A Single Negatively Charged Residue Affects the Orientation of a Membrane Protein in the Inner Membrane of *Escherichia coli* Only When It Is Located Adjacent to a Transmembrane Domain*

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Claudia Rutz‡, Walter Rosenthal†‡§, and Ralf Schülein‡¶

From the ‡Forschungsinstitut für Molekulare Pharmakologie, Alfred-Kowalke-Strasse 4, D-10315 Berlin and the ¶Institut für Pharmakologie, Freie Universität Berlin, Thielallee 66–73, D-14195 Berlin, Germany

The orientation of membrane proteins is determined by the asymmetric distribution of charged residues in the sequences flanking the transmembrane domains. For the inner membrane of *Escherichia coli*, numerous studies have shown that an excess of positively charged residues defines a cytoplasmic domain of a membrane protein (“positive inside” rule). The role of negatively charged residues in establishing membrane protein topology, however, is not completely understood. To investigate the influence of negatively charged residues on this process in detail, we have constructed a single spanning chimeric receptor fragment comprising the N terminus and first transmembrane domain of the heptahedral G protein-coupled vasopressin V₂ receptor and the first cytoplasmic loop of the β₂-adrenergic receptor. When fused to alkaline phosphatase (PhoA), the receptor fragment inserted into the inner membrane of *E. coli* with its N terminus facing the cytoplasm (N_{in}-C_{out} orientation), although both membrane-flanking domains had rather similar topogenic determinants. The orientation of the receptor fragment was changed after the introduction of single glutamate residues into the N terminus. Orientation inversion, however, was found to be dependent on the location of the glutamate substitutions, which had to lie within a narrow window up to 6 residues distant from the transmembrane domain. These results demonstrate that a single negatively charged residue can play an active role as a topogenic determinant of membrane proteins in the inner membrane of *E. coli*, but only if it is located adjacent to a transmembrane domain.

In the past decade, two related hypotheses have been proposed to define the structural features of membrane proteins that determine the establishment of their correct orientation (topology) in the bilayer. For proteins in the inner membrane of *Escherichia coli*, the “positive inside” rule (1) predicts that loops with an excess of positively charged residues are directed to the cytoplasmic face of the membrane, and many studies have demonstrated that positively charged residues indeed inhibit translocation (for review see Ref. 2). For membrane proteins in the eucaryotic endoplasmic reticulum membrane, the related “charge difference” rule postulates that the charge difference between the flanking segments determines orientation and that negatively and positively charged residues contribute equally (3). The charge difference rule was recently confirmed in yeast with fragments of the pheromone receptor Ste2p (4, 5). Folding of an N-terminal domain of a membrane protein impairs translocation and can also influence the orientation (6).

For the inner membrane of *E. coli*, it was demonstrated that the positive inside rule is based on the electrochemical membrane potential (positive and acidic outside) (7, 8). The membrane potential impairs the translocation of positively charged residues and facilitates that of negatively charged residues (7, 8). Although the strong topogenic potential of positively charged residues for such an electrophoretic mechanism has been consistently demonstrated, the significance of negatively charged residues is not so clear and has been reported to occur only under certain conditions. From experiments with the double spanning (N_{out}-C_{in}) leader peptidase (Lep) of *E. coli*, these were defined as the presence of very high numbers of negatively charged residues (3–4 Asp or Glu residues) (9) and the position-specific attenuation of positively charged residues lying in conformational vicinity to them (10). Experiments with a fusion protein consisting of the N-terminal tail of the phage Pf3 coat protein and Lep indicated that a decreased hydrophobicity of the corresponding transmembrane domain might be another requirement since, in this case, the influence of the membrane potential and hence that of negative charges increases (11). In contrast to those effects that are detectable only under certain conditions, an active and direct role of negatively charged residues as topogenic determinants in the inner membrane of *E. coli* was also reported using the single spanning (N_{out}-C_{in}) wild-type Pf3 protein (12). Negatively charged residues were found essential to promote the translocation of the N terminus of this protein and thus to establish the correct topology, even in the absence of positively charged residues (12).

Here we have investigated whether negatively charged residues can play an active role as topogenic determinants for proteins in the inner membrane of *E. coli* and whether there might be a critical distance from the transmembrane domain within which negatively charged residues are effective. As a model, we used a single spanning chimeric receptor fragment comprising the N terminus and TM₁ of the heptahedral G protein-coupled vasopressin V₂ receptor and the IL1 domain of the β₂-adrenergic receptor. This fragment inserted with an N_{in}-C_{out} orientation and was thus suited for assessment of the translocation of the N terminus in the presence of additional negatively charged residues. To determine the orientation of...

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† To whom correspondence should be addressed. Tel.: 49-30-51551-343; Fax: 49-30-51551-291; E-mail: schuelein@fmp-berlin.de.

** The abbreviations used are: TM, transmembrane domain; IL, intracellular loop; IPTG, isopropyl-1-thio-β-galactopyranosidase; V₂ receptor, human vasopressin V₂ receptor; β₂ receptor, human β₂-adrenergic receptor; PAGE, polyacrylamide gel electrophoresis.
this protein in vivo, the PhoA protein of *E. coli* (13) was fused C-terminally. PhoA is only active when translocated into the periplasm and is thus a suitable marker for membrane protein orientation (13). β-Galactosidase (LacZ) fusions, which are active only when the LacZ moiety is located in the cytoplasm (14), were used as controls. We show that even a single negatively charged residue can play an active role as a topogenic determinant. In addition, we have defined for the first time a window region and show that single negatively charged residues are only effective within a stretch of 6 residues from the transmembrane domain.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction and modifying enzymes were from New England Biolabs Inc. (Schwalbach, Germany). Nonidet P-40 was from Calbiochem (Bad Soden, Germany). Ampicillin, IPTG, phenylmethylsulfonyl fluoride, o-nitrophenyl-β-D-galactosides, p-nitrophenyl phosphate, and nitro blue tetrazolium were from Sigma (München, Germany). 5-Bromo-4-chloro-3-indolyl phosphate was from BIOMOL Research Labs Inc. (Hamburg, Germany). The Complete protease inhibitor mixture was from Roche Molecular Biochemicals (Mannheim, Germany). Oligonucleotides were from Biotez (Berlin, Germany). All other reagents were from Merck (Darmstadt, Germany). The rabbit polyclonal anti-PhoA antibody has been described (15); anti-rabbit 125I-IgG was added to a final concentration of 1 μg/ml. Materials—Restriction and modifying enzymes were from New England Biolabs. All oligonucleotides were from Biotez. Lysis of *E. coli* was accomplished with a 400-μl overnight culture of an *E. coli* CC118 clone and grown at 37 °C for 2 h with aeration. Cells were induced with 1 mM IPTG and grown for an additional 45 min. The PhoA and LacZ activity assays were based on the protocols of Brickman and Beckwith (17) and Miller (18), respectively.

To determine PhoA activity, bacteria from a 100-μl cell suspension were harvested (10 min, 8000 × g, 4 °C), and the supernatant was removed. The pellet was supplemented with 300 μl of 1 M Tris-HCl (pH 8.0), 25 μl of 0.1% SDS, and 25 μl of chloroform. Samples were stirred for 20 min at 28 °C. After phase separation, 200 μl of the aqueous upper phase were transferred into a microtiter plate well. Reactions were started by the addition of 25 μl of a solution containing 1 μg/ml 5-bromo-4-chloro-3-indolyl phosphate, 5 μM MgCl₂ (pH 8.0). Reactions were incubated for 170 min at 28 °C, and A₅₉₅ was measured on a microtiter plate reader.

To determine LacZ activity, bacteria from a 100-μl cell suspension were harvested and supplemented with 300 μl of buffer (60 mM Na₂HPO₄, 40 mM NaF, 10 mM KCl, 1 mM MgSO₄, and 50 mM β-mercaptoethanol (pH 7.0), 25 μl of 0.1% SDS, and 25 μl of chloroform. Samples were stirred for 20 min at 28 °C. After phase separation, 200 μl of the aqueous upper phase were transferred into a microtiter plate well. Reactions were started by the addition of 25 μl of a solution containing 100 mM K₂HPO₄ and 4 mg/ml o-nitrophenyl-β-D-galactoside (pH 7.0). Reactions were incubated for 170 min at 28 °C, and A₄₉₀ was measured on a microtiter plate reader.

**Topology Determination by Negatively Charged Residues**—Aliquots containing 1.5 × 10⁶ cells of an induced *E. coli* CC118 clone were washed once with phosphate-buffered saline. For lysis, cells were boiled for 3 min in 20 μl of Laemmli buffer (60 mM Tris-HCl, 2% SDS, 10% glycerin, 5% β-mercaptoethanol, and 0.01% bromphenol blue (pH 6.8)). Proteins were separated by SDS-PAGE (10% acrylamide) and blotted onto nitrocellulose filters as described (19).

For the detection of PhoA fusions, filters were blocked for 1 h with blocking buffer (10 mM Tris-HCl, 0.9% NaCl, 1% casein, and 1% gelatin (pH 7.2)), supplemented with anti-PhoA antibodies (1:4000 dilution), and incubated for 2 h at room temperature. Filters were washed three times (10 min each) with wash buffer (10 mM Tris-HCl and 0.9% NaCl (pH 7.2)) and, anti-rabbit 125I-IgG was added to a final concentration of 1 μg/ml (1 μCi/ml). Filters were incubated for 2 h at room temperature, washed with washing buffer (4 times, 5 min each) with wash buffer, dried, and exposed to x-ray film (1 day).

For the detection of LacZ fusions, filters were blocked for 1 h with buffer A (20 mM Tris-HCl, 150 mM NaCl, 5% low fat milk powder, and 1% Triton X-100 (pH 7.0)) and incubated with monoconal anti-LacZ antibodies (1:4000 dilution in buffer A) for 1 h at room temperature. Filters were washed four times (15 min each) with buffer A and incubated with alkaline phosphate-conjugated anti-rabbit IgG (1:5000 dilution in buffer A) for 1 h at room temperature. Filters were washed four times (10 min each) with buffer A; twice (10 min each) with 20 mM Tris-HCl, 150 mM NaCl, and 1% Triton X-100, (pH 7.0); and once (5 min) with 10 mM Tris-HCl, pH 9.5. Filters were incubated in staining solution (0.56 mM 5-bromo-4-chloro-3-indolyl phosphate and 0.48 mM nitro blue tetrazolium) until bands became visible.

**Fractionation of PhoA Fusion Proteins in Total Cell Lysates**—Lysis of *E. coli* CC118 Clones and Trypsin Digestion of PhoA Fusion Proteins—The protease sensitivity of solubilized PhoA fusion proteins was used to localize the PhoA moieties and to control the PhoA activity data. Prior to membrane protein solubilization, spheroplasts were prepared according to the method of Koshland and Botstein (20) to facilitate lysis. 20 ml of 10 g/liter yeast extract, 5 g/liter NaCl, 500 μg/ml ampicillin were inoculated with a 400-μl overnight culture of an *E. coli* CC118 clone and grown at 37 °C for 2 h with aeration. Cells were induced with 1 mM IPTG and grown for 45 min at 37 °C with aeration. Bacteria were harvested (10 min, 8000 × g, 4 °C) and resuspended in 900 μl of ice-cold

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2 Primer sequences are available upon request.
osmotic shock buffer (100 mM Tris-HCl, 0.5 mM EDTA, and 500 mM sucrose (pH 8.0)). After the addition of 100 μl of lysozyme solution (2 mg/ml shock buffer) and 900 μl of ice-cold H2O, cells were incubated for 5 min on ice to allow the formation of spheroplasts. The suspension was supplemented with 80 μl of 500 mM MgCl2, and spheroplasts were collected by centrifugation at 1500 x g for 15 min at 4 °C. After removal of the supernatant containing periplasmic proteins, spheroplasts were broken in 1 ml of lysis buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl, 150 mM NaCl, and 1 mM Na-EDTA, (pH 8.0)), and membrane proteins were solubilized. The solution was divided into two 500-μl aliquots. One aliquot was incubated with trypsin (1 μg/μl final concentration) for 30 min at 4 °C on an Eppendorf shaker. Protease digestion was stopped with 50 μl of Complete protease inhibitor mixture. The control sample was supplemented immediately after membrane protein solubilization with protease inhibitor. For the detection of the PhoA fusions by immunoblot analysis, 20-μg protein samples were applied per lane.

RESULTS

Construction and Orientation of V2, β2, and Chimeric Receptor Fragments—We have previously shown that a V2 receptor fragment comprising the N terminus, TM1, and IL1 (Fig. 1) is inserted with its correct Nout-Cin orientation in the inner membrane of E. coli (16). In contrast, Lacatena et al. (21) demonstrated that the corresponding fragment of the β2 receptor adopts an inverted Nout-Cin orientation. Both results are consistent with the positive inside rule; IL1 of the V2 receptor contains significantly more positively charged residues than IL1 of the β2 receptor (16, 21). To investigate the process of topology determination with high sensitivity, it is advantageous to use a model protein with similar topogenic determinants on either side of the transmembrane domain. In this case, orientation may be influenced by the addition of single charged residues. With this in mind, we constructed a β2 receptor fragment comprising the N terminus, TM1, and IL1 (PROβ66) (Fig. 1), corresponding to the previously described V2 receptor fragment PROV71 (Fig. 1) (16). We next generated chimeric receptor fragments by exchanging the IL1 domains reciprocally. PhoA or LacZ was fused C-terminally to all receptor fragments. Positively charged residues are indicated by closed circles, and negatively charged residues by open circles.

As described previously, high LacZ activity, but no PhoA activity, was found in cells expressing the V2 receptor fragment PROV71, demonstrating its correct Nout-Cin orientation (16). In contrast, and in agreement with the data of Lacatena et al. (21), high PhoA activity, but no LacZ activity, was detected in cells expressing the β2 receptor fragment PROβ66, demonstrating its inverted Nout-Cin orientation. When the IL1 domain of the β2 receptor fragment is replaced by that of the V2 receptor (chimera V2/ILβ2), the loss of PhoA activity and the acquisition of LacZ activity demonstrate that the correct Nout-Cin orientation is established. These results are consistent with the positive inside rule. The number of positively charged residues in IL1 is increased from 2 residues in the β2 receptor fragment to 5 residues in the chimeric fragment. If the IL1 domain of the V2 receptor fragment is replaced by that of the β2 receptor (chimera V2/ILβ2), the correct Nout-Cin orientation of the PhoA fusion protein is changed to the Nout-Cin orientation as demonstrated by the appearance of high PhoA activity. (No proteolytic degradation products indicative of residual Nout-Cin-oriented fragments were observed, indicating that this orientation switch is complete (see below).) The topogenic determinants of the two membrane-flanking domains of the V2/ILβ2 PhoA fusion protein, however, seem to be rather similar. This is indicated by the corresponding LacZ fusion protein, which also displayed an active phenotype, demonstrating that at least part of the V2/ILβ2 LacZ fusion protein remains in the correct Nout-Cin orientation. (Although not demonstrated directly, the virtual halving of the LacZ activity compared with that of the PROV71.LacZ receptor fragment suggests that this fusion protein adopts both orientations.) To demonstrate that the observed differences in the PhoA or LacZ activities of the fusions are not caused by different expression levels, the fusion proteins were also quantified in whole cell lysates by immunoblot analyses using polyclonal anti-PhoA and monoclonal anti-LacZ antibodies (Fig. 2B). For all PhoA fusion proteins, two specifically stained bands with apparent molecular masses of 55 and 50 kDa were detected. The observed molecular mass of the upper 55-kDa band is in good agreement with the calculated sizes of the receptor fragments plus their PhoA moieties (54.9 kDa, PROV71.PhoA; 54.5 kDa, PROβ66.PhoA; 54.8 kDa, V2/ILβ2.PhoA; and 54.7 kDa, β2/ILV2.PhoA). This band thus seems to represent the intact fusion proteins. The lower 50-kDa band most likely represents proteolytic degradation products. Proteolysis of PhoA fusion proteins was also observed in pre-
protease-resistant PhoA. The protease sensitivity of the PhoA moieties of these receptor fragments demonstrates their N_{out}/C_{in} orientation. In contrast, the PROV71.PhoA and β_{2}/ILV_{2}.PhoA receptor fragments were completely digested upon trypsin treatment, demonstrating the protease sensitivity of their PhoA moieties and thus their complete N_{out}/C_{in} orientation. In the untreated controls of these two receptor fragments, the intact 55-kDa bands were accompanied by several degradation products, which were not observed in total cell lysates. Spheroplast formation may activate additional endogenous E. coli proteases that further degrade the protease-sensitive PhoA moieties of these two receptor fragments. The fact that these additional degradation products were not detectable in the untreated lanes of the chimeric V_{2}/ILβ_{2}.PhoA receptor fragment supports the conclusion that this receptor fragment inserts completely in the N_{out}/C_{in} orientation. In summary, the results of the protase sensitivity assays are entirely consistent with those of the PhoA activity assays.

**Negatively Charged Residues Efficiently Invert the Orientation of V_{2}/ILβ_{2}.PhoA**—To assess the precise influence of negatively charged residues on protein topology determination in the inner membrane of E. coli, we used the PhoA-tagged chimeric receptor fragment V_{2}/ILβ_{2}.PhoA. It is inserted with an N_{in}/C_{out} orientation, but seems nevertheless to have rather similar topogenic determinants in its membrane-flanking sequences (see above) and should thus represent a good model for these studies. To investigate whether negatively charged residues may play an active topogenic role, we introduced clustered (1–3) negatively charged glutamate residues into the N terminus of this fragment (Fig. 4A). E. coli CC118 cells were transfected with the respective expression plasmids, and the orientation of the fusion proteins was assessed by PhoA activity assays (Fig. 4B). In the proximity of the transmembrane domain, 3 glutamate residues (L34E/L35E/L36E), 2 glutamate residues (L35E/L36E), and even a single glutamate residue (L36E) were sufficient to establish the opposite N_{out}/C_{in} orientation as demonstrated by the complete loss of PhoA activity. A
of three independent experiments. Vector-transformed E. coli CC118 cells were used as a control. The immunoblot is representative of three independent experiments.

![Fig. 3: Protease sensitivity assay with the PROV71.PhoA, PROβ66.PhoA, β/ILβ2.PhoA and V/ILβ2.PhoA receptor fragments. Mature PhoA is protease-resistant when a critical intramolecular disulfide bond is formed in the periplasm, but protease-sensitive when retained in the cytoplasm, where disulfide bonding does not occur. To assess for protease sensitivity, E. coli CC118 clones expressing the PROV71.PhoA, PROβ66. PhoA, β/ILβ2.PhoA, and V/ILβ2.PhoA receptor fragments were converted to spheroplasts and lysed in the presence of SDS and Triton X-100. Solubilized proteins were treated with trypsin (+) or left untreated (−). Immunoactive proteins were detected with rabbit polyclonal anti-PhoA antibodies and anti-rabbit 125I-IgG. Vector-transformed E. coli CC118 cells were used as a control. The immunoblot is representative of three independent experiments.](image)

![Fig. 4: Effect of individual and clustered negatively charged residues on the orientation of the chimeric V/ILβ2.PhoA receptor fragment. A, depiction of the constructs used. The residues in the N terminus that were exchanged for glutamate residues are circled. The arrows indicate a reversal of the orientation. B, PhoA activity of E. coli CC118 clones expressing the constructs depicted in A. Activity assays were performed with total lysates from 9.5 x 10⁷ cells. Each bar shows the PhoA activity in arbitrary units (V/ILβ2.PhoA without additional negatively charged residues = 1) and represents the means ± S.D. from three individual experiments, each performed in quadruplicate. C, SDS-PAGE/immunoblot analysis of the total lysates shown in B. Fusion proteins of 3 x 10⁷ cells were probed with rabbit polyclonal anti-PhoA antibodies and anti-rabbit 125I-IgG. The immunoblot is representative of three independent experiments. Vector-transformed E. coli CC118 cells were used as a control. WT, wild-type.](image)

more distantly introduced single glutamate residue (20 residues from the transmembrane domain, S21E) had only a barely detectable effect. More glutamate residues may be needed in this region to invert the orientation since 2 (P20E/S21E) or 3 (L19E/P20E/S21E) glutamate residues decreased the PhoA activity further. All mutant V/ILβ2.PhoA receptor fragments were detected in similar amounts by immunoblot analysis (Fig. 4C), demonstrating that the observed PhoA activities are not caused by different expression levels. The activities of clones expressing the corresponding LacZ fusions were used as controls for the PhoA activity data (Table 1). A strong increase in LacZ activity, indicating stabilization of the Nout-Cin orientation, was observed when the 3 glutamate residues (P34E/L35E/L36E), the 2 glutamate residues (L35E/L36E), or the single glutamate residue (L36E) was introduced in the proximity of the transmembrane domain. The more distantly introduced glutamate residue (S21E) had again only a minor effect, which appeared to increase after progressive addition of further glutamate residues to this region (P20E/S21E and L19E/P20E/S21E). The data obtained with LacZ fusions thus strongly support those obtained with PhoA fusions.

A Single Negatively Charged Residue Affects the Orientation of V/ILβ2.PhoA Only if It Is Located within a Narrow Window of 6 Residues from the Transmembrane Domain—Our results imply that there might be a critical distance within which a single glutamate residue is effective. To address this question, we introduced such residues at a distance of 2–7, 10, 13, and 20 residues from the transmembrane domain into the N terminus

| Construct | LacZ activitya |
|-----------|----------------|
| Control   | 1              |
| P34E/L35E/L36E | 1.93 ± 0.15 |
| L35E/L36E   | 1.73 ± 0.06   |
| L36E        | 2.03 ± 0.18   |
| L19E/P20E/S21E | 1.48 ± 0.08 |
| P20E/S21E   | 1.40 ± 0.05   |
| S21E        | 1.21 ± 0.10   |
| A39E        | 1.57 ± 0.03   |
| R33E        | 1.56 ± 0.08   |
| A37E        | 2.15 ± 0.1    |
| L36E        | 2.03 ± 0.18   |
| L35E        | 1.95 ± 0.05   |
| P34E        | 1.09 ± 0.03   |
| T31E        | 1.03 ± 0.04   |
| P23E        | 1.22 ± 0.1    |
| S21E        | 1.21 ± 0.1    |
| Y63R/F65R/L68R | 2.13 ± 0.08 |
| Y63R/F65R   | 1.78 ± 0.05   |
| Y63R        | 1.02 ± 0.11   |
| F65R        | 1.27 ± 0.03   |
| L68R        | 0.93 ± 0.03   |
| T70R        | 1.03 ± 0.01   |

a LacZ activities are shown in arbitrary units (V/ILβ2.PhoA without additional negatively charged residues = 1) and represent the mean ± S.D. from three individual experiments, each performed in quadruplicate. LacZ activity assays were performed with total lysates from 1.9 x 10⁶ cells.
of the chimeric V$_2$/IL$_β_2$-PhoA receptor fragment construct (Fig. 5A). E. coli CC118 cells were transformed with the expression plasmids, and the orientation of the fusion proteins was assessed by PhoA activity assays (Fig. 5B) and immunoblotting (Fig. 5C). A strong decrease in PhoA activity, demonstrating the establishment of the N$_{out}$-C$_{in}$ orientation, was observed only for those glutamate substitutions lying within 6 residues of the transmembrane domain (A39E, R38E, A37E, L36E, and L35E). The more distantly introduced glutamate residues (P34E, T31E, P28E, and S21E) had only small or negligible effects. All mutant receptor fragments were expressed in similar amounts as demonstrated by immunoblotting of total cell lysates (Fig. 5C).

The different PhoA activities are thus not caused by different expression levels. The PhoA activity data are strongly supported by those for the corresponding LacZ fusions (Table I). Here, too, a major increase in LacZ activity, indicating stabilization of the N$_{out}$-C$_{in}$ orientation, was observed only for those glutamate substitutions that lie within the 6-residue window of the transmembrane domain (A39E, R38E, A37E, L36E, and L35E). The more distantly introduced glutamate residues (P34E, T31E, P28E, and S21E) had minor effects. In summary, our results demonstrate that even a single glutamate residue can play an active role as a topogenic determinant, but only when it is present in the proximity of the transmembrane domain.

Positively Charged Residues in the IL1 Domain of V$_2$/IL$_β_2$-PhoA Affect Orientation Only When Present in Larger Numbers—We finally assessed whether positively charged residues might also serve as topogenic determinants in our experimental system. Single and clustered (1–3) arginine residues were introduced into the IL1 domain of the chimeric V$_2$/IL$_β_2$-PhoA receptor fragment (Fig. 6A), and the orientation of the fusion proteins was assessed by PhoA activity assays (Fig. 6B) and immunoblotting (Fig. 6C). Consistent with the positive inside rule, a complete loss of PhoA activity demonstrating the establishment of the N$_{out}$-C$_{in}$ orientation was observed when 2 (Y63R/F65R) or 3 (Y63R/F65R/L68R) positively charged residues were present. Individual positively charged residues (Y63R, F65R, L68R, and T70R), however, had only minor or negligible effects. The immunoblot with total cell lysates demonstrates that all mutant receptor fragments were expressed in similar amounts (Fig. 6C). For the corresponding LacZ fusions, complementary activity data were obtained (Table I), i.e. a strong activity increase occurred after the introduction of 2 (Y63R/F65R) or 3 (Y63R/F65R/L68R) positively charged residues, but only minor effects were observed after the introduction of single charges (Y63R, F65R, L68R, and T70R).

**DISCUSSION**

We have shown that the introduction of even a single glutamate residue into the N terminus of V$_2$/IL$_β_2$-PhoA can invert the orientation of this receptor fragment. In contrast, 2 or more additional arginine residues are needed in the IL1 domain to
cause an equivalent effect. The weaker influence of positively charged residues is not surprising if one assumes that the IL1 domain (see Fig. 1) and the attached 452-residue PhoA moiety are transported via the Sec machinery over the inner membrane, which seems to be the general mechanism for all translocated fragments longer than 60 residues (24). In Sec-dependent transport, translocation of positively charged residues is facilitated (25), thus clarifying their weaker influence. In contrast to the IL1/PhoA domain, the N terminus of V2/ILβ2, PhoA is most probably translocated Sec-independently. It was postulated that this is the general translocation mechanism for all N tail proteins without a signal peptide (26). In such Sec-independent translocation, charged residues are assumed to have a stronger topogenic potential, thus explaining the stronger influence of negatively charged residues in this case.

Whereas it seems clear that the electrochemical membrane potential can promote the translocation of negatively charged residues (7, 8, 11, 12), they were thought to be topogenically active only under certain conditions. Among these were the presence of high numbers of negatively charged residues (9), a decreased hydrophobicity of the corresponding transmembrane domain (11), and the position-specific attenuation of positively charged residues lying in their conformational vicinity (10). For V2/ILβ2, PhoA, a strong topogenic effect was seen even with a single glutamate residue. Taking the high hydrophobicity of the transmembrane domain of this receptor fragment into account, the first two conditions obviously do not apply here. A position-specific attenuation of positively charged residues may on first sight explain our results since a positively charged residue is present in the window region where the glutamate residues were introduced (Arg38; see Figs. 1 and 5). However, attenuation can be ruled out, at least for this arginine residue, since its replacement by glutamine leads only to a relatively minor reduction of PhoA activity (72% of the wild type) (data not shown), demonstrating that Arg38 is not essential to hold V2/ILβ2, PhoA in the N_in-C_out orientation. Furthermore, the effect of glutamate residues was observed in an uninterrupted window region, and not at specific residue positions within a certain sequence, as was the case for negatively charged residues, which attenuate positively charged residues (10). Our results thus strongly suggest that negatively charged residues can play an active and direct role as topogenic determinants and support those of Kiefer et al. (12), who demonstrated that negatively charged residues help to translocate the N terminus of the Ph3 coat protein.

We have also shown that the topogenic potential of a single negatively charged residue depends on its distance from the transmembrane domain and have defined a window region of 6 residues. Since high resolution structural data for the TM1 boundaries of V2/ILβ2, PhoA are not available, the length of this window region may vary by several residues. For a Ph3-Lep fusion protein, it was shown that when the hydrophobicity of the transmembrane domain of Lep was reduced, a negatively charged residue lying in the proximity of the transmembrane domain was necessary to translocate the N terminus, whereas a more distant residue was not (11). Although we observed the topogenic effects even with a highly hydrophobic transmembrane domain, these results suggest that a similar window region might exist for the N terminus of the Ph3 protein. The reason why single glutamate residues are effective only within a critical distance from the transmembrane domain remains obscure. The head groups of negatively charged phospholipids contribute to the retention of positively charged residues on the cytoplasmic side of the inner membrane (27). It is conceivable that these head groups interact only with residues in the proximity of the transmembrane domain. Repulsion of negatively charged residues on the cytoplasmic side may contribute to a mechanism that allows only those negatively charged residues lying within a critical distance from the transmembrane domain to influence the orientation of a membrane protein. On the other hand, the electrochemical membrane potential, which favors the translocation of negatively charged residues, and perhaps also their periplasmic retention, may also play a role. That the electrical field is of limited extension may explain the observed distance effects.

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REFERENCES

1. von Heijne, G., and Gavel Y. (1988) Eur. J. Biochem. 174, 671–678
2. von Heijne, G. (1994) Annu. Rev. Biophys. Biomol. Struct. 23, 167–196
3. Hartmann, E., Rapoport, T., and Lodish, H. F. (1989) Proc. Natl. Acad. U. S. A. 86, 5786–5790
4. Harley, C. A., and Tipper, D. J. (1996) J. Biol. Chem. 271, 24625–24633
5. Harley, C. A. Holt, J. A., Turner, R., and Tipper, D. J. (1998) J. Biol. Chem. 273, 24963–24971
6. Spiess, M. (1995) FEBS Lett. 369, 76–79
7. Andersson, H., and von Heijne, G. (1994) EMBO J. 13, 2267–2272
8. Cao, G., Kuhn, A., and Dalbey, R. E. (1995) EMBO J. 14, 866–875
9. Andersson, H., Bakker, E., and von Heijne, G. (1992) J. Biol. Chem. 267, 1491–1495
10. Andersson, H., and von Heijne, G. (1993) J. Biol. Chem. 268, 21389–21393
11. Delgado-Partin, V. M., and Dalbey, R. E. (1998) J. Biol. Chem. 273, 9927–9934
12. Kiefer, D., Hu, X., Dalbey, R., and Kuhn, A. (1997) EMBO J. 16, 2197–2204
13. Manoil, C., and Beckwith, J. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 8129–8133
14. Manoil, C. (1990) J. Bacteriol. 172, 1053–1054
15. Schulein, R., Gentschev, I., Mollenkopf, H. J., and Goebel, W. (1992) Mol. Gen. Genet. 234, 155–163
16. Schulein, R., Rutz, C., and Rosenthal, W. (1996) J. Biol. Chem. 271, 28844–28852
17. Brickmann, E., and Beckwith, J. (1975) J. Mol. Biol. 91, 307–317
18. Miller, J. H. (1972) Experiments in Molecular Genetics, pp. 352–355, Cold Spring Harbor Laboratory Press, Plainview, NY
19. Khvotche-Andersen, J. (1984) J. Biochem. Biophys. Methods 17, 105–112
20. Koshland, D., and Botstein, D. (1980) Proc. Natl. Acad. Sci. U. S. A. 86, 24625–24633