A Multiprotein Trafficking Complex Composed of SAP97, CASK, Veli, and Mint1 Is Associated with Inward Rectifier Kir2 Potassium Channels

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The function, localization, and trafficking behavior of many ion channels are regulated by interactions of their intracellular domains with members of the MAGUK protein family. MAGUK proteins have a characteristic domain structure containing up to three PDZ domains, a Src homology 3 (SH3) domain, and a catalytically inactive guanylate kinase-like domain (GK). These domains interact with numerous other proteins allowing MAGUK proteins to participate in the assembly of macromolecular signaling or transport complexes (4, 5). Kir2.1, Kir2.2, and Kir2.3 bind to the PDZ domains of selected members of the MAGUK family via their PDZ-binding motif ([T/S]X[V/I]) found at the extreme C terminus (6, 7). Kir2 channels have been demonstrated to interact with the MAGUK proteins PSD-95/SAP90 (6, 8) and SAP97 in a PDZ-dependent manner (7). Whereas PSD-95 is known to cause Kir2 channel clustering in the plasma membrane (8), the functional consequences of the interaction of Kir2 channels with SAP97 in native tissues are still unclear.

Because SAP97 is involved in the formation of macromolecular signaling complexes (9), we hypothesized that one function of SAP97 might be to assemble larger protein complexes to recruit effector molecules to Kir2 channels. SAP97 has been shown to interact with a number of proteins including another MAGUK molecule known as CASK/Lin-2 (10, 11). CASK contains a unique N terminus that interacts with Veli (also known as MALS and Lin-7) and Mint1 (also known as X11a and Lin-10), forming a tightly bound, evolutionarily conserved tripartite complex implicated in basolateral receptor localization and dendritic vesicle/receptor trafficking (12–15). Recently, Olsen et al. (16) demonstrated that the Veli-2 (hLin7-b) isoform associates with Kir2.3 in epithelial cells, suggesting that Veli-2 directly interacts with Kir2 channels. Additionally, by using immunocytochemical methods they showed that Kir2.3, Veli-2, and CASK proteins are all expressed at the basolateral membrane of renal cortical collecting duct epithelial cells (16) appropriate for complex formation. These data suggest that Kir2 channels may form a multiprotein complex with SAP97, CASK, Veli, and Mint1.

Strong inward rectifier potassium (Kir2) channels are a widely expressed family of ion channel proteins distinguished by their ability to pass K⁺ current in the inward direction more readily than outward. Kir2 channels are key components in control of neuronal excitability in brain, electrical activity in heart, vascular tone, and glial buffering of potassium (1, 2). Kir2 channels are important in the modulation of cell excitability, repolarization of the action potential, and determination of the cellular resting potential. Furthermore, Kir2 mutations are implicated in at least one genetic disease causing periodic paralysis and heart arrhythmias (Andersen’s syndrome (3)).

The function, localization, and trafficking behavior of many ion channels are regulated by interactions of their intracellular domains with members of the MAGUK protein family. MAGUK proteins have a characteristic domain structure containing up to three PDZ domains, a Src homology 3 (SH3) domain, and a catalytically inactive guanylate kinase-like domain (GK). These domains interact with numerous other proteins allowing MAGUK proteins to participate in the assembly of macromolecular signaling or transport complexes (4, 5). Kir2.1, Kir2.2, and Kir2.3 bind to the PDZ domains of selected members of the MAGUK family via their PDZ-binding motif ([T/S]X[V/I]) found at the extreme C terminus (6, 7). Kir2 channels have been demonstrated to interact with the MAGUK proteins PSD-95/SAP90 (6, 8) and SAP97 in a PDZ-dependent manner (7). Whereas PSD-95 is known to cause Kir2 channel clustering in the plasma membrane (8), the functional consequences of the interaction of Kir2 channels with SAP97 in native tissues are still unclear.

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The strong association of CASK with Kir2.2 channels led us to hypothesize that the CASK-Veli-Mint1 complex is recruited to Kir2.2 channels by SAP97 and possibly participates in channel trafficking or assembly of a signaling complex. Additionally, we identified SAP97, CASK, Veli, and Mint1 in association with Kir2.2 channels in a parallel proteomic study (17). Here we address this hypothesis by demonstrating that a complex composed of CASK, Veli, Mint1, and SAP97 indeed associates with Kir2.2 channels in brain by a combination of co-immunoprecipitations, affinity interaction assays, and co-localization studies. When expressed in polarized epithelial cells, Kir2.2 channels are targeted to the basolateral membrane. As a test of the functional role of this complex, we expressed a dominant-interfering form of CASK. The resulting mislocalization of Kir2.2 suggests that a functional role of the CASK-containing complex is in Kir2.2 trafficking and localization in the plasma membrane.

EXPERIMENTAL PROCEDURES

DNA Constructs—cDNAs encoding potassium channel C-terminal amino acids were cloned in-frame to the 3’ end of glutathione S-transferase (GST) in the bacterial expression vector pGEX-2T (Amersham Biosciences) as described previously (17). GST-rat SAP97 fusion proteins were expressed in BL21 (DE3) (Novagen). The cDNA encoding full-length SAP97 was a gift from Dr. D. S. Bredt (11), and the cDNA encoding rat SAP97 was a gift from Florin G. H. Hahn (12). The cDNA encoding mouse Kir2.1/2.2 was a gift from Dr. B. Ellisman (9) and was cloned into the pGEX-2TK vector (Amersham Biosciences) previously described (13). The cDNA encoding rat Kir2.2 was a gift from Dr. B. Ellisman (9) and was cloned into the pGEX-2TK vector (Amersham Biosciences) previously described (13).

Affi-Gel GST-Kir2.2 affinity Chromatography—GST, GST-Kir2.2, or GST-Kir2.2Δ3 fusion proteins were purified on glutathione-agarose as described (7). Fusion proteins were eluted from beads with 20 mM glutathione (Sigma), dialyzed against 200 volumes 1× PBS, and coupled to Affi-Gel-10 (Bio-Rad) (5 mg of purified GST-fusion protein per 1 ml of packed Affi-Gel-10) according to the manufacturer’s instructions. The Affi-Gel-10/GST fusion protein matrix was transferred to a column and washed consecutively with 20 volumes Buffer C (50 mM Hepes, pH 7.6, 125 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol), 20 volumes Buffer C with 2.5 mM urea, 20 volumes Buffer C with 4 mM urea, 10 volumes Buffer D with 1% Triton X-100, and finally 10 volumes Buffer D with 0.12% sodium azide and stored as 50/50 slurry at 4 °C.

A multiprotein complex associates with Kir2.2 channels

Affi-Gel GST-Kir2.2 affinity chromatography—GST, GST-Kir2.2, or GST-Kir2.2Δ3 fusion proteins were purified on glutathione-agarose as described (7). Fusion proteins were eluted from beads with 20 mM glutathione (Sigma), dialyzed against 200 volumes 1× PBS, and coupled to Affi-Gel-10 (Bio-Rad) (5 mg of purified GST-fusion protein per 1 ml of packed Affi-Gel-10) according to the manufacturer’s instructions. The Affi-Gel-10/GST fusion protein matrix was transferred to a column and washed consecutively with 20 volumes Buffer C (50 mM Hepes, pH 7.6, 125 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol), 20 volumes Buffer C with 2.5 mM urea, 20 volumes Buffer C with 4 mM urea, 10 volumes Buffer D with 1% Triton X-100, and finally 10 volumes Buffer D with 0.12% sodium azide and stored as 50/50 slurry at 4 °C. A multiprotein complex was isolated from brain extract was preclarified by incubation with 2 ml of bovine serum albumin Affi-Gel (5 mg/ml), followed by passage over a 1 ml GST-Affigel column (5 mg/ml). 50 µl of packed Affi-Gel-GST fusion protein matrix (5 mg/ml) was added to 10 ml of 1% Triton X-100 solubilated rat brain protein (~10 mg/ml) and incubated in batch over-night at 4 °C with rotation. At 4 °C, matrix was loaded onto a column (1 cm diameter) and washed with 1000 column volumes Buffer D Wash (Buffer D, 1% Triton X-100, 0.5% Igepal CA-630, 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) followed by 1000 column volumes Buffer D with 1% Triton X-100, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride. Columns were moved to room temperature, and bound proteins were incubated with 400 µl of Buffer D, 1% Triton X-100, supernatant was added, and purified by centrifugation. CPU-IH was collected.

Co-immunoprecipitation—Solubilized protein was preclarified by the addition of 1% protein A-agarose (Pierce). 5 µg of affinity-purified anti-Kir2.1/2.2 antibodies or rabbit IgG (control) was added to the preclarified solubilized protein and incubated for 1 h to collect immunocomplexes. 25 µg of protein A-agarose was added and incubated overnight with rotation. Precipitated proteins were washed four times with 25 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and co-immunoprecipitated proteins were analyzed by immunoblotting.

In Vitro Protein Interaction Assays—To determine whether CASK binds directly or indirectly with Kir2 channels, in vitro binding assays were performed. GST-SAP97 was purified as described above, and purified, full-length SAP97 was released from GST by cleavage with biotinylated thrombin followed by thrombin removal with streptavidin-Agarose (Sigma). GST-SAP97 (1 µg/ml) was added to the bacterial expression vector pET23a and expressed in BL21 E. coli. Bacteria were sonicated on ice in HEN20 IP buffer and centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatant containing CASK was used for the interaction assay. 2 µg of GST, GST-Kir2.2, or GST-Kir2.2Δ3 fusion protein attached to glutathione-agarose was incubated with or without 2 µg of purified SAP97 at room temperature for 10 min followed by addition of CASK extract and incubation overnight at 4 °C. Beads were washed twice with HEN20 IP buffer with 500 mM NaCl and twice with HEN20 IP buffer with 150 mM NaCl. Bound proteins were eluted in SDS sample buffer and processed for immunoblotting.

To test the possible role of Veli in linking CASK to the channel, identical co-immunoprecipitation assays were performed as above, except that purified Veli-1, Veli-2, or Veli-3 was substituted for SAP97. Veli-1 (MALS-1), Veli-2 (MALS-2), and Veli-3 (MALS-3) cDNAs (gifts from Dr. D. S. Bredt) were subcloned into the vector pGEX-2TK (Amersham Biosciences) previously described (13). The cDNA encoding full-length CASK was a gift from Florin G. H. Hahn (12), and was cloned into the pGEX-2TK vector (Amersham Biosciences) previously described (13).

GST Affinity Purdown assay—Fusion proteins were expressed in Escherichia coli and purified on glutathione-agarose (Sigma) as described previously (7). Cerebellum, whole brain, or COS-1 extracts were preclarified with GST (20 µg/pulldown) bound to glutathione-agarose. GST fusion protein bound to glutathione-agarose (5 µg/tissue extract pulldown) was incubated with the preclarified red blood cell lysate overnight with rotation. Beads were collected by centrifugation and washed four times with 25 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100. Beads were re-suspended in SDS-PAGE loading buffer and the eluted proteins analyzed by immunoblotting.

Immunofluorescence Microscopy—Rats were euthanized with CO2. Cerebellums were removed and immediately fixed in 60% methanol, 30% chloroform, 10% acetic acid at 4 °C for 24 h and then transferred to 100% methanol for 24 h, followed by PBS for 24 h, then 20% sucrose in 100 mM phosphate buffer, pH 7.4, for 24 h. Cerebellums were embedded in 300 bloom gelatin and frozen at -70 °C. 7-µm frozen sections were mounted on glass slides and stained with a 1:2000 diluted polyclonal (Leica CM 1850) and transferred to charged microscope slides (Fisher). Sections were incubated in blocking buffer (3% bovine serum albumin, 1% donkey serum, 0.1% Triton X-100 in 1× PBS) overnight at 4 °C. Sections were then incubated with primary antibodies diluted in blocking buffer overnight at 4 °C. Sections were
To determine whether CASK can associate with the C-terminal portion of Kir2 channels, we performed GST fusion protein affinity chromatography with C termini of Kir2.1, Kir2.2, and Kir2.3. GST was fused to ~50 C-terminal amino acids of Kir2.1, Kir2.2, and Kir2.3 (Fig. 2A). GST fusion proteins were incubated with either cerebellum or heart extracts, and bound proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an anti-CASK antibody. The C terminus of all of these Kir2 channels associated with CASK from cerebellum and heart extracts (Fig. 2B, upper panels, lanes 3, 4, and 6). Association with the Kir2.1 construct could be observed with longer film exposure times (data not shown). The interaction with CASK was specific for the C-terminal amino acids of Kir2.1, Kir2.2, and Kir2.3 because neither GST nor Kir6.2, which lacks a PDZ-binding motif (Fig. 2A), interacted with CASK (Fig. 2B, upper panels, lanes 2 and 8). To test whether the presence of a C-terminal PDZ-binding motif was sufficient for CASK association, we constructed a C-terminal fusion protein of TASK (TASK), a tandem pore potassium channel containing a similar putative PDZ-binding motif (SSV) (Fig. 2A). CASK was not detected when cerebellum or heart extracts were incubated with the TASK fusion protein, despite the presence of an intact consensus PDZ-binding motif (Fig. 2B, upper panels, lane 7).

The PDZ domain binding (TSIVXVI) motif is present in several ion channels, including Kir2 channels, and is necessary for interactions with the MAGUK proteins PSD-95 (6, 22, 23) and SAP97 (7). If CASK is binding directly to the channel via its PDZ binding domain or via another protein utilizing a PDZ domain, we predicted that deletion of the C-terminal PDZ-interacting motif (SEI) of Kir2.2 should abolish the ability of Kir2.2 to interact with CASK. Deletion of the three C-terminal amino acids of the GST-Kir2.2 C-terminal fusion construct (Kir2.2Δ3, Fig. 2A) resulted in a complete loss of CASK association, demonstrating the absolute requirement for this C-terminal PDZ binding domain for CASK association with the channel (Fig. 2B, upper panels, lane 5). It was recently demonstrated that CASK can directly associate with SAP97 (10, 11); therefore, a full-length SAP97 construct with GST fused to the N terminus of SAP97 was used as a positive control for CASK interaction. The SAP97 fusion protein interacted with CASK from both cerebellum and heart (Fig. 2B, upper panels, lane 9). Staining the immunoblot with the protein stain Ponceau S shows approximately equal amounts of GST fusion proteins were used and recovered (Fig. 2B, lower panel).

To determine whether CASK, Veli, and Mint1 are recruited to Kir2 channels, scaled up affinity interactions were performed by using GST-Kir2.2 and GST-Kir2.2Δ3 fusion proteins linked to Affi-Gel-10 matrix. SAP97, CASK, Mint1, and Veli (using a pan-Veli antibody; most likely the Veli-1 protein based on the apparent molecular weight) were all detected on immunoblots of eluates from these columns but were not observed in eluates from the control Affi-Gel-Kir2.2Δ3 columns (Fig. 2C). This shows that the association of these proteins is specific and occurs via the PDZ binding domain at the C terminus of Kir2.2. The a-amino-3-hydroxy-5-hydroxy-5-methyl-4-isoxazolepropionate glutamate receptor GluR1, which also interacts with SAP97 (24), was not isolated with the Kir2.2 column, indicating that it is probably not a component of a macromolecular complex with Kir2.2. Additionally, we have performed mass spectrometry analysis on samples from these columns, and we have confirmed the presence or absence of the proteins described above (17). These data show that CASK, Veli, and Mint1 can associate with the Kir2.2 C terminus via interaction with the channel PDZ binding motif.

To identify the Veli isoform(s) associated with the Kir2 chan-
nels, pulldown experiments were performed, and immunoblots were probed with isoform-specific anti-Veli antibodies. GST pulldown experiments with rat cerebellum extracts with Kir2.2 and Kir2.3 fusion proteins (scaled down 50-fold from the affinity columns) readily indicated that SAP97, PSD-95, CASK, Veli-1, and Veli-3 can associate with the Kir2.2 and Kir2.3 C termini (Fig. 2D, lanes 3 and 5) but not with the GST alone or the Kir2.2Δ3 construct lacking the three C-terminal amino acids (Fig. 2D, lanes 2 and 4). Veli-2 was not detected in these GST pulldown assays even though it was present in the extract (Fig. 2D, lane 1), possibly due to lower overall expression levels in this tissue. These data agree with mass spectrometry analysis, where Veli-1 and Veli-3 associated with the channel C terminus but Veli-2 was not detected (17). Mint1 was not detected in these GST pulldown assays with the Kir2 channel complex possibly due to weaker interactions or lower overall expression levels in the starting material (data not shown).

These affinity interaction experiments indicate that brain-expressed SAP97, CASK, and at least two isoforms of Veli (Veli-1 and Veli-3), and Mint1 can all associate specifically with the C-terminal PDZ-binding motif of Kir2 channels.

**CASK Associates with Kir2.2 When Co-expressed in a Cellular Environment**—To verify that Kir2.2 interacts with CASK in a cellular environment, we expressed both proteins alone and

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**Fig. 2.** The CASK-Mint1-Veli tripartite complex specifically associates with the C termini of Kir2 channels. A, schematic representation of Kir2 channel subunit topology. For use in pulldown assays, GST was fused to the C termini of Kir2.1, Kir2.2, Kir2.3, TASK, and Kir6.2. The boxed region indicates the ~50 C-terminal amino acids that were used in constructing the GST fusion proteins used in pulldown assay. Numbers represent starting amino acids of each construct, and the single letter code for the extreme C-terminal amino acids is shown. GST fused to full-length SAP97 is shown with modular domains schematically depicted. NT, N terminus. B, upper panels, glutathione-agarose charged with the respective GST fusion protein was incubated with detergent-solubilized rat cerebellum or heart proteins as indicated at the left. Bound proteins were analyzed by probing the immunoblots with anti-CASK antibodies. The Input lanes contain 1% of protein used in each binding reaction. Lower panel, to confirm that equal amounts of fusion protein were used and recovered, proteins were stained with Ponceau S. C, GST-Kir2.2 and GST-Kir2.2Δ3 fusion proteins were cross-linked to Affi-Gel-10 matrix. 50 μl of affinity matrix was incubated with 100 μg of detergent-solubilized brain extract and eluted with an excess of peptide corresponding to the last 10 amino acids of Kir2.2 (see “Experimental Procedures”). Eluates from these columns were analyzed by probing individual immunoblots with anti-SAP97 (monoclonal which also recognizes PSD-95), anti-CASK, anti-pan-Veli, anti-Mint1, or anti-GluR1 antibodies. The Input lane contains 25 μg (0.025%) of the protein loaded onto the column. D, upper panels, glutathione-agarose charged with the respective GST fusion protein was incubated with detergent-solubilized rat cerebellum extracts. Bound proteins were analyzed by probing individual immunoblots with the antibodies indicated to the right of each panel. The Input lanes contain 1% of protein used in each binding reaction. Lower panel, to confirm that equal amounts of fusion protein were used and recovered, proteins transferred to immunoblots were stained with Ponceau S.
in combination in COS-1 cells. These cells also express endogenous SAP97 and CASK. By using an antibody against the N terminus of Kir2.1/2.2, we immunoprecipitated Kir2.2 from COS-1 cell extracts. Endogenous CASK as well as transfected CASK co-immunoprecipitated with Kir2.2 (Fig. 3A, lanes 2 and 5). When CASK was co-expressed with a mutant Kir2.2 construct with the last three C-terminal amino acids deleted (Kir2.2Δ3), no CASK co-immunoprecipitation could be detected (Fig. 3A, lane 4). Additionally, CASK did not co-immunoprecipitate if Kir2.2 was not transfected or if non-immune rabbit IgG was used as the precipitating antibody (Fig. 3A, lanes 1, 3, and 6). Expression levels of Kir2.2, Kir2.2Δ3, and CASK were comparable between transfections as indicated by immunoblotting a fraction of the immunoprecipitation inputs (Fig. 3A, lower panels). This shows that full-length Kir2.2 can associate with CASK in cells, and the C terminus of Kir2.2 encoding the PDZ-binding motif is required for the association with CASK.

To determine whether Kir2.1 and Kir2.3, in addition to Kir2.2, also interact with CASK in a cellular environment, we also performed co-immunoprecipitations with green fluorescent protein (GFP)-tagged Kir2.1, Kir2.2, and Kir2.3 co-expressed with CASK in COS-1 cells. Immunoprecipitation with an anti-GFP antibody and immunoblotting for CASK show that all three GFP-Kir2 channels co-immunoprecipitate with CASK (Fig. 3B). These data indicate that Kir2.1, Kir2.2, and Kir2.3 channels containing the C-terminal PDZ-binding motif associate with CASK in cells.

Kir2.2 Associates with CASK in the Cerebellum and Heart—Do Kir2 channels and CASK associate in brain tissue? Because both Kir2.2 and CASK are abundantly expressed in the cerebellum (25–29), we performed co-immunoprecipitations with the anti-Kir2.1/2.2 antibody from rat cerebellum extracts. Because there is very little Kir2.1 expressed in the cerebellum (25, 26), most of the primary precipitating protein should be Kir2.2. Co-immunoprecipitation of CASK was detected with the anti-Kir2.1/2.2 immunoprecipitate but not with control rabbit IgG (Fig. 3C, upper panel). Stripping and reprobing the same blot showed that SAP97 co-immunoprecipitated in these extracts, as shown previously (Fig. 3C, upper panel) (7). This demonstrates that Kir2.2 is associated with CASK in the cerebellum and provides evidence that CASK and SAP97 may be associated together with Kir2 channels.

We also performed an identical co-immunoprecipitation with the anti-Kir2.1/2.2 antibody from rat heart extracts. Co-immunoprecipitation of CASK was detected with the anti-Kir2.1/2.2 immunoprecipitate but not with control rabbit IgG (Fig. 3C, lower panel). These data demonstrate that the Kir2 channel association with CASK is not restricted to brain but may be important in many different tissues. Furthermore, the data suggest that a similar Kir2 channel complex containing CASK and SAP97 also forms in heart tissue.

The C Terminator of Kir2.2 Interacts with CASK through Its Direct Interaction with SAP97—There are two possibilities for how CASK associates with Kir2.2. It is either associated directly with the channel or binds through interactions with other proteins. We demonstrated previously that the second PDZ domain of SAP97 binds to the Kir2.2 C terminus (7) and recent reports (10, 11) have detected the interaction of SAP97 with CASK in epithelial cells. This leads to the hypothesis that SAP97 binds to both CASK and Kir2.2 simultaneously, thus acting as a bridging scaffold. To determine whether SAP97 and CASK associate in brain, extracts were immunoprecipitated with anti-SAP97 antibodies followed by immunoblotting for CASK. Here we show that CASK robustly co-immunoprecipitates with SAP97 in brain using two different anti-SAP97 antibodies (Fig. 3D).

PDZ domains have been classified into three different sub-families based on their ligand specificity. Class I PDZ domains have the consensus ligand binding sequence (S/T)X(E/D)-COOH; class II PDZ domains have the consensus ligand sequence ΨXφ-COOH; and class III PDZ domains have the consensus ligand sequence (E/D)XW(C/S)-COOH where Ψ indicates a hydrophobic residue) (30, 31). CASK contains a class II PDZ domain, which makes it an unlikely candidate for direct interaction with Kir2 channels. Class II PDZ domains have a preference for tyrosine or hydrophobic amino acids at the C-terminal –2 position of binding polypeptides (31), whereas the Kir2 channel C terminus contains a serine at this position (class I ligand binding sequence SEI). This led to the prediction that CASK does not interact directly with Kir2 channels but is tethered via its association with SAP97, which directly interacts with the class I PDZ-binding motif of Kir2 channels. To address this hypothesis, we tested the ability of bacterially expressed recombinant proteins to interact with the C terminus of Kir2.2 in vitro. GST, GST-Kir2.2, or GST-Kir2.2Δ3 fusion proteins attached to glutathione-agarose beads were incubated with or without bacterially expressed and purified full-length SAP97 followed by incubation with bacterially expressed CASK extracts. Unbound protein was washed away, and then bound proteins were separated by SDS-PAGE and immunoblotted with anti-CASK antibodies. CASK only was detected in the pulldown with the intact Kir2.2 C terminus with the addition of SAP97 (Fig. 4, lane 6). The Kir2.2 fusion construct did not associate with CASK without added SAP97 (Fig. 4, lane 3). Neither GST nor the Kir2.2Δ3 construct associated with CASK in the presence or absence of SAP97 (Fig. 4, lanes 2, 4, 5, and 7), which demonstrates that association of CASK with the Kir2.2 C terminus requires the intact PDZ-binding motif. As a positive control, a GST-SAP97 construct lacking the GK domain (SAP97ΔGK, shown to interact with CASK more robustly than full-length SAP97 (11)) efficiently pulled down CASK (Fig. 4, lane 8). These data demonstrate that CASK does not bind directly the Kir2 channel C terminus but is associated with the potassium channel via its interaction with SAP97.

SAP97 Mediates the Association of Kir2.2 with the CASK-Veli-Mint1 Complex—Previous data demonstrated that the N terminus of SAP97 is critical for interaction with CASK, with the SH3 domain also contributing to the association in epithelial cells (10, 11). To confirm that these interactions occur in brain, we performed affinity pulldowns using GST fusion proteins encoding different domains of SAP97 (Fig. 5A). CASK was detected in all the pulldowns using constructs containing the SAP97 N terminus (Fig. 5B, lanes 3 and 5–7) including a SAP97NT construct that encodes only the N-terminal amino acids up to but not including PDZ1 (Fig. 5A). This is consistent with a previous report indicating that CASK interacts with SAP97 via its N terminus in epithelial cells (10). No association was observed with GST alone or when the N terminus was deleted (SAP97PDZ1–2) (Fig. 5B, lanes 2 and 4). An identical interaction pattern to CASK was observed for Veli-1 and Veli-3 with the N terminus of SAP97 being sufficient for association. Mint1 was also found to associate with SAP97 (Fig. 5B, lanes 6 and 7), but only the full-length SAP97 and SAP97ΔGK constructs were able to associate, suggesting that the SH3 domain of SAP97 may participate in strengthening the association with Mint1. This further supports the idea that Mint1 associates with Kir2 channels via interactions mediated by SAP97. Importantly, we were also able to pull down Kir2.2 from these...
CASK was detected as above. The co-immunoprecipitations with the anti-Kir2.1/2.2 antibody or control rabbit IgG, and detergent-solubilized rat heart protein was used for co-immunoprecipitation. CASK was immunoblotted with the anti-CASK antibody. The input lane contains 1% of the protein used in each immunoprecipitation.

Glutathione-agarose charged with the respective GST fusion protein was incubated with detergent-solubilized CASK bacterial lysate with (+) or without (−) added purified recombinant SAP97. The immunoblot of the bound protein was probed with the anti-CASK antibody. The input lane contains 3.5% of the protein used in each affinity pulldown.

To test further the hypothesis that SAP97 associates with the CASK-Veli-Mint1 complex in brain, we performed co-immunoprecipitations with an anti-SAP97 antibody from brain extract. Co-immunoprecipitated proteins were detected with antibodies for Mint1, pan-Veli, CASK, and a pan-MAGUK antibody that recognizes the MAGUKs SAP97, PSD-99/Chapsyn-110, and PSD-95. Of these proteins, Mint1, CASK, and Veli-1 (based on apparent molecular mass: Veli-1 ~27 kDa, Veli-2 ~23 kDa, and Veli-3 ~22 kDa) co-immunoprecipitated with SAP97 (Fig. 5C). Immunoblot analysis using the pan-MAGUK antibody showed co-precipitation of SAP97 only, and not the lower molecular weight proteins PSD-93 and PSD-95, which are also recognized by the antibody.

Veli-1, Veli-2, and Veli-3 Can Directly Associate with the C Terminus of Kir2.2—To determine whether Kir2.2 and individual isoforms of Veli do indeed interact in cells, we co-transfected Kir2.2 pairwise with FLAG-tagged Veli-1, Veli-2, and Veli-3. Immunoprecipitations were performed on detergent-solubilized COS-1 cell extracts with an anti-FLAG antibody followed by immunoblot analysis with the Kir2.2 antibody. Veli-1, Veli-2, and Veli-3 all associated with Kir2.2 (Fig. 6A, lanes 1, 3 and 5, upper panel). When the control Kir2.2/3 construct was co-transfected with any of the Veli isoforms, no co-immunoprecipitation with Kir2.2/3 could be detected (Fig. 6A, lanes 2, 4, and 6, upper panel). Equal amounts of Kir2.2 and Veli proteins were expressed in the cells used in these experiments (Fig. 6A, input, lower panels). These data indicate that all three isoforms of Veli can associate with Kir2.2 in a cellular environment, and this interaction requires the three C-terminal amino acids of the channel protein.

Because Veli proteins contain a single class I PDZ domain that has been shown to interact with NMDA receptors in brain (20) and Kir2.3 in epithelial cells (16), we hypothesized that at least the Veli-1 and Veli-3 isoforms, shown to associate with used in each immunoprecipitation. D, detergent-solubilized protein from rat cerebellum was used for co-immunoprecipitations with anti-SAP97-A antibody, anti-SAP97-B antibody, or control rabbit IgG. Immunoprecipitated CASK was detected by immunoblotting with a monoclonal anti-CASK antibody. The input lane contains 1% of the protein used in each immunoprecipitation.
Kir2.2 C terminus from brain extracts (Fig. 2, C and D), may interact with the C terminus of Kir2.2 directly. To determine a direct interaction with the channel C terminus, we expressed all three isoforms of Veli as His6-tagged proteins in bacteria and purified the proteins with nickel-agarose affinity chromatography. We then incubated these recombinant proteins with GST, Kir2.2, Kir2.2Δ3, or SAP97 fusion proteins. The Kir2.2 construct interacted directly with Veli-1, Veli-2, and Veli-3 (Fig. 6B, lane 3), but the recombinant Veli proteins did not interact with GST alone, Kir2.2Δ3, or SAP97 (Fig. 6B, lanes 2, 4, and 5). These data demonstrate that Veli-1, Veli-2, and Veli-3 are capable of directly interacting with the Kir2.2 C terminus. In addition, these proteins do not interact directly with SAP97, indicating that Veli association with SAP97, in vivo must occur via another linking protein, most likely CASK.

Veli-1 and Veli-3 but Not Veli-2 Recruit CASK to the C Terminus of Kir2.2—We have already demonstrated that SAP97 can link CASK to the Kir2.2 C terminus (Fig. 4). Because the Veli proteins bind directly to the C-terminal Kir2.2 construct (Fig. 6B) and have been shown to interact with the L27C domain of CASK (10), we postulated that all three Veli
isoforms could also recruit CASK to Kir2.2. Kir2.2 fusion proteins attached to glutathione-agarose beads were combined with recombinant CASK extracts with and without recombinant Veli-1, Veli-2 or Veli-3. Bound proteins were separated by SDS-PAGE and probed with an anti-CASK antibody. The control protein constructs (GST alone or Kir2.2/H9004) showed no detectable CASK interaction with or without added Veli proteins (Fig. 7, lanes 2, 4, 6, and 8). CASK did not interact with Kir2.2 directly (Fig. 7, lane 3) but could be detected when either Veli-1 (Fig. 7, lane 7, upper panels) or Veli-3 (Fig. 7, lane 7, lower panels) were added. Surprisingly, the addition of the recombinant Veli-2 did not facilitate recruitment of CASK (Fig. 7, lane 7, middle panels). The same immunoblots were stripped and reprobed for Veli-1, Veli-2, or Veli-3, revealing that each Veli protein associated with the Kir2.2 construct in these assays (Fig. 7, lane 7). This demonstrates the ability of Veli-1 and Veli-3, like SAP97, to bind directly to the Kir2.2 C terminus and recruit CASK to the channel. The SAP97ΔGK construct was used as a positive control for interaction with CASK, which bound CASK both in the absence and presence of Veli (Fig. 7, lanes 5 and 9). Surprisingly, the Veli isoforms were not recruited to SAP97ΔGK in the presence of bound CASK (Fig. 7, lane 9). These data indicate that Veli-1 and Veli-3 bind directly to the Kir2.2 C terminus and participate in linking CASK to the channel.

**Different Isoforms of Veli Participate in Distinct Multiprotein Complexes**—To determine which isoforms of Veli are likely to be involved in complex with CASK, SAP97, Mint1, and Kir2.2 in brain, co-immunoprecipitation experiments using rat cerebellum or brain extracts were performed with isoform-specific anti-Veli antibodies. Fig. 8 shows results from co-immunoprecipitations from cerebellar extracts (Fig. 8A) and whole brain extracts (Fig. 8B). Immunoblots indicate that CASK associates with all three Veli proteins (Fig. 8, A and B, lanes 3–5). However, only anti-Veli-1 and anti-Veli-3 antibodies co-immunoprecipitated SAP97 (Fig. 8, A and B, lanes 3 and 5), with much more SAP97 detected in conjunction with Veli-3. SAP97 could not be detected in co-immunoprecipitations with anti-Veli-2 antibodies either from cerebellum or whole brain (where Veli-2 is more abundant) (Fig. 8A and B, lane 4). Mint1 co-immunoprecipitated with both anti-Veli-2 and anti-Veli-3 antibodies (Fig. 8, A and B, lanes 4 and 5); however, Mint1 could not be detected in immunoprecipitates with anti-Veli-1 antibody (Fig. 8, A and B, lane 3). This indicates that Mint1 probably only associates with Veli-2 or Veli-3 in brain. The data from these co-immunoprecipitations suggest that the most likely Kir2.2-
The cerebellum, known for its role in motor control and learning, has an invariant structure with well-characterized anatomy in normal animals (35). We focused on the outer layers of the cerebellum where strong Kir2.2 expression was observed previously (7, 25). Kir2.2 shows a striking punctate localization and stability of the Kir2.2-SAP97 protein complex. Therefore, co-expression of a dominant negative CASK (CASK-612)) with Kir2.2 in MDCK cells should result in a Kir2.2

Kir2.2 Displays an Overlapping Expression Pattern with SAP97, CASK, Veli-1, Veli-2, Veli-3, and Mint1 in the Cerebellum—Kir2.2, SAP97, CASK, Veli-1, Veli-2, Veli-3, and Mint1 have all been reported to be expressed in the cerebellum (7, 26, 27, 29, 32–34). Our GST fusion protein affinity pulldown and co-immunoprecipitation experiments from cerebellar extracts provide biochemical evidence for the association of these proteins in a macromolecular complex. Because this complex of proteins was identified in the cerebellum, we predicted that their expression patterns would overlap. To address this hypothesis, we performed immunofluorescence confocal microscopy on fixed rat cerebellar tissue sections.

The cerebellum, known for its role in motor control and learning, has an invariant structure with well-characterized anatomy in normal animals (35). We focused on the outer layers of the cerebellum where strong Kir2.2 expression was observed previously (7, 26). Kir2.2 shows a striking punctate labeling pattern along radial processes extending from the Purkinje cell layer to the molecular layer along with punctate labeling in the granule cell layer (Fig. 9A) (7, 26). Previously, we demonstrated that the Kir2.2 expression was co-localized with the glial cell marker GFAP in Bergmann glia in the molecular layer and astrocytes in the granule cell layer, thus indicating the presence of Kir2.2 in glial cells of the cerebellar cortex (7). CASK immunostaining appeared to be ubiquitous, present in all three cerebellar layers uniformly (Fig. 9B). As observed previously (7, 34), SAP97 showed intense labeling in the molecular layer as well as expression in the granule cell layer (Fig. 9C). Co-localization of CASK with SAP97 showed overlapping distribution throughout the cerebellum (Fig. 9, B and C), consistent with the intimate association of these two proteins supported by the robust co-immunoprecipitation data (Fig. 3D) (10, 11). Mint1 appeared to be expressed in all three cerebellar layers, including the molecular layer and granule cell layer (Fig. 9G), both areas that express Kir2.2 (Fig. 9A) (7, 25). Veli-1 expression was clearly seen in the Purkinje cell bodies with prominent labeling in the granule cell layer (Fig. 9D). Staining with the anti-Veli-2 antibody was relatively weak, with very little staining in the molecular layer and only slightly more in the granule cell layer (Fig. 9E). Veli-3 labeling appeared to be highly expressed in the molecular layer (Fig. 9F) in an inverse pattern to Veli-1. These Veli immunostaining patterns are in agreement with previous studies (33). Background staining of the cerebellum was negligible when the primary antibodies were omitted (secondary antibodies: anti-rabbit FITC (Fig. 9H, FITC) or anti-mouse CY3 (Fig. 9F, CY3)). Comparison of the immunoprecipitation data (Fig. 8) with the immunocytochemical distribution suggests that Kir2.2, CASK, and SAP97 associate with Veli-1 primarily in the granule cell layer and with Veli-3 primarily in the molecular layer. Because Mint1 was only detected in association with SAP97, CASK, and Veli-3 (Fig. 8, A and B, lane 5), it is likely that a complex with Kir2.2, SAP97, CASK, Veli-3, and Mint1 would primarily be found in the molecular layer.

Dominant Negative CASK Alters Basolateral Localization of Kir2.2 in Polarized Epithelial Cells—To address the role of CASK in Kir2 channel trafficking, we focused on the polarized kidney epithelial MDCK cell line, in which membrane proteins containing PDZ-binding motifs often specifically target and localize to the basolateral membrane (16, 36, 37). To determine the membrane localization of Kir2.2, we imaged transfected, polarized MDCK cells in both the XY and XZ focal planes by immunofluorescence confocal microscopy. Indeed, stable transfection of the GFP-Kir2.2 construct in MDCK cells resulted in basolateral expression of the channel (Fig. 10, A and D) in agreement with Kir2.3 localization in these cells (16). The GFP-Kir2.2A3 construct, which lacks the PDZ-binding motif, displayed incomplete basolateral localization, with much of the channel expressed intracellularly and mislocalized to the apical surface (Fig. 10, G and J). This suggests that the PDZ-binding motif of Kir2.2 is important for targeting and/or stabilization of the channels to the basolateral plasma membrane. The targeting and stabilization of GFP-Kir2.2 to the basolateral membrane suggests that PDZ domain-containing interacting proteins may play a role in these functions. Because both of the Kir2.2-associated proteins SAP97 and CASK are expressed at the basolateral membrane in MDCK cells (Fig. 10, C, F, I, and L) (10, 16), these proteins could possibly play roles in channel targeting and membrane stabilization. Lee et al. (10) has demonstrated that a mutant form of CASK that lacks the final 297 amino acids (CASK-1–612) acts in a dominant negative fashion and causes the mislocalization of SAP97 in MDCK cells. Because SAP97 directly interacts with Kir2.2 (Fig. 4), we hypothesized that CASK plays a central role in the localization and stabilization of the Kir2.2-SAP97 protein complex. Therefore, co-expression of a dominant negative CASK (CASK-1–612) with Kir2.2 in MDCK cells should result in a Kir2.2
A Multiprotein Complex Associates with Kir2.x Channels

Fig. 10. Basolateral membrane localization of Kir2.2 is altered by expression of dominant negative CASK in polarized epithelial cells. MDCK cells were stably transfected with GFP-Kir2.2 (A–P) or GFP-Kir2.2Δ3 (G–L) and allowed to polarize at confluency and then fixed and co-stained with anti-GFP (A, D, G, and J) and anti-SAP97 (C, F, I, and L) antibodies. Dominant negative CASK (mycCASK–(1–612)) (M–R) or overexpressed full-length SAP97 (O/E SAP97) (S–X) constructs were transiently transfected into GFP-Kir2.2 expressing MDCK cells and allowed to polarize at confluency and then fixed and co-stained with anti-GFP (M and P) and anti-Myc (O and R) or anti-GFP (S and V) and anti-SAP97 (U and X) antibodies. MDCK cell monolayers were imaged in both the XY (left panels) and XZ (right panels) planes by indirect immunofluorescence confocal microscopy. XZ images are depicted with the apical surface at the top. Colored images are merged with anti-GFP in green and anti-SAP97 or anti-Myc in red; yellow indicates overlapping labeling. Scale bars, 10 μm.

phenotype in which the channels are mislocalized from the basolateral membrane. Transfection of the Myc-tagged CASK–(1–612) construct into stably transfected GFP-Kir2.2 MDCK cells resulted in an accumulation of intracellular Kir2.2 protein as well as apical membrane localization (Fig. 10, M and P), whereas neighboring cells not transfected with the dominant negative CASK remained basolateral (Fig. 10, P). As a control, overexpression of SAP97 construct (Fig. 10, U and X) did not affect the GFP-Kir2.2 localization (Fig. 10, S and V), indicating that the effect of the dominant negative CASK was not simply an effect of protein overexpression. These data indicate that a functional role for the association of Kir2.2 with the CASK–SAP97 complex in polarized epithelial cells is in subcellular trafficking and/or localization of channels at the plasma membrane.

DISCUSSION

In this study we demonstrate for the first time that the inward rectifier potassium channels Kir2.1, Kir2.2, and Kir2.3 associate in brain with a complex of proteins that include SAP97, CASK, Veli-1, Veli-3, and Mint1. Our results indicate that the complex of associated proteins is recruited to Kir2 channels via direct binding of either SAP97 or Veli. Furthermore, our results show that the three isoforms of Veli participate in the formation of distinct protein complexes in brain. Veli-1 associates with Kir2 channels, SAP97, and CASK, whereas Veli-3 associates with Kir2 channels, SAP97, CASK, and Mint1. Veli-2 does not appear to form detectable amounts of complex with Kir2 channels or SAP97. A dominant interfering CASK construct resulted in the redistribution of Kir2.2 from the basolateral membrane of MDCK cells and an accumulation of intracellular Kir2.2, indicating an essential role for CASK and its interacting proteins in Kir2 channel trafficking.

A Kir2 Channel Complex with SAP97, CASK, Veli, and Mint1—Affinity pulldown assays and co-immunoprecipitations demonstrated that the three C-terminal amino acids of the Kir2.2 channel that encode a PDZ interaction motif (SEI) are required for the association of any of the identified complex proteins (SAP97, CASK, Veli-1, Veli-3, and Mint1) with the channel (Figs. 2, 3, 4, 6, and 7). Co-immunoprecipitation experiments using detergent-solubilized cerebellum extracts showed that the previously identified Kir2 channel-interacting protein SAP97 interacts with CASK, and both of these proteins associate with Kir2.2 in native tissue (Fig. 3, C and D). Further experiments utilizing GST-SAP97 pulldown assays as well as co-immunoprecipitations with anti-SAP97 antibodies demonstrated that the CASK-Veli-Mint complex associates with SAP97 as well as Kir2.2 (Fig. 5). In vitro protein interaction assays showed that CASK cannot bind to the C terminus of Kir2 channels directly but associates with the channel through its direct interaction with SAP97 (Fig. 4). We also investigated the association of the three Veli protein isoforms with Kir2.2, and we demonstrated that the proteins co-immunoprecipitate when co-expressed in heterologous cells as well as directly interact with the Kir2.2 C terminus using in vitro interaction assays (Fig. 6). This interaction is most likely mediated via its PDZ domain and does not require any other scaffolding pro-
proteins. Like SAP97, we also demonstrated that both Veli-1 and Veli-3 can recruit CASK to the Kir2 channel C terminus (Fig. 7).

From these data, we hypothesize that two alternate complexes of Kir2 with SAP97-CASK-Veli-Mint1 are present in brain, with different proteins linking Kir2 to the channel-associated proteins (Fig. 11). In one complex, SAP97 forms the link (Fig. 11A); our data support a model in which the PDZ2 domain of SAP97 interacts directly with the Kir2 channel C terminus and recruits CASK (Fig. 4) (7). This interaction is likely to occur through binding of the N-terminal domain of SAP97 to the N-terminal L27C domain of CASK (10, 13, 14, 32). Co-immunoprecipitation data (Figs. 5C and 8) and affinity interaction assays (Figs. 2C and 5B) suggest that Veli and Mint1 associate with this complex, most likely by Veli interacting with the L27C domain of CASK (10) and Mint1 interacting with the calmodulin kinase II-like domain of CASK (13, 14, 32) (Fig. 11A). In the second complex, isoforms of Veli bind directly to the channel; our data support a model (Fig. 11B) in which the PDZ domain of Veli-1 or Veli-3 interact directly with the Kir2 channel C terminus and recruit CASK (Fig. 7), presumably through binding of the Veli L27 domain to the CASK C-terminal L27C domain (10, 14). In this alternative complex, SAP97 and Mint1 are also recruited to the Kir2 channel (Fig. 11B), via interactions with the CASK N terminus (10).

Kir2 channels associated with both Veli-1 and Veli-3 in the brain, and these Veli isoforms formed distinct protein complexes. Co-immunoprecipitations with brain extracts using isoform-specific anti-Veli antibodies indicate that only Veli-1 and Veli-3 associate with SAP97 (Fig. 8). Of these two isoforms, only Veli-3 stably associated with Mint1. These data suggest that Kir2 channel complexes that contain Veli-1 exclude Mint1, whereas Kir2 complexes that contain Veli-3 include Mint1 (Fig. 11). We could not detect Veli-2 association with the C terminus of Kir2.2 or Kir2.3 (Fig. 2D) or complex formation with SAP97 (Fig. 5B and Fig. 8) in GST pulldown assays using brain extract. This indicates that a complex containing Kir2 channels and SAP97 is unlikely to contain Veli-2 in the brain. Overlapping expression in adult rat cerebellum of Kir2.2, SAP97, CASK, Mint1, and Veli-1, in the granule cell layer, and of Kir2.2, SAP97, CASK, Mint1 and Veli-3, in the molecular layer, was established using immunofluorescence microscopy. These tissue distributions suggest that distinct Kir2.2-associated complexes occur in specific layers of the cerebellum.

Functional Implications of Kir2-associated SAP97-CASK-Veli-Mint1 Complexes—The MAGUK proteins SAP97 and CASK seem to have similar and/or complementary functions in trafficking and targeting ion channels and receptors in cells. Both of these proteins have been studied in epithelial cells where they are targeted and localized to the basolateral membrane at sites of cell-cell contact. The first 65 amino acids of SAP97 were found to be critical for this targeting (38). Most interesting, this is the same region of SAP97 that interacts with the L27N domain of CASK (10). Lee et al. (10) also used a CASK deletion mutant lacking the GK domain to observe its effects on SAP97 localization. This dominant negative CASK construct caused SAP97 to deviate from its normal basolateral location in MDCK epithelial cells (10). Functional genetic analyses of CASK and SAP97 in Drosophila (39, 40) and Caenorhabditis elegans (12, 41, 42) also implicate these proteins in targeting and localization in polarized cells. Taken together, it is apparent that both SAP97 and CASK have functional roles in the trafficking and targeting of ion channels and receptors.

We expressed a GFP-Kir2.2 construct in MDCK cells and, like Kir2.3 (16), found the channels to traffic and localize to the basolateral membrane (Fig. 10, A and D). Expression of GFP-Kir2.2:2.3 construct lacking the PDZ-binding motif displayed a lack of specific membrane localization with much of the channel expressed intracellularly (Fig. 10, G and F). Kir2.2 channels associated with both Veli-1 and Veli-3 in the brain, and these Veli isoforms formed distinct protein complexes. Co-immunoprecipitations with brain extracts using isoform-specific anti-Veli antibodies indicate that only Veli-1 and Veli-3 associate with SAP97 (Fig. 8). Of these two isoforms, only Veli-3 stably associated with Mint1. These data suggest that Kir2 channel complexes that contain Veli-1 exclude Mint1, whereas Kir2 complexes that contain Veli-3 include Mint1 (Fig. 11). We could not detect Veli-2 association with the C terminus of Kir2.2 or Kir2.3 (Fig. 2D) or complex formation with SAP97 (Fig. 5B and Fig. 8) in GST pulldown assays using brain extract. This indicates that a complex containing Kir2 channels and SAP97 is unlikely to contain Veli-2 in the brain. Overlapping expression in adult rat cerebellum of Kir2.2, SAP97, CASK, Mint1, and Veli-1, in the granule cell layer, and of Kir2.2, SAP97, CASK, Mint1 and Veli-3, in the molecular layer, was established using immunofluorescence microscopy. These tissue distributions suggest that distinct Kir2.2-associated complexes occur in specific layers of the cerebellum.

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studies demonstrated increased plasma membrane stabilization by Veli-2 of Kir2.3 expressed in Xenopus oocytes (16). These studies suggest that for Kir2 channels expressed in brain, Velis could function in a similar manner, acting to stabilize channels once they are trafficked to the plasma membrane. Because we could not detect Veli-2 associated with Kir2 channels or SAP97 in brain, this may reflect a preference of the channels for Veli-1 and Veli-3 in brain.

The interaction of Kir2 channels with the SAP97-CASK-Veli-Mint1 complex may serve to attach channels to cytoskeletal elements. Many MAGUK proteins, including SAP97 and CASK have an alternatively spliced short peptide sequence (known as the protein 4.1 binding domain or HOOK domain) between their SH3 and GK domains with the capability of interacting with protein 4.1 (44–46). Protein 4.1 is known for its role in linking plasma membrane components to the actin/spectrin cytoskeleton (47). In a recent study, CASK was demonstrated to bind to a neuronal form of protein 4.1 and nucleate filamentous actin on the C termini of neurexin membrane proteins (46). In addition, motor and motor-like proteins have been found to associate with SAP97 including GAKIN (48), myosin VI (49), and dynein light chain (50). These data support the idea that Kir2 channels may be linked via SAP97, CASK, and associated proteins to either the actin- or microtubule-based cytoskeleton (47). In a recent study, CASK was demonstrated to associate with SAP97 including GAKIN (48), myosin VI, and dynein light chain (50). These data support the idea that Kir2 channels may be linked via SAP97, CASK, and associated proteins to either the actin- or microtubule-based cytoskeleton (47).

Another role of the Kir2-associated SAP97-CASK-Veli-Mint1 complex may be to form a molecular scaffold that recruits signaling molecules to Kir2 channels, which may then mediate the channel activity. MAGUKs have been demonstrated to associate with a number of signaling molecules including protein kinase A (9), Src family tyrosine kinases (51, 52), and GTPase-associated proteins (53, 54).

How might this Kir2 channel-associated complex be regulated? An intriguing possibility is that the Kir2-associated complex is regulated by phosphorylation. It was shown previously that protein kinase A phosphorylation of the serine within the PDZ-binding motif at the C terminus of Kir2 channels abolishes the ability of the channels to interact with MAGUK proteins (6, 7). An interesting possible scenario is that Kir2 channels are trafficked to the plasma membrane by the SAP97-CASK-Veli-Mint1 complex, transiently phosphorylated by protein kinase A to release Kir2 channels from the complex, followed by interaction with a membrane-associated MAGUK such as PSD-95, which would stabilize, anchor, and cluster the channels. Additionally, it is likely that this complex may be regulated by intramolecular and/or intermolecular interactions as well as post-translational modifications and cytoplasmic calcium levels (29, 32, 55–59).

In summary, we have identified novel macromolecular complexes containing SAP97, CASK, Veli-1, Veli-3, and Mint1 that are assembled at the C termini of Kir2 channels in brain. These complexes are capable of binding to Kir2 channels through two possible mechanisms: one bound to the C terminus of Kir2 channels through SAP97, and the other with the complex associated via recruitment by Veli. In addition, distinct protein complexes are recruited by different Veli isoforms. Veli-1 is associated with CASK, SAP97, and Kir2 channels, whereas Veli-3 is associated with Kir2 channels, CASK, SAP97, and Mint1. These Kir2-associated proteins have been suggested to be involved in ion channel targeting, trafficking, and localization (15, 16, 20, 60). CASK is a key member of this complex possibly functioning in conjunction with other complex proteins to traffic and stabilize channels at the plasma membrane. These studies have important implications in the regulation of Kir2 channels, and hence a host of physiological processes from neuronal excitability to cardiac arrhythmia. Further studies such as knockdown of individual genes using RNA interference should provide insight into the precise roles of SAP97, CASK, Veli, and Mint1 in trafficking and function of Kir2 channels.

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