Molecular Determinants for Assembly of G-protein-activated Inwardly Rectifying K⁺ Channels*

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Kir3.1 and Kir3.2 associate to form G-protein-activated, inwardly rectifying K⁺ channels. To identify regions involved in the coassembly of these subunits, truncated Kir3.1 polypeptides were coexpressed with epitope-tagged subunits in an in vitro translation system. N-terminal, C-terminal, and core region polypeptides were coimmunoprecipitated with both Kir3.2 and Kir3.1, suggesting that multiple elements distributed throughout the Kir3.1 polypeptide contribute to inter-subunit binding interactions. The Kir3.2 C-terminal polypeptide coimmunoprecipitated with the Kir3.1 C-terminal polypeptide, but neither region recognized the N-terminal domain and core region of the Kir3.1 subunit. This suggests that within Kir3 channels the C-terminal domains of neighboring subunits interact. Coexpression of the truncated polypeptides with Kir3.1 and Kir3.2 in Xenopus oocytes reduced functional expression of the heteromeric channels. Constructs encoding the core region plus N-terminal and proximal C-terminal regions competed more effectively than the core region alone, which supports the contribution of all three regions to intersubunit binding interactions. Proximal and distal segments of the C-terminal domain were as effective at inhibiting functional expression as the entire C-terminal domain.

Potassium-selective ion channels are the most functionally diverse group of ion channels. Molecular cloning has revealed two major families: the voltage-gated channels (Kvs) with six putative transmembrane domains (TMDs) and the inwardly rectifying channels (Kirs), with two putative TMDs. Voltage-gated K⁺ channels have a tetrameric arrangement of Kv subunits contributing to a central conductance pore (1, 2). Channel diversity is increased by the ability of different subunits to heteromultimerize to form channels with functional properties that differ from those of the homomeric channels (3–8). Kir subunits can also form homomeric and heteromeric complexes with distinct functional properties. The number of subunits composing a functional channel has been suggested to be four (9–12), although Krapivinsky et al. (13) have reported that Kir3 channel complexes, immunoprecipitated from atrial myocytes, can be much larger than would be predicted for a tetramer. The two families of channel subunits are further divided into subfamilies on the basis of amino acid sequence similarity (14–17). Heteromultimerization of Kv subunits is predominantly subfamily selective (18), although there are a few examples where subunits from different subfamilies have been shown to associate in heterologous expression systems (19). An important determinant of subfamily-selective associations is a region within the cytoplasmic N-terminal domain of the Kv subunits (2, 20–22). It has been named the N-terminal A and B box (NAB), according to the nomenclature of Drewe et al. (23), and is highly conserved within subfamilies.

Heteromultimerization of Kir subunits has been shown to occur both within and between subfamilies in heterologous expression systems (9, 12, 24). Little is known about the molecular determinants of Kir subunit associations. Studies with chimeric channels have suggested that the N-terminal domains and core regions of Kir subunits may be involved in this process. Fink et al. (25) have shown that substituting the N-terminal domain of Kir3.2 for that of Kir2.3 promotes the coassembly of these two subunits. Tucker et al. (24) made chimeras between Kir3.1 and Kir3.4 and reported that the transmembrane domains dictate the potentiation of coassembled channels.

Kir3 channels couple to G-protein-linked receptors and are activated by a direct interaction with G-protein βγ-subunits. Several Kir3 channel subunits have been cloned from mammalian heart and brain (13, 26–28). Kir3.1 and Kir3.4 associate to form the muscarinic receptor-coupled channel in atria (13). The neuronal channels are thought to be formed from Kir3.2 and Kir3.1, both of which are expressed in several areas of the central nervous system (28–31). Members of the Kir3 subfamily produce relatively small, G-protein-activated currents when expressed individually in Xenopus oocytes. However, the coexpression of Kir3.1 with either Kir3.4 or Kir3.2 produces much larger inwardly rectifying K⁺ currents (13, 28, 31–33). Hedin et al. (34) showed that Kir3.1, expressed alone, coassembles with an endogenous oocyte subunit, Kir3.5, to form a functional channel. Immunofluorescence labeling of Kir3.1 subunits showed no detectable expression at the surface of oocytes injected with Kir3.1 cRNA alone and high expression at the surface of oocytes coinjected with Kir3.1 and Kir3.2 cRNAs. This suggests that Kir3.1 homomeric complexes either are not formed or are not targeted to the plasma membrane. A recent study by Kennedy et al. (35) has shown that Kir3.1 expressed in COS cells is localized to internal cytoskeletal structures. In contrast, oocytes injected with Kir3.2 cRNA alone showed similar immunofluorescence labeling of Kir3.2 compared with the coinjected oocytes, suggesting that Kir3.2 homomeric channels are assembled and delivered to the plasma membrane with relative efficiency.

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1 The abbreviations used are: TMD, transmembrane domain; NAB, N-terminal A and B box; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

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We have investigated the structural elements in Kir3 channels that are involved in the coassembly of subunits. This was approached by coexpressing truncated Kir3.1 polypeptides with full-length Kir3.1 and Kir3.2 subunits in an *in vitro* system and in *Xenopus* oocytes. N-terminal, C-terminal, and core region polypeptides were communoprecipitated with the full-length subunits and were able to inhibit functional expression of heteromeric channels. This suggests that these elements of Kir3 channels all contribute to intersubunit binding interactions.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology—**Standard PCR procedures were used to construct a strong Kozak consensus sequence (36), GCGCCGACC, immediately upstream of the ATG initiation codon in cDNA clones for Kir3.1, Kir3.2, and Kir3.4. These constructs were then subcloned into the EcoRI site of the pBG7.2 vector (gift from R. W. Aldrich), which provides the 5'- and 3'-untranslated regions of the *Xenopus* β-globin gene. In addition, a FLAG tag sequence corresponding to the amino acid sequence MDYKDDDDK was added to the extreme N terminus of Kir3.1 and Kir3.2 using standard PCR procedures. The cDNA for Kir3.1 was subdivided into three portions, encoding the N terminus (1–85), the core region (87–190), and the C terminus (191–501), using standard PCR procedures. An additional construct was made by PCR, which added residues 60–80 of Kir2.1 to the Kir3.1 region 80–190 by incorporation of the corresponding Kir2.1 sequence into the 5' PCR primer. PCR reactions were performed with Vent polymerase (New England Biolabs) according to the manufacturer's instructions. The sequences of all PCR-generated clones were verified using dideoxy- DNA sequencing using the Sequenase II enzyme (U.S. Biochemistry Corp.) according to the manufacturer's instructions.

A further series of deletion clones were made in the Kir3.1 cDNA using a nested deletion strategy, gradually deleting more DNA from the 3'-end of the open reading frame. A linker sequence flanked by KpnI and BamHI sites that had internal TAG stop signals in each of the three reading frames was introduced into the pBG7.2Kir3.1 multiple cloning site after the native stop codon. NotI- and KpnI-linearized DNA generated suitable ends for unidirectional nested deletion library construction. A library of random length deletion clones was generated using the Exo-Size kit (New England Biolabs) following the manufacturer's instructions.

**Antisera Production—**Rabbit polyclonal antisera were raised against peptides derived from unique sequences in Kir3.1, at the N terminus (amino acids 5–20) and at the C terminus (amino acids 485–499). Both sequences had an additional cysteine residue added to the C-terminal end to facilitate covalent linkage to keyhole limpet hemocyanin using the m-maleimidobenzoic acid N-hydroxysuccinimide ester method (37).

**In Vitro Translation and Immunoprecipitation—**Full-length and truncated construct plasmid cDNAs were coexpressed in the TNT coupled transcription/translation system (Promega), according to the manufacturer's instructions, in the presence of translation grade [35S]methionine (Amersham Corp.) and either in the presence or absence of canine pancreatic microsomes.

Twenty-four microliters of the translation mix was solubilized with 200 μl of solubilization buffer (150 mM NaCl, 1 mM EDTA, 3 mM diethyldithitol, 2% (v/v) Triton X-100, 10 mM HEPES, pH 7.5) for 30 min, and the solute lysate was then incubated on ice for 15 min with 10 μl of protein G-coated Sepharose beads (Pharmacia Biotech Inc.). The beads were cleared, and the supernatant was incubated with either 150 ng of anti-FLAG monoclonal antiserum (IBI Kodak) or a 1:2000 dilution of anti-Kir3.1 polyclonal antiserum for 1 h on ice. 20 μl of a slurry of 50% protein G-coated Sepharose beads was then added, and the incubation continued for 1 h. Immunoprecipitated material was sedimented by centrifugation at 13,000 × g for 20 s and washed four times in 800 μl of solubilization buffer at 4 °C. Immunoprecipitates were boiled in Laemmli sample buffer (38) and resolved by SDS-PAGE followed by fluorography of the dried gels.

**Immunoprecipitated in Vitro Translation Products—**SDS-PAGE gels of [35S]-labeled translation products were dried and exposed to a phosphor screen overnight. A densitometric analysis of the amounts of labeled polypeptides was made on a Molecular Dynamics PhosphorImager using the ImageQuant software.

**In Vitro Transcription—**Capped cRNA was made from NdeI-linearized cDNA using the T7 polymerase mMESSAGE mMACHINE Kit (Ambion) according to the manufacturer's instructions.

**Preparation and Microinjection of Oocytes—**Oocytes were surgically removed from *Xenopus laevis* anesthetized with 3-amino benzoic acid (Sigma) and dissociated from connective tissue using 0.3% collagenase (Sigma) in Ca<sup>2+</sup>-free buffer (82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl<sub>2</sub>, 5 mM HEPES, pH 7.6). Isolated oocytes were microinjected with 50-ncRNAs dissolved in water. Spectroscopic determinations of cRNA concentrations indicated that undiluted cRNAs were between 30 and 50 ng/50 μl. In all experiments, the human M<sub>3</sub>-muscarnic receptor cRNA was coinjected. Oocytes were incubated in ND96 at 18 °C.

**Electrophysiology—**Two-electrode voltage clamp recordings were performed 3–6 days after microinjection using an OC-725B amplifier (Warner Instruments), interfaced to a Macintosh Quadra 700 computer using an ITC16 A/D board (Instrutech) with pulse acquisition software (version 7.89; HEKA). Microelectrodes filled with 3 mM KCl had resistances ranging between 0.5 and 2 megaohms. Oocytes were continuously perfused with standard recording solution (90 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 1 mM HEPES, pH 7.4). Currents were recorded in the presence of 10 μM acetylcholine before and after application of 300 μM Ba<sup>2+</sup>. Current records were filtered at 1 kHz and digitized at 5 kHz. Experiments were done at room temperature (22–24 °C).

**Immunocytochemistry—**Oocytes were microinjected with 50 nl of cRNAs and incubated at 18 °C in ND 96 for 5–6 days. Batches of 8–10 oocytes were fixed for 4 h in methanol at −20 °C and cryopreserved in PBS, pH 7.4, 30% (v/v) sucrose at 4 °C overnight. Oocytes were then embedded in octyltransferase (Gurr) on cryostat chucks sitting on dry ice. 16-μm slices were cut on a Leica CM3000 cryostat, and the sections were mounted onto poly-L-lysine-coated microscope slides and left to air-dry. Slices were then blocked in PBS, pH 7.4, containing 1% bovine serum albumin at 4 °C overnight. Samples were incubated in primary antiserum diluted 1:2000 in PBS, pH 7.4, plus 1% bovine serum albumin overnight at 4 °C and then washed with several changes of PBS. They were then incubated for 3 h with a fluorescein isothiocyanate-conjugated secondary antiserum (Vector) at room temperature. Following a series of washes in PBS, the samples were mounted in DPX medium (BDH) and left to dry in the dark. Samples were viewed on a Zeiss Axiohot UV microscope using a ×20 objective.

**Statistics—**Statistical analyses were performed with a unpaired Student’s t test using InStat software (version 2.01, GraphPad). Data were assumed to be normally distributed. A significant difference was accepted when the two-tailed p value was less than 0.01. Averaged data were presented as mean ± S.E.

**RESULTS**

Different members of the Kir3 family that were cotranslated *in vitro* in the presence of microsomal membranes were shown to associate by the communoprecipitation of both subunits using antiserum that recognized only one of the subunits (Fig. 1).
The subunit specifically precipitated contained a FLAG epitope added to the N terminus. This did not alter the functional characteristics of the channels expressed in *Xenopus* oocytes (results not shown), suggesting that it does not interfere with the coassembly of subunits. Kir3.1 gave two products, a 56-kDa species and a 58-kDa glycosylated species, whereas Kir3.2 and Kir3.4 both gave a single product of molecular size 54 and 47 kDa, respectively. Fig. 1 shows the coprecipitation of...
Kir3.1 with FLAG-Kir3.2 and the coprecipitation of Kir3.4 with both FLAG-Kir3.1 and FLAG-Kir3.2.

To investigate which regions of Kir3.1 are involved in subunit coassembly, we made a series of truncated constructs with progressively larger deletions from the C terminus (Fig. 2A) and tested their ability to coprecipitate with full-length subunits containing the FLAG epitope. We looked at binding to both Kir3.1 and Kir3.2 to see if we could identify structural elements that distinguish heteromultimerization from Kir3.1 homomultimerization, and the results are shown in Fig. 2B. The polypeptides 1–347, 1–200, and 1–160, which have the distal half of the C-terminal domain, almost the entire C-
terminal domain, and the C-terminal domain plus TMD2 deleted, respectively, were all coprecipitated by full-length Kir3.1 and Kir3.2. All three constructs gave two products, the glycosylated and unglycosylated species, indicating that these polypeptides incorporated into the microsomal membranes. The N-terminal segment, Kir3.1-(1–85), which has the entire core region and C-terminal domain deleted, did not appear to coprecipitate with either Kir3.1 or Kir3.2 (results not shown). This region contains just a single methionine and consequently would only produce a very weak fluorographic signal. We therefore added a FLAG epitope to the 1–85 construct and tested the ability of the full-length subunits without the FLAG epitope to coprecipitate with this segment. In the absence of microsomal membranes, both Kir3.1 and Kir3.2 coprecipitated with Kir3.1-(1–85). However, when microsomes were included in the translation mixture we were unable to detect coprecipitation of either subunit (results not shown). One possible explanation for this difference is that the presence of membranes favors associations between the membrane-incorporated full-length subunits while reducing the rate of encounter between the soluble Kir3.1-(1–85) peptide and the subunits.

We next tested the ability of the core and C-terminal segments to bind to Kir3.1 and Kir3.2 subunits. Kir3.1-(77–190) starts a few residues upstream of the proposed TMD1 and includes H5 and TMD2. When translated in the presence or absence of microsomal membranes, this construct gave only a single product of molecular size 15 kDa, which was not coprecipitated with either Kir3.1 or Kir3.2 (Fig. 2C). The lack of a detectable glycosylated species suggests that the Kir3.1-(77–190) polypeptide did not incorporate correctly into the microsomal membranes. In the absence or presence of microsomes, the polypeptide was sedimented by spinning the translation mixture at 100,000 × g for 10 min, suggesting that it forms insoluble aggregates (results not shown). To obtain a polypeptide that included only the core region of the Kir3.1 subunit but had sufficient residues upstream of TMD1 to incorporate into membranes, we made a chimeric construct encoding residues 60–80 of Kir2.1 and residues 80–190 of Kir3.1 (Kir3.1-(80–190)). This gave both a glycosylated and unglycosylated product, which were coprecipitated with Kir3.1 and Kir3.2 (Fig. 2C). This suggests that the core region of Kir3.1 recognizes and binds to the full-length subunits, although we cannot rule out an involvement of the 20 Kir2.1 residues. Kir2.1 has been reported not to readily associate with Kir3.1 in the Xenopus oocyte expression system (39). We found that coinjecting an excess of Kir3.1 cRNA with Kir2.1 cRNA, reduced the functional expression of Kir2.1 (results not shown), suggesting that there is some recognition between the two subunits.

The 35-kDa C-terminal domain (Kir3.1-(180–501)) coprecipitated with both Kir3.1 and Kir3.2 in the presence and absence of microsomal membranes. The constructs Kir3.1-(180–307) and Kir3.1-(308–501) divide up the C-terminal domain at the start of a region showing some sequence similarity to pleckstrin homology domains (40). Such domains are found in proteins that may interact with Gα-subunits (41). Both polypeptides were individually coprecipitated with the full-length subunits and particularly effectively with Kir3.1.

These results suggest that regions involved in subunit coassembly are distributed throughout the Kir3.1 polypeptide, within the proximal and distal segments of the C-terminal domain, the core region and possibly also the N-terminal domain. All regions recognized both Kir3.1 and Kir3.2 subunits and there was no evidence to suggest that any part of the Kir3.1 polypeptide inhibits its homomultimerization.

A densitometric scan of gels provided a quantitative analysis of the relative amounts of each product in the translation reactions and in the immunoprecipitates. Representative scans of immunoprecipitated products are shown in Fig. 3, the top row showing truncated proteins coprecipitated with Kir3.1 and the bottom row showing proteins precipitated with Kir3.2. Whereas Kir3.1-(1–160) and Kir3.1-(1–347) appear to coprecipitate to a similar extent with both Kir3.1 and Kir3.2, there is clearly very much more Kir3.1-(308–501) coprecipitated with Kir3.1 compared with Kir3.2. Correcting for the number of methionines in each polypeptide, the relative amounts of each construct polypeptide in the immunoprecipitates and corresponding translation reactions, expressed as a percentage of the total product, are given in Table I. The amount of construct coprecipitated per mol of Kir3.1 or Kir3.2 was dependent upon its proportion in the translation reaction. For Kir3.1-(1–347), a 6:1 molar ratio of construct to full-length subunit gave a maximal amount of construct polypeptide in the precipitate. We therefore aimed to achieve at least this molar ratio in all cotranslation reactions, although Kir3.1-(180–501) was translated relatively inefficiently and lower ratios were obtained. This low translation efficiency may explain why the amount of the C-terminal domain coprecipitated with either subunit was low compared with the amount of constructs 180–307 and 308–501 coprecipitated. The polypeptides containing regions within the C-terminal domain of Kir3.1 or Kir3.2 were all coprecipitated in significantly higher amounts with Kir3.1 than with Kir3.2, whereas there was no significant difference for the
other polypeptides. This suggests that the relative contribution of the C-terminal domain to the overall intersubunit binding interaction is different for Kir3.1 homomeric complexes than for heteromeric complexes.

To test whether the C-terminal domain of the Kir3.1 subunit interacts with either N- or C-terminal regions of neighboring subunits, two constructs with minimal sequence overlap, Kir3.1-(1–200) and Kir3.1-(180–501), were cotranslated and immunoprecipitated with an antibody directed to the N-terminal region of Kir3.1. The C-terminal polypeptide failed to co-precipitate with Kir3.1-(1–200) (Fig. 4). Similarly, polypeptide Kir3.2-(192–425), which contains the entire C-terminal region of Kir3.2, did not coprecipitate with Kir3.1-(1–200), although it did coprecipitate with both full-length Kir3.1 and the Kir3.1 C-terminal polypeptide (Fig. 4). None of the C-terminal segments (Kir3.1-(180–501), (180–307), and (308–501) and Kir3.2-(192–425)) coprecipitated with the N-terminal polypeptide Kir3.1-(1–85) (results not shown). This suggests that in heteromeric and homomeric complexes, the C-terminal regions of neighboring subunits interact.

To locate the interaction sites for the proximal and distal C-terminal segments on the Kir3.1 polypeptide we tested the ability of both segments to coprecipitate with construct 1–347, which has 154 residues of the distal C-terminal region deleted. Both segments coprecipitated with Kir3.1-(1–347), although the amount of Kir3.1-(308–501) coprecipitated with Kir3.1-(1–347) was less than the amount coprecipitated with full-length Kir3.1 subunits (Fig. 2). This indicates that binding of this segment to Kir3.1 involves an interaction with both proximal and distal C-terminal regions of the subunit.

To test the ability of the truncated polypeptides to compete with the full-length subunits during the assembly of Kir3 channels in a cell, they were coexpressed with Kir3.1 and Kir3.2 in Xenopus oocytes. The coexpression of Kir3.1-(1–347) with Kir3.1 and Kir3.2 cRNAs along with the deletion construct cRNAs indicated. Kir3.1 and Kir3.2 cRNAs were injected at different dilutions (undiluted, 3-fold, 10-fold, and 100-fold); the dilution factor is indicated in parentheses. All deletion construct cRNAs were injected undiluted. Currents were normalized to recordings from control oocytes injected with Kir3.1 and Kir3.2 cRNAs alone. The total number of recordings from test oocytes is indicated above each bar. For each experiment we recorded from an equal number of control oocytes. Statistical significance was assigned using Student’s t test; an asterisk represents p < 0.01, while n/s indicates not significant. B, immunofluorescence staining of oocytes using an anti Kir3.2 C terminal antiserum 5 days after coinjection with the cRNAs indicated.
Kir3.2 and the M_{2}-muscarinic receptor produced no basal or agonist-stimulated Kir currents (results not shown), indicating that the truncated polypeptide was unable to substitute for Kir3.1 in the formation of functional heteromeric channels. Five days after injecting the cRNAs, the antibody raised against Kir3.2 was unable to detect any channel expression at the oocyte plasma membrane. In contrast, oocytes from the same batch that were injected with Kir3.2 plus M_{2}-muscarinic receptor cRNAs alone showed intense immunofluorescence staining of Kir3.2 at the cell surface (Fig. 5B). This suggests that Kir3.1-(1–347) coassembled with Kir3.2, thereby inhibiting the formation of Kir3.2 homomeric channels. The complexes formed were not delivered to the cell surface, indicating that the distal C-terminal segment of the Kir3.1 polypeptide plays a crucial role in this process. The truncated constructs were tested for their ability to reduce the size of the Kir3.1/Kir3.2 heteromeric channel currents rather than the Kir3.2 homomeric currents because the heteromeric currents were much larger. The Kir3.1 and Kir3.2 cRNAs were injected either undiluted or diluted 3-, 10-, or 100-fold, with or without the undiluted construct cRNAs. Currents recorded at −80 mV from oocytes expressing the truncated polypeptides were normalized to currents recorded from oocytes expressing Kir3.1 and Kir3.2 alone (Fig. 5A). The amount of inhibition was concentration-dependent, increasing as the ratio of construct cRNA to Kir3.1/Kir3.2 cRNAs increased. The greatest inhibition was seen with Kir3.1-(1–347), followed by Kir3.1-(1–200) and then the chimeric core region construct, Kir3.1-(1–85). It appears that the core region alone can bind to the full-length subunits and that adding the N-terminal and proximal C-terminal regions increases the overall binding affinity. This supports the contribution of regions 1–79, 80–190, and 200–347 to intersubunit binding interactions. Kir3.1-(1–347) was less effective at inhibiting Kir3.1/Kir3.2 heteromultimerization than Kir3.2 homomultimerization. Coinjected with undiluted Kir3.1/Kir3.2 cRNAs, it reduced the current amplitude by 44%. This suggests that Kir3.2 subunits prefer to bind Kir3.1 subunits rather than themselves. Construct Kir3.1-(1–200) competed much less effectively than Kir3.1-(1–200), suggesting that TMD2 makes an important contribution to the coassembly of subunits. Immunofluorescence labeling of Kir3.2 in oocytes expressing Kir3.1-(1–200) with the 10-fold diluted cRNAs showed that the truncated polypeptide reduced cell surface expression of this subunit (Fig. 5B).

The soluble N- and C-terminal polypeptides all reduced the size of the currents when the Kir3.1/Kir3.2 cRNAs were diluted 100-fold. Kir3.1-(180–307) and Kir3.1-(308–501) were as effective as the entire C-terminal domain, which supports the in vitro translation/coimmunoprecipitation data.

**Discussion**

Kir3.1 subunits coassemble with other members of the Kir3 subfamily to form G-protein-activated, inwardly rectifying K⁺ channels. By analyzing the binding interactions between truncated Kir3.1 polypeptides and full-length Kir3.1 and Kir3.2 subunits in an in vitro expression system and in Xenopus oocytes, we have identified several regions distributed throughout the Kir3.1 polypeptide that appear to contribute to intersubunit binding interactions. These regions lie within the N-terminal, core, and C-terminal domains, suggesting that they are all involved in the coassembly of subunits. Kir3.1 and Kir3.2 C-terminal polypeptides recognized each other but did not appear to recognize either the N-terminal or core regions of Kir3.1. This suggests that within the Kir3 channel complex, the C-terminal regions of neighboring subunits interact as do the more N-terminal regions.

Coprecipitation experiments showed that all of the truncated Kir3.1 polypeptides bound to both Kir3.1 and Kir3.2, suggesting that Kir3.1 subunits are capable of forming homomeric as well as heteromeric complexes. These homomeric complexes are not processed to the plasma membrane in Xenopus oocytes, which suggests that they may have a different quaternary structure from that of the heteromeric complexes that are appropriately delivered to the cell surface. The quantitative analysis of the amount of coprecipitated products showed that there was a difference in the amount of Kir3.1 C-terminal polypeptides coprecipitated with Kir3.2 compared with Kir3.1, suggesting that this region plays a discriminatory role in the coassembly of Kir3 subunits. Our studies with Kir3.1 and Kir3.2 chimeric subunits suggest that both the cytoplasmic tails and the core region of the Kir3.1 subunit inhibit the expression of homomeric channels at the cell surface.

The ability of Kir3.1-(80–190) to coprecipitate with Kir3.2 in vitro and to reduce functional expression of the Kir3.1/Kir3.2 channels in oocytes suggests that the Kir3.1 TMDs can recognize and bind to Kir3.2 in the absence of the cytoplasmic tails. This is assuming that the short, hydrophilic Kir2.1 region is not responsible for the binding interaction and that the difference seen between Kir3.1-(77–190) and the chimeric core construct is caused by the inability of Kir3.1-(77–190) to incorporate into the microsomal membranes. The relative contributions of TMD1 and the N-terminal region to the binding interaction is unclear. In the in vitro expression system, Kir3.1-(1–160) was coprecipitated in the presence of microsomes, whereas Kir3.1-(1–85) was not. This suggests that the TMD1 and H5 region plays an important role in the coassembly of subunits. Kir3.1-(1–160) was glycosylated, indicating its incorporation into microsomes. The contribution of the TMD1 and H5 region may have been simply to incorporate the polypeptide into the microsomal membranes, thereby increasing its rate of encounter with the full-length subunits and promoting the binding of the N-terminal domain, or it may have included a direct involvement of this region in intersubunit binding interactions.

In oocytes, Kir3.1-(1–160) was similar to Kir3.1-(1–85) in only producing a significant reduction in channel expression when coinjected with the 100-fold diluted Kir3.1/Kir3.2 cRNAs. It is possible that the 1–85 peptide had a greater opportunity to bind to the full-length subunits when expressed in oocytes rather than in vitro due to the spatial localization of the translational machinery and the endoplasmic reticulum, and thus the role of TMD1 in increasing the rate of association was reduced. Alternatively, the reduction in current amplitude produced by Kir3.1-(1–85) may have been partly a functional inhibition caused by binding to G-protein βγ-subunits. A similar mechanism was proposed by Dascal et al. (42) to account for the inhibition of Kir3.1 functional expression by the myristoylated C-terminal tail of Kir3.1. Kir3.1-(1–200) was much more effective than Kir3.1-(1–160) in inhibiting channel expression in oocytes, suggesting that TMD2 directly participates in intersubunit binding interactions.

In voltage-gated K⁺ channels, the NAB region within the N-terminal domain appears to have two functions in regulating α-subunit coassembly. Within subfamilies it provides a recognition site for coassembly and stabilizes the tetrameric complex, whereas between subfamilies it prevents coassembly taking place. Lee et al. (43) showed that deleting this N-terminal region enabled two subunits from different subfamilies to form functional heteromeric complexes. Our results suggest that within the Kir3 subfamily the N-terminal domain does not have a dominant role. Regions within the core and the C-terminal domain are involved in subunit recognition, and whether or not they have an additional role in creating subunit incompatibility remains to be determined.
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Note Added in Proof—While this paper was undergoing editorial review, Tinker et al. [Tinker, A., Jan, Y. N., and Jan, L. Y. (1996) Cell 87, 857–868] reported that the proximal C-terminus and TMD2 deter-

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