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Mapping of the Physical Interaction between the Intracellular Domains of an Inwardly Rectifying Potassium Channel, Kir6.2*

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The amino-terminal and carboxyl-terminal domains of inwardly rectifying potassium (Kir) channel subunits are both intracellular. There is increasing evidence that both of these domains are required for the regulation of Kir channels by agents such as G-proteins and nucleotides. Kir6.2 is the pore-forming subunit of the ATP-sensitive K^+ (K_{ATP}) channel. Using an *in vitro* protein-protein interaction assay, we demonstrate that the two intracellular domains of Kir6.2 physically interact with each other, and we map a region within the N terminus that is responsible for this interaction. "Cross-talk" through this interaction may explain how mutations in either the N or C terminus can influence the intrinsic ATP-sensitivity of Kir6.2. Interestingly, the "interaction domain" is highly conserved throughout the superfamily of Kir channels. The N-terminal interaction domain of Kir6.2 can also interact with the C terminus of both Kir6.1 and Kir2.1. Furthermore, a mutation within the conserved region of the N-terminal interaction domain, which disrupts its interaction with the C terminus, severely compromised the ability of both Kir6.2 and Kir2.1 to form functional channels, suggesting that this interaction may be a feature common to all members of the Kir family of potassium channels.

Inwardly rectifying potassium (Kir)[1] channels are essential for determining the resting membrane potential, and regulating transmembrane K^+ fluxes, of many excitable and nonexcitable cells (1–3). Since the cloning of the first Kir channel in 1993, several important subfamilies with differing physiological roles have been identified. For example, Kir3.1 and Kir3.4, members of the G-protein-gated subfamily, coassemble to form I_{KACh}, the current which slows the heart rate in response to vagal nerve stimulation (3). In the pancreatic β-cell, the ATP-sensitive (K_{ATP}) channel is formed by coassembly of Kir6.2 and the sulfonylurea receptor, SUR1, which is a member of the ABC-transporter superfamily (4, 5). K_{ATP} channels are sensitive to the levels of intracellular adenine nucleotides and thereby couple the metabolic status of the cell to its electrical activity. This provides the link between changes in blood glucose and insulin secretion by the β-cell.

Like other K^+ channels, Kir subunits have been shown to form a tetrameric K^+-selective pore, and they exhibit significant sequence homology to other K^+-channels within the pore-forming region (2, 3, 6). However, in contrast to the six-transmembrane domain (TM) structure of the voltage-gated K^+ and related cation channels, Kir channel subunits have only two transmembrane domains (1). Kir channels also appear to differ in the structural elements which define subunit assembly and the specificity of subunit heteromultimerization. For voltage-gated K^+ channels these properties are largely defined by the first TM and an N-terminal "tetramerization" domain (2, 3). By contrast, the domains that determine the subunit assembly of Kir subunits are less well understood. Different studies have implicated multiple regions in both the N and the C termini, as well as in the TMs, which are involved in subunit assembly and processing (7–11). There are also a number of studies that suggest physical interactions between the N and C termini of Kir channels may have important functional effects (11–20).

A direct physical interaction between the N- and C-terminal domains of the G-protein-gated Kir3.0 subunits has been shown to enhance $G_{\beta\gamma}$ binding, supporting the now accepted view that $G_{\beta\gamma}$ subunits gate the channel by interacting with a complex binding site formed by both the N and C termini (11, 13, 17–19). Similarly, the ability of Kir1.1 to respond to changes in intracellular pH involves conformational changes in both the N and C termini (16). There are also reports that both the N and C termini of Kir6.2 participate in regulating K_{ATP} channel function (12, 14, 15, 21).

In this study we demonstrate a direct physical interaction between the N and C termini of Kir6.2. More importantly, we map a highly conserved region within the N terminus which is responsible for this interaction. We also show that this highly conserved "interaction domain" is capable of interacting with the C termini of other Kir subunits and that disruption of this interaction severely compromises the ability of both Kir6.2 and Kir2.1 to form functional channels.

MATERIALS AND METHODS

Molecular Biology—Relevant N-terminal fragments with in-frame restriction sites were generated by polymerase chain reaction and subcloned between the *Eco*RI and *Sal*I sites of the GST-fusion expression vector, pGEX-5X-1 (Amersham Pharmacia Biotech). All N-terminal constructs included a hexahistidine tag at the C terminus. Relevant C-terminal fragments representing amino acids 170–391 for Kir6.2 and equivalent regions for Kir6.1 (amino acids 180–424) and Kir 2.1 (amino acids 182–428) were also generated by polymerase chain reaction and subcloned in-frame between the *Eco*RI and *Sal*I sites of the pET-28a vector (Novagen). This vector directs protein expression under the control of the T7 promoter. Site-directed mutagenesis was performed by subcloning appropriate fragments into the pALTER-1 vector and using the Altered Sites *in vitro* mutagenesis system (Promega). Constructs for the *Shaker* N termini (22) were generously provided by Dr W. N. Zagotta (University of Washington, WA).

Protein Production—N-terminal GST-(HIS_6) fusion constructs were transformed into the *BL21(DE3) Escherichia coli* strain, proteins were induced with 0.25 mM IPTG, and cultures were grown for 3–4 h at

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[1] The abbreviations used are: Kir, inwardly rectifying potassium; TM, transmembrane domain; GST, glutathione *S*-transferase; IPTG, isopropyl-1-thio-b-D-galactopyranoside; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; NID, N-terminal interaction domain; DTNB, 5,5′-dithiobis(nitrobenzoic acid); NDSB, dimethylbenzylammonium, propane sulfonate.

20 °C. Cultures were harvested by centrifugation, resuspended in buffer S (150 mM Tris, pH 7.8, 50 mM NaCl, 25 mM imidazole, 1% NDSB-256, 0.5% CHAPS, 0.2% Tween 20), lysed by sonication, and the insoluble material precipitated by centrifugation at 10000 × *g* for 15 min. N-terminal fusion proteins were then purified from the supernatant on a Ni^{2+}-agarose column and eluted with 100 mM EDTA. [^{35}S]methionine-labeled C-terminal constructs were synthesized using the TNT T7 Quick Coupled Transcription Translation system (Promega), according to the instructions of the manufacturer. After synthesis, the reaction was stopped by adding 500 μl of buffer S per 50 μl of reaction volume, and insoluble material was precipitated by centrifugation at 100,000 × *g* for 30 min before use in the binding assay.

Binding Assay—In vitro binding assays were carried out in a 1.5-ml microcentrifuge tube by adding 20 μl of GST-fusion protein (15 μg), 10 μl of bovine serum albumin (10 mg/ml), 15 μl of glutathione-agarose beads (60% slurry; Amersham Pharmacia Biotech), 200 μl of buffer S, and 250 μl of the relevant radiolabeled C terminus (prepared as above). Tubes were then mixed by constant rotation for 40 min at room temperature, and the beads then washed three times for 15 min in 1 ml of buffer S at room temperature. After the final wash, all supernatant was removed and the beads were resuspended in 15 μl of 2× protein sample buffer. A 10-μl aliquot was then subjected to 10% SDS-polyacrylamide gel electrophoresis and autoradiography.

Electrophysiology—Oocytes were prepared and whole-cell currents were recorded by two-electrode voltage clamp as described previously (23). Mean steady-state whole-cell currents were recorded at −110 mV during a 250-ms pulse from a holding potential of −10 mV. The recording solution contained (in mM): 90 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 Hepes (pH 7.4 with KOH). All experiments were performed at room temperature.

Alignments and Secondary Structure Prediction—Sequences for the N termini were aligned and homology indicated using the GCG PILEUP and PRETTYPLOT programs. Full-length sequences for the relevant Kir subunits were submitted to the Predict Protein server (available on the WWW) for secondary structure prediction (*PHDsec*) using the programs default settings (24, 25).

RESULTS

The N- and C-terminal Domains of Kir6.2 Physically Interact—To assess whether the intracellular domains of Kir6.2 interact, we used an *in vitro* protein-protein interaction assay that exploits the ability of recombinant glutathione *S*-transferase (GST) fusion proteins to interact with [^{35}S]methionine-labeled *in vitro* translated proteins. If the two proteins interact, then the radiolabeled protein can be purified using glutathione-Sepharose beads (see "Materials and Methods"). To assess the specificity of such a potential interaction, we have used the N-terminal "tetramerization" domain of the *Shaker* K^+ channel as both a positive and negative control (22), as well as the GST protein by itself.

Fig. 1 shows that neither GST alone, nor the N terminus of Kir6.2 (residues 1–53), nor the very distal C terminus of Kir6.2 (residues 349–391) exhibited association with the radiolabeled *Shaker* N terminus. However, as expected, the *Shaker* N-terminal GST-fusion protein did associate with the radiolabeled *Shaker* N terminus. By contrast, when these same GST-fusion proteins were screened against the radiolabeled Kir6.2 C terminus (residues 170–391), only the Kir6.2 N terminus was found to interact; the *Shaker*-N terminus did not bind, and neither did residues 349–391 of Kir6.2 nor GST alone. Together these results provide strong evidence that the N- and C-terminal domains of Kir6.2 specifically associate with one another.

Mapping the Interaction Domain within the N Terminus of Kir6.2—To identify the region within the N terminus of Kir6.2 which is responsible for interaction with the C terminus of the channel, we made serial truncations of the N terminus, and tested the ability of the truncated proteins to bind to the C terminus. Fig. 2 shows that the N terminus of Kir6.2 still retained the ability to interact with the C terminus when either residues 1–29, or residues 47–53, were deleted. Further deletions resulted in a loss of binding. This implicates the region encompassing residues 30–46 (highlighted in Fig. 3) as critical

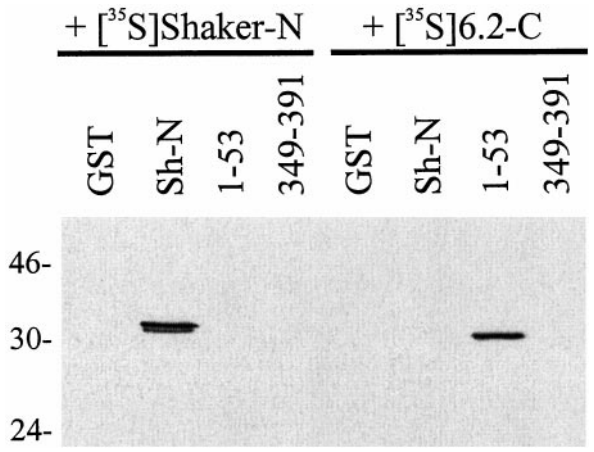

FIG. 1. **Physical association between the N- and C-terminal domains of Kir6.2.** Individual GST-fusion proteins as labeled above each lane (*GST*, GST alone; *Sh-N*, *Shaker* N terminus; *1–53*, Kir6.2 N terminus residues 1–53; *349–391*, Kir6.2 C terminus residues 349–391) were tested for their ability to interact with *in vitro* translated [^{35}S]Met-labeled *Shaker* N terminus ([^{35}*S*]*Shaker-N*), or residues 170–391 of the Kir6.2 C terminus ([^{35}*S*]*6.2-C*).

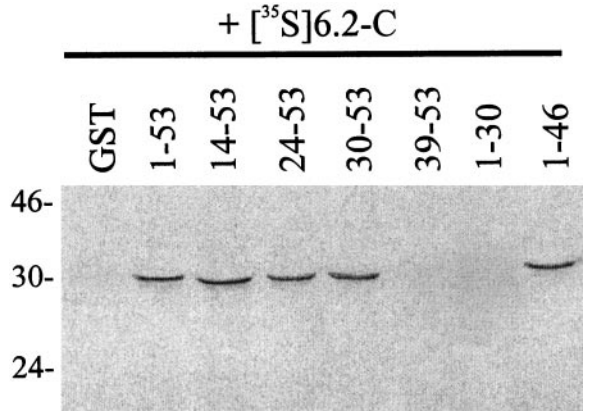

FIG. 2. **Mapping the interaction domain within the N terminus of Kir6.2.** The effect of different truncations on the ability of Kir6.2 N-terminal GST-fusion proteins to interact with the [^{35}S]Met-labeled C terminus of Kir6.2 (residues 170–391). The residues used for each individual GST-fusion protein are indicated above each lane (*GST*, GST alone).

for the ability of the N terminus of Kir6.2 to associate with the C terminus. These results are summarized in Fig. 3, where the individual constructs are displayed against a sequence alignment of the N termini of several different Kir subunits. Above the alignment in Fig. 3 is shown a secondary structure prediction for Kir6.2 calculated using the *PHDsec* program (see "Materials and Methods").

The region of greatest sequence conservation between different Kir subfamilies (highlighted in Fig. 3) is predicted to contain two β-strand structures. The proximal N terminus is predicted to be α-helical. The *PHDsec* program does not predict any other regions of significant secondary structure within the N terminus of Kir6.2. Similar predictions for the secondary structure of the N terminus of Kir6.1, Kir2.1, and Kir3.4 were also obtained using the *PHDsec* program (not shown). The sequence homology and secondary structure conservation within the region highlighted in Fig. 3 suggests that this interaction may be common to all Kir subunits.

Interaction between Different Kir Subunits—We tested whether the GST-fusion protein containing residues 25–53 of Kir6.2, which encompasses this interaction domain, was capable of interacting with the C termini of other Kir subunits. Fig. 4 shows the N terminus of Kir6.2 was able to bind to the C

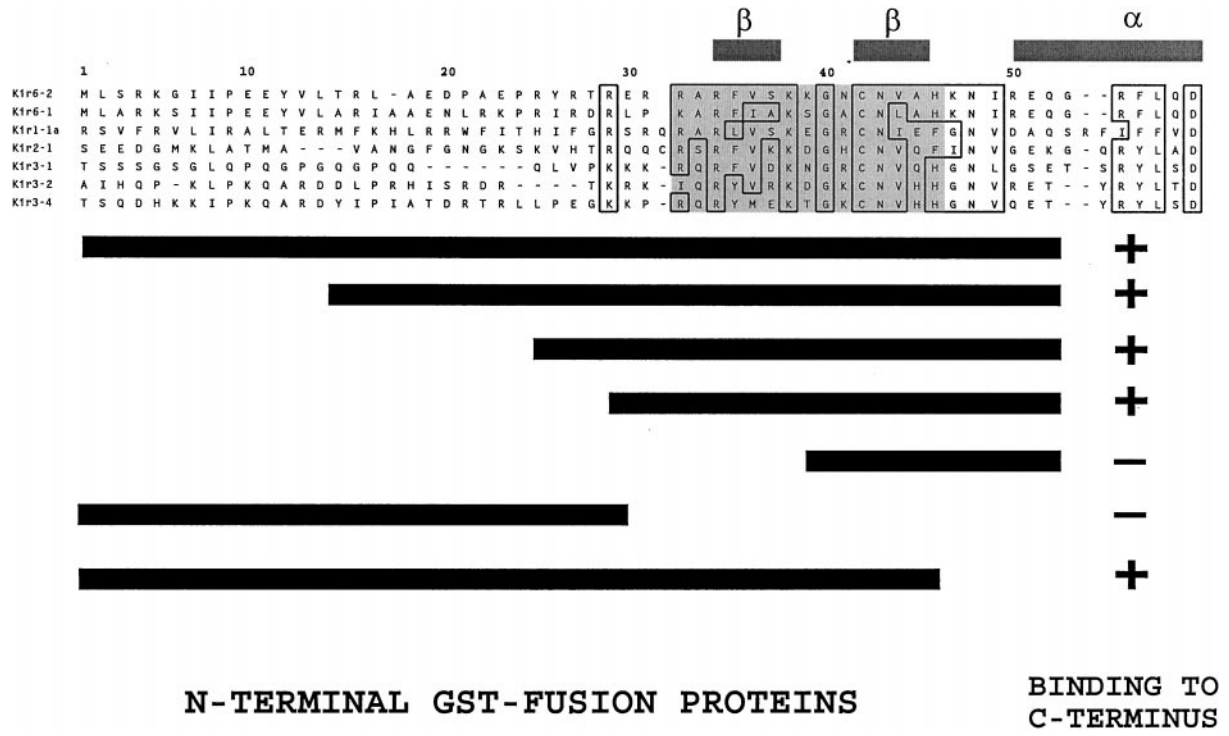

FIG. 3. **The interaction domain is highly conserved among Kir channels.** *Top*, a sequence alignment of the N termini of several different Kir channels showing the predicted secondary structure for the N terminus of Kir6.2 (β, predicted β-strand; α, predicted α-helix). The *numbering* refers to Kir6.2. The *boxed regions* indicate areas of sequence conservation across subfamilies. *Bottom*, a schematic representation of the data presented in Fig. 2. The ability to interact is indicated with either (+) for a positive result or (−) for a negative result. These data indicate that the region *highlighted* in the sequence alignment is critical for this interaction.

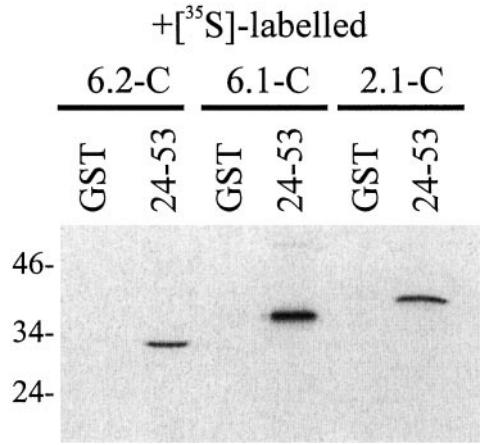

FIG. 4. **Interaction of the Kir6.2 N terminus with the C terminus of other Kir channels.** The N terminus of Kir6.2 (residues 24–53) was tested for interaction with the [^{35}S]Met-labeled C terminus of Kir6.2, Kir6.1, and Kir2.1 (exact residues are given under "Materials and Methods"). *GST*, GST alone.

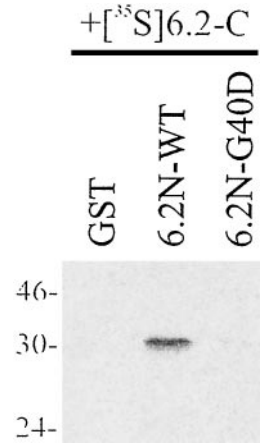

FIG. 5. **A mutation within the N-terminal interaction domain disrupts association with the C terminus of Kir6.2.** The mutation G40D in the N terminus of Kir6.2 severely impairs the ability of the N-terminal GST-fusion protein (residues 25–53) to interact with the [^{35}S]Met-labeled *in vitro* translated C terminus of Kir6.2 (residues 170–391).

terminus of Kir6.1, as well as to the C terminus of the more distantly related Kir2.1. Likewise, the equivalent regions of the N terminus of both Kir6.1 and Kir2.1 were also able to interact with the C termini of Kir6.2, Kir6.1, and Kir2.1 (not shown).

Mutation of the N-terminal Interaction Domain—We assayed the effect of mutation of a highly conserved glycine residue (Gly-40) within the interaction domain of Kir6.2. Fig. 5 shows that substitution of this residue with aspartate (G40D) severely disrupted the ability of the N-terminal interaction domain to bind to the C terminus of Kir6.2. To address the functional consequences of disrupting this interaction, we examined the effect of the G40D mutation on Kir6.2 function.

K_{ATP} currents were measured by two-electrode voltage clamp recordings from *Xenopus* oocytes coinjected with Kir6.2 + SUR1 mRNA. It is necessary to coinject SUR1 with Kir6.2 as this subunit does not properly traffic to the plasma membrane except in association with a sulfonylurea receptor (26, 27). In control recording solution, K_{ATP} currents were extremely small because of block by intracellular nucleotides. We therefore used the metabolic inhibitor sodium azide to activate the channel (23). Fig. 6 shows that, after perfusion for 10 min with 3 mM sodium azide, large inward currents can be recorded (28.1 ± 2.9 μA, n = 8) from oocytes injected with wild-type Kir6.2 + SUR1 mRNA. By contrast, only background level currents were recorded from oocytes injected with equivalent amounts of

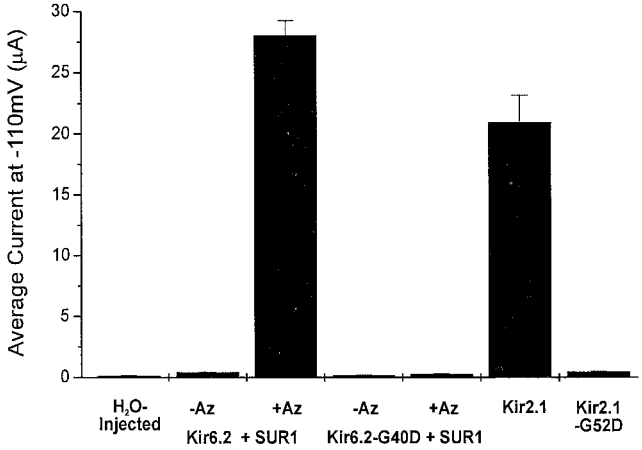

FIG. 6. **A mutation within the interaction domain severely impairs the ability of Kir6.2 and Kir2.1 to form functional channels.** Mean steady-state currents recorded at −110mV from a holding potential of −10mV for *Xenopus* oocytes injected with equal amounts of mRNA encoding SUR1 and either wild-type or mutant Kir6.2-G40D (*left*) or with wild-type or mutant Kir2.1-G52D. *Error bars* represent the S.E., the number of oocytes used was eight in each case. K_{ATP} currents were activated by metabolic poisoning and measured 10 min after addition of 3 mM sodium azide (+*Az*).

mRNAs encoding Kir6.2-G40D + SUR1, even in the presence of sodium azide (297 ± 102 nA, $n = 8$).

The equivalent mutation in Kir2.1 (G52D) also severely compromised the ability of this subunit to form functional channels. Unlike K_{ATP} currents, it is not necessary to metabolically inhibit the oocyte to record Kir2.1 currents. Fig. 6 shows that robust inward currents could be recorded from oocytes injected with mRNA encoding wild-type Kir2.1 (20.96 ± 4.9 μA, $n = 8$). However, for oocytes injected with an equivalent amount of Kir2.1-G52D mRNA, the currents were reduced to almost background levels (460 ± 110 nA, $n = 8$).

DISCUSSION

The results we present here define a highly conserved domain within the N terminus of the inwardly rectifying K^+ channel Kir6.2 which determines physical association with the C terminus of the channel. We also demonstrate that this interaction is likely to be common to all members of the Kir channel family and that its disruption severely compromises the ability of the Kir subunit to form functional channels.

Physical Association of the N and C Terminus Is Required for Functional Kir Channels—The N-terminal interaction domain (NID) that we have identified in Kir6.2 appears to be highly conserved across different Kir subfamilies (see Fig. 3), whereas the more distal regions of the N terminus show little sequence conservation. In addition, the *PHDsec* program predicts a similar β-strand secondary structure for this region in Kir6.1, Kir6.2, Kir3.4, and Kir2.1. This suggests that the NID domain of a given Kir channel may be able to substitute for that of another. In support of this idea is the ability of the NID of Kir6.2 to interact with the C-terminal domains of both Kir6.1 and Kir2.1, and *vice versa.* Kir subunits associate as tetramer, and whereas heteromerization of different subunits may occur within subfamilies, it does not usually occur between different subfamilies. Therefore, although the NID may contribute to subunit assembly, because Kir6.0 and Kir2.1 do not heteromerize to form functional channels (9), the NID is unlikely to be a major determinant of the specificity of heteromeric Kir channel assembly. Furthermore, deletion of this region from Kir2.1, or Kir1.1, does not affect the ability of these subunits to tetramerize (8, 9).

A key question is whether the NID is involved in a purely structural association between the N and C termini or if it has a role in Kir channel function. It is not possible to answer this question from our data nor to distinguish between an *intra-* or *inter*-subunit association. However, there is evidence that the NID is required for the formation of functional Kir channels. Truncation of up to 30 amino acids from the N terminus of Kir6.2 can be made without compromising channel activity (15, 21), but larger deletions which remove the NID result in a loss of Kir6.2 currents (21).[2] Deletion of 71 residues from the N terminus of Kir2.1 also results in a nonfunctional channel (9). Similar studies with Kir1.1a reveal that deletion of residues 3–38 from the N terminus (which leaves the predicted interaction domain intact) does not affect channel function (8). However, deletion of residues 39–68 (which remove the interaction domain) results in a nonfunctional phenotype. Taken together, these data demonstrate that deletion of the NID results in the loss of functional Kir channels.

Does the Physical Interaction between the N and C Termini Modulate Kir Channel Function?—Our demonstration of a direct physical association between the N- and C-terminal domains of Kir6.2 may elucidate the mechanism by which ATP interacts with Kir6.2. Previous studies have implicated both N and C termini in ATP inhibition. First, mutations that affect ATP-sensitivity (without altering the channel gating) are found in both intracellular domains of Kir6.2 (20, 28). Second, mutations in both domains, *e.g.* R50G and K185Q, have also been shown to reduce the photoaffinity labeling of Kir6.2 by 8-azido-ATP (29). Finally, when a mutation in the N terminus of Kir6.2 is combined with one in the C terminus of Kir6.2, there is an additive reduction in ATP sensitivity (14, 21). These observations suggest that the ATP-binding site is either formed by residues contributed by both N and C termini or that the binding site on one of these domains can be allosterically regulated by the other. Although we cannot exclude the possibility that these allosteric effects are mediated via the TMs, the simplest mechanism would be through a direct physical interaction between the N and C termini, such as that we describe.

There is also evidence that implicates the NID in other aspects of K_{ATP} channel gating. A region of Kir6.2 (residues 37–45), which comprises part of the NID of Kir6.2, has been shown to be partly responsible for the ability of K_{ATP} channels to open spontaneously when ATP is removed (30). In addition, a cysteine residue (Cys-42) within the NID of Kir6.2 has been identified as the target for sulfydryl-reactive reagents: modification of Cys-42 by pCMPS causes an irreversible inhibition of channel activity (31). The effect of pCMPS was also state-dependent, indicating that this residue is only accessible in the closed state. Similarly, the equivalent cysteine in Kir1.1 (Cys-49) can also be modified by the sulfydryl reagent DTNB, which causes irreversible channel inhibition. Access to this residue is also state-dependent (16). These observations suggest that the interaction of the NID with the C terminus is not static but that it moves during channel gating

It is therefore tempting to speculate that a dynamic interaction between the N and C termini of Kir6.2 is also involved in channel gating and that the NID may play an important role in this interaction. Such a speculation would be consistent with the reported involvement of both intracellular domains of Kir3.0 in the gating of channel activity by $G_{\beta\gamma}$ subunits (11, 13, 17–19) and also with the interactions between the N and C termini that underlie the nucleotide regulation of the distantly related cyclic nucleotide-gated channels (22). However, further work is required to determine which regions within the C terminus are involved in this interaction and how association of

[2] S. J. Tucker, unpublished observations.

the N and C termini contribute to the various aspects of Kir6.2 channel gating.

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