylated, are released rapidly to the medium during exponential growth (Kornacker *et al.*, 1989). In either case, however, the mechanism of transport of pullulanase across the outer membrane or the role of other *pul* genes in the final stages of secretion are completely unclear.

IgA1 Proteases

IgA1 proteases are secreted by some strains of *Neisseria gonorrhoeae* and *Haemophilus influenzae* and are able to cleave the heavy chain of human IgA1. The enzyme is synthesized with an N-terminal signal sequence which is cleaved as it crosses the inner membrane. The active enzyme is normally found in culture supernatants as a 106-kD polypeptide (corresponding to the N-terminus region of the molecule), compared with the primary gene product of about 169-kD. Pohlner *et al.* (1987) investigated the mechanism of transport of the IgA1 protease across the outer membrane and demonstrated that an active form accumulated in the periplasm if portions of the C-terminal region had been deleted. Moreover, the authors were able to detect a 45-kD polypeptide corresponding to the C-terminal domain of IgA1 protease in the outer membrane of gonococci. In consequence, as shown in Fig. 1, it is proposed that, quite remarkably, the C-terminal domain integrates into the outer membrane and acts as a "helper or accessory" protein to promote in some way the transport of the N-terminal (protease active) domain across the membrane. As final steps, autocatalytic cleavage leads to release of the N-terminal domain into the medium followed by other cleavage events, resulting in the formation of the fully active, 106-kD protease.

Other Secreted Proteins

Several other secreted proteins have been described in a wide range of Gram-negative bacteria, and in the great majority of cases such proteins are initially synthesized with an N-terminal signal sequence, and periplasmic intermediates can be detected in mutants defective in specific secretion functions (see Review by Pugsley, 1988; Hirst and Welch, 1988). One other example is, however, worthy of note. Delepelaire and Wandersman (1989) (see also Nakahama *et al.*, 1986) have recently reported that a protease secreted by *Erwinia chrysanthemi* does not contain an N-terminal signal sequence but depends instead upon a C-terminal signal sequence. This is

similar to the *E. coli* hemolysin HlyA described in detail below. Moreover, secretion is apparently dependent upon two genes with considerable homology to the hemolysin export genes, *hlyB* and *hlyD* (C. Wandersman, personal communication).

Secretion of Hemolysin (HlyA) by *E. coli*

Four Linked Genes Control the Production and Secretion of HlyA2001

For the purpose of this review we shall consider primarily the mechanism of secretion of the hemolysin HlyA2001 studied in this laboratory. Details of other, closely related hemolytic determinants and aspects of the regulation of hemolysin syntheses have been recently reviewed (Holland *et al.*, 1989a). As shown in Fig. 2, HlyA2001 is secreted to the medium, late in the exponential phase of growth, as a highly labile 107-kD polypeptide.

Four genes, *hlyD,A,B,D* are required for the synthesis and secretion of HlyA2001 (Mackman *et al.*, 1986). HlyC encodes a 20-kD polypeptide responsible for post-translational modification of the 107-kD, HlyA molecule to the active toxin. HlyC plays no role in the secretion process while in contrast HlyB and HlyD are absolutely required for translocation of HlyA to the medium.

As indicated in Fig. 3, the four *hly* genes are located on a contiguous stretch of DNA (the Hly determinant) present in the chromosome of the urinopathogenic strain *E. coli* LE2001. The Hly determinant was previously cloned into the low copy number, temperature-amplifiable vector pOU71 (Larson *et al.*, 1984), to form the recombinant plasmid pLG570 (Mackman and Holland, 1984). The *hlyC,B* genes and the majority of *hlyA* have been sequenced (Nicaud *et al.*, 1985; unpublished data, this laboratory) and are almost identical to those genes of a chromosomal determinant from *E. coli* strain J96 reported by Felmlee *et al.* (1985a).

Structure of HlyA and the Identification of a C-terminal Secretion Signal

As first reported by Felmlee *et al.* (1985b) HlyA does not possess an N-terminal signal sequence and as described by the same group the N-terminus is not subjected to proteolytic processing. In fact, contrary to earlier reports (see Hartlein *et al.*, 1983) the 107-kD primary gene product of *hlyA* is apparently secreted to the medium without any proteolytic processing although the removal of 5–10 amino acids from the C-terminus might have escaped detection.

HlyA is a member of the so-called RTX group of toxins, characterized by the presence of several tandem repeats of a glycine-rich nonapeptide,

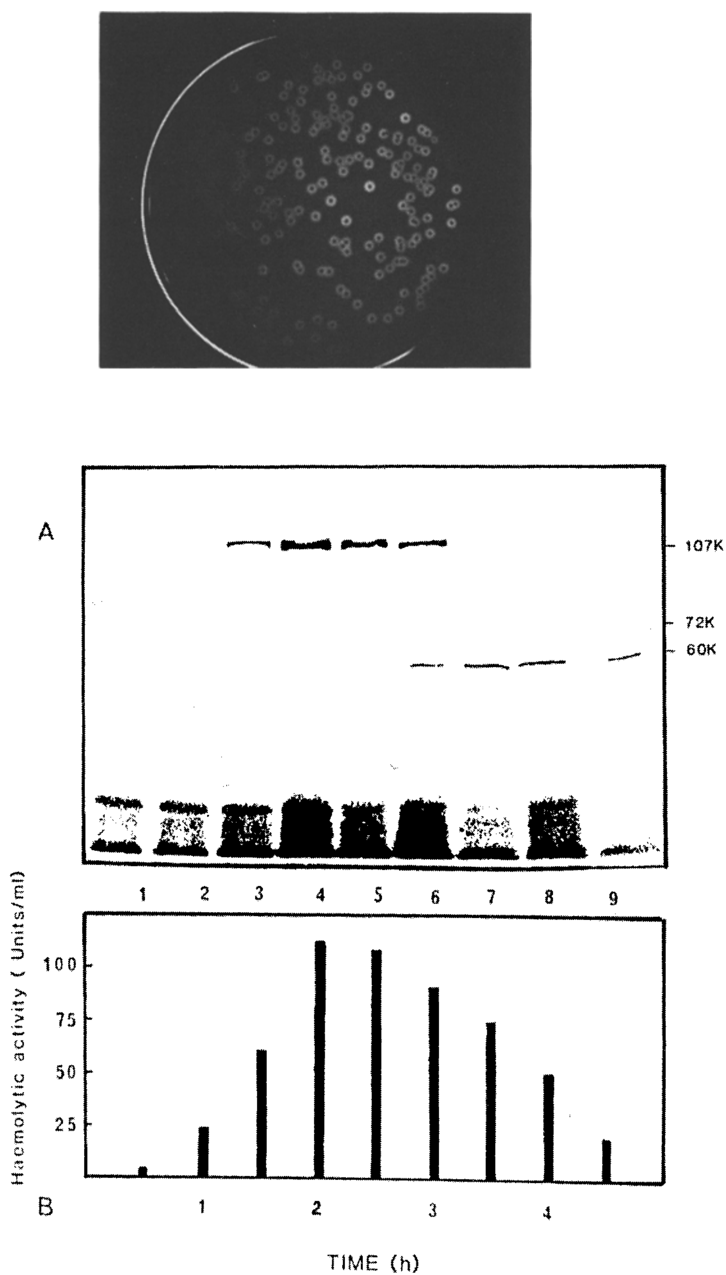

Fig. 2. Secretion of hemolysin (HlyA) by *E. coli*. The upper panel shows the halos produced by *E. coli* (*hly*+) colonies grown on plates containing sheep erythrocytes. Panel A shows the release of the 107-kD hemolysin during a brief window of exponential growth and its eventual breakdown during the early stationary phase. Panel B shows the parallel accumulation and loss of hemolytic activity in supernatants of *E. coli* carrying the Hly2001 determinant.

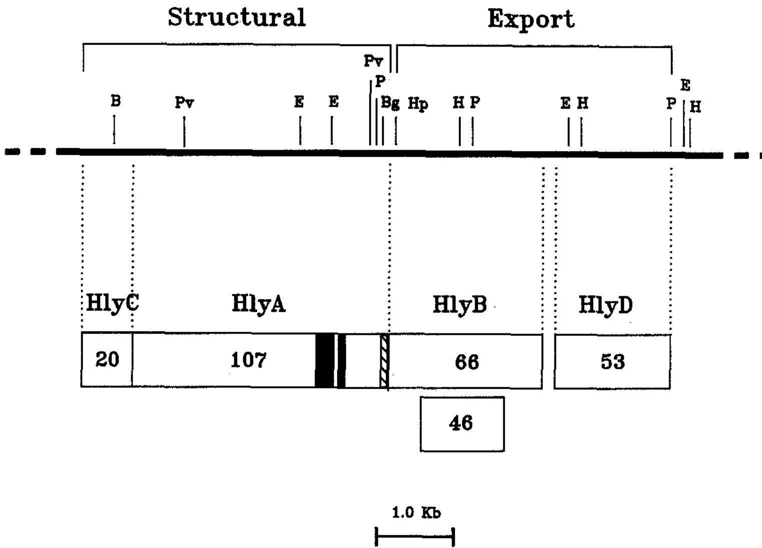

Fig. 3. Organization of the Hly genes. The figure shows the genes encoding HlyC and HlyA required for synthesis of an active toxin and the genes encoding HlyB and HlyD, essential for secretion of HlyA to the medium. The molecular weights (kD) of the proteins are indicated including the alternative form (46-kD) of HlyB detected in SDS-PAGE analysis. The presence of 14 (nona) peptide repeats in two blocks in HlyA is indicated (solid shading) and the position of the HlyA secretion signal (hatched shading).

consensus GGBGBBXLX. HlyA2001 has two blocks of nine and five repeats, respectively, between residues 722 and 873 (see Fig. 3). In the case of HlySF4000 the removal of 11 out of 14 of these repeats results in greatly reduced hemolytic activity, and the kinetics of secretion are somewhat reduced (Felmlee and Welch, 1988). However, these repeats are apparently not essential for recognition and hence secretion by the translocation complex. Thus, several studies including those involving the deletion of up to 90% of the N-terminal portion of HlyA2001, the fusion of heterologous polypeptides to the C-terminal 218 or 114 amino acids of HlyA2001 and the deletion of the C-terminal 27 amino acids, have now shown that a specific secretion or targeting signal exists very close to the C-terminus of the molecule (Nicaud *et al.*, 1986; Gray *et al.*, 1986; Mackman *et al.*, 1987; Holland *et al.*, 1989b). There have been other reports that targeting signals resembling mitochondrial signal peptides are also present at the extreme N-terminus of HlyA and that these might be involved in initial steps in the translocation process (Erb *et al.*, 1987; see also Pugsley, 1988). Since the whole of this region can readily be deleted or even replaced by several heterologous

polypeptides (including chloramphenical acetyl transferase, CAT) without preventing high levels of secretion (Holland *et al.*, 1989b; unpublished data), the presence of such secretion signals can be discounted.

Close examination of the amino acid sequence at the C-terminus of the *E. coli* hemolysin as shown in Fig. 4 clearly indicates that the secretion signal represents a novel type of targeting signal, quite distinct from those described previously. The sequence at the C-terminus of other *E. coli* hemolysins (Felmlee *et al.*, 1985a; Hess *et al.*, 1986) and hemolysin secreted by *Proteus* strains (Koronakis *et al.*, 1989), like the rest of the molecule, are highly conserved. However, when compared to more distantly related toxins, such as leukotoxin (Lo *et al.*, 1987), adenylcyclase toxin (Glaser *et al.*, 1988), or an *Erwinia chrysanthemi* protease (Delepelaire and Wandersman, 1989) or the closely related protease from *Serratia chrysanthemi* (Nakahama *et al.*, 1986), there is little conservation of primary sequence. Nevertheless, both leukotoxin and adenlycyclase toxin (cyclolysin) are apparently recognized

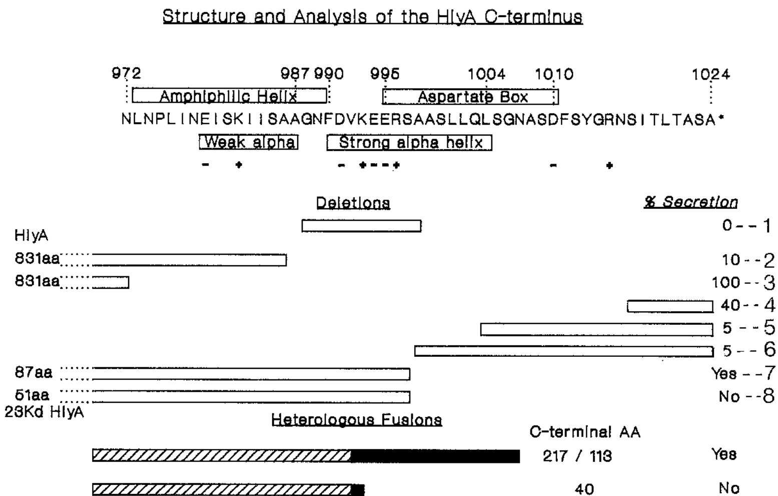

Fig. 4. Amino acid sequence of the HlyA molecule and localization of the targeting signal. The amino acid sequence shown is for HlyA2001 with the predicted secondary structures and charged residues as indicated. The "aspartate box" (see text) is a feature apparently conserved in a number of distantly related toxins. Deletion of the terminal 27 amino acids was previously shown to block secretion (Gray *et al.*, 1986). The C-terminal deletions shown here are from Koronakis *et al.*, 1989; deletions 1, 2, 3 shown here are also from the same source. Deletions 7 and 8 which differ in upstream end points and the two OmpF fusion to the C-terminal 217 or 113 amino acids of HlyA were described by Mackman *et al.*, 1987. Similar fusions to prochymosin have been described (Holland *et al.*, 1989b) which were secreted, while fusion of the terminal 40 amino acids of Hly is insufficient to promote secretion of prochymosin (unpublished data). Finally, not shown, the 114-residue C-terminus of HlyA is the smallest fragment reported to be secreted alone, in an HlyB, HlyD dependent manner (Mackman *et al.*, 1987).

and translocated by the HlyB, D complex in *E. coli* (Strathdee and Lo, 1989; Masure *et al.*, 1989). Interestingly, Swinkels *et al.* (1988) have described a form of phosphoglycerate kinase which appears to carry a C-terminal targeting signal for glycosomes, with some general features similar to those of the HlyA signal region. In contrast, the short C-terminal SKL motif involved in targeting several polypeptides to peroxisomes (see Miyazawa *et al.*, 1989) appears to be completely unrelated to the hemolysin signal sequence.

In the absence of processing events which conveniently (from the investigator's point of view) remove the signal sequence, the *precise* boundaries of targeting sequences are difficult to establish. In particular, in the case of C-terminal signals the construction of internal deletions to define proximal boundaries may either fortuitously recreate particular secondary structural motifs inherent to the signal or lead to structural changes resulting in occlusion of the C-terminal signal by the N-terminal domain.

With these reservations in mind, we summarize in Fig. 4 our current knowledge of the location of the C-terminal signal of the hemolysin molecule. The figure also indicates a number of secondary structural and other features which may form part of the targeting signal. The predicted α helices and the cluster of charged residues between residues 990 and 997 are conserved in the *E. coli* and *Proteus* HlyA molecules so far analyzed (Felmlee *et al.*, 1985a; Hess *et al.*, 1986; Koronakis *et al.*, 1989; and for HlyA2001, this study). Koronakis *et al* (1989) have also identified a similarly conserved amphiphilic helix in *E. coli* and *Proteus* hemolysins. This feature is, however, apparently absent in the cyclolysin (Glaser *et al.*, 1988), and the cluster of positive and negatively charged residues is not conserved in the cyclolysin, leukotoxin, or the protease from *Erwinia* or *Serratia*. On the other hand, a block of 12–14 small, hydroxylated residues, usually flanked by aspartic acid residues (aspartate box, Fig. 4) appears to be largely conserved close to the C-terminus of all these molecules.

A number of strategies have been used in attempts to more precisely localize the targeting sequence. Thus, from the construction of C-terminal deletions, sequences essential for secretion have been identified particularly between residues 998 to 1016 (Gray *et al.*, 1986; Koronakis *et al.*, 1989). In addition, Koronakis *et al.* (1989) have shown that deletion of the charged residues between 988–998 in the Hly determinant encoded by pANN202-312 (Hess *et al.*, 1986) completely blocks secretion, suggesting that this region constitutes an essential part of the signal sequence. However, initial studies with site-directed mutagenesis indicate that at least some individual charged residues are dispensable (unpublished data, this laboratory). Other data also indicate that the results of the effect of internal deletions on secretion should be interpreted with care. Thus, we have previously shown that a deletion

extending upstream from residue 997 of HlyA2001 has a maximal effect upon secretion depending upon the length of the deletion towards the N-terminus (Mackman *et al.*, 1987). This variation could not be explained by the presence of upstream sequences specifically required for secretion. Rather, we propose that internal deletions, by bringing together normally well-separated sequences, may lead to long- or short-range overall structural changes within the deleted polypeptide, which may result in varying degrees of occlusion of the targeting signal at the C-terminus. A normal function of the glycine-rich repeats, for example, might be to ensure the structural independence of the C-terminal domain of HlyA.

Detailed analysis of the HlyA secretion signal has so far clearly demonstrated the importance of residues 998–1016 in the apparent recognition of the HlyB,D translocation complex. Other flanking regions essential for signal function have not, however, been excluded and the precise structural features which constitute the targeting function remain to be established. Attempts to analyze this further are now being undertaken through the generation of random and directed point mutations and will be the subject of a future publication.

Localization and Membrane Organization of HlyB,D

We have previously identified the *hlyD* product as a 53-kD polypeptide in minicells which fractionates primarily with the inner membrane (Mackman *et al.*, 1985). In addition, we have now shown that although resistant to proteases in intact cells, HlyD is extremely sensitive to protease when the outer membrane is first removed (manuscript in preparation). These results indicate that HlyD straddles the periplasm, perhaps also forming an association with the outer membrane.

HlyB2001 encodes a 66-kD polypeptide *in vitro* and in minicells. Both forms are truncated when Tn5 is inserted into the middle of the gene (Mackman *et al.*, 1985). Surprisingly, the open reading frame of *hlyB* predicts a polypeptide of 79.9-kD, suggesting that HlyB2001 migrates abberently during electrophoresis. A similar discrepancy between the observed size and that predicted from DNA sequences has been reported for the homologous leukotoxin (*lktB*) gene product (Strathdee and Lo, 1989). On the other hand, Felmlee *et al.* (1985a) detected 79-kD protein under their gel conditions in addition to a 46-kD product of *hlyB* from *E. coli* J96 carrying the *hly* determinant subclone pSF4000.

A number of explanations have been proposed that might account for the two forms of HlyB which have been observed, including an internal translational start at Met^{286}, or cleavage of a putative signal peptidase site

at Ala^{307} (Fig. 5) in the N-terminal membrane binding domain (Felmlee *et al.*, 1985a). However, subcloning an *hlyB* fragment commencing with the *met*^{286} codon does not result in expression of the 46-kD polypeptide (unpublished data this laboratory). In addition, the predicted N-terminal protein fragment resulting from a specific signal peptidase cleavage has not been detected and, moreover, the 46-kD polypeptide is produced *in vitro* in the absence of membrane vesicles (Mackman *et al.*, 1985; unpublished data). In pulse-chase experiments in minicells we have found no evidence that the smaller protein is derived from the 66-kD polypeptide by proteolysis (unpublished data), and in view of the widely varying relative amounts of the two proteins observed in our studies, we propose rather that the two forms may represent different conformations of the molecule induced by differential SDS binding.

In detailed localization studies we have now clearly identified HlyB (66 and 46 kD) as an inner membrane protein, although small but significant amounts also fractionate with the outer membrane (Wang *et al.*, manuscript in preparation). Protease studies are also consistent with the localization of HlyB to the inner membrane, while topology analysis, using the β-lactamase topology probe (Broome-Smith and Spratt, 1986), indicate that HlyB is a polytopic inner membrane protein with a large cytoplasmic, C-terminal domain (Wang *et al.*, in preparation).

HlyB, a Member of a New Family of ATP-Dependent Translocators

HlyB displays extensive homology with the mammalian P-glycoprotein (Mdr or multdrug resistance protein). P-glycoprotein (reviewed by Endicott and Ling, 1989; Blight and Holland, 1990) is expressed at high levels in some mammalian cells or tumors which have been exposed to anticancer drugs. Overexpression appears to result from a gene amplification mechanism and in some cases the selection of variants which confer resistance to specific drugs. However, P-glycoprotein normally confers resistance to a wide range of antitumor drugs which nevertheless share some structural relationship (Pearce *et al.*, 1989). On the basis of the similarity with HlyB, Gerlach *et al* (1986) proposed that the Mdr protein acted as an ATP-dependent pump to re-export (secrete) drugs which had already entered the cell. Subsequent studies have shown that this export process may take place in two steps, initial ATP-dependent binding of the drug to the membrane domain of the translocator followed by translocation across the membrane accompanied by further hydrolysis of ATP (see Gottesman and Pastan, 1988).

The basic stuctures of HlyB and P-glycoprotein share an N-terminal membrane domain and a large, approximately 27-kDa, cytoplasmic C-

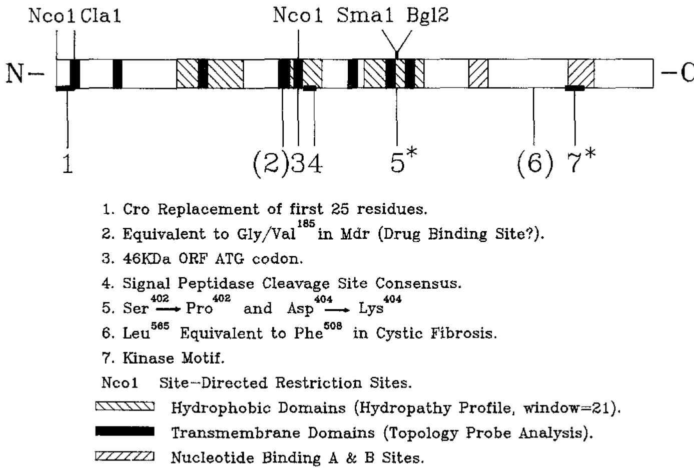

Fig. 5 Structure and function of HlyB. The map indicates the transmembrane regions predicted by β-lactamase fusion analysis, the core sequences for ATP binding in the highly conserved cytoplasmic domain, and the positions of various mutations so far studied in HlyB or its homologues. The asterisk indicates mutations in HlyB which abolish secretion, while (2) and (6) indicate the equivalent position of mutations in the Mdr protein which define a drug-binding site or defective transport in CF, respectively. The figure also indicates sites which have been proposed to give rise to the 46-kD form of HlyB; see also text.

terminal membrane domain. In fact P-glycoprotein is a tandem duplication of such a structure with highly homologous halves. HlyB and P-glycoprotein show extensive conservation, in particular within the cytoplasmic domain which contains typical nucleotide or ATP-binding folds (Fig. 5). The N-terminal, membrane domains show relatively little conservation of primary sequence but may show very similar organization within the membrane (Wang *et al.*, in preparation).

As summarized in Table I, HlyB and P-glycoprotein have now been shown to have many homologues in both prokaryotes and eukaryotes, which remarkably are responsible for the translocation of an astonishingly wide range of compounds, for which we propose the general term allocrite, from large polypeptides to small hydrophobic drugs and probably chloride ions in the case of the CF (cystic fibrosis) protein. The structure and possible function of many of these translocators and their relationship has been reviewed recently (Blight and Holland, 1990) and we shall not consider them further in this article.

Table I. Membrane Polypeptides with Homologies with Haemolysin B

System	Protein	Allocrite	Reference
Escherichia coli	HlyB	Haemolysin A	Felmee *et al.* (1985b)
Pastuerella haemolytica	LktB	Leukotoxin A	Strathdee and Lo (1989)
Bordetella pertussis	CyaB	Adenyl cyclase toxin	Glaser *et al.* (1988)
Rhizobium meliloti	NdvA	β-1,2 glucan	Stanfield *et al.* (1988)
Agrobacterium tumefaciens	ChvA	β-1,2 glucan	Cangelosi *et al.* (1989)
Drosophila melanogaster	White[a]	pigment	O'Hara *et al.* (1984)
Drosophila melanogaster	Brown[a]	pigment	Dresson *et al.* (1988)
Drug tumor cells	Mdr[b]	Therapeutic drugs	Gerlach *et al.* (1986)
Secretory epithelia-mammals	Mdr[b]	Excretion/drugs	Cordon-Cordo *et al.* (1989)
Plasmodium falciparum	Pfmdr[b]	Chloroquine	Foote *et al.* (1989)
Human cystic fibrosis	CF[b]	Chloride ions	Riordan *et al.* (1989)
Saccharomyuces cerevisiae	Ste6[b]	peptide pheromone	

[a]The ATP domain appears at the N-terminus of the molecule in this case although the relative position of ATP and membrane domains is maintained. These systems are in fact involved in *uptake* of pigment precursors.
[b]Tandem duplications of an HlyB-like molecule.

Genetic Analysis of HlyB2001 and Its Homologues

Detailed genetic analysis of HlyB is still in its early stages, but some interesting mutants have been obtained and these will be briefly described (see Fig. 5).

Previous studies by Koronakis *et al.* (1988) have shown that highly conserved residues, Gly^{605} and Gly^{608} in the ATP-binding fold, are essential for HlyB function, consistent with an energy-generating role for this domain. The deletion of a single Phe residue between the highly conserved ATP binding domains in one-half of the molecule has been identified in 68% of CF mutants, apparently leading to defective chloride ion transport. Site-directed mutageneis and DNA replacement with respect to the periplasmic loop most proximal to the cytoplasmic domain and the N-terminus of HlyB, respectively, have been carried out, resulting in loss of function in the former but no effect in the latter (Wang *et al.*, in preparation). Using *in vitro* mutagenesis, we have recently isolated several ts mutations in *hlyB*, and the prospects are excellent therefore for a detailed analysis of the structure and function of HlyB.

Finally, an interesting mutation (a Gly^{185}-Val change) in P-glycoprotein which leads to enhanced resistance to colchicine (as compared with other drugs) has been identified. Choi *et al.* (1988) suggest that this mutation may have pinpointed a specific region involved in the initial binding of the drug prior to translocation. From such data, together with the similar organization of different HlyB homologues that we can now predict (Wang *et al.*,

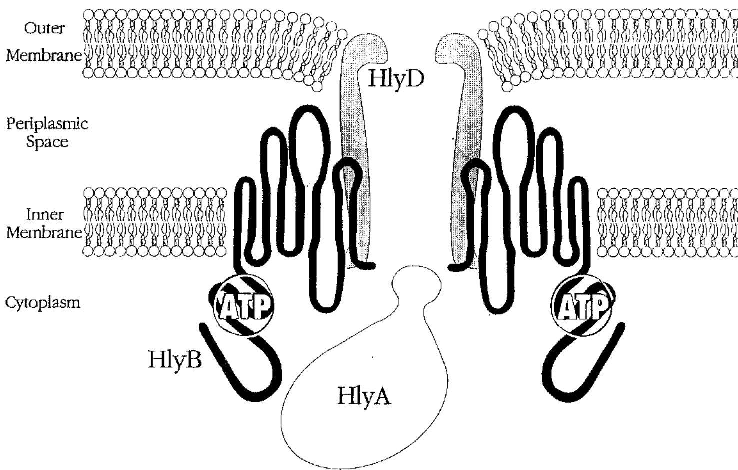

Fig. 6. A model for the secretion of HlyA. The C-terminal signal of HlyA recognizes the HlyB,D translocator complex and is translocated to the medium by a mechanism involving energy generation by the C-terminal domain of HlyB.

in preparation), we are able to provide a rational basis for site-directed mutagenesis of *hlyB* in order to identify specific regions within the membrane domains which might be involved in initial *recognition* (cytoplasmic domains) or subsequent *translocation* (periplasmic domains) of HlyA.

Mechanism of HlyA Translocation

In contrast to other proteins carryng an N-terminal signal sequence, we have found no evidence for a periplasmic intermediate in the secretion of the *E. coli* hemolysin. Some earlier evidence (Wagner *et al.*, 1983; Mackman and Holland, 1984) indicated that such periplasmic intermediates did occur, but it is likely that this resulted from spurious "hemolytic" activity under certain conditions in osmotic shockates unrelated to hemolysin. In several careful studies we have been completely unable to detect hemolytic activity or the hemolysin protein in osmotic shockates or spheroplast supernatants from strains expressing wild-type Hly genes or with a mutant of HlyA deleted for the secretion signal (Gray *et al.*, 1986, 1989; Baker, 1987). Moreover, the same studies completely failed to detect a periplasmic intermediate when either *hlyB* or *hlyD* were absent, although in all cases when secretion was blocked the hemolysin was detected in the cytoplasmic fraction. These results are in contrast to the report of Wagner *et al.* (1983) in which studies we suspect that the periplasmic "activity" detected was not hemolysin. Studies with other Hly determinants have now confirmed the absence of detectable

periplasmic intermediates in the normal secretion pathway or when secretion is blocked by deletion of the C-terminus of HlyA (Felmlee and Welch, 1988; Koronakis *et al.*, 1989; Oropeza-Wekerle *et al.*, 1989). These findings taken together with the apparently immediate secretion of newly sythesized HlyA to the medium (Felmlee and Welch, 1988; our unpublished data) led us to propose that HlyA is secreted directly to the medium across both membranes. Consequently, we propose (see also Mackman *et al.*, 1986; Holland *et al.*, 1989a) that the hemolysin translocator, composed of HlyB and HlyD, forms a trans-envelope complex at junctions between the inner and outer membrane (see Fig. 6). Such junctions might be identical to Bayer adhesion sites or might form directly due to the structural organization of HlyB and HlyD. Interestingly, the Tol C protein, a membrane protein involved in colicin uptake and general integrity of the outer membrane (Morona and Reeves, 1982), is now reported to be essential for hemolysin secretion (C. Wandersman, personal communication), perhaps as a constituent of adhesion sites.

The role of HlyD in hemolysin secretion remains a mystery. The presence of this protein, however, has only been observed in bacterial systems in association with HlyB-like proteins involved in polypeptide translocation. It remains a possibility therefore that HlyD confers specificity for protein transport on the HlyB translocator. Studies on the topological organization of HlyD suggest that it has a short (approximately 60 residues) cytoplasmic domain at the N-terminus with the great majority of the molecule on the periplasmic side of the membrane (Wang *et al.*, in preparation). This would be consistent with extensive interactions between HlyB and HlyD to form a specific translocation complex spanning both membranes at adhesion sites. HlyD might equally be specifically required to mediate the transport of hemolysin through the outer membrane after initial translocation involving HlyB. However, it is notable that in the absence of HlyD, hemolysin accumulates in the cytoplasm, suggesting that both proteins are involved in the coordinated translocation of hemolysin through both membranes.

The actual process of hemolysin transport and the precise role of the HlyB,D complex remains unclear. Nevertheless, some interesting possibilities are indicated. The presence of a specific secretion signal at the extreme C-terminus of the molecule and the fact that the first 80% at least of HlyA can be replaced by completely heterologous sequences (including the majority of the CAT protein), strongly suggests that the transport process is truly post-translational. On the basis of our knowledge of HlyB in particular, it seems highly likely that this protein is indeed involved directly as a translocator. Given the degree of conservation of the C-terminal domain of HlyB and its homologues, we may argue that specificity for transport of a given allocrite is located in the N-terminal domain. As argued above, we may then propose as a working hypothesis that cytoplasmic loops in this domain will be

involved in recognition of the HlyA signal, while periplasmic loops are more likely to be involved in the final states of translocation.

Finally, regarding the mechanism of movement of HlyA through the membrane, two major possibilities present themselves: via a proteinaceous channel formed by HlyB and HlyD, presumably necessitating considerable *unfolding* of HlyA, or alternatively by some form of "revolving door" mechanism. In the latter case, movement through the envelope might be achieved through conformational changes in the translocator(s), allowing HlyA to be secreted in largely folded form. We are now attempting to address these possibilities.

Acknowledgments

We are pleased to acknowledge the invaluable support of the MRC, project grant G8604617CB, and the SERC, project grants GR/0/82593 and GR/C81148. Mark A. Blight also wishes to acknowledge the receipt of an MRC studentship.

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