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Predicting the orientation of eukaryotic membrane-spanning proteins

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ABSTRACT **We have developed a rule to predict the orientation of the first internal signal–anchor sequence in eukaryotic transmembrane proteins synthesized on the rough endoplasmic reticulum. The difference in the charges of the 15 residues flanking the first internal signal–anchor determines its orientation, with the more positive portion facing the cytosol. In proteins that span the membrane more than once, the orientation of all subsequent transmembrane segments would be determined by that of the most N-terminal one.**

In eukaryotic cells most transmembrane proteins acquire their final membrane orientation during or immediately after synthesis on the rough endoplasmic reticulum. The orientation of a protein is determined by certain of its sequences of amino acids, the so-called topogenic sequences. However, the way in which the sequence of a protein determines its transmembrane orientation is not known. Membrane proteins may span the membrane a single time or multiple times, and with the N terminus facing either the cytoplasmic or the exoplasmic face of the membrane ($N_{cyt}C_{exo}$ or $N_{exo}C_{cyt}$ orientation, respectively; for review see refs. 1, 2).

If the protein has a cleaved N-terminal signal sequence, the new N terminus is generally located in the lumen of the endoplasmic reticulum (ER). In many cases, however, an internal, uncleaved signal sequence, which also functions as a membrane anchor, directs the transmembrane insertion of the nascent polypeptide. A signal–anchor sequence can have either transmembrane orientation, $N_{exo}C_{cyt}$ or $N_{cyt}C_{exo}$. In eukaryotes, insertion of virtually all integral proteins occurs cotranslationally, but only the most N-terminal signal–anchor sequence interacts with signal-recognition particle (SRP) and the SRP receptor (3, 4). Thus, the directionality of insertion of the first internal signal–anchor sequence would determine the transmembrane topology of the entire multi-spanning polypeptide (Fig. 1). In particular, a transmembrane segment can be integrated in either direction depending on the number of transmembrane segments preceding it (4).

We have noted a striking correlation of transmembrane orientation with the disposition of charged residues surrounding the most N-terminal membrane-spanning and presumed signal sequence, and we suggest that this charge difference is a major determinant for the membrane orientation of proteins. An implication of this theory is that the topogenic sequence extends well beyond the 22 or so most hydrophobic residues that span the membrane and that function during biosynthesis as an internal signal–anchor sequence. Changes in the charged residues on one side or the other of an uncleaved signal–anchor sequence can affect its transmembrane orientation (5–8), and our theory explains the orientation of virtually all of these recombinant proteins.

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RESULTS AND DISCUSSION

Our analysis focused initially on those naturally occurring proteins that do not undergo proteolytic processing and whose transmembrane topology is reasonably well established (Table 1). Altogether 91 entries were considered. In order to locate the N-terminal signal–anchor sequence, several programs were used that are based on the hydrophobicity scales of Eisenberg *et al.* (62) and Kyte and Doolittle (65) or on the method of Rao and Argos (64). Since the boundaries of the signal–anchor sequences are often difficult to define, we employed different procedures, which, however, gave essentially the same results. In the most predictive method, used for the data in Tables 1 and 2, we started with the middle of the signal–anchor sequence and searched for the first charged residue in both directions; these were taken to be the boundaries of the membrane-spanning segment. Starting from the boundaries, the charges at both sides of the signal–anchor sequence were summed up for a given distance. Arginine, lysine, and the N-terminal NH_2 group were given a value of +1, histidine +0.5, and aspartate and glutamate −1. For our analyses, we assumed that all NH_2 groups were unmodified; similar results were obtained, however, by assuming that all the N-termini were blocked and therefore uncharged.

Table 1 shows the striking correlation between membrane topology of the first signal–anchor sequence and the difference in charge of the 15 C-terminal and N-terminal flanking amino acids. In all proteins where the first signal–anchor sequence has an $N_{exo}C_{cyt}$ orientation, the segment C-terminal to the signal–anchor is positive with respect to the N-terminal flanking region [$\Delta(C - N) \geq 0$]; in one case the charge difference is zero. In contrast, for all but three proteins (considered below) where the first signal–anchor has an $N_{cyt}C_{exo}$ orientation, the C-terminal flanking segment is negative with respect to the N-terminal [$\Delta(C - N) < 0$]. By variation of the distance from the boundaries of the signal–anchor sequence, we have found that the best correlation is obtained when 10–15 residues on each side are taken into account (Fig. 2). It is important to note that within that range most proteins (around 90%) remain in the same class. The fact that the correlation is less pronounced if the distance becomes too long suggests that only the charge difference around the signal–anchor sequence is important for the determination of the orientation.

A positive charge difference [$\Delta(C - N) \geq 0$] appears to be necessary but not sufficient for the $N_{exo}C_{cyt}$ orientation of the signal–anchor sequence, since three proteins with an N_{cyt}-C_{exo} orientation for the first membrane-spanning helix are predicted to have the opposite polarity (Table 1). An $N_{cyt}C_{exo}$ orientation can be thought of as a "default" situation for an internal uncleaved signal–anchor sequence; an $N_{exo}C_{cyt}$ ori-

Abbreviations: ER, endoplasmic reticulum; SRP, signal-recognition particle.

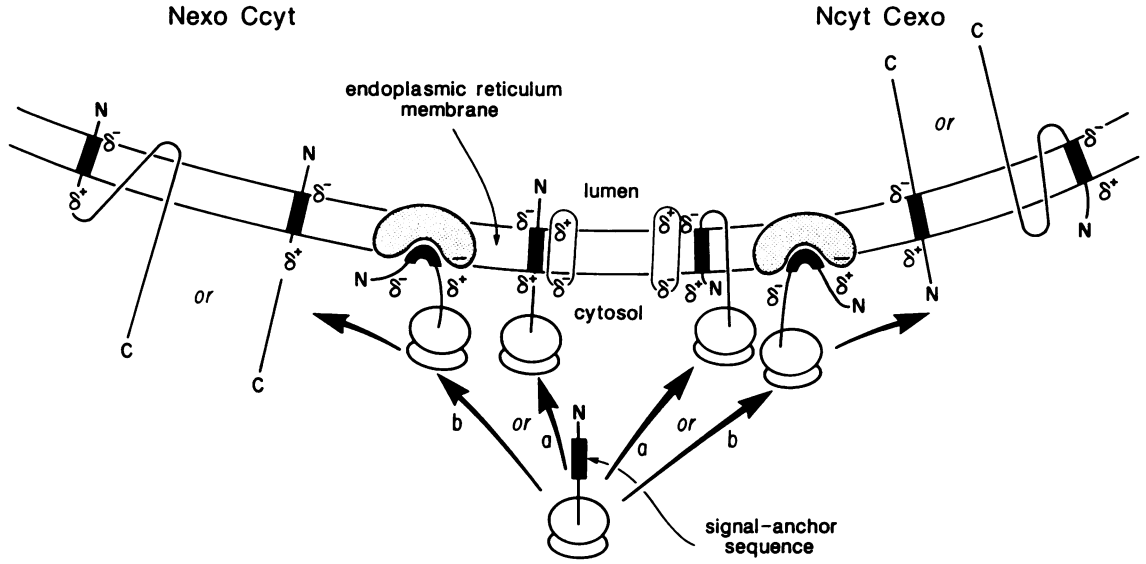

FIG. 1. Model for insertion of signal–anchor sequences. The N-terminal signal–anchor sequence on a nascent polypeptide is brought to a receptor on the ER membrane, presumably by SRP and SRP receptor. A membrane-spanning protein (or local concentrations of lipid head groups) could provide a membrane potential that the nascent chain would "sense" (arrows a). The hydrophobic signal–anchor sequence inserts in the membrane with the more positive flanking segment on the cytoplasmic face. Alternatively, there may be competition between the two flanking regions for translocation (arrows b). The more positively charged sequence could bind tighter to a negatively charged partner, which would impair its translocation competence. If there are no other signal sequences in the protein, the insertion mechanisms generate single-spanning proteins with $N_{exo}C_{cyt}$ orientation (left) or an $N_{cyt}C_{exo}$ orientation (right). The orientation of other membrane-spanning sequences in multispanning proteins would be fixed by the orientation of the most N-terminal signal–anchor.

entation would occur only if $\Delta(C - N)$ were ≥ 0 and if certain other criteria were met.

Another conclusion from the data in Table 1 is that with three exceptions all signal–anchor segments have a positive net charge in the cytoplasmic (i.e., nontranslocated) flanking region. The exceptions are all $N_{cyt}C_{exo}$; two of these have zero charge on the cytosolic face. A net positive charge is, in itself, not predictive of the orientation, since many proteins have a net positive charge in both flanking sequences.

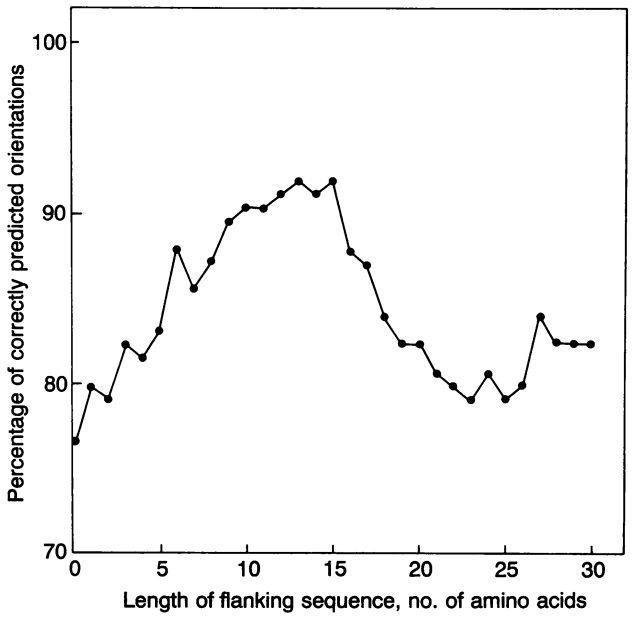

FIG. 2. Predictive value of the charge-difference rule is dependent on the length of the flanking region. The percentage of correct prediction of the protein orientation was calculated as a fraction of the length of the sequences flanking the signal–anchor. All naturally occurring (Table 1) and genetically engineered proteins were considered; constructs from the following sources were taken into account: refs. 4–8, 57, and 66–72.

Other features of the proteins do not correlate well with their membrane orientation. For example, there is no correlation with the length of the N-terminal segment preceding the signal–anchor sequence or with the occurrence of hydrophilic or hydrophobic clusters of amino acids at both flanking regions. Also, the number of positive charges in both flanking sequences, as suggested by von Heijne and Gavel (73), does not appear to be decisive. Most importantly, there is no clear-cut correlation of the net charges on either side of the transmembrane helix with membrane topology. The correlation with the charge difference, rather than with the actual number of positive or negative charges on one side or another of the first hydrophobic segment, may be seen, for example, if one compares two $N_{exo}C_{cyt}$ proteins: the hamster β-adrenergic receptor and the influenza B NB protein (see Table 1). The β-adrenergic receptor has 4 net negative charges N-terminal to the first hydrophobic membrane-spanning segment and 1 positive net charge C-terminal [$\Delta(C - N) = +5$]. The NB protein has 1.5 positive charges N-terminal and 3 positive charges C-terminal [$\Delta(C - N) = +1.5$]. Likewise, among the cytochrome P-450 species there are some with positive N termini and others with negative ones (see Table 1). Nevertheless, the charge difference is always in agreement with an $N_{exo}C_{cyt}$ orientation.

The charge-difference rule explains the topology of most artificial (genetically engineered) proteins in which an N-terminal signal–anchor sequence is placed in various surrounding sequence contexts. In several cases the orientation of the signal–anchor sequence is reversed (5–8). For instance, the normally $N_{exo}C_{cyt}$ orientation of the cytochrome P-450 signal–anchor can be inverted either by introducing more acidic residues in the C-terminal flanking segment ($p450_{1-20}$ pGH_{21-216}; ref. 8) or by introducing more basic residues in the N-terminal flanking sequence (7). We analyzed 38 cases (included in Fig. 2) where the majority of the newly made protein inserted with a defined orientation. The charge-difference rule generated four erroneous predictions (Table 2). In three other cases the charge difference is zero; since natural proteins generally do not have a zero charge difference, the charge-difference rule cannot predict an un-

Table 1. The orientation of the most N-terminal membrane-spanning segment correlates with the charge difference of the flanking regions

$N_{exo}C_{cyt}$ orientation					$N_{cyt}C_{exo}$ orientation				
Protein	*N*	*C*	Δ(*C* − *N*)	Ref.	Protein	*N*	*C*	Δ(*C* − *N*)	Ref.
Hamster β-adrenergic receptor	−4.0	+1.0	+5.0	9	Human P-glycoprotein	+0.5	−4.0	−4.5	32
Influenza B NB protein	+1.5	+3.0	+1.5	10	Mouse erythroid band 3	+1.0	0.0	−1.0	33
Human α-adrenergic receptor	0.0	+2.0	+2.0	11	Rat sodium channel 1	+3.5	−1.0	−4.5	34
Human β-adrenergic receptor 1	−2.0	+2.0	+4.0	12	Rat sodium channel 2	+3.5	−1.0	−4.5	34
Human β-adrenergic receptor 2	−3.0	+1.0	+4.0	13	Rat sodium channel 3	+3.5	−1.0	−4.5	35
Bovine substance K receptor	−2.0	+2.5	+4.5	14	Pig Na^+/K^+-ATPase α subunit	+1.0	−4.0	−5.0	36
Rat serotonin receptor	−2.0	+1.5	+3.5	15	Bovine lens fiber mp26	0.0	+1.5	+1.5	37
Rat M3 muscarinic receptor	−1.5	+3.0	+4.5	16	Hep G2 glucose transporter	+3.0	0.0	−3.0	38
Yeast α receptor (STE2)	+1.0	+3.0	+2.0	17	Rat brain glucose transporter	+3.0	+0.5	−2.5	39
Yeast a receptor (STE3)	+2.0	+2.0	0.0	18	Rat liver glucose transporter	0.0	−1.5	−1.5	40
Bovine opsin	−1.0	+3.5	+4.5	19	Rat muscle glucose transporter	−2.0	+1.0	+3.0	41
Drosophila opsin	0.0	+2.0	+2.0	20	Mouse band 3-related protein	+1.5	−1.0	−2.5	42
Human blue opsin	+0.5	+4.0	+3.5	21	Human band 3-related protein	+1.5	−2.0	−3.5	43
Human green opsin	+1.0	+4.5	+3.5	21	Human transferrin receptor	+2.0	−1.0	−3.0	44
Human red opsin	+1.0	+4.5	+3.5	21	Human asialoglycoprotein receptor H1	+2.0	−1.0	−3.0	45
Avian infectious bronchitis virus E1 protein	−2.0	+3.0	+5.0	22	Human asialoglycoprotein receptor H2a	+2.0	+1.5	−0.5	46
Mouse multipotent colony-stimulating factor, "long"	−1.5	+2.0	+3.5	5	Human asialoglycoprotein receptor H2b	+2.0	0.0	−2.0	46
Rat cytochrome P-450 reductase	−4.0	+2.0	+6.0	23	Human HLA-DR invariant γ chain	+3.0	0.0	−3.0	47
Rabbit cytochrome P-450 reductase	−3.0	+2.0	+5.0	24	Human IgE receptor	+2.0	−0.5	−2.5	48
Human glycophorin C	−2.0	+4.0	+6.0	25	Mouse 1,4-β-galactosyltransferase	+2.0	+1.0	−1.0	49
Influenza A M2 protein	−1.0	+1.5	+2.5	26	Rabbit sucrase–isomaltase	+3.0	−1.0	−4.0	50
Cytochrome P-450, bovine 17a	+1.0	+4.5	+3.5	27	Rabbit neutral endopeptidase	+3.0	0.0	−3.0	51
Cytochrome P-450, bovine C21	+1.0	+4.0	+3.0	27	Rat γ-glutamyl transpeptidase	+3.0	+1.5	−1.5	52
Cytochrome P-450, chicken PB	0.0	+2.0	+2.0	27	Rat asialoglycoprotein receptor	+3.0	0.0	−3.0	53
Cytochrome P-450, human 4 MC	0.0	+4.0	+4.0	27	Respiratory syncytial virus G glycoprotein	+2.5	+0.5	−2.0	54
Cytochrome P-450, human HLP glu.	−1.0	+3.0	+4.0	27	Pig Na^+/K^+-ATPase β subunit	+2.0	0.0	−2.0	36
Cytochrome P-450, human j EtOH	+1.0	+1.5	+0.5	27	Chicken hepatic lectin	+3.0	+1.0	−2.0	55
Cytochrome P-450, human NF25	−1.0	+3.0	+4.0	28	Influenza neuraminidase A BD	+2.0	−0.5	−2.5	56
Cytochrome P-450, human P1 MC	0.0	+4.0	+4.0	27	Influenza neuraminidase A DA	+2.0	−0.5	−2.5	56
Cytochrome P-450, human C21	+1.0	+3.5	+2.5	27	Influenza neuraminidase A DG	+2.0	+0.5	−1.5	56
Cytochrome P-450, human 17a	0.0	+5.0	+5.0	27	Influenza neuraminidase A M1	+2.0	+1.5	−0.5	56
Cytochrome P-450, mouse P1 MC	0.0	+4.0	+4.0	27	Influenza neuraminidase A M2	+2.0	−0.5	−2.5	56
Cytochrome P-450, mouse P3 MC	0.0	+4.0	+4.0	27	Influenza neuraminidase A S2	+2.0	+0.5	−1.5	56
Cytochrome P-450, mouse C21	+1.0	+5.5	+4.5	27	Influenza neuraminidase A TO	+2.0	0.0	−2.0	56
Cytochrome P-450, rabbit 1 PB	0.0	+3.0	+3.0	27	Influenza neuraminidase A TR	+2.0	+1.5	−0.5	56
Cytochrome P-450, rabbit IA1 TCDD	−1.0	+5.0	+6.0	29	Influenza neuraminidase A pNA	+2.0	+1.0	−1.0	57
Cytochrome P-450, rabbit 3a EtOH	+1.0	+1.5	+0.5	27	Rat Golgi sialyltransferase	+4.0	0.0	−4.5	58
Cytochrome P-450, rabbit 3b con.	0.0	+5.0	+5.0	27	Mouse glycoprotein PC1	+1.0	+4.0	+3.0	59
Cytochrome P-450, rabbit LM2 PB	0.0	+4.0	+4.0	27	Human neutral endopeptidase	+4.0	0.0	−4.0	60
Cytochrome P-450, rabbit LM4 MC	0.0	+6.0	+6.0	27	Human intestinal aminopeptidase	+3.0	+1.0	−2.0	61
Cytochrome P-450, rat d MC	0.0	+4.0	+4.0	27					
Cytochrome P-450, rat b PB	0.0	+4.5	+4.5	27					
Cytochrome P-450, rat f con.	0.0	+5.0	+5.0	27					
Cytochrome P-450, rat j EtOH	+1.0	+2.0	+1.0	27					
Cytochrome P-450, rat LW	+2.0	+3.0	+1.0	27					
Cytochrome P-450, rat c MC	0.0	+4.0	+4.0	27					
Cytochrome P-450, rat p PCN	−1.0	+4.5	+5.5	27					
Cytochrome P-450, rat *e* PB (PB4)	0.0	+4.5	+4.5	27					
Cytochrome P-450, rat PB1 con.	0.0	+3.0	3.0	27					
Cytochrome P-450, rat LM2	0.0	+4.5	+4.5	30					
Yeast ALG1	+1.0	+3.0	+2.0	*					

The analysis included naturally occurring proteins that do not undergo proteolytic processing and in which the transmembrane topology ($N_{exo}C_{cyt}$ or $N_{cyt}C_{exo}$) of the N-terminal signal–anchor is reasonably well established. The first transmembrane segment was searched with the programs HELIXMEM (62), SOAP (63), and RAOARGOS (64) contained in the software system PC/Gene. The most N-terminal hydrophobic membrane anchor was taken as the one identified by at least two of the programs. This segment corresponded in all but four cases to that proposed in the literature (15, 33, 38, 39). For the exceptions the anchor identified in the literature was accepted. The middle of this transmembrane segment could be identified with little ambiguity. From this point the first charged residues on either side were taken as the beginning of the flanking regions. The net charge was calculated for a segment of 15 amino acids beginning with this first charged residue. The table gives the net charge in the N-terminal (*N*) and C-terminal (*C*) flanking regions, as well as the difference [Δ(*C* − *N*)].

*C. F. Albright and P. W. Robbins, personal communication.

ambiguous orientation. The four exceptions were also cases where the charge-difference rule predicted an $N_{exo}C_{cyt}$ orientation but where the opposite occurred (see Table 2). Again, as with the naturally occurring proteins, in the majority of cases (three exceptions) the nontranslocated flanking segment has a positive net charge.

Table 2. List of proteins with falsely predicted orientation

Protein	Orientation	*N*	*C*	Δ(*C* − *N*)	N_{tot}	Ref.
Naturally occurring proteins						
Bovine lens fiber mp26	$N_{cyt}C_{exo}$	0.0	+1.5	+1.5	5.0	37
Rat muscle glucose transporter	$N_{cyt}C_{exo}$	−2.0	+1.0	+3.0	5.0	41
Mouse glycoprotein PC1	$N_{cyt}C_{exo}$	+1.0	+4.0	+3.0	20.0	59
Constructs translated *in vitro*						
Influenza neuraminidase A NA23-122	$N_{cyt}C_{exo}$	+2.0	+3.5	+1.5	2.0	57
Human transferrin receptor H30	$N_{cyt}C_{exo}$	+1.0	+1.5	+0.5	20.5	69
Human transferrin receptor H8	$N_{cyt}C_{exo}$	−2.5	+1.0	+3.5	18.0	69
Human transferrin receptor p26	$N_{cyt}C_{exo}$	−4.0	+2.0	+6.0	17.5	69

A total of 126 proteins, natural and artificial, were analyzed according to the charge-difference rule as explained in Table 1 for naturally occurring ones (for refs. see Table 1 and Fig. 2). The 7 proteins listed do not fit the rule. N_{tot} gives the total number of charged residues in the N-terminal sequence preceding the first membrane anchor.

There are two mechanistic models that could explain the role of a charge difference in determining the topology (Fig. 1). According to a "dipole moment" theory (74), the N-terminal sequence as it emerges from the ribosome and is brought to the ER membrane (by SRP; ref. 75) would "sense" an electrochemical potential, cytoplasmic face negative. Such a potential could be due either to a local electric potential across the ER membrane produced by membrane proteins (a in Fig. 1) or to a local concentration of negative charges, such as phospholipid head groups, on the cytoplasmic surface. A bulk electrical potential is unlikely to be of importance since ionophores do not have an effect on the *in vitro* insertion of membrane proteins into microsomes (refs. 76 and 77; unpublished observations). The actual insertion process could be aided by ER proteins (78). In any case, the dipole across the first hydrophobic segment would cause membrane insertion so as to have the positive pole facing the cytosol.

An alternative model assumes a competition between translocation competence of the segments at both sides of the N-terminal signal–anchor sequence (b in Fig. 1). The more positively charged segment will be more difficult to translocate across the membrane, perhaps because of binding to a negatively charged partner, and the signal sequence will orient accordingly. Other features of the protein may also contribute to the translocation competence of the flanking regions. One important factor may be the total number of charged residues that would have to be transferred across the membrane. Indeed, comparing proteins with about equal lengths of N-terminal flanking regions, we found a maximum of 11 total translocated charges for the $N_{exo}C_{cyt}$ cases and up to 20.5 total charges for the $N_{cyt}C_{exo}$ cases. It is remarkable that among the seven $N_{cyt}C_{exo}$ proteins that are predicted to have the opposite orientation by the charge-difference rule, there are four cases where there are 17.5 or more total charges at the N terminus, a fact that could explain why they are not translocated (Table 2).

Another aspect that has been neglected by applying the charge-difference rule is the possible involvement of protein folding. Folding of the N-terminal flanking region is likely to prevent its translocation across the membrane, but it is not obvious why folding in the exceptional cases (Table 2) would be different from the majority of proteins.

Whatever the model, it is assumed that in multispanning proteins, the second helix would insert with an orientation opposite to that of the first, and so on with the subsequent transmembrane helices (Fig. 1). As discussed above and in Fig. 1, our model proposes that the signal–anchor sequence inserts directly in the ER membrane with its final orientation. There is no need to postulate, as did Monier *et al.* (8) that the signal–anchor inserts initially in an $N_{cyt}C_{exo}$ orientation and then "flips" to an $N_{exo}C_{cyt}$ topology.

Cleaved signal sequences are generally thought to insert into the ER membrane with the "default" $N_{cyt}C_{exo}$ orientation—the loop model for signal-sequence insertion (79, 80). Our charge-difference rule was developed to explain the orientation of signal sequences that contain a sufficient number of hydrophobic residues to span the lipid bilayer. We did test the charge-difference rule on a set of 200 randomly selected proteins with cleavable signal peptides. In 149 cases the signal sequence was identified as a membrane anchor by the computer algorithms used (see Table 1), although in the majority of cases the hydrophobic segment was shorter than the average for the integral membrane proteins. The charge-difference rule predicted for all but 19 proteins an $N_{cyt}C_{exo}$ orientation. There were also 17 cases where the calculated charge difference was ≤0.5. Importantly, the percentage of errors was much smaller when only signal sequences with long predicted "transmembrane" segments (21 residues) were taken into account (3 incorrectly predicted orientations out of 38). Most cleaved N-terminal signals are preceded by a positively charged N terminus, as was previously noted by von Heijne (81) for this $N_{cyt}C_{exo}$ orientation.

Our analysis has not considered bacterial proteins, since the mechanism of membrane insertion may be different from that in eukaryotes and may involve prior folding of the polypeptide chain and an electrochemical membrane potential (82–84). Nevertheless it appears that the charge-difference rule may also be applicable to prokaryotic secretory proteins. von Heijne (81) has noted that the hydrophobic region of bacterial signal sequences is generally preceded by positive charges and succeeded by a region containing an abundance of negative charges. Furthermore, when positive charges were engineered into the mature region of alkaline phosphatase, the protein was not properly translocated and was presumed to adopt the reverse orientation (31).

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