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Positively charged amino acid residues can act as topogenic determinants in membrane proteins

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ABSTRACT **When alkaline phosphatase is fused to the periplasmic domain of a cytoplasmic membrane protein, it is efficiently exported to the periplasm. Such a hybrid protein exhibits high alkaline phosphatase enzymatic activity. When alkaline phosphatase is fused to the cytoplasmic domain of a membrane protein, it remains, for the most part, in the cytoplasm. Such fusions exhibit low enzymatic activity. However, stable retention of alkaline phosphatase in the cytoplasm requires the presence in the fusion protein of the cytoplasmic loop ordinarily present in that position in the native, unfused protein. Using oligonucleotide-directed mutagenesis, we have shown that positively charged amino acids are required for the stable cytoplasmic localization of the fused alkaline phosphatase. We propose that, in addition to hydrophobic transmembrane segments, positively charged amino acids in the hydrophilic cytoplasmic domains of a membrane protein are determinants of the protein's topology.**

The problem of predicting protein structure from amino acid sequence is a fundamental one in molecular biology. Complex integral membrane proteins may constitute a class of proteins for which this problem is somewhat simpler than for soluble proteins. Since these proteins cross the plane of the membrane many times, a description of their topological relationship to the membrane severely limits the number of possible three-dimensional structures they can attain. This property of membrane proteins facilitates the task of predicting their three-dimensional structures. The membrane-spanning stretches of such proteins usually can be identified as long sequences composed mostly of hydrophobic amino acids. Algorithms for recognizing the appropriate hydrophobic stretches and predicting topological structure have met with considerable success (1, 2). However, features of the amino acid sequence other than hydrophobic regions may contribute to the topology of proteins in membranes (3, 4). In this paper, we describe an experimental approach that allows us to identify characteristics of the amino acid sequence in addition to hydrophobicity that may function to determine topological structure.

Blobel and Friedlander (5, 6) have proposed that the topological structure of integral membrane proteins may be accounted for by two different classes of determinants. The first class includes signal sequences that initiate transfer of parts of the peptide across the membrane; the second includes sequences that stop this transfer. A number of studies have shown that the hydrophobic nature of the membrane-spanning stretches is an important feature of both types of topogenic determinants (7–12).

We have presented evidence that the hydrophilic cytoplasmic domains of complex integral membrane proteins also play a role in determining topology (4). In particular, features of the amino acid sequences of cytoplasmic domains ensure their stable cytoplasmic localization. This conclusion came from studies on protein fusions between MalF, an *Escherichia coli* cytoplasmic membrane protein, and alkaline phosphatase (4). Topogenic determinants of integral membrane proteins can direct efficient localization of fused alkaline phosphatase to either side of the membrane as if it were part of the membrane protein (13).

The important feature of the analysis with alkaline phosphatase fusions is that the protein only has enzymatic activity when it has been transferred into the periplasm or to the periplasmic face of the cytoplasmic membrane. It has been shown in numerous cases that assaying alkaline phosphatase activity gives a direct measure of the amount of the protein that has been exported (4, 13–19).

As a result, alkaline phosphatase fusions can be used to detect topogenic determinants; the pattern of specific activities of a set of fusion proteins indicates the topological structure of the membrane protein (13). Fusions of alkaline phosphatase to periplasmic domains of membrane proteins give high enzymatic activity, while fusions to cytoplasmic domains give low activity.

However, in some cases, the level of alkaline phosphatase activity of a fusion depends on its position within a cytoplasmic domain (4). With the MalF protein, alkaline phosphatase fusions to the amino termini of cytoplasmic loops have much higher alkaline phosphatase activity than do fusions to the carboxyl termini of the same loops (see, for example, Fig. 1, fusions L and M). Using proteases to determine the location of the alkaline phosphatase in these strains, we have shown that the activity difference is correlated with the fraction of fusion alkaline phosphatase that is actually transferred across the membrane (ref. 4; C. A. Lee, D.B., and J.B., unpublished data). In other words, with fusion M, nearly all the alkaline phosphatase is localized to the cytoplasm; with fusion L, most of the alkaline phosphatase is exported. Since the cytoplasmic domains are deleted in the first class of fusions (as in L) and not in the second (as in M), we concluded that the cytoplasmic loops play a role in determining the cytoplasmic localization of carboxyl-terminally fused alkaline phosphatase. In addition, we proposed that these sequences play a role in determining the topology of the native membrane protein.

von Heijne (3) has suggested that the net positive charge characteristic of short cytoplasmic loops of bacterial inner membrane proteins is involved in determining the cytoplasmic localization of these loops. In this paper, we describe the use of an *in vitro* mutagenic approach to alter the charge within a cytoplasmic loop of an MalF–alkaline phosphatase fusion protein. The results obtained support the proposal of an important role for positively charged amino acids as topogenic determinants.

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MATERIALS AND METHODS

The materials and methods, including DNA sequencing and construction of single-copy derivatives of the fusions, are as described (4) except that plasmids were inserted in the chromosome of DHB85. The primer used for DNA sequencing corresponds to a sequence within Tn*phoA* as described (4). DHB85 is DHB4 carrying pACYC*lacI*^{Q} (4). The plasmid p*lacI*^{Q} was obtained from James C. Wang (Harvard University). Mutagenesis was carried out as described but without mung bean nuclease and using a uracil-containing single-stranded template prepared by growing f1R408 (20) on DHB5371. DHB5371 is derived from CJ236 *dut ung thi rel* (21) and carries p*lacI*^{Q} and a compatible pBR322-derived plasmid of the type described (4) carrying the *malF–phoA* fusion M (also called IV-958) as well as the F factor pCJ105 (21). For mixed oligonucleotide mutagenesis, the mutagenic 57-mer oligodeoxynucleotide complementary to the sequence shown in Table 2 was synthesized with the following substitutions: base 10, 80% G, 10% C, and 10% A; bases 19 and 37, 91% C and 9% G; bases 20 and 38, 91% G and 9% A; bases 21 and 39, 91% C and 9% A; base 25, 80% A, 10% C, and 10% G; base 47, 80% A and 6.7% each G, C, and T. The use of this mixed oligonucleotide should result in an average of one mutation per oligonucleotide and conversion of each charged residue both to a neutral one and to an oppositely charged one in a fraction of the population. The oligodeoxynucleotide mixture was synthesized by Alex Nussbaum (Harvard Medical School).

Site-specific oligonucleotide-directed mutagenesis was done similarly but using unique oligodeoxynucleotides encoding specific mutations synthesized by M. Moore (Microchemical Facility, University of California, Berkeley). The mutations in Table 3 were constructed with 23- to 28-mer oligodeoxynucleotides. The constructs were verified by DNA sequencing.

To restore the sequence of mutants M1, M7, M59, and M62 plasmids to the parental type, we used a 35-mer that corresponded to the sequence of the original M fusion from base 6 to base 40 as numbered in Table 1. Phage f1R408 lysates were grown on 8–17 transformant colonies obtained from each mutagenesis experiment. The lysates, which contained packaged plasmid DNA were characterized by plating transductants on 5-bromo-4-chloro-3-indolyl phosphate indicator plates. For all four mutants, ≈50% of the transformants formed pale blue colonies that were indistinguishable from the colonies made by the original M fusion. The remaining colonies exhibited the dark blue color of the starting mutant strain. No pale blue colonies were seen in a control experiment without the oligonucleotide.

Alkaline phosphatase assays were carried out essentially as described (4). The difference between the previously reported value of 1.5 units of activity for the M fusion and the value of 2.4 units reported here results from a difference in assay protocol. Assay mixtures with a small number of cells incubated for 24 hr or more and assay mixtures with a large number of cells incubated for 30–60 min both give results that underestimate the amount of activity determined in assays with an intermediate number of cells incubated for an intermediate time. Assays were repeated at least twice with each strain. The average variation in specific activities was generally no more than 10%.

RESULTS

Our proposal for the topology of the MalF protein (Fig. 1) is based on (*i*) the amino acid sequence (22), (*ii*) the properties of alkaline phosphatase fusions to MalF (4), and (*iii*) the properties of β-galactosidase fusions to MalF (23). There is, as yet, no biochemical evidence for this model of the arrangement of MalF in the membrane. The L and M fusions of MalF (Fig. 1) to alkaline phosphatase have fusion junction points at the beginning and end of the third proposed cytoplasmic domain of the membrane protein, respectively. They exhibit 21 and 2.4 units of alkaline phosphatase activity, respectively, whereas fusions to periplasmic domains exhibit up to 30 units of activity. We have shown directly that the level of activity is proportional to the fraction of the fusion alkaline phosphatase that is exported; 30 units corresponds to efficient export (C. A. Lee, D.B., and J.B., unpublished data). This pattern, in which fusions to the beginning of a cytoplasmic domain exhibit considerably higher alkaline phosphatase activity than fusions to the end of the same domain, was seen with fusions to the other cytoplasmic domains of the MalF protein (4) and in similar studies with *E. coli* leader peptidase (24, 25).

We have tested the hypothesis that the charged residues in the cytoplasmic segment present in the M fusion protein are responsible for the more efficient localization of the alkaline phosphatase moiety to the cytoplasm. We have used an oligodeoxynucleotide corresponding to a 57-base-pair segment of a region including the M fusion joint region to alter the charged amino acids in this region. In this oligodeoxynucleotide, we have incorporated mixtures of bases at nine positions in the five codons for charged residues in the region so that each of the five is replaced by codons for neutral or oppositely charged residues. The proportion of mutant base pairs in the oligonucleotide was such that we would expect an average of about one mutation per molecule. Each of the codons is mutated in ≈20% of the population.

After mutagenesis, we examined the colonies to detect those that exhibited either higher or lower activity than the parent fusion strain. This was done by spreading bacteria transformed with mutagenized plasmid DNA on agar medium containing the alkaline phosphatase indicator 5-bromo-4-chloro-3-indolyl phosphate, and identifying colonies with darker or lighter blue color than the parent strain. Colonies were picked from two plates. On one with ≈500 colonies, 30 colonies appeared darker blue and 10 were paler blue than the average colony. On another plate with 200 colonies from an independent mutagenesis, 15 colonies with color variations were detected. On restreaking, only about half of these colonies remained distinguishable from the parent fusion. Unmutagenized transformant colonies all exhibited the same pale blue color.

DNA sequence data for the region was obtained with 15 plasmids, 1 of which had not been mutagenized. Of the 14 mutagenized plasmids, 12 exhibited changes in the DNA sequence of the mutagenized region. The sequences of 11 of these, which represent 8 different alterations, is shown in Table 1. Mutant plasmids carrying the sequences found in M2, M5, and M51 originate from both independent sets of mutants, while M50 and M59 originate from only one set. The 12th altered plasmid, which conferred the highest level of

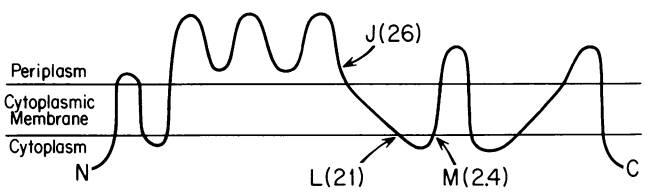

FIG. 1. Topological model of MalF. The model is shown together with arrows pointing to the positions of the J, L, and M fusion joints. The numbers beside the names of the fusion proteins indicate the alkaline phosphatase activity measured when the fusion is present in a single copy (4). The alkaline phosphatase moiety of the J fusion protein is rapidly exported and fully active. The alkaline phosphatase moiety of the L fusion protein is exported more slowly and has lower activity.

Table 1. Mutational alteration of a cytoplasmic domain of MalF: DNA sequence changes obtained with the mixed oligonucleotide

Allele	Sequence
	10 20 30 40 50
M (parental)	GTGCAGTGGGAAGCGTTGCGCGGCAAAGCGGTCTATCGCGTCCctgactcttatac
M1	.....C....G.....
M2, M5, M51	.....A....G.....
M4	.....------G.....
M7	.....G.....G.....
M53	.....GA.....
M50, M59	.....C.....
M58	.....CA.....
M62	.....C.....
L	....------.....

DNA sequences of the mutant alleles of the M fusion are shown together with the parental sequence (first line) and the L fusion sequence. In the first line, which is the complement of the 57-mer primer, capital letters represent *malF* sequences and lowercase letters represent Tn*phoA* sequences. The underlined bases are those that were mutagenized. In subsequent lines, bases identical to parental are represented by dots, deleted bases (or bases not present in the case of the L fusion) are represented by dots, and mutant bases are indicated by capital letters.

alkaline phosphatase (3 times more than any other MalF–PhoA fusion), contained a large deletion with one end in the mutagenized region and the other near the DNA coding for the amino terminus of MalF. Two mutagenized plasmids, obtained from strains that exhibited the parental M fusion phenotype, also showed the parental M fusion DNA sequence as did the one nonmutagenized plasmid. All base substitutions found were at mutagenized positions, but in four cases a cytidine not deliberately specified in the mutagenic mixture was found at position 20. In one case, both a mutagenized and an adjacent nonmutagenized codon were deleted.

Thus, we have obtained mutations at three of the five mutagenized codons. We have recombined the altered fusions into the bacterial chromosome in single copy (4) and assayed the strains to determine the effect of the mutations on alkaline phosphatase activity. The amino acid sequence changes and the enzymatic activities are presented in Table 2.

We find that mutants with decreased net positive charge in the cytoplasmic domain exhibit considerably higher levels of alkaline phosphatase activity than the parent M fusion. Some of these altered fusions give alkaline phosphatase activities similar to those found with fusions of the enzyme to periplasmic domains of MalF. This higher activity indicates that, in these fusions, the alkaline phosphatase is less stably localized to the cytoplasm and substantial amounts of it cross the membrane into the periplasmic space. Conversely, one mutant with increased net positive charge has lower activity, indicating even more stable cytoplasmic localization.

While the results present a striking pattern in terms of the relationship between charge and alkaline phosphatase export, the method of mutagenesis has clearly introduced unexpected mutational changes and could have resulted in undetected changes in other parts of the protein. The pattern itself makes this seem unlikely. However, to eliminate this possibility, we have taken four of the altered fusion strains, M1, M7, M59, and M62, and replaced the DNA sequence corresponding to the cytoplasmic loop with the wild-type sequence. In all cases, restoration of the wild-type cytoplasmic loop sequence restored the parental phenotype (see *Materials and Methods*).

Our results reveal that of the three positively charged amino acids in the cytoplasmic loop, at least the first arginine plays a role, since all the derivatives with higher levels of alkaline phosphatase export carried a change at this amino acid. These data do not clearly indicate the role of the nearby

Table 2. Mutational alteration of a cytoplasmic domain of MalF: Amino acid changes obtained with the mixed oligonucleotide

Allele	Units	Sequence	Net charge
		1 7 9 13	
M	2.4	ValGlnTrpGluAlaLeuArgGlyLysAlaValTyrArgValProAspSerTyrThr	+1
M1	25	.....Pro...Glu.....	−2
M2	20	.....His...Glu.....	−2
M4	20	.....------Glu.....	−2
M7	22	.....Gly...Glu.....	−2
M53	17	.....Asp.....	−1
M50	13	.....Pro.....	0
M58	12	.....Pro.....	0
M62	1.2	.....Gln.....	+2
L	20	....------.....	−2

The protein sequence of the cytoplasmic loop region of the M fusion protein is shown in the first line. Mutagenized residues are indicated by their charge, either + or −. In subsequent lines, unchanged residues are represented by dots and deleted residues (or those not present in the L fusion) are represented by dashes. Changed residues and their charge are indicated by each allele. Histidine is considered to be uncharged. Alkaline phosphatase assays were done on strains carrying the fusions in single copy on the chromosome. The net charge is the sum of the charged residues shown, except for L, in which a glutamic acid residue 12 amino acids from the fusion joint in the Tn*phoA* linker region is counted.

lysine and give no information on the role of Arg-13. To further probe the role of the positively charged amino acids, we have used oligonucleotide-directed mutagenesis to obtain derivatives of the M fusion carrying several mutations not found by the random mutagenesis experiment. Three of these, changing single amino acids (M3.1, M6.1, and M6.2 in Table 3), result in little or no change in alkaline phosphatase activity, while the fourth, which changes both Arg-7 and Lys-9 to neutral residues, results in an intermediate level of activity. The combined results from these experiments (Table 3) and those from Table 2 show that altering the first arginine, Arg-7, has the most significant effect, while only a moderate increase in alkaline phosphatase export is seen when Lys-9 is changed to Gln. However, altering both Arg-7 and Lys-9 when the latter is changed to Glu gives close to maximal export of alkaline phosphatase. No, or only a very slight, increase occurs when Arg-13 is altered to either Gly or Gln.

DISCUSSION

We have used gene fusions to study the determinants of membrane protein topology. This approach to analysis of membrane protein topology has proved successful with several integral membrane proteins of known topology (13, 25, 26). That is, the alkaline phosphatase specific activity of a fusion protein is closely correlated with the cellular location, in the native protein, of the domain to which the fusion is made. We propose that this correlation holds because the same features of the primary sequence that determine the topology of the native membrane protein direct the localization of the fused alkaline phosphatase. Here we have used localized mutagenesis to determine which features of the primary sequence of a fusion protein maintain alkaline phosphatase in the cytoplasm.

The striking result is that when the positive charge of the cytoplasmic domain is reduced, export of carboxyl-terminally fused alkaline phosphatase is increased. This finding supports the suggestion of von Heijne (3) that arginine and lysine residues that follow transmembrane stretches contribute to the determination of their orientation. Since the mutagenesis was confined to only the charged residues, we can say nothing about the role of other residues in the region. However, the observed results do conform to those predicted by von Heijne's proposal. This is seen most markedly in the mutants with changes at both Arg-7 and Lys-9. In this case, the export of alkaline phosphatase is at a level similar to that of the L fusion, in which the entire cytoplasmic domain is deleted.

The properties of our mutants suggest that Arg-7 and Lys-9 may play the dominant roles in determining cytoplasmic localization in this fusion protein. The only mutants detected with substantially higher levels of alkaline phosphatase were those in which one or both of these two amino acids were affected. Direct alteration of Arg-13 gave no, or only a very slight, increase in alkaline phosphatase export. These results are reminiscent of those found in studies on altered versions of the normal structure gene for alkaline phosphatase (19). Those studies showed that the introduction of an Arg-Ile-Arg sequence immediately following the normal signal peptide resulted in a severe block in the transfer of alkaline phosphatase across the cytoplasmic membrane. In that case and in the present one, a hydrophobic sequence is closely followed by two positively charged amino acids, which are separated by only one uncharged amino acid. In both cases, the result is that alkaline phosphatase is localized predominantly to the cytoplasm. Also, in both cases, mutations that reduce the net positive charge result in a substantial increase in alkaline phosphatase export. The results suggest that, to maintain cytoplasmic location, the positively charged amino acids must follow closely after the hydrophobic segment.

von Heijne (3) has suggested that arginine and, to a lesser extent, lysine residues that follow transmembrane segments may exert an effect on the orientation of these segments because penetration of the membrane by these residues would be kinetically unfavorable. Alternatively, hydrophobic sequences, including both signal sequences and transmembrane segments, may orient themselves in the membrane in response to the electrochemical gradient, which results in a greater negative charge inside the cytoplasm than outside the cytoplasmic membrane. Thus, the greater positive charge that follows the hydrophobic segments described here would favor the positioning of these segments with their positive charges inside the cell. Further experiments will be required to determine over what distance from the hydrophobic stretch charged residues can exert their effects.

While we have focused on the two positively charged amino acids following the hydrophobic segment, we note that these amino acids are preceded by a negatively charged glutamic acid at position 4. This amino acid apparently does not interfere significantly with the effect of the arginine and lysine residues. However, the change of Glu-4 to Gln does result in 2 times lower activity of alkaline phosphatase. This weak effect may be due to a reduction in a slight charge neutralization of the arginine or lysine residues by the glutamic acid residue. Neither the substitution of proline residues at Arg-7 nor the deletion of Arg-7 and Gly-8 was deliberately specified in the mutagenic oligonucleotide. These mutations may have been inadvertently introduced into the oligonucleotide or they may have arisen during the mutagenic procedure.

We do not know the actual frequency of mutagenesis in this experiment, only the frequency of mutations that alter the phenotype—i.e., ≈5%. It may well be that many of the rest of the colonies harbor altered plasmids that have no effect on phenotype. Since the upper limit for expected mutagenesis in this experiment is ≈63% (assuming 100% substitution of

Table 3. Site-specific mutagenesis of the cytoplasmic loop

Allele	Units	Sequence	Net charge
		1 7 9 13	
M	2.2	ValGlnTrpGluAlaLeuArgGlyLysAlaValTyrArgValProAspSerTyrThr	+1
M2.2	11	..... Gly ... Gln	−1
M3.1	3.2	..... Gln	0
M6.1	2.2*	..... Gly	0
M6.2	2.4*	..... Gln	0

The units of alkaline phosphatase activity were normalized to those presented in Table 1, using the J fusion as a standard. The units for J, M, and M1 were consistently ≈40% higher at the time the assays were done compared to those in Table 1.

*Despite the low levels of activity, colonies of these strains consistently formed bluer colonies on 5-bromo-4-chloro-3-indolyl phosphate indicator medium.

thymidine-containing oligonucleotide and a Poisson distribution of oligonucleotide with parental sequence), the actual mutagenesis frequency must be between 5% and 63%. Thus, the frequency of recovery of mutant plasmids is within the range normally seen after such oligonucleotide-directed mutagenesis.

With such high mutation frequency and the occurrence of mutations not deliberately specified in the mutagenized region, it is important to show that the mutations sequenced are actually responsible for the phenotype. We have done this by reverting several of the mutants by using an oligonucleotide with the parental sequence. Restoration of the parental phenotype indicates that the sequenced mutations are responsible for the mutant phenotype.

Our results indicate that, in addition to the membrane-spanning segments proposed by Blobel and Friedlander (5, 6), positively charged amino acids also act as topogenic determinants in membrane proteins. The hydrophobic stretch and the positively charged residues may function together like the shank and fluke parts of an anchor to stabilize the localization of alkaline phosphatase to the cytoplasm. We propose that a similar process takes place during the assembly of the native protein and, perhaps, in concert with other factors contributes to the determination of its topology.

We have suggested elsewhere (24) that membrane protein assembly occurs in two stages, in some ways similar to those outlined by Engelman and coworkers (27). In the first stage, a correct, but possibly unstable, topological structure is achieved as the result of the effects of features of the amino acid sequence on the orientation or rate of transfer to the periplasm of different segments of the polypeptide chain. In the second stage of membrane protein assembly, the final stable structure is achieved by interactions between different domains of the protein, for instance by packing of membrane helices as proposed by Engelman and coworkers (27).

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