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Use of *phoA* Fusions To Study the Topology of the *Escherichia coli* Inner Membrane Protein Leader Peptidase

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A topology of the *Escherichia coli* leader peptidase has been previously proposed on the basis of proteolytic studies. Here, a collection of alkaline phosphatase fusions to leader peptidase is described. Fusions to the periplasmic domain of this protein exhibit high alkaline phosphatase activity, while fusions to the cytoplasmic domain exhibit low activity. Elements within the cytoplasmic domain are necessary to stably anchor alkaline phosphatase in the cytoplasm. The amino-terminal hydrophobic segment of leader peptidase acts as a weak export signal for alkaline phosphatase. However, when this segment is preceded by four lysines, it acts as a highly efficient export signal. The coherence of in vitro studies with alkaline phosphatase fusion analysis of the topology of leader peptidase further indicates the utility of this genetic approach to membrane protein structure and insertion.

The *Escherichia coli* leader peptidase is a cytoplasmic membrane protein responsible for the cleavage of leader or signal peptides from many exported proteins. Proteolytic treatment of various *E. coli* membrane preparations has suggested the arrangement of the protein shown in Fig. 1 (19, 23). The existing data are consistent with a model in which the first 22 amino acids (H1) of leader peptidase span the membrane with the amino-terminal methionine on the periplasmic face of the membrane. This location and orientation of H1 are based on the known arrangement of the rest of the protein and on the highly hydrophobic nature of this stretch. In addition, studies of various deletion derivatives of the *lep* gene show that (i) the hydrophobic segment 2 (H2) is sufficient to promote export of the periplasmic domain and (ii) the cytoplasmic domain (amino acids 23 through 61) acts as a poison sequence that prevents H2 from acting as an export signal when H1 is not present (22).

In addition to proteolytic and biophysical studies, recently developed genetic approaches to membrane protein topology have proved fruitful (16). In particular, the use of alkaline phosphatase fusions has provided information on the topology of a number of cytoplasmic membrane proteins, including Tsr, MotB, MalF, SecY, and GlpT (1, 3, 5, 9, 15). The alkaline phosphatase fusion approach is based on the dependence of the enzymatic activity of this protein on its cellular location; the enzyme is active when it is in the periplasm but inactive in the cytoplasm (2, 17). Thus, alkaline phosphatase is highly active when fused to periplasmic domains of membrane proteins but exhibits low or no activity when fused to cytoplasmic domains. This distinction allows one to determine the location of the different extramembranous domains of cytoplasmic membrane proteins. It has now been verified for many different cases that the amount of alkaline phosphatase activity is directly correlated with amount of the protein exported to the periplasm (1–3, 13, 15). Even the low activity found with cytoplasmic fusions is due to a small amount of export of the fused alkaline phosphatase.

So far, the proteins that have been analyzed by using gene fusions of alkaline phosphatase have been ones in which the amino terminus of the protein has been in the cytoplasm. To further extend this approach, we have obtained a set of fusions of alkaline phosphatase to leader peptidase. The results of these studies indicate the general usefulness of the gene fusion approach and also provide additional details on the mechanism of leader peptidase assembly.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The *E. coli* K-12 strain KS300 (*lacX74 galE galK* Str^r *phoA PvuII recA1*) was obtained from Kathy Strauch (Harvard Medical School). It carries on the chromosome a *phoA* gene with a *PvuII* fragment deleted from it (3). Plasmid pRD8, a pBR322 derivative in which the synthesis of leader peptidase is under the control of the inducible arabinose B promoter (6), was used for constructing alkaline phosphatase gene fusions. Bacteriophage λ::Tn*phoA* (10) was used as a source of Tn*phoA* (Tn*5* IS*50*$_L$::*phoA*).

Media and reagents. TYE, LB, and M63 media (18) were used throughout. Antibiotics were used at the following final concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml. All cultures were grown aerobically at 37°C. Colonies producing PhoA fusions were selected on solid media containing 5-bromo-4-chloro-3-indolyl phosphate (XP) at 40 μg/ml. Alkaline phosphatase enzyme activity was determined in cells grown in LB medium by using paranitrophenylphosphate, as described by Manoil and Beckwith (15). The fusions of alkaline phosphatase to leader peptidase were induced for 1 h with arabinose. [^{35}S]methionine (1,500 Ci/mmol) and [α-^{32}P]dATP (800 and 3,000 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, Ill.). Restriction endonucleases, Klenow fragment, T4 DNA ligase, T4 DNA polymerase, and nuclease S1 were obtained from New England Biolabs, Inc. (Beverly, Mass.). IgGSORB was

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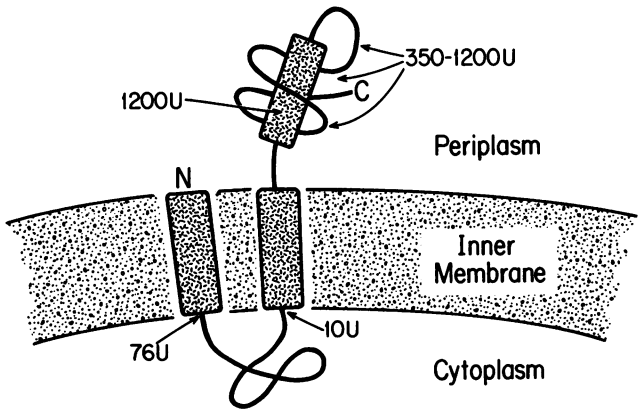

FIG. 1. Topology of leader peptidase with activity of *phoA* fusions. Numbers represent units of alkaline phosphatase activity of different fusions.

obtained from the Enzyme Center, Inc. (Boston, Mass.). All reagents were used by the recommendations of the manufacturer.

Isolation and characterization of Tn*phoA* insertions. Alkaline phosphatase gene fusions were obtained by infecting strain KS300(pRD8) with λ::Tn*phoA* as described previously (10). Independently mutagenized cultures were plated on TYE agar containing 300 μg of kanamycin per ml in addition to ampicillin to select Tn*phoA* transpositions onto the multicopy plasmid. Resistant colonies were pooled, and plasmid DNA was obtained, which was used to transform strain KS300 with selection for Ap^r Km^r on TYE agar containing XP and 0.2% arabinose. Transformants which produced blue ($PhoA^+$) colonies were isolated, and those in which the expression of alkaline phosphatase was induced by arabinose were retained for further analysis. Because the maps of pRD8 (6) and Tn*phoA* (14) are known, the sites of Tn*phoA* insertions could be inferred from the electrophoretic pattern of appropriate digests of the Tn*phoA*-carrying derivatives. Transpositions onto pRD8 were mapped by digestions with *Pst*I, *Hin*dIII, and *Eco*RI (Fig. 2). Plasmids were stabilized by deleting DNA between the *Sma*I site within Tn*phoA* and the *Sma*I site at the linker between the *lep* gene and pBR322 DNA (Fig. 2). This procedure removed IS*50R* from Tn*phoA*. The DNA sequence of the *lep-phoA* fusion joints was determined by the chain termination method (20) on the stabilized plasmids. We used a 16-mer-Tn*phoA* primer corresponding to a sequence beginning 70 nucleotides into Tn*phoA*.

Construction of *lep-phoA* fusions in vitro. To obtain fusions in which PhoA was attached to the cytoplasmic domain of leader peptidase (Lep), we have used oligonucleotide-directed mutagenesis to generate specific deletions as described previously (3, 7). A *Pst*I-*Xho*II fragment from plasmid pSAN111, containing the entire gene fusion, was subcloned into M13tg131 (12) that had been cut with *Pst*I and *Sal*I. Cells infected with this phage produce dark blue plaques on TYE solid medium containing XP and IPTG (isopropyl-β-D-thiogalactopyranoside). Oligonucleotides were synthesized that contained 20 nucleotides from the end of Tn*phoA* that generates the fusion and 20 from a sequence corresponding to the cytoplasmic domain of leader peptidase. We prepared two oligonucleotides which allowed us to make deletions from the fusion joint in SAN111 (nucleotide 268) to nucleotide 67 or 184 of the coding sequence. After annealing and extension of the templates, the nonhybridizing segments were eliminated by S1 nuclease treatment (7). These mixtures were used to transform Ca^{2+}-treated cells, and plaques that showed different colors on XP-containing media were selected. Some white plaques were analyzed and found to correspond to phages with aberrant deletions. Blue plaques corresponded to phage with the parent sequence. Only the pale blue plaques yielded phage with the restriction pattern corresponding to the desired deletions. Deletions were confirmed by sequencing the fusion joint on the M13 derivative by using a Tn*phoA*-specific primer. The two new gene fusions obtained were cloned in plasmid pSAN116; this is a pACYC184 derivative harboring the fusion SAN111, including the *araC* gene and *araB* promoter, inserted in site *Ava*I. The new gene fusions were cloned by replacing a *Pst*I-*Hin*dIII fragment in pSAN116. We selected Cm^rTc^s pale blue colonies on XP medium, different from the dark blue colonies produced by cells harboring pSAN116.

Insertion of four lysines in fusion SAN119. Oligonucleotide-directed mutagenesis was used to insert four lysyl residues between positions 4 and 5 of the leader peptidase-PhoA fusion (see Fig. 4). To make this mutation, the *Hin*dIII-*Pst*I fragment of pSAN119 was subcloned into M13mp9. The oligonucleotide, 5′-ATGGCGAATATGAAGAAGAAGAA GTTTGCCCTGATT-3′, was annealed and extended by *E. coli* DNA polymerase (large fragment) and ligated by T4 DNA ligase. After transformation into JM103, the plaques were screened by plaque hybridization with the ^{32}P-labeled oligonucleotide, and then the DNA from the positive plaques was sequenced to confirm the mutation. The *Hin*dIII-*Pst*I leader peptidase-PhoA fragment containing the 4-lysine insertion was then inserted back into the expression vector by ligating it to the pSAN119 *Hin*dIII and *Pst*I large fragment (alkaline phosphatase treated).

[^{35}S]methionine labeling and immunoprecipitation conditions. For detecting Lep-PhoA hybrid proteins, cells were grown at 37°C in M63 amino acids with ampicillin. Cultures were induced with 0.2% arabinose and, 1 h later, were pulse-labeled for 30 s with 100 μCi of [^{35}S]methionine per ml.

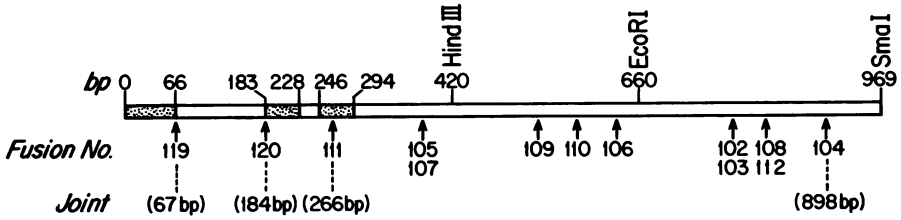

FIG. 2. Position of *phoA* fusion joints in the *lepA* gene. The positions were determined by restriction mapping and, in the case of SAN119, SAN120, SAN111, and SAN104, by DNA sequencing. Estimated distances determined by restriction mapping are shown for the fusions which were not sequenced. ▩, Hydrophobic sequences in the protein. bp, Base pairs.

Pulse-labeling conditions, preparation of cell extracts, and immunoprecipitations were as described by Ito et al. (11). Proteins immunoprecipitated with anti-PhoA were analyzed by electrophoresis as previously described (14). Gels were fixed and treated for fluorography by the method of Chamberlain (4).

RESULTS

Using alkaline phosphatase fusions to study membrane protein topology. We will describe the procedure we have developed for using alkaline phosphatase fusions to analyze membrane protein topology, since improper use of data can lead to mistaken conclusions. This approach is based on the following considerations. Alkaline phosphatase fusions to the periplasmic domains of membrane proteins produce hybrid proteins where most, if not all, of the protein made exhibits normal alkaline phosphatase enzymatic activity. While fusions to cytoplasmic domains of the same proteins produce much lower activity, they nearly always show some activity. This activity is due to a smaller amount of export of alkaline phosphatase. Thus, simply finding alkaline phosphatase activity with a fusion is not evidence that a particular domain is normally localized to the periplasm.

What is important to determine is the efficiency with which various fusion proteins localize the alkaline phosphatase portion to the periplasm. This can be done by relating the alkaline phosphatase enzymatic activity to the total amount of hybrid protein made. This calculation of specific activity is critical because the rates of synthesis of different hybrid proteins produced by alkaline phosphatase fusions may vary. This can be true even among fusions to the same membrane protein (see data in this paper). However, there is a problem in such an analysis. In many cases, hybrid proteins in which the alkaline phosphatase is localized to the cytoplasm are unstable; the alkaline phosphatase moiety is rapidly degraded. In such situations, if one were to measure steady-state levels of hybrid protein (e.g., with Western blots [immunoblots]), one would only be measuring that fraction which was exported and had assembled into the stable active form. The specific activity of such a hybrid protein, if measured on the basis of steady-state levels of protein, would be similar to that of a periplasmic fusion protein. For these reasons, we estimate specific activities based on the rates of synthesis of the fusion proteins by using pulse-label experiments (see Table 1). Ordinarily, the half-life of the unstable proteins is such that after a 30 s or 1-min pulse, practically no degradation has taken place. These calculations will indicate how much of the alkaline phosphatase that is initially made is exported.

In our experience, when calculated in this way, the specific enzymatic activities of a variety of fusion proteins in which alkaline phosphatase is localized to the periplasm are quite similar. The specific activities of hybrids in which alkaline phosphatase is fused to a cytoplasmic domain are, for the most part, at least 20 times lower.

Determining the specific activity of hybrid proteins on the basis of the rate of protein synthesis seen in pulse-labeling experiments has some limitations of its own. The enzymatic activity measured represents the accumulation of enzyme resulting from the period during the growth of the culture when the gene fusion is expressed. For instance, in experiments described in this paper the fusion is induced for 1 h with arabinose, but the labeling is done at the end of the 1-h period. If there should be any variation in expression of the gene fusion during the growth of the culture, estimating rates of synthesis at one time point with pulse-labeling would give an inaccurate picture of the specific activity as we describe it above. Nevertheless, in several cases in which proteins with known topology have been analyzed, the pattern of specific activities predicts the correct topological model. Therefore, while this reservation should be kept in mind in the case of genes whose expression may be variable, overall it does not seem to be a serious weakness in the approach.

Isolation and characterization of leader peptidase-alkaline phosphatase fusion proteins obtained by Tn*phoA* transposition. Studies of proteolysis of leader peptidase have led to the model for its topology shown in Fig. 1. *Lep-phoA* gene fusions were obtained by Tn*phoA* mutagenesis of plasmid pRD8, in which the *lep* gene is transcribed from the *araB* promoter (6). The PhoA enzyme activity was monitored by using the chromogenic substrate XP added to the plates. Twelve independent clones in which the expression of alkaline phosphatase was inducible by arabinose were isolated.

To determine the site of fusion of alkaline phosphatase to leader peptidase, the plasmids containing the transposon insertions that were blue on XP-containing media were analyzed by restriction mapping. In 11 plasmids, the insertion mapped within the *lep* gene, and in 1 plasmid (SAN101), the transposon was upstream of *lep* (Fig. 2). Cells harboring this last plasmid produce pale blue colonies.

The initial plasmids were stabilized by deleting IS*50R*. Levels of alkaline phosphatase activity were assayed on strains carrying these derivatives that were deleted for the chromosomal *phoA* gene. The fusion strains were induced with arabinose for 1 h. These plasmids directed the synthesis of proteins which were precipitated by antiserum against the PhoA protein (Fig. 3). The apparent molecular weights of these proteins were in good agreement with that predicted from the restriction analysis for the corresponding *lep-PhoA* gene product (Table 1). Some of the strains also showed degradation product of the hybrid proteins. However, in pulse-chase experiments, these proteins were relatively stable, with little degradation after 45 min (data not shown).

The exact site of the fusion joint was determined by DNA sequencing only for the extreme fusions, SAN111 and SAN104 (Fig. 2). The sequencing results agreed with those of restriction analysis. The maintenance of the correct reading frame in the fusion joint was also confirmed for these two fusions.

The results of the analysis of the fusion strains is summarized in Table 1. All but one of the fusions exhibited high levels of alkaline phosphatase activity (from 400 to 1,200 U), with SAN111 (1,200 U) and SAN104 (900 U) being the most active. In all of the fusions, the site of the fusion joint was within the proposed periplasmic domain of the leader peptidase (23).

Fusion SAN109, which showed a lower activity (3 to 10% of the high-activity fusions) also had a fusion joint in the periplasmic domain. However, this low activity is due to a lowered production of the hybrid protein after induction of its synthesis. While cells carrying plasmid pSAN109 produce a correct-sized hybrid protein that was stable in pulse-chase experiments, their viability is severely affected by the presence of arabinose in the medium. In fact these cells could not grow in minimal M63 medium supplemented with glycerol when arabinose was added. Presumably, the interference with cell growth prevents long-term induction by arabinose of the synthesis of full amounts of the protein. The hybrid protein that is produced has about the same specific activity as the other periplasmic hybrids studied (Table 1). Thus, the

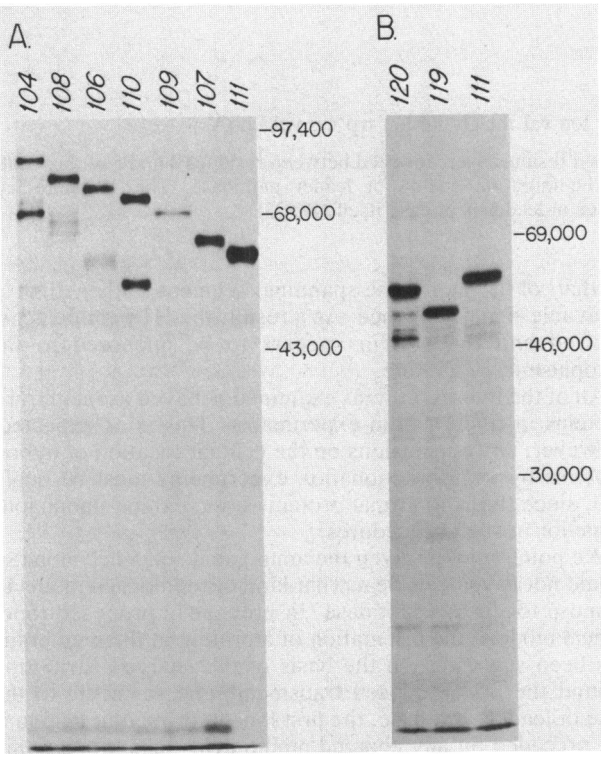

FIG. 3. Immunoprecipitated pulse-labeled fusion proteins. Extracts (see Materials and Methods) were precipitated with anti-alkaline phosphatase antibody after a 30-s pulse-label. The molecular weight standards (in order of decreasing molecular weight) were phosphorylase B, bovine serum albumin, and ovalbumin (A) and bovine serum albumin, ovalbumin, and carbonic anhydrase (B). Since the standards were obtained at different times, the recorded molecular weights for the derivatives of bovine serum albumin and ovalbumin differed.

TABLE 1. Characterization of *lep-phoA* gene fusions

Fusion	Fusion site[a]	Fusion protein[b]	Alkaline phosphatase activity (U)	Sp act[c]
In vivo fusions				
SAN101	ND	ND	10	ND
SAN111	266	60,000	1,200	1,200
SAN105				
SAN107	ND	63,000	350	ND
SAN109	ND	70,000	30	882
SAN110	ND	73,000 (55,000)	500	ND
SAN106	ND	76,000	450	850
SAN102				
SAN103	ND	ND	500	ND
SAN108				
SAN112	ND	80,000	650	ND
SAN104	898	85,000 (70,000)	900	1,011
In vitro fusions				
SAN119	67	52,500	76	168
SAN120	184	57,000	10	15
119-4-lys	67	52,500	1,270	1,164

[a] Positions of the Tn*phoA* inserts are relative to the estimated translation start point of the *lep* gene.

[b] Molecular weight of the hybrid proteins and the presumed degradation products estimated from position on gels.

[c] In most cases, the initial rates of hybrid protein synthesis as measured after pulse-labeling were comparable, indicating that high-activity strains had similar alkaline phosphatase activities. The exceptions were fusions SAN109, SAN119, and SAN120. In the case of SAN109, the rate of synthesis was much lower (see text). In the cases of SAN119 and SAN120, the rates were approximately 30 and 60% lower, respectively. These lower apparent rates may be due to the instability often seen with cytoplasmically located fusions. The specific activities were determined by dividing the alkaline phosphatase measured by the amount of radioactivity found in gel bands for the particular protein as determined with a densitometer. The amount of radioactivity in fusion SAN111 was taken as 1, and the rest were normalized to that of SAN111.

[d] ND, Not determined (these fusions were not sequenced, and the fusion joint was estimated by restriction analysis).

alkaline phosphatase in SAN109 is exported to the same extent as in the other periplasmic fusions.

Gene fusions obtained in vitro. We obtained no fusions by transposition in which alkaline phosphatase was directly attached to the proposed cytoplasmic domain of leader peptidase. It appeared possible that such fusions could not be detected because they exhibited no alkaline phosphatase activity. Therefore, we decided to generate fusions in the cytoplasmic domain of leader peptidase by producing oligonucleotide-directed deletions starting with a Tn*phoA* fusion to the periplasmic domain. This technique has been previously used in the study of MalF topology (3).

We prepared two oligonucleotides that contained 22 nucleotides from the end of Tn*phoA* and 22 corresponding to the end of the first hydrophobic domain (nucleotides 46 to 67 of the gene), or to the end of the cytoplasmic domain (nucleotides 163 to 184, Fig. 2). In this way, we hoped to obtain alkaline phosphatase fusions to both extremes of the cytoplasmic domain by deleting fragments of the leader peptidase gene coming from nucleotide 67 or 184 to the insertion site in a previously obtained fusion. The mutant oligonucleotides were annealed to single-stranded M13tg131 DNA containing the fusion SAN111. After extension, ligation, treatment with S1 nuclease and transformation, we selected pale blue single plaques. Deletions were verified by sequencing and were cloned in plasmids under the control of the arabinose promoter.

Plasmids containing the two new fusions were used to determine their alkaline phosphatase activity. The levels of alkaline phosphatase activity depended on the fusion site within the cytoplasmic domain (Table 1). A fusion at the beginning of this domain, SAN119, produced 76 U of alkaline phosphatase activity, while a fusion at the end of the domain, SAN120, produced only 10 U. Although the alkaline phosphatase activities of these strains were low, the amounts of the proteins produced upon pulse-labeling were no more than about twofold lower than that of a periplasmic fusion such as SAN111 (Fig. 3 and Table 1). This is in contrast to the results with fusion SAN109. Thus, with these two fusions, the specific activity of the hybrid protein was substantially lower than those of the periplasmic fusions. The sizes of the hybrid proteins established by immunoprecipitation with anti-PhoA antibody and sodium dodecyl sulfate-gel electrophoresis were as predicted (Fig. 3 and Table 1). These two proteins behaved as membrane proteins in fractionation experiments (data not shown).

Converting H1 to a signal sequence. Von Heijne et al. (22) have shown that H1 will behave as a strong signal sequence if it is preceded by four positively charged amino acids. The fusion of alkaline phosphatase to H1 itself yields a hybrid protein in which only a small fraction of the alkaline phosphatase is exported to the periplasm. To determine whether positively charged amino acids preceding H1 would also result in the efficient export of alkaline phosphatase, we have constructed the fusion shown in Fig. 4. When this fusion strain (119-4-lys) was assayed, it was found to export high levels of alkaline phosphatase (Table 1). As with the other

(lys lys lys lys)

↓

Met ala asn met phe ala leu ile leu val ile ala thr leu val thr gly ile leu trp cys val-PhoA

FIG. 4. Construction of a 4-lysine insertion with fusion SAN119. Four lysyl residues were inserted between residues 4 and 5 of the leader peptidase-PhoA fusion, SAN119. The first methionine corresponds to the initiation codon for leader peptidase. The 22-amino-acid hydrophobic sequences presented here is followed by a hydrophilic sequence in leader peptidase itself (19, 23).

hybrid proteins, the enzymatic activity was associated with the membrane fraction.

DISCUSSION

The properties of alkaline phosphatase fusions to leader peptidase suggest a model for the membrane topology of the protein which is identical to that proposed on the basis of proteolytic studies. Fusions to the proposed periplasmic domain of leader peptidase exhibit high alkaline phosphatase enzymatic activity, while fusions to the cytoplasmic domain show 10- to 100-fold-lower activities. The one apparent exception to this rule, SAN109, a periplasmic fusion, showed low alkaline phosphatase activity. However, this low activity was due to low amounts of the protein present in the cells, not to a hybrid protein with low specific activity. The findings with SAN109 demonstrate the importance of careful analysis of specific activities when alkaline phosphatase fusions are used to analyze membrane protein topology. The low amounts of SAN109 hybrid protein may be explained by the loss of viability conferred on the cell by its induction. While induction of several of the hybrid proteins resulted in some degree of growth inhibition, SAN109 had the greatest effect. We do not understand why this particular fusion has such strong effects on cell growth.

The two fusions to the cytoplasmic domain of leader peptidase show significant differences in their activities. Fusion SAN120, which includes the entire cytoplasmic domain, produces only about 1% of the activity seen with the average periplasmic fusion, while fusion SAN119, which is missing the cytoplasmic domain, produces about 10-fold more activity. This same difference in activity, a difference which depends on the position within the cytoplasmic domain of the fusion joint, was seen with fusion of alkaline phosphatase to three cytoplasmic domains of the MalF protein (3). We believe that the explanation for this difference is that the positively charged amino acids following H1 help to orient this hydrophobic segment as indicated in Fig. 1. In fusion SAN120, which contains none of these charges, H1 may alternate in its orientation so that in a significant proportion of the molecules, alkaline phosphatase is exposed to the periplasm.

According to this model, H1, by itself, acts as a weak signal sequence. Von Heijne et al. (22) have shown that H1 will behave as a strong signal sequence if it is preceded by four positively charged amino acids. We have found analogous results with the H1-alkaline phosphatase fusion. Thus, it appears that simply inserting a long hydrophobic stretch before a secreted protein, while allowing some export, does not confer efficient transfer across the membrane. Positively charged amino acids may be essential for determining the orientation of such segments and, thus, their role as export signals. The introduction of positively charged amino acids before a hydrophobic segment in a eucaryotic system has also been shown to convert that segment to an export signal (21). While H1 is acting as a signal in this case, its structure is that of a membrane-spanning segment rather than a cleavable signal sequence. As a result, the H1 peptide is not cleaved and the protein appears to be anchored to the cytoplasmic membrane.

All of the hybrid proteins examined behaved as membrane proteins in fractionation experiments. This is as expected. However, any conclusions on the cellular location of hybrid proteins by using fractionation experiments must be qualified, since these abnormal proteins often exhibit anomalous behavior in such procedures.

We point out that given the amino acid sequence alone, it would not have been clear what kind of topological model to propose for leader peptidase. In previously proposed topological models, the orientation of a protein in the membrane has been suggested on the basis of the charge distribution around the first proposed transmembrane segment. In the case of leader peptidase, the first long hydrophobic region is not preceded by any charged amino acids. The finding that the properties of alkaline phosphatase fusions lead to a correct prediction for the structure indicates that the method may be generally useful for such analyses with proteins of unknown topology. Moreover, this method and the complementary fusion approach with β-galactosidase (8) may give an overall picture of the topology of many cytoplasmic membrane proteins by using genetic criteria.

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