Secretion and Circular Dichroism Analysis of the C-Terminal Signal Peptides of HlyA and LktA†

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ABSTRACT: The secretion of the 107 kDa hemolysin A (HlyA) from *Escherichia coli* is mediated by membrane proteins hemolysin B (HlyB) and hemolysin D (HlyD). The signal for transport has been mapped to the C-terminal 60 amino acids of the HlyA molecule. We have shown previously that the C-terminal 70 amino acids of leukotoxin (LktA) from *Pasteurella hemolytica* can substitute functionally for the HlyA signal sequence. This 70 amino acid peptide contains little primary sequence similarity to the HlyA signal sequence, and we have hypothesized that these signal sequences assume a similar higher-order structure which is recognized by the HlyB/D transporter. In the present study, we have expressed and purified small peptides containing the C-terminal 61 amino acids of HlyA and the C-terminal 70 amino acids of LktA. We show that these signal peptides are sufficient for secretion from *E. coli* in a HlyB/D dependent manner. Circular dichroism analyses show that both molecules exhibit common biophysical properties. In aqueous solution, they appear to be mainly unstructured, but in a membrane mimetic environment they assume a helical secondary structure. The conformational change observed for both peptides going from an aqueous to a membrane mimetic environment may be an important feature of these signal sequences necessary for their recognition and transport.

The 107 kDa α-hemolysin (HlyA) protein is transported directly across both inner and outer membranes of *Escherichia coli* without an apparent periplasmic intermediate [for a review, see Holland et al. (1990)]. The signal for transport is located within the C-terminal 60 amino acids of HlyA (Koronakis et al., 1989; Hess et al., 1990; Stanley et al., 1991), which is not cleaved upon translocation (Felmlee et al., 1985). The transport is mediated by three proteins: two inner membrane proteins, HlyB and HlyD, encoded in the hemolysin operon, and an *E. coli* outer membrane protein TolC (Juranka et al., 1992; Wang et al., 1991; Wandersman & Delepelaire, 1990). There are several HlyB/D like transporters in gram negative bacteria responsible for the secretion of toxins such as *Pasteurella* leukotoxin, *Bordetella* cyclolysin, *E. coli* colicin V, and *Erwinia* metalloprotease. It has been found that the HlyB/D transporter is capable of transporting these toxic proteins (Strathdee & Lo, 1989; Highlander et al., 1990; Masure et al., 1990; Delepelaire & Wandersman, 1990; Fath et al., 1991; Šebo & Ladant, 1993). HlyB belongs to the ABC (ATP binding cassette) transporter superfamily. Members of this family exist among all organisms examined to date and are responsible for transporting a large variety of substrates; for example, the bacterial permeases for importing amino acids or sugar molecules, the yeast transporter STE6 for a-factor pheromone secretion, mammalian P-glycoprotein for multidrug resistance, TAP proteins for transporting antigenic peptides into the ER to complex with the MHC, and the cystic fibrosis-related ion channel CFTR [for a review, see Childs and Ling, (1994)].

The mechanism involved in HlyA transport is not well understood. It has been demonstrated that transport is an energy-dependent process, and both membrane potential and proton gradient are required (Koronakis et al., 1991). Genetic studies suggest that HlyA interacts with the HlyB protein (Zhang et al., 1993a). Deletions within the C-terminal signal region cause severe reduction in HlyA transport; however, point mutations have failed to identify a discrete, continuous sequence responsible for transport (Stanley et al., 1991; Kenny et al., 1992). Replacement of the C-terminal signal sequence of 58 amino acids of HlyA with the C-terminal 70 amino acids of LktA in *Pasteurella* showed that the chimeric protein can be transported by the hemolysin transporter with efficiency equal to wild-type HlyA (Zhang et al., 1993b). This indicated that the C-termini of HlyA and LktA are functionally equivalent despite different primary sequences. Secondary structure prediction revealed a common motif of helix−turn−helix and strand−loop−strand in both the HlyA and LktA C-termini. Deletion mutations which removed these predicted structural motifs in the HlyA signal sequence largely abolished transport, whereas mutations that maintain the predicted structural motifs exhibited less impairing effects on transport. These deletion studies showed a correlation between the predicted structural motif and transport function. Thus, we have hypothesized that the HlyA and LktA C-terminal signal sequences assume a similar higher-order structure (Zhang et al., 1993b).

In the present study, we have tested the above hypothesis by expressing and purifying small peptides which contain the C-terminal signal of HlyA and LktA. We show that these peptides are secreted specifically in a HlyB/D dependent manner and thus are sufficient for recognition and transport. Moreover, circular dichroism analyses showed that these two

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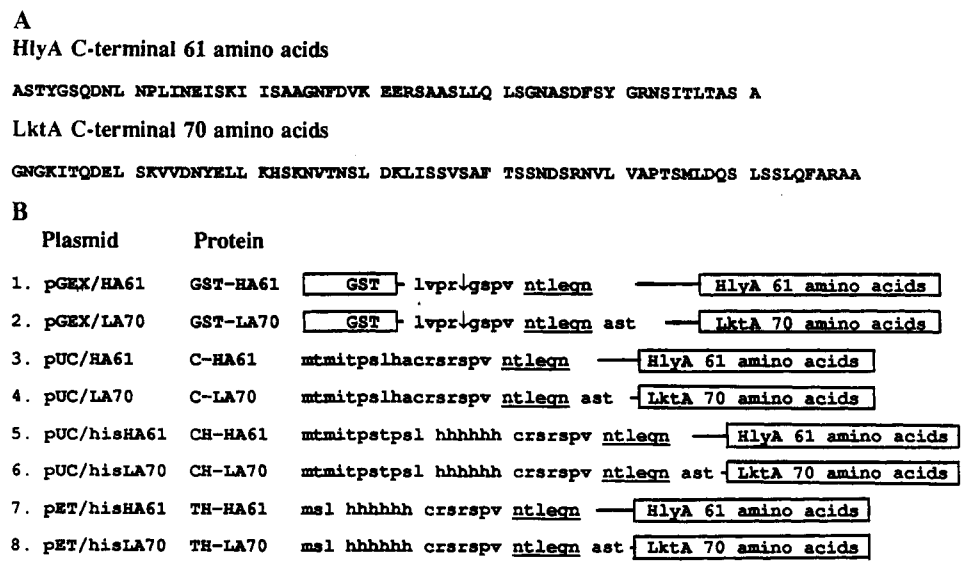

FIGURE 1: Constructions of HlyA and LktA signal sequences. (A) Amino acid sequences of the transport signals of HlyA and LktA. (B) List of fusion proteins with the C-terminal signal sequences of HlyA or LktA. The underlined sequence NTLEGN is the epitope of antibody C494. In plasmids 1 and 2, arrows indicate the predicted thrombin cleavage site.

C-terminal signal peptides exhibited similar biophysical properties. These findings support the hypothesis that different C-terminal signal sequences possess a common higher order structure which is recognized by the HlyB/D transporter.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* strain JM83 was used for all DNA manipulations and expression of most plasmids. *E. coli* strain BL21(DE3) was used for the expression of pET-based plasmids. Plasmids pLG583sk, pHA/lkt70 (Zhang et al., 1993b), pUC19, pGEX-2T, and pET-16b were used to clone the signal sequences of HlyA and LktA. HlyB/HlyD or HlyD were expressed from the pACYC-derived plasmids pLG575 (Mackman et al., 1985a) or pLG594 (Mackman et al., 1985b), respectively. Where appropriate, cells with plasmids were grown in the presence of 25 μg/mL chloramphenicol, 50 μg/mL ampicillin, and 50 μg/mL kanamycin.

Construction of Plasmids Containing Transport Signal Sequences of HlyA and LktA. An epitope of the antibody C494 epitope (Georges et al., 1990) was introduced into the HlyA molecule by inserting the pair of two complementary oligonucleotides, A-Sal-C494 (5′-TCGATCGTGAACAC-CTTGGAAGGTAACGCG) and B-Sal-C494 (5′-TC-GACGCGTTACCTTCCAAGGTGTTCACGA), at the *Sal*I site of pLG583sk. The correct orientation results in a 5′ *Pvu*I site and a 3′ *Sal*I site flanking the C494 epitope sequence. The resulting plasmid was designated pLG583sk/#2.

The eight plasmids containing the C-terminal signal sequences of HlyA or LktA shown in Figure 1B were constructed as follows: pGEX/HA61 was obtained by ligating the small *Pvu*I(blunt ended)/AatII fragment of pLG583sk/#2 with the large *Sma*I/*Aat*II fragment of pGEX-2T. pGEX/LA70 was constructed by ligating the large *Sal*I/*Kpn*I fragment of pGEX/HA61 with the small *Sal*I/*Kpn*I fragment of pHA/lkt70.

pUC/HA61 or pUC/LA70 was constructed by ligating the small *Bam*HI(blunt ended)/*Kpn*I fragment of pGEX/HA61 or pGEX/LA70 with the large *Sal*I(blunt ended)/*Kpn*I fragment of pUC19. The six-histidine-tag was then introduced by inserting a pair of synthetic oligonucleotides (puchis1 5′-AGCTTGCATCACCATCACCATCACTGCA and puchis2 5′-GTGATGGTGATGGTGATGCA) at the *Hin*dIII site. The resulting plasmids were designated pUC/hisHA and pUC/hisLA, respectively.

Construction of pET/hisHA61 and pET/hisLA70 involved three steps. First, a *Kpn*I linker (New England Biolabs) was inserted at the *Bam*HI (blunt ended) site of pET-16b, which results in plasmid pET-16k. Second, a *Hin*dIII−*Nco*I adaptor (5′-AGCTACCCATGGGT) was inserted at the *Hin*dIII site of pUC/hisHA61 or pUC/hisLA70 to create pUC/hisHA-h/n or pUC/hisLA-h/n, respectively. pET/hisHA61 or pET/hisLA70 was constructed by ligating the small *Nco*I/*Kpn*I fragment of pUC/hisHA-h/n or pUC/hisLA-h/n with the large *Nco*I/*Kpn*I fragment of pET-16k. Routine cloning procedures were carried out essentially as described by Sambrook et al. (1989).

Expression and Purification of the GST[1] *Fusion Proteins.* *E. coli* JM 83 cells harboring pGEX/HA61 or pGEX/LA70 were grown in LB with 50 μg/mL ampicillin. At an OD_{600} of about 0.6, IPTG was added to a final concentration of 200 μM. Two to three hours after induction, cells (OD_{600} = 1.5−2) were harvested by centrifugation at 6000*g* for 20 min. The purification was performed as described by Smith and Johnson (1988). Cells (from 800 mL culture) were resuspended in 24 mL of sonication buffer (10 mM Tris pH 8, 1 mM EDTA, 2 mg/mL lysozyme) and were broken by a brief sonication. The lysate was treated with 0.49 mL of 1 M $MgCl_2$ and 50 μg of DNAse for 5 min at room temperature, and then 6 mL (750 mM NaCl, 210 mM Tris, pH 8, and 5% Triton X100) was added. The lysate was spun at 6000*g* for 10 min at 4 °C to get rid of cell debris. The supernatant was transferred to a new tube and incubated with

[1] Abbreviations: CD, circular dichroism; IPTG, isopropyl β-D-thiogalactopyranoside; GST, glutathione S-transferase; MeOH, methanol; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TFE, trifluoroethanol.

0.6 mL of 50% glutathione-agarose beads (Sigma) for 30 min at 4 °C with slow rotation. The beads were washed three times with 150 mM NaCl, 50 mM Tris, pH 8, and 1% Triton. Fusion proteins were eluted with 5 mM reduced glutathione in 50 mM Tris (final pH 7.5).

Expression and Purification of the Signal Peptides TH-HA61 and TH-LA70. BL21(DE3) cells harboring pET/hisHA61 and pET/hisLA70 were grown in 2× YT medium with 50 μg/mL ampicillin. At an OD_{600} of about 0.6, IPTG was added to a final concentration of 250 or 500 μM. Two to three hours after induction, cells (OD_{600} = 1.5−2) were harvested by centrifugation at 6000*g* for 20 min. Purification under a denaturing condition is as follows: cell pellets (500 mL culture) were resuspended with 30 mL of buffer A (6 M guanidine, 10 mM Tris, 0.1 M NaP_i, 0.1% Tween20, 10 mM β-mercaptoethanol, pH 8.0) and incubated at room temperature for 1 h with gentle stirring. The mixture was centrifuged at 3000*g* for 10 min to discard the cell debris. Cell supernatants were mixed with 3 mL bed volume Ni-NTA resin (QIAGEN Inc.) for 1 h. After a brief centrifugation to discard cell supernatants, resins were washed twice with 10 mL of buffer A and then loaded onto a column. All subsequent steps were performed at 4 °C. The column was washed with 15 mL of buffer A, followed by a 30 mL linear gradient established with 15 mL of buffer A and 15 mL of buffer S (25 mM NaP_i, 250 mM NaCl, 10 mM β-mercaptoethanol, pH 8.0) and followed by 10 mL of buffer S. The column was eluted with linear gradient (pH8−4.4) achieved with 15 mL of buffer S and 15 mL of buffer E (25 mM NaP_i, 250 mM NaCl, 10 mM β-mercaptoethanol, pH 4.4) and then 2 mL of buffer E alone. The signal peptides were eluted at pH 5.0−5.5.

Transport Assay. *E. coli* cells harboring pLG575 (expressing HlyB and HlyD) and the desired signal peptide bearing plasmids were grown in LB medium. The control cells harbored pLG594 (expressing hlyD) and signal peptide containing plasmids. IPTG was added at an OD_{600} of about 0.6 to a final concentration of 200 μM to the cells harboring IPTG-inducible plasmids. At an indicated time, growth media were collected by removing cells with centrifugation at 3000*g* for 10 min. Trichloroacetic acid was added to the supernatant to a final concentration of 10% (w/v), and the sample was incubated on ice for over 1 h. After centrifugation (8000*g*, 15 min), the precipitated proteins were dissolved in 250 mM NaOH and 50 mM Tris-Cl and separated by SDS−PAGE. The signal peptides were detected by Western blot analysis.

SDS−PAGE and Western Blot Analysis. Proteins were separated by SDS−PAGE (Laemmli, 1970) using a 3% stacking and a 16% separating gel (unless indicated otherwise) and visualized by staining with Coomassie Brilliant Blue R-250. For Western blot analysis, prestained molecular weight markers (BRL) were included as standards. Western blotting was undertaken using antibody C494 (Georges et al., 1990) and anti-mouse IgG (whole molecule) conjugated to horseradish peroxidase (Sigma) and detected by enhanced chemiluminescence (ECL) (Amersham), according to the manufacturer's instructions.

Preparation of Lipid Vesicles. Phospholipids used in this study were PC, dipalmitoylphosphatidylcholine (Sigma); PG, dipalmitoylphosphatidylglycerol (Sigma); PS, bovine brain phosphatidylserine (Avanti Polar Lipids, Inc.). Vesicles were prepared by dispersing powdered phospholipids in a 200 mM NaCl, 20 mM NaP_i buffer at pH 7 in a bath sonicator under a N_2 atmosphere [modified from Jain et al. (1986)].

Table 1

plasmid	cell	induction	yield (mg/L)	secretion
pGEX/HA61	JM83, DH5α	IPTG	5−10	no
pGEX/LA70	JM83, DH5α	IPTG	5−10	no
pUC/HA61	JM83			yes
pUC/LA70	JM83			yes
pUC/hisHA61	JM83, DH5α, MC4100		0.5	yes
pUC/hisLA70	JM83, DH5α, MC4100		0.5	*a*
pET/hisHA61	BL21(DE3)	IPTG	5−10	yes
pET/hisLA70	BL21(DE3)	IPTG	2−3	yes

[a] This peptide could not bind to the antibody C-494; therefore, the transport assay could not be performed.

Circular Dichroism Spectra. CD were recorded on a Jasco model J-700 spectropolarimeter (Japan Spectroscopic Co. Ltd.) using a 0.1 cm cell at room temperature. Spectra were the average of four scans from 190 to 250 nm at a rate of 50 nm/min. All spectra were background corrected, smoothed, and converted to mean residue elipticity $[\theta]$m (deg cm^2 dmol^{-1}). Protein concentration for CD was determined by a modified Lowry method (Markwell et al., 1978). NaCl and β-mercaptoethanol, which are used for maintaining the purified signal peptides in a monomeric soluble form, cause a high level of noise at 190−203 nm. Therefore, measurements in Figure 7 were done between 200 and 250 nm.

RESULTS

GST Fusion Proteins Containing HlyA and LktA Signal Sequences. At the outset we wished to establish a cell expression system capable of producing a high yield of functional signal peptides which may be purified for biophysical studies. Ideally, we would like to have yields of 10 mg of peptide per liter of cell culture. Such a system has the advantage that peptides can be conveniently labeled with isotopes for future NMR analyses. As a first approach, we constructed fusion proteins of glutathione transferase (GST) with signal sequences of HlyA or LktA (Figure 1, plasmids 1 and 2), with the intention of purifying the fusion proteins on a glutathione affinity column and then releasing the signal peptides by cleavage at a unique thrombin site. The epitope of antibody C494 (Georges et al., 1990) was inserted prior to the signal sequence, allowing the molecule to be identified specifically by Western blot analysis. A previous experiment has demonstrated that such an insertion in the intact HlyA molecule caused little effect on secretion or hemolytic activity (data not shown). The expression levels of these fusion proteins were relatively high with yields of 5−10 mg/L of culture (Table 1).

To test the functional activity of the signal peptides, we determined if the GST fusion proteins can be transported. Previously, it has been reported that different passenger proteins containing 60 or more amino acids of the HlyA C-terminal sequence can be transported by the HlyB/D system (Hess et al., 1990; Kenny et al., 1991). We coexpressed GST-HA61 or GST-LA70 with the HlyB/D transporter but found that no secretion of the fusion proteins could be detected in the growth medium (data not shown). It is possible that if a passenger protein is not properly unfolded, the translocation of that fusion protein would be blocked (Gentschev & Goebel, 1992). In fact, GST is known

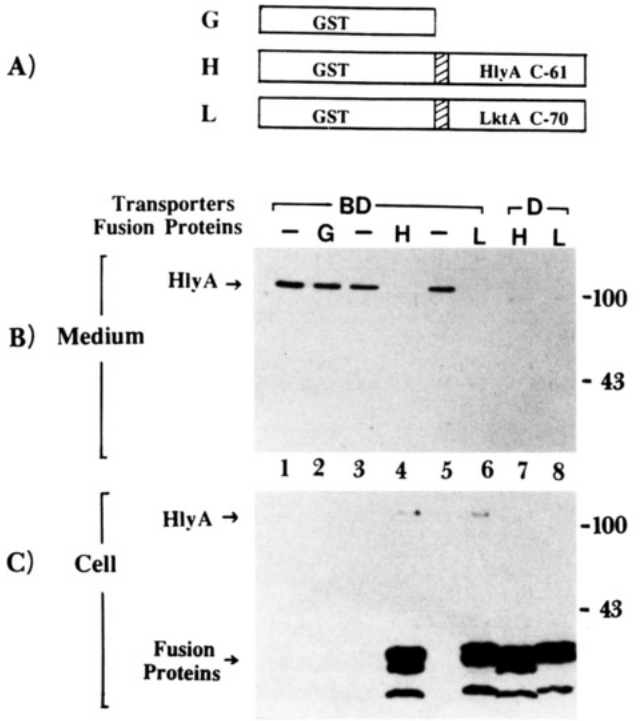

FIGURE 2: Competition of HlyA transport by GST-fusion Proteins with different C-terminal sequences. (A) Diagram of competing proteins: GST fusion proteins contain antibody C494 epitope (dashed box) with either HlyA C-terminal 61 amino acids or LktA C-terminal 70 amino acids. *E. coli* cells expressing HlyA with HlyB/D or HlyD alone, and different GST fusion proteins were grown in LB media. At cell density OD = 0.5, each sample was divided into two tubes. IPTG was added to 0.2 mM in one of the tubes. All cells were harvested at OD = 0.9. Cell media were TCA precipitated. Samples from cell pellets (C) and growth medium (B) from 1 mL of culture were subjected to SDS−PAGE and Western blot analysis. G, GST; H, GST-H61; L, GST-L70.

to be in a dimer configuration in the cell (Walker et al., 1993), and this could account for its inability to be transported as a passenger protein of the HlyA and LktA signal peptides.

If secretion is blocked at the stage of translocation, one may expect that the interaction between the functional signal sequences in the GST fusion proteins and the HlyB/D transporter is still able to take place. To test this possibility, we performed a competition experiment for transport *in vivo* with GST fusion proteins against intact HlyA. We used *E. coli* cells harboring pLG583sk#2 (intact HlyA), pLG575 (HlyB,D) with either pGEX/HA61 (GST-HA61) or PGEX/LA70 (GST-LA70) or PGEX-2T (GST) (Figure 2A). The expression of GST or GST fusion proteins were induced by the addition of IPTG. Both GST-HA61 (Figure 2C, lanes 4 and 7) and GST-LA70 (Figure 2C, lanes 6 and 8) can be detected by the antibody C-494 (the induction of GST in lane 2 was confirmed by anti-GST antiserum, data not shown). As shown in Figure 2B, without IPTG (lanes 1, 3, and 5) the intact HlyA was readily detected in the growth medium in the presence of the HlyB/D transporter. In the presence of IPTG, secretion of intact HlyA was largely abolished in cells expressing either GST-HA61 (lanes 4) or GST-LA70 (lanes 6). This abolition is specific, since the secretion of the intact HlyA was not affected in cells expressing the GST protein (lane 2) or the GST fusion protein containing only the C-terminal 29 amino acids of HlyA (unpublished observation). This experiment shows that both GST fusion proteins can compete for the secretion of the intact HlyA molecule, suggesting that the HlyA and LktA C-terminal peptides interact specifically with the HlyB/D transporter.

Since the above competition studies indicated that the C-terminal signal sequences of HlyA and LktA in the GST fusion proteins were expressed in a conformationally functional state, we proceeded to undertake the purification of these fusion proteins. Western blot analysis using the C494 antibody showed that the GST fusion proteins induced by IPTG occurred as multiple bands in *E. coli* (Figure 2C, lanes 4, 6, 7, and 8). The majority of these components migrating close to 30 kDa differ from each other by about 1 kDa, while one of the components migrated at around 20 kDa which was observed only in some experiments. All the bands from cells which express GST-LA70 (which is 12 amino acids longer than GST-HA61) migrated slower than their counterparts from cells which express GST-HA61. This size difference suggests that the C-termini of these fusion proteins are intact in these observed bands. In addition, Western blot analysis using an antiserum against GST protein showed that GST protein, expressed under the same condition as the fusion proteins, also exhibited multiple bands (data not shown). We suspected that these multiple bands are due to proteolytic cleavages in the GST region of the fusion proteins; however, we have tested many *E. coli* strains including a protease deficient strain SG21173 (Gottesman, 1990) for the expression of GST fusion proteins and found no major difference in the yield and degradation pattern of the GST fusion proteins. Using glutathione affinity chromatography (Smith & Johnson, 1988), we were able to obtain 5−10 mg of fusion proteins from 1 L of cell culture (Table 1). After this chromatograghy step, only the multiple bands around 30 kDa were observed in an SDS−PAGE gel stained with Coomassie Blue; the 20 kDa band was not detected from the purified fraction (data not shown). This 20 kDa peptide has lost most of the GST protein; therefore, it could not bind to the glutathione column.

To release the C-terminal peptides from the purified fusion proteins, we used thrombin for proteolytic cleavage. We found, however, that the commercial source of thrombin did not cleave exclusively at the advertised unique site but also cut inside both HlyA and LktA signal sequences (data not shown). Therefore, using GST fusion proteins as a purification scheme was abandoned.

Secretion and Purification of Small HlyA and LktA Signal Peptides. Our second approach was to determine if small peptides containing essentially only the signal sequence of HlyA and LktA may be expressed fuctionally and at a sufficiently high level for purification. Previously it has been shown by mutational analysis that the C-terminal 60 amino acid signal of HlyA is necessary for transport (Koronakis et al., 1989; Hess et al., 1990; Stanley et al., 1991). However, it has not been demonstrated that C-terminal signal sequences of HlyA and LktA alone are sufficient for transport. In order to investigate whether the signal sequence containing a minimum of foreign sequence can be transported by the HlyB/D system, we cloned the C-terminal 61 amino acids of HlyA or 70 amino acids of LktA into the plasmid pUC19. Sequences of the resulting peptides are shown in Figure 1 plasmids 3 and 4. In order to test the secretion of these signal peptides, C-HA61 or C-LA70 were coexpressed with the transporter proteins HlyB and HlyD in JM83 cells. Cell

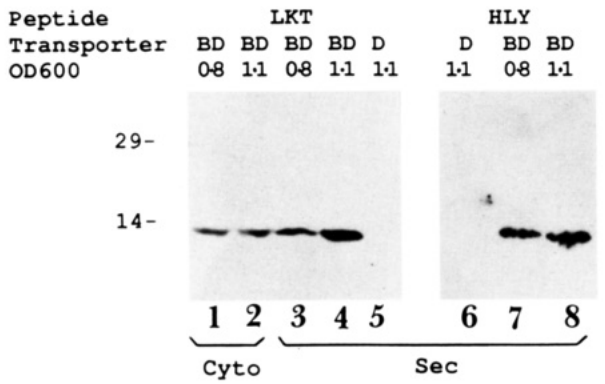

FIGURE 3: HlyB/D dependent secretion of the signal peptides of HlyA and LktA. *E. coli* Jm83 cells expressing the transporter proteins HlyB, D and the signal peptide C-HA61 or C-LA70 were grown in liquid culture and harvested at the cell density (OD_{600}) indicated. Samples from culture supernate (lanes 3−8) and cell pellet (lanes 1 and 2), which were equivalent to 0.4 mL of cell culture, were subjected to Western blot analysis using antibody C494. Peptide: LKT, C-LA70; HLY, C-HA61. Transporter: B, HlyB; D, HlyD.

media were taken at different points during the growth curve. The secreted signal peptides can be detected by Western blot analysis using C494 antibody. As shown in Figure 3 lanes 3, 4, 7, and 8, signal sequence peptides of HlyA and LktA were found in the medium. This secretion requires the presence of both HlyB and HlyD. As shown in Figure 3 lanes 5 and 6, expression of HlyD alone promotes no secretion. Comparing the secreted signal peptides (Figure 3, lanes 3 and 4) with the amount left inside the cells (Figure 3, lanes 1 and 2), we estimated that approximately 60% of the peptides were secreted. Thus, these data show that the signal sequence bearing peptides can be transported by the HlyB/D transporter in a specific manner.

We then introduced a tag of six contiguous histidine residues at the N-terminal region of the signal peptides (Figure 1, plasmids 5 and 6) in order to facilitate their purification via nickel affinity chromatography. The resulting CH-HA61 peptide can be transported by the HlyB/D transporter as shown by Western blotting of the culture medium (Table 1). This indicated that the insertion of six histidine residues at the N-terminal region of the peptide does not affect transport activity. Using nickel affinity chromatograhy, we obtained about 0.5 mg of CH-HA61 peptide per liter of cell culture (Table 1). In the case of the CH-LA70 peptide, we could obtain the same quantity of purified peptide as CH-HA61 (Table 1). This purified peptide could be detected in an SDS−PAGE gel with Coomassie Blue stain, where it migrated a little slower than the CH-HA60 peptide (12 amino acid difference in length between these two peptides). However, this CH-LA70 peptide could not be detected by the antibody C494 in Western analysis. We have ruled out the possibility of an error in DNA sequence; therefore, our conclusion is that some amino acid sequence in that peptide blocks the binding of the C494 antibody to the epitope sequence. We were therefore unable to test the CH-LA70 peptide for transport as our assay depends on Western analysis. A 30 kDa *E. coli* protein also binds to the Ni column and coelutes with the HlyA, LktA peptides. This represents 20−40% of the eluted material (data not shown).

In order to increase the yield of His-tagged signal peptides, we employed the pET derived plasmids and the BL21(DE3)

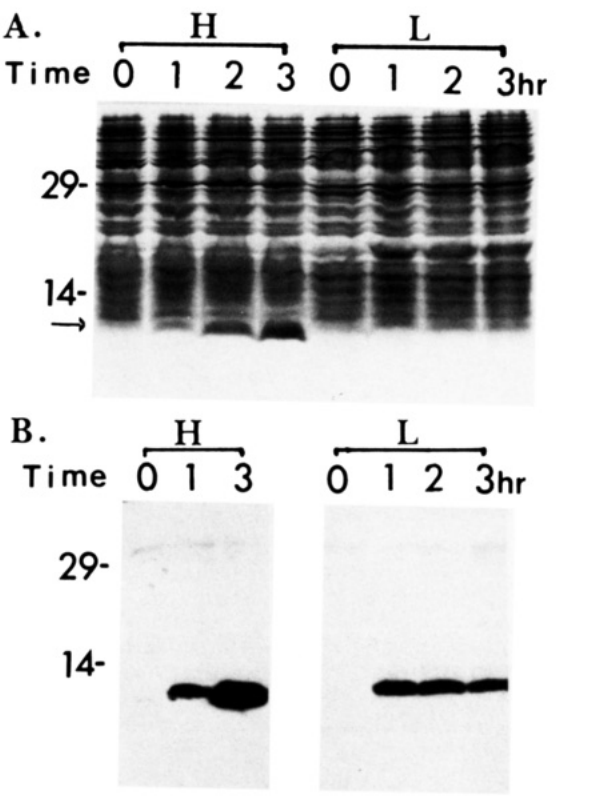

FIGURE 4: Expression of TH-HA61 and TH-LA70 in *E. coli* BL21-(DE3) cells. BL21(DE3) cells harboring pET/hisHA61 or pET/hisLA70 were grown in liquid culture. IPTG was added at cell density $OD_{600} = 0.6$. Aliquots of cell culture were taken at 0, 1, 2, and 3 h after addition of IPTG. Equal OD units of cells were subjected to SDS−PAGE. (A) Coomassie Blue staining; (B) western blot analysis. H, TH-HA61; L, TH-LA70.

cell system which is commonly used for inducible overproduction of foreign proteins (Studier et al., 1990). The genes for the signal peptides are placed under the control of the bacteriophage T7 promoter. Addition of IPTG induces the expression of T7 polymerase in BL21 (DE3), which transcribes genes under the control of its promoter exclusively. Signal sequences were cloned into pET-based plasmids (Figure 1, plasmids 7 and 8). The N-terminal sequences of the resulting peptides, TH-LA61 and TH-LA70, are eight amino acids shorter than CH-HA61 and CH-LA71, respectively (Figure 1). Figure 4 shows the induction of signal peptides in *E. coli* upon the addition of IPTG. The HlyA peptide can be readily visualized on Coomassie Blue stained SDS−PAGE gels (Figure 4A) and confirmed to be the signal peptide by Western blot analysis (Figure 4B). The accumulated level of the LktA signal peptide is lower than that of HlyA; however, time course of expression indicated that during the initial induction (1 h upon the addition of IPTG) the levels of HlyA peptide and LktA peptide are similar (Figure 4B). To test the transport ability of these peptides, we used the BL21(DE3) cells harboring the HlyB/D bearing plasmid pLG575. Upon induction, both peptides can be detected in the growth medium (Figure 5A and Table 1). The fact that at this expression level a large proportion of the signal peptides (60−80%) was in inclusion bodies made it difficult to assess accurately transport efficiency.

The principle for purification of histidine-tagged proteins is based on the binding of a contiguous histidine sequence to nickel. However, we found that, under nondenaturing conditions, the histidine-tagged signal peptides did not bind

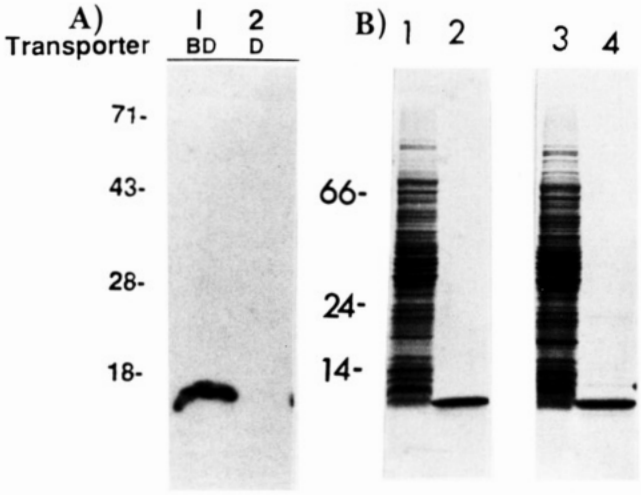

FIGURE 5: (A) Secretion of the HlyA signal peptide TH-HA61. *E. coli* cells expressing HlyA signal peptide TH-HA61 and the transporter proteins HlyB or HlyD as indicated were grown in liquid culture. IPTG was added at a cell density $OD_{600} = 0.6$. Cells were harvested at a cell density $OD_{600} = 0.9$. Culture supernatants samples were subjected to SDS−PAGE, analyzed by Western plotting. (B) SDS−PAGE of cell lysates and purified peptides. 1, lysates of the TH-HA61 expressing cells; 2, purified TH-HA61; 3, lysate of TH-LA70 expressing cells; 4, purified TH-LA70.

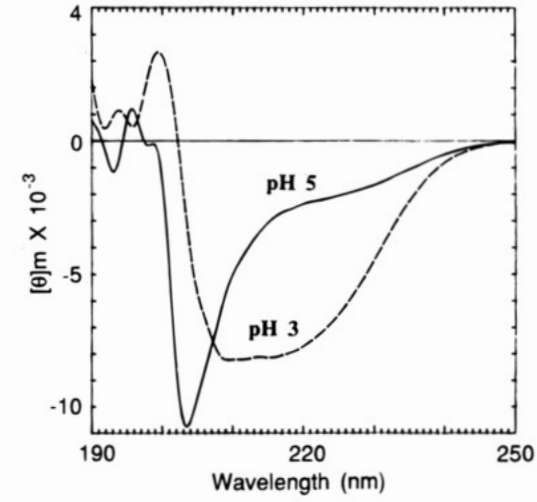

FIGURE 6: CD spectra of the HlyA signal peptide in low pH. The peptide concentration of TH-HA61 was 600 μg/mL. The buffer contains 250 mM NaCl, 25 mM NaP_i, and 10 mM β-mercaptoethanol. The measurement is performed at 23 °C. Lines: solid, pH 5.5; dashed, pH 3. NaCl and β-mercaptoethanol cause high level of noise at 190−203 nm (see Materials and Methods).

to the nickel affinity column. It is possible that the histidine sequence was not exposed. To circumvent this problem, we chose a purification procedure under denaturing conditions. This approach also has the advantage of obtaining the maximum amount of signal peptide by eliminating protease degradation during purification. Cells were dissolved with 6 M guanidine, and histidine-tagged proteins were adsorbed to the nickel affinity column. Proteins were renatured slowly on the column by a gradual removal of guanidine. Signal peptides were eluted at around pH 5 by a pH gradient. The yield of purified HlyA signal peptide was about 5−10 mg/L of culture, and LktA signal peptide was 2−3 mg/L of culture. The 30 kDa *E. coli* nickel-binding protein noted above was not readily observed in this system. It would represent less than 5% of the purified signal peptides (Figure 5B).

Circular Dichroism Properties of Small Signal Peptides. The C-terminal signal sequences of HlyA and LktA share very little sequence similarity despite their functional similarity in mediating transport. This suggests that the function of these signal peptides may not be governed by a specific amino acid sequence but by similar biophysical properties and common higher-order structural features. We examined the secondary structures of the purified signal peptides in different environments by circular dichroism (CD). In aqueous solution, the CD spectra of both HlyA and LktA reflect a predominantly random conformation (Figures 6 and 7). There is no observed structural change between 23 and 37 °C. A broad range of pH changes (pH 4.5−8) do not affect the structure of either HlyA or LktA signal peptide. However, at pH 3, the CD spectrum of the HlyA signal peptide changed to a double minimum at 209 and 216 nm with an isodichroic point at 207 nm (Figure 6), which represents the presence of α-helix and possibly β-strand conformation. This acidic pH-induced conformational change was peptide concentration dependent. A serial dilution showed that, at a peptide concentration of 40 μg/mL, low pH did not induce any change in the HlyA peptide (data not shown); therefore, the conformational change at higher protein concentrations is likely a result of intermolecular interactions. The acidic pH-induced conformation change did not occur in the LktA peptide at similar concentrations.

Since the C-terminal signals direct molecules to translocate through the membrane, they are likely to interact with the inner membrane. Indeed, it has been shown that when a cytoplasmic protein was fused with the HlyA signal sequence, the protein was found in the membrane fraction of *E. coli* (Gentschev & Goebel, 1992). Thus, testing the biophysical properties of these signal peptides in a membrane mimetic environment may be more appropriate than in aqueous solution. Previous studies of N-terminal signal peptides have shown that membrane mimetic environments stabilized the ordered structure of signal molecules which are mainly in a random configuration in aqueous solution [for a review, see Gierasch (1989)]. In such studies, membrane mimetic reagents such as the organic solvent TFE, micelles of the detergent SDS, or phospholipid membranes are commonly used to assess biophysical properties of peptides (Batenburg et al., 1988a; Bruch et al., 1989; Rizo et al., 1993; McKnight et al., 1989; Hoyt & Gierasch, 1991).

We first tested peptides in the organic solvents methanol and TFE. In 50% methanol, the HlyA peptide exhibited an atypical CD spectrum indicating that HlyA forms some other nonrandom conformation in 50% methanol (Figure 7A). The CD spectrum of the LktA peptide in methanol (Figure 7B) is characterized by minima at 208 and 222 nm, indicating adoption of partial α-helical structure. In 25% TFE, the CD spectra of both HlyA and LktA signal peptides showed formation of helices (Figure 7C,D). These results indicate a propensity of both peptides to form helical structures in a more hydrophobic environment. However, natural membranes are interfacial in character containing both hydrophilic and hydrophobic components. Therefore, we measured CD spectra of the peptides in SDS micelles which provide a simple hydrophobic/hydrophilic interface. In 20 mM SDS, both signal peptides showed helix formation with similar double minima curves as in TFE/H_2O (Figure 7A,B). In contrast, Tween20, a nonionic detergent, did not induce any conformational change (data not shown). These observations

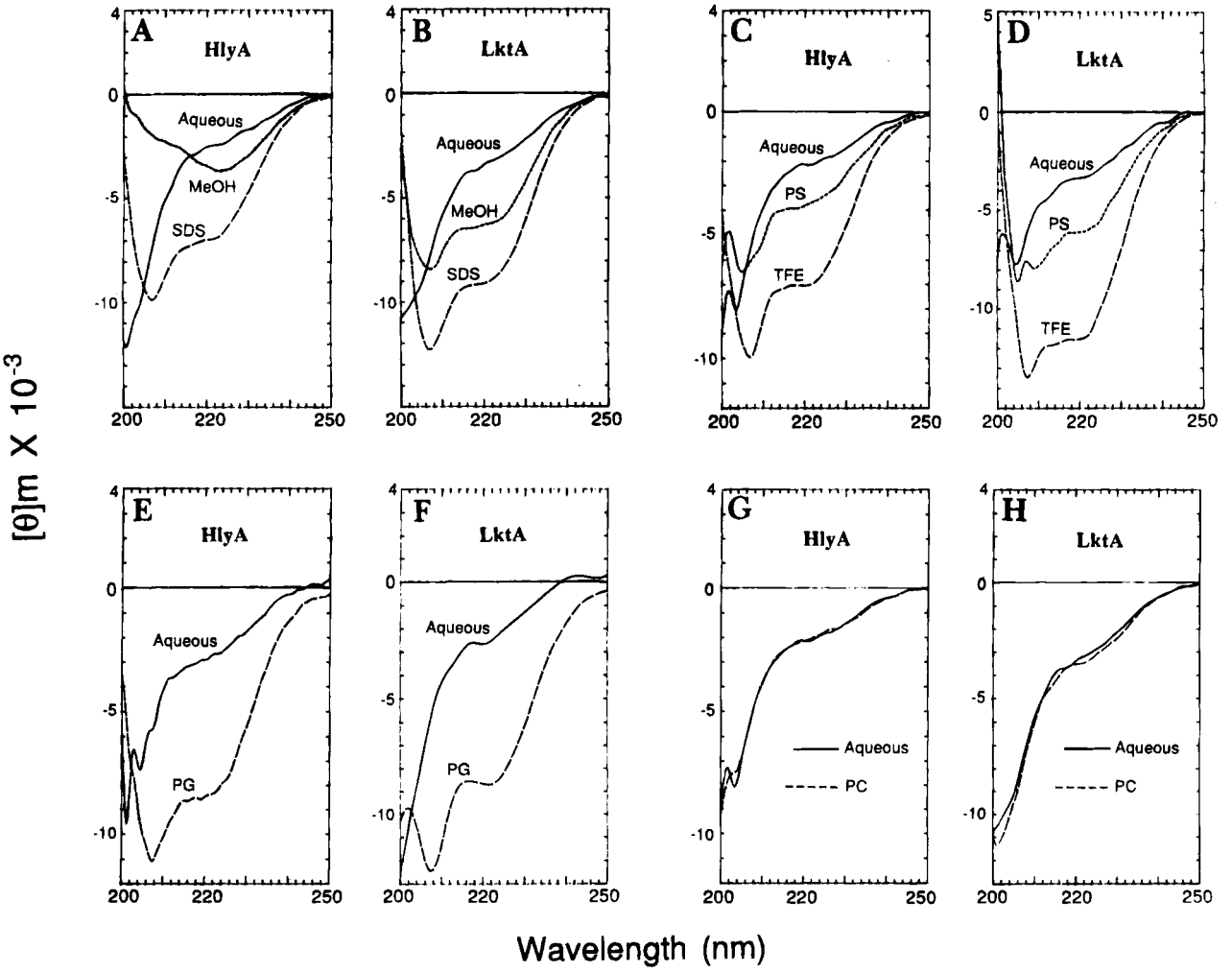

FIGURE 7: CD spectra of signal peptides of HlyA and LktA in aqueous versus membrane mimetic environments. Peptide concentrations are as follows: A, B, E, and H, 150 μg/mL; C and G, 200 μg/mL; D, 100 μg/mL; F, 50 μg/mL. Concentrations of membrane mimetic reagents were 50% MeOH, 25% TFE, 20 mM SDS, 1.5 mM PS, 1.7 mM PG, 1.4 mM PC. Buffer contains 200 mM NaCl, 20 mM NaP_i, and 7 mM β-mercaptoethanol (pH 7). All the measurements were performed at pH 7 and 23 °C.

imply a requirement for charge in a micellar environment to induce helix formation in the two signal peptides.

Finally, we tested the conformational behaviors of HlyA and LktA signal peptides in phospholipid vesicles, a lipid bilayer environment which more closely resembles a membrane compared to the other reagents tested. Uncharged phospholipids PC could not induce an ordered structure (Figure 7G,H), while the anionic phospholipids (PS, PG) induced formation of α-helices in both peptides (Figure 7C–F). The observation that anionic phospholipids but not neutral ones could induce conformational changes suggests the involvement of a charge interaction between both peptides and phospholipid vesicles. Taken together, all these findings indicate that under different conditions both the HlyA and LktA C-terminal peptides behave with similar biophysical properties.

DISCUSSION

We have established a system for the overexpression of functional HlyA and LktA signal peptides, an essential step for producing quantities of purified material for structural analysis of these signal sequences. We have demonstrated that small peptides which contain either the C-terminal 61 amino acids of HlyA or C-terminal 70 amino acids of LktA can be secreted in a HlyB/D dependent manner. This confirms that these short sequences contain information sufficient for recognition and transport, and that no other sequences are required. In addition, we have shown that GST fusion proteins with either the HlyA or LktA C-terminal signal sequences cannot be secreted but are able to compete for the secretion of the intact HlyA molecule. This suggests that these C-terminal signal sequences can interact with the HlyB/D transporter even though the rest of the molecule may not be in a proper conformation for transport.

The C-terminal signal peptides of HlyA and LktA exhibit similar biophysical behaviors. In aqueous solution, both molecules are mainly in a random conformation. Membrane mimetic environments such as TFE/H_2O, SDS micelles, and negatively charged phospholipid vesicles induce formation of α-helices. Our results indicate that both signal peptides undergo a similar conformational change upon interaction with phospholipid membranes and membrane mimetic environments.

Vesicles of neutral phospholipids do not induce ordered structure in the signal peptides suggesting a requirement for the involvement of electrostatic interactions between the signal peptides and charged head groups of the lipid. A nonspecific attraction between the signal sequences and negatively charged lipids could enhance the binding affinity between the signal sequences and the membrane surface,

which in turn may induce ordered structure in the signal sequences. Alternatively, a specific interaction of certain charged residues in the signal sequences and charged head groups of lipids may be required to maintain an ordered structure in the signal sequences. Charge pattern is not well conserved between the C-terminal signal sequences of HlyA and LktA. However, charges may be arranged in a similar way in the context of secondary structure. Investigation using NMR is underway to further define the structure of HlyA and LktA signal sequences (Yin et al., manuscript in preparation). During the preparation of this manuscript, Wolff et al. (1994) have reported their studies on the C-terminal signal of an *Erwinia chrysanthemi* protease whose transporter is homologous to HlyB/D. They observed by CD and NMR analyses that the signal peptide helical content also increased in a membrane mimetic environment, such as in the presence of TFE and dodecyl β-D-maltoside detergent. However, whether or not this C-terminal signal peptide behaves differently in neutral and charged phospholipids is not known. It would be of interest to determine if the biophysical properties of the protease signal sequence in lipid vesicles are similar to those exhibited by the HlyA and LktA signals.

It is interesting to compare the C-terminal signal in the hemolysin transport system with the N-terminal signal directed transport pathway which is dependent on multiple *Sec* proteins [for a review, see Joly and Wickner (1993)]. Both N- and C-terminal signal peptides are unstructured in aqueous solution and adopt ordered structures in membrane mimetic environments. It has been observed that many N-terminal peptides adopt α-helix conformation in both TFE/H_2O and SDS micelles (Briggs & Gierasch, 1984; Batenburg et al., 1988a; Bruch et al., 1989; Rizo et al., 1993). In PG containing vesicles, the N-terminal peptides of *E. coli* proteins, LamB (McKnight et al., 1989) and OmpA (Hoyt & Gierasch, 1991), were shown to form α-helices. The negatively charged phospholipid PG is one of the main lipid components in the inner membrane of *E. coli* (75% PE, 20% PG, and 5% cardiolipin) (Burner et al., 1980). The presence of negatively charged lipids increases the penetration of N-terminal signal peptides into the lipid membrane (Batenburg et al., 1988b; Demel et al., 1990). It has been shown by both *in vivo* and *in vitro* studies that PG is required for the N-terminal signal-directed transport process (de Vrije et al., 1988; Kusters et al., 1991; Phoenix et al., 1993). The requirement of negatively charged phospholipids for α-helix formation indicates a resemblance between the biophysical properties of the C-terminal and the N-terminal signal peptides. This similarity may represent a common theme in these two different transport mechanisms. It is possible that a fundamental step in protein translocation in bacteria requires a signal peptide to switch conformation allowing it to penetrate the membrane and interact with the appropriate transporter complex.

A model of hemolysin transport may be proposed from the above data. The C-terminal signal of HlyA may be largely unstructured in the cytoplasm, and, as a first step for transport, the signal sequence associates with the membrane, likely binding to the membrane surface via a charge interaction. Gentschev and Goebel (1992) have demonstrated that the HlyA signal sequence can direct an otherwise cytoplasmic protein into association with the membrane. The amphipathic helix predicted in many C-terminal signal sequences have also been postulated to interact with the inner membrane (Stanley et al.,1991). Biophysical evidence presented here for the HlyA and LktA signal peptides and for the signal peptide of *E. chrysanthemi* protease G (Wolff et al., 1994) show that these peptides all have a propensity to interact with membranes and undergo a conformational switch from unstructured to helix. As noted above, this kind of conformational change may be a necessary step for the association of a signal peptide with the transporter complex. Such a step allows the molecule to search for a transporter in two-dimensional space which is more efficient than in three-dimensional space (i.e., the cytoplasm).

Association with the membrane may induce in the C-terminal signal an appropriate structure for binding to HlyB. Our previous genetic complementation study has suggested that interaction of the HlyA signal sequence with HlyB is located in a region close to the cytoplasmic face of the inner membrane (Zhang et al., 1993a). On the basis of the observation of Kenny et al. (1994) and Zhang et al. (1993a,b), we speculate that the binding of a signal with the transporter may occur in two distinct steps. The first step involves a "loose" association, in which the transporter recognizes the signal by its three-dimensional shape. In this step, the C-terminal signal of HlyA and LktA may present similar three-dimensional shapes to the HlyB/D transporter. The second step is close contact between the signal sequences and the transporter, which likely involves specific amino acid interactions. By mutational analyses, Kenny et al. (1992, 1994) have identified a few critical "contact" amino acids scattered throughout the length of the signal sequence of HlyA, which are thought to interact with the HlyB/D transporter. The contact amino acids in the signal of LktA are unlikely to be the same since the sequences of these two signals are different. Our complementaion experiments have also shown that some point mutants of HlyB affect the transport of molecules with a LktA signal but not a HlyA signal (Zhang et al., 1993a). Therefore, recognition of the HlyA and LktA signals by HlyB may involve distinct, though possibly overlapping, amino acid contacts.

The secretion of small peptides containing either the C-terminal 61 amino acids of HlyA or 70 amino acids of LktA by the HlyB/D transporter has indicated that these signal sequences are sufficient for both recognition and translocation by the transporter. However, it has been reported that different passenger proteins require different lengths of HlyA C-terminal sequences from 60 to 217 amino acids for efficient transport (Kenny et al., 1991; Hess et al., 1990), and not all chimeric proteins can be transported (Gentschev & Goebel, 1992; this paper). We have shown in this report that GST/signal fusion proteins are not transported but are able to compete for transport of the intact HlyA molecule. This suggests that the GST fusion proteins are able to bind to the transporter but are impaired at the stage of translocation. It is possible that after the fusion protein is targetted by the signal sequence to the HlyB/D transporter, the translocation of the rest of the molecule requires additional steps for unfolding. In the *E. chrysanthemi* metalloprotease B transporter system which involves a HlyB/D like transporter, a 6 kDa peptide containing the C-terminal 39 amino acids of PrtB can be secreted (Delepelaire & Wandersman, 1990); however, longer sequences including the glycine-rich repeat domain are required to drive secretion of a larger molecule (Létoffé & Wandersman,

1992). A glycine-rich repeat domain is located at the C-terminal region of many bacterial toxins such as hemolysin, leukotoxin, metalloprotease, and cyclolysin (the so-called RTX toxins) and is involved in Ca^{2+} binding. It has been postulated that the glycine-rich repeat domain may be in an unfolded configuration in the cytoplasm (Baumann et al., 1993), which may help the unfolding of passenger proteins.

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