Molecular Determinants Responsible for Differential Cellular Distribution of G Protein-gated Inwardly Rectifying K⁺ Channels*

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Activation of the heteromeric G protein-gated inwardly rectifying K⁺ channel (GIRK) GIRK1 and GIRK4 subunits gives rise to I

K,ACh, which controls excitability in atrial tissue. Although homomeric GIRK4 channels localize to the plasma membrane and display moderate function, GIRK1 channels fail to localize to the cell surface and do not exhibit significant function as homomers. Using oocytes to express GFP-tagged GIRK1 and GIRK4 and chimeras between these two proteins, we have identified two regions, one in the proximal C terminus and another in the distal N terminus that are critical for their subcellular localization. Replacement of both of these regions in GIRK1 with corresponding regions from GIRK4 was required for efficient expression of GIRK1 on the plasma membrane. Replacement of either region by itself was ineffective. The distal N terminus and proximal C terminus have been previously suggested to play important roles in ER-export and subunit co-assembly respectively in this family of channels. Our data indicate for the first time that both of these regions need to work in concert to mediate efficient targeting of these channels to the plasma membrane.

Inwardly rectifying potassium (Kir)
³ channels control cell excitability and, therefore, play pivotal roles in insulin release, heart rate, vascular tone, neuronal firing, and transmitter release (1). These channels fall into seven subfamilies, each of which is composed of one or several members. Two transmembrane domains characterize all Kir channels, a pore loop with intracellular N and C termini assembling into tetramers. Some of these channels form homomers that are fully functional, such as Kir2.1 or Kir1.1 (2). Others co-assemble with accessory proteins to form functional channels, as in the case of Kir6.2, which co-assembles with sulfonylurea receptors (members of the ABC transporter family) to form the ATP-sensitive K⁺ channel (3). Yet, other inward rectifiers co-assemble co-as specifically with members within their own subfamily. Such is the case for the Kir3 family, in which Kir3.1 and one of the other Kir3 family members (Kir3.2–4) co-assemble to form functional channels (4). This class of inward rectifiers, also known as the G protein-regulated inwardly rectifying K⁺ (GIRK) channels, is directly activated by the βγ-subunits of G proteins (3–7).

The processing and assembly of Kir channels is very diverse, and primary sequences that are crucial for their membrane targeting and co-assembly have been identified in several of these channels (8–10). Tinker et al. (11) identified parts of the proximal C terminus and the second transmembrane domain to be required not only for co-assembly of these channels but also for compatibility between channels and prevention of promiscuous interactions. As for the trafficking of these channels, Zarangue et al. (12) found endoplasmic reticulum (ER) retention signals imbedded in Kir6 channels that hinder their surface expression. Co-expression of sulfonylurea receptors masks these retention signals, thus allowing proper processing and surface expression. Other channels, such as Kir2.1, contain forward transport signals that lead them out of the ER, resulting in efficient surface expression (13). In the case of GIRK channels, the processing and assembly has been studied in detail by several groups (13–18). Several studies have suggested that multiple regions within GIRK channels are crucial for their co-assembly. These findings have been largely confirmed by the crystal structure of the N and C termini of the GIRK1 channel (19). As for trafficking, it has been shown that, whereas GIRK4 channels target properly to the membrane, homomeric GIRK1 channels are retained in the ER and are associated with cytoskeletal elements (13, 14). Co-expression of GIRK4 leads to expression of GIRK1 on the membrane (4). Kennedy and colleagues used truncation mutants to show that residues 375–399 in GIRK4 are required for its ability to lead GIRK1 to the membrane (17). Further truncations showed that residues 350–375 are required for proper membrane targeting of GIRK4 channels. Recently, Ma et al. (20) performed a comprehensive analysis of the distribution patterns of GIRK channels. They used a combination of cell staining, surface labeling, and electrophysiological recordings in Cos-7 cells as well as in neurons to show that, whereas GIRK1 is mainly retained in the ER, both GIRK2 and GIRK4 target efficiently to the membrane. ER forward transport signals were identified in the distal N termini of both GIRK2 and GIRK4. Furthermore, these investigators identified signals in the distal C termini that are required for transport of these channels from endosomes to the cell membrane. They used a forward transport signal from Kir2.1 to rescue GIRK1 from the ER. Although GIRK1 exited the ER, it failed to reach the membrane, a result that indicates further defects in the trafficking of this channel subunit.

Clearly, because co-expression of GIRK4 rescues GIRK1 surface expression, there are elements that are unique to GIRK4 that are absent in GIRK1. The recently developed surface-labeling technique of Kir channels using hemagglutinin-tags has been essential in finding elements that are critical in their cellular processing and localization (12). However, tagging

* This work was supported by National Research Service Award HL10307 (to T. M.) and National Institutes of Health Grant HL 54185 (to D. E. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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³ The abbreviations used are: Kir, inwardly rectifying potassium; GIRK, G protein-regulated inwardly rectifying K⁺; ER, endoplasmic reticulum; GFP, green fluorescent protein.
GIRK4 channels with a hemagglutinin-epitope in the extracellular loop between TM1 and the pore loop altered their trafficking (20). Therefore, in this study, we pursued a chimeric approach using GFP-tagged channels to address this question. Previous studies have shown that tagging GIRK channels in the C terminus with GFP does not affect their function (21). Using GFP-tagged chimeras between GIRK1 and GIRK4, we find that replacement of the distal N terminus and proximal C terminus of GIRK1 with that of GIRK4 leads to efficient targeting of this channel to the plasma membrane.

**Fig. 1. Expression of wild-type GFP-tagged GIRK1 and GIRK4 in *Xenopus* oocytes.** a, no GFP signal is observed in uninjected oocytes. Low background auto-fluorescence can be seen. b, GIRK4 channels tagged with GFP in the C terminus localize mainly to the plasma membrane. c, GIRK1 channels tagged with GFP in the C terminus localize mainly to cytoplasmic regions. d, oocytes expressing GIRK1-GFP and a non-tagged GIRK4 display fluorescence that mainly resides in the membrane (compare with Fig. 1c). e, immunostaining of a FLAG-tagged GIRK1 channel expressed in Cos-1 cells. GIRK1 resides mainly in cytoplasmic regions. f, co-expression of GIRK4 targets GIRK1 to the membrane in Cos-1 cells.
EXPERIMENTAL PROCEDURES

The C termini of GIRK1 and GIRK4 were each tagged with enhanced green fluorescent protein (Clontech) and subcloned into pGEMHE for expression in oocytes. Chimeras between GIRK1 and GIRK4 were made by using the splice-by-overlap extension method; all chimeras were confirmed by DNA sequencing. The DNA constructs were linearized, and cRNA was synthesized from each one by using the mMessage mMachine in vitro transcription kit (Ambion, Austin, TX).

Detection of GFP-tagged channels in oocytes has been described previously in detail (22). Briefly, 2–4 days after injections, Xenopus oocytes were fixed overnight at room temperature in 4% paraformaldehyde in phosphate-buffered saline. Fixed oocytes were embedded in 3% agarose, and 50-μm sections were cut by using a Vibratome. The cut sections were mounted on coverslips and imaged by using a Leica TCS confocal microscope. To compare fluorescence intensities between different oocyte sections, image acquisition parameters such as pinhole size, intensity, and offset were kept constant.

Two-electrode voltage-clamp recordings were carried out as described previously (23). Briefly, the oocytes were placed in a chamber and perfused with a solution containing a high level of potassium (96 mM). Currents were recorded by using a voltage step protocol from −100 to +100 mV in 10 mV steps (control current). Barium (3 mM) was used to measure the left over current that was not inwardly rectifying (barium-insensitive current). In the absence of receptor stimulation, we determined the basal inwardly rectifying current by subtracting barium-insensitive current from the control current.

For mammalian cell expression, Cos-1 cells were grown on coverslips and transfected using lipofection with FLAG-GIRK1 alone or FLAG-GIRK1 and GIRK4. Two days after the transfections, cells were fixed and permeabilized. Cells were first incubated with blocking solution containing bovine serum albumin and goat serum and then with mouse-IgG anti-FLAG primary antibody, washed, and then stained with Alexa Fluor 594 chicken anti-mouse-IgG. Coverslips were washed and mounted onto coverslides for confocal microscopy; a Rhodamine filter was used to visualize red staining.

RESULTS

Distribution Pattern of GIRK1 and GIRK4—To determine the basis for the difference in the membrane targeting of GIRK1 and GIRK4 channels, we tagged both GIRK1 and GIRK4 with the green-fluorescent protein and expressed them in Xenopus oocytes. Little background fluorescence was detected in the uninjected oocytes (Fig. 1a). Although GIRK4 was clearly in the membrane (Fig. 1b), GIRK1 was predominantly cytoplasmic (Fig. 1c). We have shown previously that only a small proportion of the GIRK1 interacts with the oocyte native GIRK channels (XIR), resulting in a large number of GIRK1 channels unable to target to the membrane (24), as is evident from the data shown. Furthermore, we expressed GIRK1-GFP along with non-tagged GIRK4, and the GIRK1-GFP was efficiently targeted to the membrane because of the co-expression of GIRK4 (Fig. 1d). These findings in oocytes were consistent with those obtained when these channels were expressed in mammalian cells (Fig. 1, e–f).

Role of the C Terminus—Previous studies have suggested a role for the distal C terminus of GIRK4 in targeting to the plasma membrane and in co-assembly with GIRK1 (17). In those studies, GIRK4 was truncated and its distribution was tested. Here, we have taken a different approach by replacing parts of the C terminus of GIRK4 with that of GIRK1 and testing for its localization. Fig. 2a, left shows the schematic of one of the chimeras that we made in the C-terminal replace-
GIRK4 with that of GIRK1 (Fig. 2 left, right). G4(181–187)/G1(181–200)-GFP is poorly localized to the membrane and mainly resides within vesicles in the cytoplasm. a, left, schematic showing the chimera G4(1–206)/G1(200–501)-GFP. This chimera includes only 19 amino acids from GIRK4 in the proximal C terminus; the rest of the C terminus is from GIRK1. Right, G4(1–206)/G1(200–501)-GFP localizes mainly to the plasma membrane and shows a distribution pattern that resembles that of wild-type GIRK4. The difference between G4(1–206)/G1(200–501)-GFP and G4(1–206)/G1(200–501)-GFP underscores the critical role of the proximal C terminus in channel distribution.

Expression of the GFP-tagged chimeras G4(1–249)/G1(243–501) showed a distribution pattern similar to that of GIRK1 (Fig. 4 top, right). This chimera contained most of the C terminus of GIRK1 and only 19 residues from the GIRK4 C terminus. Furthermore, this region is highly conserved, exhibiting only four amino acid differences between the two subunits. The Proximal C Terminus Is Not Sufficient in Determining Channel Localization—In trying to assess the role of the region (181–200) in GIRK channel targeting, we replaced only these amino acids in GIRK1 with the corresponding ones in GIRK4. This chimera (G1(1–181)/G4(187–206)/G1(200–501)-GFP) showed a distribution pattern similar to that of GIRK1 (Fig. 4a), indicating that this region was not sufficient to target GIRK1 to the membrane. To assess whether this chimera could be expressed to produce viable channels, we co-expressed it with GIRK4 in oocytes and measured barium-sensitive inwardly rectifying K+ currents. The robust currents recorded from these oocytes indicated proper functional co-assembly between GIRK4 and G1(1–181)/G4(187–206)/G1(200–501)-GFP (Fig. 4b), whereas expression of either channel by itself did not produce large currents (data not shown). Furthermore, replacing this region in GIRK4 with the corresponding GIRK1 residues (chimera G4(1–187)/G1(181–200)/G4(206–419)-GFP) led to proper membrane distribution (Fig. 4c), similar to the wild-type GIRK4. This result indicated that, although the proximal C terminus is critical in GIRK channel distribution, it is not sufficient in targeting GIRK1 to the membrane. Furthermore, this region is not in-

Fig. 3. The proximal C terminus is critical in GIRK channel localization. a, left, schematic showing the chimera G4(1–187)/G1(181–200)-GFP; right, G4(1–187)/G1(181–501)-GFP. The chimera G4(1–187)/G1(181–501)-GFP is poorly localized to the membrane and mainly resides within vesicles in the cytoplasm. b, left, schematic showing the chimera G4(1–206)/G1(200–501)-GFP. This chimera includes only 19 amino acids from GIRK4 in the proximal C terminus; the rest of the C terminus is from GIRK1. Right, G4(1–206)/G1(200–501)-GFP is poorly localized to the membrane and mainly resides within vesicles in the cytoplasm. Expression of the GFP-tagged G4(1–200)/G1(200–501)-GFP showed that it mainly resided in the membrane, behaving more like G4(1–249)/G1(243–501) than like G4(1–187)/G1(181–501) (Fig. 3b, right). This chimera contained most of the C terminus of GIRK1 and only 19 residues from the GIRK4 C terminus. Furthermore, this region is highly conserved, exhibiting only four amino acid differences between the two subunits.

The Proximal C Terminus Is Critical—Next, we replaced the entire C terminus of GIRK4 with that of GIRK1 to produce the chimera G4(1–187)/G1(181–501)-GFP (Fig. 3a, left). When this chimera was expressed in oocytes, it mainly localized to the cytoplasm (Fig. 3a, right). This pattern was clearly different from both GIRK4 and the other C-terminal chimeras that we tested above. This result suggested that the proximal C terminus plays a role in the targeting of GIRK4 channels to the plasma membrane. To further narrow the critical region involved, we produced the chimera G4(1–206)/G1(200–501) that splits the C-terminal region between residues 181 and 243 (Fig. 3b, left).
dispensable for proper distribution of GIRK4. Together, the findings from these two chimeras suggest that other factors in both GIRK1 and GIRK4 dictate their distribution.

Role of the N Terminus—Recent reports have shown the presence of ER transport signals in inwardly rectifying K⁺ channels (9, 13, 20). One such signal was found in the N terminus of GIRK4. Although GIRK1 and GIRK4 show 46% overall identity and 73% similarity, the distal N terminus is highly variable between the two proteins. This region in GIRK4 could be grafted into unrelated proteins and lead to their escape from the ER (20). To test the role of this region in the apparent difference in distribution between GIRK1 and GIRK4, we produced chimeras G1₁₋₄₂/G4₁₈₇₋₂₀₆/G₁₂₀₀₋₅₀₁-GFP and GIRK4 showing robust inwardly rectifying K⁺ currents. For the G4₁₋₄₉/G₁₂₀₀₋₅₀₁-GFP, this result indicated that the lack of the ER transport signal can be partially overcome by GIRK4. For the G4₁₋₄₉/
G1(42–501)-GFP chimera, this result indicated either that deficiency in additional domains in GIRK1 prevents the newly inserted ER transport signal from rescuing it or that it could only escape the ER but lacked proper domains for targeting to the plasma membrane. Co-expression of either one of these chimeras with a non-tagged GIRK4 led to their proper targeting to the membrane (data not shown), indicating that the wild-type channel could rescue them. Furthermore, co-expression of G4(1–49)/G1(42–501)-GFP with GIRK4 produced viable channels with robust barium-sensitive inwardly rectifying potassium currents when compared with GIRK4 alone (Fig. 5c), thus suggesting proper functional interactions between the two.

**Insertion of the Proximal C Terminus and Distal N Terminus of GIRK4 into GIRK1 Guide It to the Membrane—** Next, we placed the two critical regions identified, the proximal C terminus and the distal N terminus of GIRK4, into GIRK1 (Fig. 6, left) and tested for its distribution. The chimera G4(1–49)/G1(42–501)-GFP was efficiently targeted to the membrane (Fig. 6, right). This chimera encompasses two domains in inward rectifiers that have been previously identified as important in channel distribution.

**Fig. 5. The distal N terminus plays an important role in GIRK channel localization.** a, left, schematic showing the chimera G1(1–42)/G4(49–419)-GFP; right, replacing the non-conserved region in the distal N terminus of GIRK4 by the corresponding region of GIRK1 increases the amount of channel in the cytoplasmic regions as compared with the wild-type GIRK4 (see Fig. 1b). b, left, schematic showing the chimera G4(1–49)/G1(42–501)-GFP; right, the first 46 amino acids of GIRK4 contain an ER export signal. G4(1–49)/G1(42–501)-GFP where the first 42 amino acids of GIRK1 are replaced by those of GIRK4 is not efficiently targeted to the membrane. c, current-voltage plot from oocytes expressing G4(1–49)/G1(42–501)-GFP and GIRK4. Although expression of GIRK4 by itself in oocytes results in negligible current levels, co-expression of G4(1–49)/G1(42–501)-GFP with GIRK4 in oocytes gives large inwardly rectifying K+ currents, indicating proper functional interaction between the two.
The distal N terminus has been indicated in ER export (20), and the proximal C terminus is critical in channel assembly (11). These regions seem to work in concert to target these channels to the membrane.

DISCUSSION

GIRK1 channels require assembly with another GIRK subunit for proper surface localization and functional expression. GIRK1 expressed alone shows little to no surface localization, and this may serve as a quality checkpoint so that only functional heteromers are processed. We have used a chimeric approach to show that defects in two regions of GIRK1 are responsible for its failure to reach the membrane. Replacement of these regions with the corresponding regions from GIRK4 led to proper surface expression.

Role of the C Terminus—By replacing progressively larger regions in GIRK4 with those of GIRK1, we show that the entire C terminus of GIRK4 needs to be replaced to prevent it from reaching the plasma membrane. Keeping only 19 amino acids following the second transmembrane domain of GIRK4 and replacing the rest of the C terminus with that of GIRK1 maintained robust surface expression. Although this does not rule out a role for the distal C terminus of GIRK4 in channel co-assembly and localization, it clearly shows that elements within the GIRK1 C terminus can successfully fulfill these requirements. Kennedy et al. (17) have suggested that residues 375–399 in GIRK4 are required for its co-assembly with GIRK1. This was delineated based on a truncation mutant of GIRK4 that failed to target GIRK1 to the membrane. Furthermore, the same study showed that further truncation in GIRK4 drastically reduced its own surface expression. These findings were corroborated by another study in which an acidic cluster in the C terminus of GIRK4 was shown to play an important role in post-ER trafficking and, ultimately, exiting the endosomes for surface expression (20). Although this acidic cluster plays a crucial role in surface expression of GIRK4, our data clearly indicate that the GIRK1 C terminus can compensate for this region in the context of a chimera between GIRK1 and GIRK4. Our findings directly support a role for the proximal C terminus of GIRK4 for proper targeting of the tetramer. However, efforts to exchange surface expression phenotypes between GIRK1 and GIRK4 by swapping this region were unsuccessful. This result indicates that, whereas this region is important in the context of the chimeras that we have produced, there are additional elements that are required to target GIRK1 to the membrane. Additionally, other trafficking motifs in GIRK4 can overcome any defect that is introduced by replacing the proximal C terminus with that of GIRK1. The proximal region of the C terminus has been shown to be important in the co-assembly of homomeric Kir6.2 as well as heteromeric Kir4.2/Kir5.1 (25, 26). It has been suggested that this region interacts directly with the N terminus of these channels. Furthermore, this region is rich in positively charged residues that have been shown to interact with the membrane phospholipid PIP2 that is required for activation of all inwardly rectifying K channels (27). A recent crystal structure of the GIRK1 intracellular regions sheds some light on this by showing extensive interaction between the N and C termini of this protein in the tetramer (19). Interestingly, there are only four amino acid differences between GIRK1 and GIRK4 in the proximal C-terminal region that we have identified. Mutation of any single one of these mutants in the context of the larger chimera G4–206/G1–200–501–GFP did not affect its subcellular distribution (data not shown). One of these residues, Glu-198, is within 2.75 Å of Lys-49 in the N terminus, and there may exist an H-bond between the carbonyl oxygen in glutamate and the nitrogen on the side chain of lysine. Unfortunately, most of the C-terminal elements and all of the N-terminal regions that we have identified in this study are missing in the crystal structure, thus limiting our ability to further examine inter- and intra-molecular interactions that may be critical in channel processing.

Role of the N Terminus—Ma et al. (20) showed that a forward trafficking signal resides in the distal N terminus of GIRK2 and GIRK4 channels. This region is not well conserved between the different members of this family and the specific elements that were identified in GIRK4 and are missing in GIRK1. We show here that, where this region in GIRK4 was replaced with the corresponding region in GIRK1, a chimera showed substantial cytoplasmic localization and is clearly different from the wild-type GIRK4. However, a chimera where this region in GIRK1 was replaced with the corresponding region in GIRK4 did not reside in the membrane. These data show that, although the ER transport signal in GIRK4 is critical for proper surface expression, it is not sufficient to localize GIRK1 to the surface. This is consistent with data where placement of the Kir2.1 forward transport signal onto GIRK1 led to its exit from the ER, but this chimera also failed to localize to the surface (20).

Both N- and C-terminal Elements Are Required for Channel Processing—A chimera between GIRK4 and GIRK1, where the first 42 amino acids in the N terminus and the first 19 amino acids in the C terminus of GIRK1 were replaced by those of GIRK4, contained the minimal regions that could overcome targeting defects in GIRK1.

This finding indicates that GIRK1 has deficiencies in both N-
and C-terminal elements that are present for processing of GIRK4. Given previous data on the importance of the regions that we have identified in GIRK1, it is conceivable that the N-terminal regions are responsible for GIRK1 retention in ER, and the proximal C-terminal regions are critical in proper assembly and insertion into the plasma membrane. This may be a built-in mechanism to prevent GIRK1 channels from reaching the cell surface unless they are assembled into a heterotetramer. Therefore, there are two quality checkpoints for GIRK1 surface expression: one in the ER, and another one before insertion into the cell membrane. Although the N terminus may be critical in ER retention, C-terminal elements may control the final steps in proper targeting of these channels to the membrane.

Acknowledgments—We thank Drs. Kim W. Chan and Elizabeth Buck for help in the preliminary stages of this project.

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