The inhibitory glycine receptor is a member of the ligand-gated ion channel superfamily of neurotransmitter receptors, which are composed of homologous subunits with four transmembrane segments (M1-M4), each. Here, we demonstrate that the correct topology of the glycine receptor α1 subunit depends critically on six positively charged residues within a basic cluster, RFR-RKRR, located in the large cytoplasmic loop (designated M3-M4 loop) following the C-terminal end of M3. Neutralization of one or more charges of this cluster, but not of other charged residues in the M3-M4 loop, led to an aberrant translocation into the endoplasmic reticulum lumen of the M3-M4 loop. However, when two of the three basic charges located in the ectodomain linking M2 and M3 were neutralized, in addition to two charges of the basic cluster, endoplasmic reticulum disposition of the M3-M4 loop was prevented. We conclude that a high density of basic residues C-terminal to M3 is required to compensate for the presence of positively charged residues in the M2-M3 ectodomain, which otherwise impair correct membrane integration of the M3 segment.

The anion-conductive inhibitory glycine receptor (GlyR) is a member of the ligand-gated ion channel superfamily of neurotransmitter receptors that includes the closely related inhibitory γ-aminobutyric acid, type A receptors, as well as the cation-permeable nicotinic acetylcholine receptors and 5-hydroxytryptamine type 3 receptors. Four GlyR genes encoding ligand binding α subunits (α1-α4) and a single gene for the structural β subunit are known in vertebrates (for review see Refs. 1 and 2). These subunits form homopentameric and heteropentameric chloride channels (3, 4), which mediate postsynaptic inhibition in the spinal cord and other regions of the mammalian central nervous system, thus controlling motor and sensory pathways. All α subunit isoforms assemble into functional homopentameric GlyRs upon heterologous expression in Xenopus oocytes or mammalian cells (5–8).

Like other proteins of the ligand-gated ion channel superfamily, GlyR subunits are multispanning (polytopic) type I membrane proteins with an N-terminal cleavable signal sequence, which targets the nascent polypeptide to the ER and drives its insertion into the lipid bilayer. Mature GlyR subunits as released by signal peptidase cleavage are modular polypeptides, composed of 1) a large glycosylated N-terminal ectodomain that forms the agonist binding site; 2) four transmembrane segments (M1-M4), which between M1 and M3 are connected by short hydrophilic loops and between M3 and M4 by a large cytoplasmic loop of ~85 amino acids (designated M3-M4 loop in this paper); and 3) a short extracellular C-terminal tail.

The classic model of how multispanning membrane proteins insert cotranslationally into the ER membrane assumes that the overall topology of the mature protein is determined by the orientation of the signal sequence, which is inserted first and initiates the translocation of the following peptide segments (9, 10). Accordingly, downstream hydrophobic sequences simply serve as alternate stop transfer and signal anchor sequences, which cause the nascent polypeptide to passively follow the lead of the preceding transmembrane segment and thereby direct the sequential insertion of polytopic proteins. In prokaryotes, the transmembrane orientation of the most N-terminal hydrophobic sequence, i.e. the cleavable signal sequence in case of type I proteins, has been found to depend on the flanking charged residues; the more positively charged end is retained on the cytoplasmic (Cis) side of the membrane, as described by the “inside- or Cis-positive rule” (11). Electrostatic interaction of arginine and lysine residues with negatively charged head groups of phospholipids (12), and, at least in prokaryotes, the negative-inside transmembrane potential (13) determines the transmembrane orientation. Because of their low average degree of ionization at physiological pH, histidines have almost no effect on peptide topology (14). Negatively charged residues affect the topology of prokaryotic proteins only when present in high numbers (15). In contrast, the orientation of the first transmembrane segment of eukaryotic proteins correlates best with the charge difference hypothesis, which considers both positively and negatively charged amino acids by proposing that a net negative cytoplasmic charge dictates a luminal disposition (16). Besides charges, the length and hydrophobicity of the signal sequence (17, 18), as well as glycosylation at sites near the signal sequence (19), have all been documented to affect transmembrane topology. Experimental support for the classical insertion model has been provided for a variety of membrane proteins (20–23). There is, however, increasing evidence that the initial translocation events may not necessarily dictate the topology of the entire mature protein (24–26). Rather, additional topogenic sequence...
information in subsequent transmembrane segments or internal loops appears to be required for the correct positioning of some transmembrane segments of multipassing proteins (for a recent review see Ref. 27).

In this paper, we show that a cluster of basic residues located in the cytoplasmic loop of the GlyR α1 subunit approximately eight residues C-terminal to the transmembrane segment M3 constitutes an important determinant of proper membrane insertion. Based on the usage of three naturally occurring, though normally inaccessible N-glycosylation sites of the M3-M4 loop, we demonstrate that the unbiased orientation of M3 and the ensuing hydrophilic loop depends on the presence of these positively charged residues. The other positively charged amino acids of the M3-M4 loop located outside of this basic cluster exert little or no effect, indicating that a high density of positive charges rather than a net charge difference determines topology.

**EXPERIMENTAL PROCEDURES**

**cDNA Constructs**—The cDNA construct encoding an α1-His with a C-terminal hexahistidyl tag (His) has been described previously (8). Mutations were inserted using the QuickChange™ site-directed mutagenesis kit (Stratagene) and confirmed by sequencing. All amino acids were numbered according to their position in the mature protein sequence.

**Oocyte Expression**—Defolliculated *Xenopus* oocytes were injected with ∼50 nl aliquots of capped cRNAs (0.5 µg/µl) and kept at 19°C in sterile frog Ringer’s solution (90 mM NaCl, 1 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM Hepes, pH 7.4) supplemented with 50 µg/ml gentamycin as described (28). One to three days after cRNA injection, incubation with L-[35S]methionine (Amersham Biosciences) at about 100 MBq/ml (0.1 MBq per oocyte) in frog Ringer’s solution at 19°C and chased with 1 mM unlabeled methionine as indicated. His-tagged proteins were then purified by Ni²⁺-NTA-agarose chromatography from [35S]methionine-labeled *Xenopus* oocytes (Fig. 2, A and B, lane 1). Treatment with Endo H reduced the mass of the α1-His subunit isolated directly after the pulse by 3 kDa to the 45-kDa protein core (Fig. 2B, lane 2). This mass shift is consistent with the presence of a single N-glycan, resulting from usage of the sole N-glycosylation motif, [35S]NVS, in the N-terminal extracellular domain of the α1 polypeptide (Fig. 1A) (8). After replacement of the acceptor Asn³⁸ by glutamine, the α1-His polypeptide migrated at 45 kDa, and no mass shift upon Endo H treatment was observed (results not shown).

In contrast to the parent α1-His polypeptide, both the 316–320 A-α1-His and the 316–322,325 A-α1-His mutants migrated as double bands of apparent masses of 48 and 54 kDa when analyzed by reducing Tricine-PAGE under the same conditions (Fig. 2A, lanes 2 and 3). Strikingly, Endo H treatment of the 316–320 A-α1-His (Fig. 2B, lane 5) and the 316–322,325 A-α1-His (not shown) mutants shifted both the 48- and the 54-kDa polypeptides to the 45-kDa α1-His protein core. The same result was obtained with PNGase F (Fig. 2B, lane 6). This indicates that the 48- and the 54-kDa polypeptides possess the same protein mass but differ in their number of N-glycans.

To determine the number of N-glycans that give rise to the 54-kDa polypeptide, the 316–320 A-α1-His was incubated with increasing concentrations of Endo H (Fig. 2C, lanes 7–12). Because of partial deglycosylation at intermediate Endo H concentrations, a ladder-like pattern of four bands was generated (lanes 8 and 9). Neighboring bands differ in mass by 3 kDa, the mass of an N-linked oligosaccharide side chain. The 45-kDa band represents the core protein, whereas the 48-, 51-, and 54-kDa bands correspond to polypeptides with one, two, and three N-glycans, respectively. Because [35S]NVS is the only potential acceptor site for N-linked glycosylation on the extracellular part of the α1-His polypeptide, the two additional N-glycans must originate from usage of other endogenous sequences that are topologically inaccessible in the non-mutated GlyR α1 polypeptide. Indeed, the GlyR α1 subunit sequence carries a total of four consensus N-glycosylation sites, three of which reside on the cytoplasmic M3-M4 loop, [35S]NVS, [35S]NNS, and [35S]NTT (cf. Fig. 1A). These sites should be glycosylated only when the M3-M4 loop translocates into the ER lumen, thus allowing for formation of the hyperglycosylated 54-kDa form.

The glycosylation of only two of the three sequences of the
Fig. 1. Sequence and topology of the human GlyR α1 subunit. A, the membrane topology and predicted boundaries of the transmembrane segments are based on the original model (see Ref. 2). NX(T/S) sequons are indicated with gray symbols combined with white lettering. Notably, only one of the sequons (38NVS) is located on the predicted ectodomain, whereas the three others (335NFS, 358NNS, 361NTT) are located on the
M3-M4 loop could signify either that one of the closely adjacent sequons 358NNS and 361NTT remains unused for steric or other reasons or that acceptor Asn335 is exposed to the cytoplasm. To discriminate between these possibilities, we substituted Asn335 of the 318,321A/H92511-His mutant with glutamine. The 318,321A/H92511-His mutant behaved virtually identical to the 316–320A/H92511-His mutant (Fig. 2D, lane 2) (see also below). Consistent with the occupancy of Asn 335 of the 54-kDa form with an N-glycan, elimination of Asn335 resulted in a 3-kDa shift to 51 kDa (Fig. 2D, lane 3). After further 3-kDa shift was observed when 358NNS and 361NTT were simultaneously eliminated in addition to Asn 335 (Fig. 2D, lane 4). This indicates that the two N-glycans of the M3-M4 loop of the 54-kDa α1-His mutant polypeptides are located at Asn335 and Asn358 (or Asn361), each contributing a 3-kDa oligosaccharide side chain to the total mass of 54 kDa. Because of the highly polar nature of the amino acid residues between the end of M3 (position 307) and Asn335, the simultaneous usage of both Asn335 and Asn358 (or Asn361) further implies that virtually the entire M3-M4 loop was translocated into the ER lumen upon charge neutralization in mutants. Accordingly, the preceding lipophilic segment, M3, fails to integrate properly into the membrane upon neutralization of positive charges of the M3-M4 loop.

GlyRs with Mixed Topology of the M3-M4 Loop Have an Impaired Assembly Capacity and Are Unable to Leave the Endoplasmic Reticulum—To analyze the effect of a mixed topology of the M3-M4 loop on subunit assembly, we resolved natively purified α1-His mutants by BN-PAGE, a method which displays the oligomeric nature of receptor proteins (8, 32). Regardless of the number of basic charges neutralized downstream to M3, all mutants analyzed migrated as perfectly assembled homopentamers when the [35S]methionine pulse was cytoplasmic M3-M4 loop. Basic residues (Lys, Arg) are highlighted with filled black symbols and white lettering, whereas acidic residues (Glu, Asp) are highlighted in gray. B and C, survey about the GlyR α1 subunit mutants used in this study. Amino acid residues are designated by the single letter code with basic residues highlighted in boldface. Numbers indicated by indices correspond to positions of the mature α1 sequence, i.e. after cleavage of the 28-amino acid-long signal peptide. B, GlyR α1 subunit constructs carrying mutations solely in the M3-M4 loop. The entire sequence of the M3-M4 loop is shown without the flanking transmembrane segments M3 and M4. Charged residues were replaced by alanine, whereas asparagine was replaced by glutamine. C, the amino acid sequence of the hydrophobic stretches thought to represent the transmembrane segments M2 and M3 (boxed) are shown together with the linking sequence (M2-M3 ectodomain) and the N-terminal part of the M3-M4 loop encompassing the cluster of basic residues.

Fig. 2. N-Glycan status of GlyR α1-His subunits with neutralized basic charges downstream to M3. Oocytes were injected with indicated cRNAs, labeled overnight with [35S]methionine, and extracted with digitonin. Proteins were natively purified by Ni2+-NTA-agarose chromatography, denatured with Tricine-SDS sample buffer, and resolved by reducing Tricine-SDS-PAGE (4%/10%/13% acrylamide). Autoradiographs of the gels are shown. A, the wild-type GlyR α1-His subunit migrates as a 48-kDa polypeptide. Neutralization of positively charged amino acids leads to the appearance of an additional 54-kDa polypeptide. B, the same samples as in A were denatured with reducing Tricine-SDS sample buffer and then incubated for 2 h with Endo H or PNGase F as indicated. The 54-kDa form of the 316–320A-α1-His mutant was reduced to the 45-kDa protein core by deglycosylation with Endo H or PNGase F. C, the indicated polypeptides were incubated with increasing amounts of Endo H (in percent of maximum amount of enzyme used). D, elimination of N-glycosylation sequons located in the M3-M4 loop results in mass shifts, which corroborate that the misfolded 54-kDa polypeptides carries N-glycans at Asn335 and Asn358 (or Asn361).
followed by an overnight chase interval (results not shown). If, however, the GlyRs were purified directly after a 4-h \[^{35}\text{S}\text{]methionine pulse, a propensity of the mutants to aggregate became apparent, as indicated by the appearance of high molecular weight \(\alpha_1\)-His protein that migrated at a broad range of masses above that of the pentameric receptor (Fig. 3A). Quantitative scanning of the protein bands resolved by BN-PAGE showed also that the wild-type GlyR \(\alpha_1\)-His subunits existed partially in an aggregated form shortly after synthesis (Fig. 3B). However, the amount of aggregates was markedly higher when one charge of the basic cluster downstream of M3 was neutralized (R319A, K320A; see lanes 3 and 5) and increased further upon neutralization of four basic charges (RFRRK\(^{316-320}\)AFAAA; see lane 7). Most likely, these aggregates are formed primarily of \(\alpha_1\) subunits with luminally exposed M3-M4 loop. The aggregates of both the wild-type and the mutant GlyR \(\alpha_1\) subunits disappeared during a subsequent chase interval despite the continued presence of the hyperglycosylated 54-kDa form (not shown) (cf. Fig. 3C). Evidently, the luminally exposed M3-M4 loop delays but does not prevent proper GlyR assembly. The lack of a marked effect of the wrongly folded M3-M4 loop on assembly can be reconciled with the known location of the assembly domains of ligand-gated ion channel subunits in the N-terminal ectodomain (1).

To examine whether \(\alpha_1\)-His GlyRs with aberrantly folded M3-M4 loop are able to leave the ER, we exploited that N-glycans in general become complex-glycosylated and hence resistant to Endo H during passage of the Golgi apparatus en route to the cell surface. Accordingly, the glycosylation status of the 48- and the 54-kDa polypeptides was determined after a chase interval (Fig. 3C). Consistent with previous observations, the wild-type 48-kDa \(\alpha_1\)-His chain was entirely Endo H-resistant when isolated after a 24-h chase interval (Fig. 3C, lane 2). In contrast, the hyperglycosylated 54-kDa polypeptide generated from the \(\alpha_1\)-His construct persisted entirely in the Endo H-sensitive form, indicating that \(\alpha_1\)-His chains with aberrantly folded M3-M4 loop are incapable of leaving the ER. This view is supported by a relative decrease in the amounts of the additional 35- and 13-kDa polypeptides, which represent proteolytic cleavage products generated in a lysosomal compartment from GlyRs that are endocytotically retrieved from the cell surface, and hence are indicative of the plasma membrane insertion of

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**Fig. 3. Assembly and ER exit of GlyR \(\alpha_1\)-His subunits with neutralized basic charges downstream to M3.** A, \(\alpha_1\)-His GlyRs natively purified from cRNA-injected oocytes immediately after a 4-h \[^{35}\text{S}\text{]methionine pulse were resolved by BN-PAGE (4–12% acrylamide). Where indicated, samples were partially denatured by a 1-h incubation at 8 M urea and 56 °C. B, quantitative profiles of the protein bands of the lanes shown in A obtained by PhosphorImager analysis reveal an increased propensity of the \(\alpha_1\)-His mutants to aggregate (hatched areas). C, \(\alpha_1\)-His GlyRs natively purified from cRNA-injected oocytes after a 4-h \[^{35}\text{S}\text{]methionine pulse and an additional 36-h chase interval were denatured with reducing Tricine-SDS sample buffer and then incubated for 2 h with Endo H or PNGase F as indicated. The monoglycosylated 48-kDa polypeptide was entirely Endo H-resistant, indicating that it had reached the Golgi apparatus. In contrast, the 54-kDa polypeptide persisted in the Endo H-sensitive form, consistent with retention in the ER.
GlyRs (33). Quantification by PhosphorImager analysis revealed that 57% of the wild-type γ1-His subunit, but only 16% of the 316–320A-γ1-His mutant, was proteolytically cleaved into the 35- and 13-kDa products. We conclude from these results that GlyRs with aberrantly folded M3-M4 loop are not exported to the cell surface.

Neutralization of a Single Basic Residue Downstream to M3—To determine how many basic residues can be removed without disturbing membrane topology, GlyR γ1 mutants with only one or two alanine substitutions in the 316RFRRKRRHHK motif were generated. Surprisingly, substitution of only a single basic residue was already sufficient to create a mixed topology of the M3-M4 loop, as evidenced by the synthesis of the hyperglycosylated 54-kDa polypeptide (Fig. 4A). The faint 54-kDa band isolated with the parent GlyR γ1-His subunit indicates that even a minor portion of the wild-type γ1 subunit adopts the incorrectly folded conformation (Fig. 4A, lane 1; see also Fig. 2D, lane 1). Quantification by PhosphorImager analysis revealed that 5–10% wild-type γ1 subunits possessed a luminaly oriented M3-M4 loop shortly after synthesis.

Neutralization of a Single Basic Residue Downstream to M3 Is Sufficient to Disturb Topology of the M3-M4 Loop—To determine how many basic residues can be removed without disturbing membrane topology, GlyR γ1 mutants with only one or two alanine substitutions in the 316RFRRKRRHHK motif were generated. Surprisingly, substitution of only a single basic residue was already sufficient to create a mixed topology of the M3-M4 loop, as evidenced by the synthesis of the hyperglycosylated 54-kDa polypeptide (Fig. 4A). The faint 54-kDa band isolated with the parent GlyR γ1-His subunit indicates that even a minor portion of the wild-type γ1 subunit adopts the incorrectly folded conformation (Fig. 4A, lane 1; see also Fig. 2D, lane 1). Quantification by PhosphorImager analysis revealed that 5–10% wild-type γ1 subunits possessed a luminaly oriented M3-M4 loop shortly after synthesis.

Positional Effect of Basic Residues on M3-M4 Loop Topology—Quantification by PhosphorImager analysis of the 48- and 54-kDa bands revealed a striking positional effect of single charge neutralizations of the M3-M4 loop. Neutralization of Lys320, located at a distance of 12 residues from the end of the M3 domain (cf. Fig. 1), produced the largest effect among all single mutants investigated, with a fraction of 48% aberrantly folded 54-kDa polypeptide (Fig. 4C). To examine the effect of charge neutralization over the entire M3-M4 loop, a set of double mutants was generated in which two consecutive positively charged amino acids were systematically replaced by alanine. The aberrantly folded 54-kDa polypeptide was most abundant relative to the normal 48-kDa polypeptide when Arg318 and Arg 321 or Arg 319 and Lys 320 were neutralized by alanine substitution (Fig. 4, B (lane 8) and D). Thus, the positive charges in the center of the basic cluster are of particular importance for the topology of the M3-M4 loop. Neutralization
of >two positive charges within the cluster (up to seven positive charges as in the \(316^{\text{R}}-322^{\text{R}}\)A-a1-His mutant; see Fig. 4B, lane 3) did not further increase the relative amount of the 54-kDa polypeptide. Notably, charge neutralizations close to the M3 segment (Fig. 4B, lane 6) or in the C-terminal half of the M3-M4 loop (lanes 11–14) had only little or no effect. Even when a total of five basic residues was replaced by alanines in the C-terminal half of the M3-M4 loop, no 54-kDa polypeptide was formed (lane 15).

In a further attempt to assess the role of charges for the disposition of the M3-M4 loop, we used the \(318^{\text{A}}-321^{\text{A}}\)-a1-His mutant to also neutralize three negative charges, EDE, at positions 326–329 immediately downstream of the basic cluster. The resulting \(318^{\text{A}},321^{\text{A}},326^{\text{A}},329^{\text{A}}\)-a1-His mutant displayed a mixed topology of the M3-M4 loop (Fig. 4B, lane 5). Quantification revealed a relative decrease of the misfolded 54-kDa polypeptide from 60% (parent \(318^{\text{A}},321^{\text{A}}\)-a1-His mutant) to 48% (\(318^{\text{A}},321^{\text{A}},326^{\text{A}},329^{\text{A}}\)-a1-His mutant), indicating that increasing the net charge difference (positive charges minus negative charges) can only partially compensate for neutralization of residues within the basic cluster.

Neutralization of Basic Charges in the M2-M3 Ectodomain Rescues GlyR a1 Mutant Topology—The presence of several positive charges on both sides of a transmembrane segment can prevent its membrane insertion (34). We, therefore, considered that positively charged residues in the 14-amino acid-long hydrophilic loop connecting M2 and M3 may impede membrane insertion of M3 in our charge neutralization mutants. Indeed, alanine substitution of one of these charges, Lys\(^{276}\) or Lys\(^{281}\) (Fig. 1C), markedly reduced the fraction of newly synthesized \(318^{\text{A}}\)-a1-His mutant with luminal M3-M4 loop orientation (Fig. 5, A (lanes 3 and 4) and B). Simultaneous neutralization of both Lys\(^{276}\) and Lys\(^{281}\) almost fully abolished formation of the 54-kDa polypeptide (lane 5), suggesting that the basic cytoplasmic cluster C-terminal to M3 is required to counteract the three basic charges in the M2-M3 ectodomain. Two-electrode voltage-clamp measurements in oocytes demonstrated that the K276A and K281A mutations result in 29-fold decrease in glycine potency (Fig. 5C). A related mutation, K276A, which is associated with startle disease, a rare neurological disorder, is known to produce a similar substantial decrease in glycine sensitivity (35) by impairing the opening of the channel rather than the binding of glycine (36).

**DISCUSSION**

The present study shows that the correct topology of the M3-M4 loop of the GlyR a1 subunit depends on a cluster of positively charged residues, \(\text{RXRKRKR}\), immediately downstream of the hydrophobic region M3. If one or more positive charges are neutralized within this motif, the GlyR a1 subunit adopts two topologies, the correct one with the hydrophobic region M3 spanning the membrane and the connecting loop being localized cytoplasmically, and an aberrant one with M3 being non-spanning and the M3-M4 loop being translocated into the ER lumen. This indicates that the cluster of basic residues contains topogenic information. Both the correctly and the incorrectly folded a1-His subunits assembled to homopentamers, but solely homopentamers consisting of correctly folded a1 subunits were exported to the plasma membrane, suggesting that the aberrant M3-M4 loop is recognized by the cellular quality control system. Also, when in our experiments the metabolic labeling period was followed by a 20-h chase, the intensity of the aberrant 54-kDa polypeptide decreased relative to that of the correctly folded a1 subunit. Together these data indicate that incorrectly folded receptor proteins are not only unable to reach the plasma membrane but are also subject to increased proteolytic degradation. The plasma membrane-bound mutant GlyRa, on the other hand, exhibited an electrophysiological phenotype identical to that of the parent a1 GlyR, indicating that the basic charges C-terminal to M3 are not directly involved in receptor functioning.

**Functional Importance of the Basic Cluster for Correct GlyR Subunit Topogenesis**—According to the classic model of membrane integration of polytopic proteins, the hydrophobic M3 region should follow the lead of the preceding transmembrane segment, M2. Because M2 has a \(N_{\text{sys}}-C_{\text{exo}}\) orientation, M3 should adopt passively the opposite \(N_{\text{exo}}-C_{\text{sys}}\) orientation, thus acting as a stop-transfer sequence that halts further translocation of the polypeptide chain across the membrane. Here, failure of the M3 segment of 30–80% of the newly synthesized a1 chains to span the membrane was demonstrated by the aberrant \(N\)-glycosylation of the M3-M4 loop after neutraliza-
Impair the Stop-transfer Function of the Apolar M3 Segment

the 14-amino acid-long M2-M3 loop may impose constraints to positioning itself correctly. The three positive amino acids of by itself into the lipid bilayer. From our observation that un-

Hydropathy analysis based on the Kyte-Doolittle algorithm

The failure of the hydrophobic M3 region to integrate into the membrane after neutralization of only a single positively charged residue was nevertheless unexpected, because seven positively charged residues are left within the 15 residues flanking the C-terminal end of the hydrophobic M3 region. When considering all charges within the flanking 15-residue windows on each side of the M3 segment, there remains a marked net positive charge difference $\Delta (C-N)$ of +4 (with net charges of +2 and +6, respectively) for the single charge neutralization mutants. According to the charge difference rule, this should be more than sufficient to dictate an $N_{\text{exo}}$-C$_{\text{cyt}}$ orientation of a signal anchor sequence. However, despite this excess of positive charges only a minor fraction of the M3 segment adopted the correct $N_{\text{exo}}$-C$_{\text{cyt}}$ orientation, and most of the M3-M4 loop was translocated incorrectly. This is also surprising in view of an excess of 11 positive charges within the entire M3-M4 loop, which harbors a total of 20 positively and nine negatively charged residues. In conclusion, neither the charge difference rule nor the positive inside rule provide any indication for a biased topology of the apolar M3 domain subsequent to neutralization of one or several positive charges of the cluster. This suggests that the M3 segment by itself is unable to reliably halt translocation.

Positive Charges on the Short M2-M3 Ectodomain Seem to Impair the Stop-transfer Function of the Apolar M3 Segment—Hydropathy analysis based on the Kyte-Doolittle algorithm with a window of five amino acids provided no indication that the M3 domain may not be sufficiently hydrophobic to partition by itself into the lipid bilayer. From our observation that unbiased folding can be restored by neutralization of positive charges on the M2-M3 ectodomain, we infer that the positive charges on the M2-M3 ectodomain prevent the M3 domain from positioning itself correctly. The three positive amino acids of the 14-amino acid-long M2-M3 loop may impose constraints to the M3 segment to adopt a $N_{\text{cyt}}$-C$_{\text{exo}}$ orientation, which is obviously incompatible with the $N_{\text{cyt}}$-C$_{\text{exo}}$ orientation of the preceding M2 domain. Therefore, the hydrophobic M3 domain may remain in an unstable state unless the downstream topogenic cluster of basic residues imposes the correct $N_{\text{exo}}$-C$_{\text{cyt}}$ orientation. The extreme sensitivity of M3 transmembrane orientation to neutralization of single basic residues within the $\cdots$RR$\cdots$ sequence suggests that the particular high density of positive charges is essential for keeping the M3-M4 loop on the Cis-side of the membrane. Hence, the basic cluster seems to serve as an accessory cytoplasmic stop-transfer signal which, by blocking translocation of the M3-M4 loop, forces the apolar M3 segment to insert properly into the membrane. Once inserted, hydrophobic interactions with the lipid bilayer may suffice to stabilize the M3 domain in the correct $N_{\text{exo}}$-C$_{\text{cyt}}$ orientation.

Positive charges appear to be more easily translocated through the ER than through bacterial membranes, most likely because of the absence of a membrane potential in the ER (23). Our findings imply that the more frequent translocation of positive charges in eukaryotic systems requires additional topogenic signals, such as the basic cluster described here. Interestingly, clusters of basic charges have been shown recently (40) by computer analysis to occur more frequently in cytoplasmic loops of proteins near the cytoplasmic membrane surface than predicted from the abundance of Arg and Lys residues. It is likely that topogenic basic clusters act through electrostatic interactions with negative charges of lipids or proteins (or both). Proteins of the translocation machinery appear not to harbor relevant charged residues (27), but anionic phospholipids, at least in prokaryotes, are determinants of membrane protein topology because of the electrostatic interaction of their head groups with arginine and lysine residues (12, 41, 42). Interestingly, successful solubilization of the GlyR but not nicotinic acetylcholine receptors has been found to depend stringently on the presence of exogenous phospholipids (43). This is consistent with a role of lipids in the conformational stabilization of GlyR subunits. Whether the topogenic basic cluster identified here contributes to phospholipid stabilization of GlyR structure by ensuring its proper topology remains to be determined.

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