

## N-terminal PDZ-binding domain in Kv1 potassium channels

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**Abstract** We have investigated the interactions of prototypical PDZ domains with both the C- and N-termini of Kv1.5 and other Kv channels. A combination of *in vitro* binding and yeast two-hybrid assays unexpectedly showed that PDZ domains derived from PSD95 bind both the C- and N-termini of the channels with comparable avidity. From doubly transfected HEK293 cells, Kv1.5 was found to co-immunoprecipitate with the PDZ protein, irrespective of the presence of the canonical C-terminal PDZ-binding motif in Kv1.5. Imaging analysis of the same HEK cell lines demonstrated that co-localization of Kv1.5 with PSD95 at the cell surface is similarly independent of the canonical PDZ-binding motif. Deletion analysis localized the N-terminal PDZ-binding site in Kv1.5 to the T1 region of the channel. Co-expression of PSD95 with Kv1.5 N- and C-terminal deletions in HEK cells had contrasting effects on the magnitudes of the potassium currents across the membranes of these cells. These findings may have important implications for the regulation of channel expression and function by PDZ proteins like PSD95.

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**Key words:** PSD95; PDZ; Potassium channel; Kv1.5

## 1. Introduction

Ion channels are not distributed randomly across cellular surfaces. In fact, their specialized localization is frequently essential to cell function. In neurons for example, Kv2.1 localizes to high-density clusters on the soma and proximal dendrites in the hippocampus [1], voltage-gated K<sup>+</sup> channels co-localize with Na<sup>+</sup> channels at the nodes of Ranvier [2], and the NMDA receptor is found almost exclusively at the post-synaptic density [3]. Implicated in the clustering of NMDA receptors [4] and *Shaker*-type K<sup>+</sup> channels [5] is PSD95. PSD95, the prototypical mammalian PDZ-containing protein [6] contains three of the domains in tandem. Originally identified as a 95-kDa protein highly enriched in the post-synaptic density, PSD95 binds NMDA receptors via their NR2 subunits [4,7,8], and this binding is most likely responsible for the channels' post-synaptic clustering. Other channels localized by interaction with PDZ proteins include muscle Na<sup>+</sup> channels [9], inwardly rectifying K<sup>+</sup> channels [10–12] and *Shaker*-type channels such as Kv1.4, a K<sup>+</sup> channel the clustering of which in neurons may also be dependent on linkage to PSD95 [13].

PDZ domains are present in a variety of dissimilar proteins

and are among the most common protein motifs [14]. Consisting of 80–120 amino acids, they are involved in protein–protein interactions, mostly with components of the cytoskeleton and associated structures. Proteins as diverse as syntrophins, PSD95 and neuronal nitric oxide synthase all contain the motifs [15]. Most interactions with PDZ domains involve the C-terminal 4 amino acids of the interacting protein and a peptide-binding groove in the PDZ motif which ends with a conserved carboxylate-binding loop [16]. The NR2 subunit of the NMDA receptor and Kv1.4 both bind PSD95 in this way. But other binding mechanisms have been described. A few proteins bind via PDZ–PDZ interactions. The neuronal and muscle isoform of nitric oxide synthase, nNOS, binds the PDZ domains of PSD95 and  $\alpha$ 1-syntrophin [17] by a mechanism that involves a  $\beta$ -finger that essentially mimics a canonical C-terminal PDZ-binding motif [18]. Other proteins bind PDZs by as yet undetermined mechanisms. For example, the PDZ motif of the actinin-associated LIM protein, ALP, binds to the internal spectrin repeats of  $\alpha$ -actinins [19,20].

To further our understanding of PDZ domain interactions with ion channels, we have utilized archetypal PDZ domains from the protein PSD95, to investigate potential interactions with the C- and N-termini of a *Shaker*-type K<sup>+</sup> channel, Kv1.5. We have found that, as with Kv1.4, these domains bind a C-terminal fragment of the channel, very probably the C-terminal sequence ETDL. Significantly, we have also uncovered an interaction between the PDZ domains and the N-terminus of Kv1.5. This second binding site localizes to the Kv1.5 T1 domain and appears to be independent of  $\beta$ -finger involvement. This N-terminal binding is not restricted to Kv1.5 but occurs also with other Kv1-type channels.

## 2. Materials and methods

### 2.1. DNA constructs and site-directed mutagenesis

DNA encoding the 240-amino acid Kv1.5 N-terminus and internal deletions were cloned in frame with the GST-tag into pET42 (Novagen, Madison, WI, USA). Sequence-confirmed PCR-derived segments encoding N-terminal fragments of Kv1.1 (aa 1–167), Kv1.2 (aa 1–64), Kv1.3 (aa 1–182), Kv1.4 (aa 90–305) and Kv4.2 (aa 1–183) were similarly cloned into pET42. Deletion mutations in the Kv1.5 N-terminus were made by restriction digests or internal digestion followed by incubation with nuclease *Bal31* for varying times. Digestions were stopped by addition of 20 mM EGTA, and the DNA was ligated and recovered after transformation into *Escherichia coli*. A 412-amino acid N-terminal portion of PSD95, containing the protein's three PDZ domains, was cloned in frame with the T7 tag into pET28a.  $\alpha$ -Actinin2, minus amino acids 1–11, was similarly cloned into pET42. For immunocytochemical detection, Kv1.5 and its truncation mutants were N-terminally T7 tagged in pcDNA3. Briefly, Kv1.5 was sub-cloned as a *HindIII*–*NotI* fragment into pET28-a. The tagged channel was then recovered by digesting the resultant plasmid with *NdeI* plus *NotI* and cloning the T7-tag-Kv1.5 fragment into *EcoRV*–*NotI*-di-

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gested pcDNA3. In all cases, the presence of in-frame fusions with the glutathione-S-transferase (GST) tag or T7 tag was confirmed by DNA sequencing. Site-directed mutagenesis was performed using Stratagene's Quikchange kit with appropriate primers. Deletion mutations were produced using PCR-based strategies. The presence of the targeted mutations was confirmed by DNA sequencing. GFP-tagged PSD95 was made by subcloning the 2.1-kb PSD95 *SacII*–*EcoRI* fragment from a clone in pGEX-2T into *HindIII*–*EcoRI*-digested pGFP [21]. Kv1.5 deletion mutants for expression in HEK293 cells were made by deletion of the *NcoI*–*NcoI* fragment of Kv1.5 (Kv1.5ΔN209) or by deletion of the coding sequence downstream of the internal *BamHI* site of Kv1.5 (Kv1.5ΔC51). The former lacks the first 209 amino acids of the channel; the latter lacks amino acids 563–613. Kv1.5ΔETDL was produced by PCR using primers such that the C-terminal ETDL sequence was specifically deleted.

## 2.2. Preparation of GST- and T7-tagged proteins

Recombinant proteins were expressed in *E. coli* strain BL21(DE3) and purified using BugBuster extraction reagent (Novagen). Proteins detected in the soluble fraction by Coomassie staining of SDS–PAGE gels were purified using a T7-tag affinity purification kit (Novagen) or a GST-binding resin and buffer kit (Novagen), as appropriate. Expressed proteins appearing in the insoluble fraction were washed and solubilized using Novagen's solubilization buffer containing 0.3% *N*-lauroylsarcosine according to the company's recommendations. The solubilized proteins were then dialyzed against 20 mM Tris–HCl to remove residual detergent.

## 2.3. In vitro binding assays

Approximately 2 μg (normalized by comparison to standards on Coomassie-stained SDS–PAGE gels) of GST, GST N-terminus of Kv1.5 GST–C-terminus of Kv1.5, other GST Kv1.5 N-terminal and actinin fragments were individually combined with 2 μg of aT7 PSD95 PDZ-domain construct comprising amino acids 1–412 of PSD95 in binding buffer (2 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, 0.1% Triton X-100) [22]. The mixtures were incubated at room temperature for 1 h with periodic mixing. Glutathione–Sepharose beads 4B (Amersham Pharmacia) pre-washed in binding buffer were added to each tube and incubated with mixing for 30 min at room temperature. The mixtures were spun for 5 min at 1000 rpm in a microcentrifuge and the pelleted beads were washed and re-pelleted four times in wash buffer (25 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.5 mM dithiothreitol) [22]. The pelleted bead–protein complexes were boiled in SDS sample buffer for 5 min and aliquots containing 0.2 μg of the GST fusion were then resolved by SDS–PAGE. The proteins were transferred to PVDF membranes and probed as Western blots with horseradish peroxidase-labeled monoclonal anti-T7 antibodies (Novagen). Antibody binding was detected using a chemiluminescent reagent (Renaissance, New England Nuclear). To check for equivalent loading of constructs, identical quantities of GST fusion proteins were subjected to PAGE on a separate gel and stained with Coomassie Blue.

## 2.4. Yeast two-hybrid experiments

The Kv1 channel N-terminal fragments and the PSD95 fragment were cloned into pGAD424 and pGBD-C1, respectively. β-Galactosidase assays were conducted in Y190. β-Galactosidase activity was measured spectrophotometrically at OD<sub>420</sub>, where 1 β-gal unit = 1000 × OD<sub>420</sub> / [OD<sub>600</sub> × time (h) × volume of initial culture used (ml)]. All assays were performed on two to three different yeast transformants on several different experimental days.

## 2.5. Co-immunoprecipitation

HEK293 cells were co-transfected with individual T7-tagged Kv1.5 clones and a GFP-tagged PSD95 clone. Supernatants from cell extracts were made as previously reported [23], with the exception that 0.5% IGEPAL CA-630 (Sigma) was used in place of Triton X-100 in the Lysis buffer. The supernatants were precleared with 20 μl of pre-swelled Sepharose CL4B in bead buffer (20 mM HEPES, pH 7.4, 5% glycerol, 100 mM NaCl, 0.1 mM EDTA), then transferred to fresh tubes. 5–10 μl of the appropriate antibody (anti-T7, Novagen, or anti-GFP, Torrey Pines Biolabs; buffer in control) was added to each tube and incubated on ice for 1 h with mixing. 15 μl pre-swelled protein A-Sepharose (Sigma) was added to each tube in bead buffer and incubated on ice for 1 h with mixing. The mixtures were spun 15 s in the

microfuge and the pelleted beads were washed two times each with wash buffer (10 mM Tris–HCl, 140 mM NaCl, 0.1% Triton X-100), once with Tris–saline (10 mM Tris–HCl, 140 mM NaCl), then once with 50 mM Tris–HCl (pH 6.8). The pellets were then boiled in 20 μl SDS–PAGE loading buffer and run on SDS–PAGE. After transfer to PVDF, membranes were probed with either HRP-conjugated mouse anti-T7 antibody (Novagen) or with rabbit anti-GFP primary antibody (Torrey Pines Biolabs) and HRP-conjugated goat anti-rabbit IgG (Jackson Laboratories). Antibody binding was detected using a chemiluminescent reagent (Renaissance, New England Nuclear).

## 2.6. Deglycosylation experiments

T7-tagged Kv1.5 was immunoprecipitated as above. After the final wash with 50 mM Tris, samples were resuspended in denaturing buffer (5% SDS, 10% β-mercaptoethanol) and boiled for 10 min. To one aliquot, 1/10 volume of EndoH buffer (0.5 M sodium citrate, pH 5.5) and 1500 U Endoglycosidase H (New England Biolabs) was added. 1/10 volume PNGaseF buffer (0.5 M sodium phosphate, pH 7.5), 1/10 volume of 10% NP-40 and 1500 U peptide-*N*-glycosidase F (New England Biolabs) was added to a second aliquot. A third aliquot was left untreated. Samples were incubated for 1 h at 37°C, then subjected to Western analysis.

## 2.7. Imaging

Stable cell lines were generated from HEK293 cells transfected with appropriate T7-tagged Kv1.5 constructs in pcDNA3 using LIPO-FECTAMINE<sup>™</sup> 2000 (Invitrogen, Carlsbad, CA, USA). Three days after transfection, 0.5 mg/ml geneticin was added to the growth media, and after 10 days the cells were tested for expression of the tagged full length and truncated channel proteins.

For co-expression studies, the stable Kv1.5 lines were transiently transfected with full length PSD95 in pcDNA3 and incubated for 24 h prior to fixation. The cells were rinsed and fixed with 4% paraformaldehyde for 12 min at room temperature (RT). After three 5-min washes with 1× phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>), cells were incubated in a blocking solution (PBS containing 2% BSA and 0.2% Triton X-100) for 30 min at RT. A mouse monoclonal antibody to the T7 Tag (1:1000; Novagen) or a rabbit polyclonal anti-PSD95 (1:500; Zymed Laboratories) was diluted in blocking solution and incubated at 4°C overnight or for 2 h at RT. Cells were then washed three times, 5 min in PBS on a rotator before incubation with secondary antibodies, Alexa 594-conjugated goat anti-mouse IgG antibody and Alexa 488-conjugated goat anti-rabbit IgG antibody (1:1000; Molecular Probes) for 1 h on the rotator at RT. Coverslips were once again washed three times with PBS prior to mounting with 10 μl of a 90% glycerol, 2.5% w/v DABCO–PBS solution. Images of labeled cells were taken using a Bio-Rad radiance plus on an inverted Zeiss Axiovert microscope using BioRad LaserSharp 2000 software. Images were later viewed and prepared using NIH Image and PhotoShop software packages.

## 2.8. Electrophysiological procedures

Stable line HEK293 cells were transfected with either pGFP or PSD95:pGFP and the experimenter was blinded to transfection cDNA group. Coverslips containing cells were removed from the incubator before experiments and placed in a superfusion chamber (volume 250 μl) containing the control bath solution at ambient temperature (22–23°C), and perfused with bathing solution throughout the experiments. Transfected cells were selected for using fluorescence microscopy. Whole-cell current recording and data analysis were done using an Axopatch 200A amplifier and pClamp 8 software (Axon Instruments, Foster City, CA, USA). Patch electrodes were fabricated using thin-walled borosilicate glass (World Precision Instruments, Sarasota, FL, USA). Electrodes had resistances of 1–3 MΩ when filled with control filling solution. Capacity compensation and 80% series resistance compensation were used in all whole cell recordings. No leak subtraction was used when recording currents, and zero current levels are denoted by the dotted lines in the current tracings in Fig. 6. Data were sampled at 10–20 kHz and filtered at 5–10 kHz. Membrane potentials have not been corrected for small junctional potentials between bath and pipet solutions. Patch pipets contained (in mM): NaCl, 5; KCl, 135; Na<sub>2</sub>ATP, 4; GTP, 0.1; MgCl<sub>2</sub>, 1; EGTA, 5; HEPES, 10; and was adjusted to pH 7.2 with KOH. The bath solution contained (in mM): NaCl, 135; KCl, 5; HEPES, 10; sodium acetate, 2.8; MgCl<sub>2</sub>, 1; CaCl<sub>2</sub>, 1; and was adjusted to pH 7.4 with

NaOH. All chemicals were from Sigma Aldrich Chemical (Mississauga, ON, Canada). Data are presented as mean ± S.E.M. Statistical significance was determined using a two-tailed Student's *t*-test.

### 3. Results

#### 3.1. PDZ domains bind both Kv1.5 C- and N-termini

The C-termini of many *Shaker*-type K<sup>+</sup> channels are known to bind to the PDZ domains of proteins like PSD95 and SAP97. Although hKv1.5 includes a canonical sequence for PDZ binding at its extreme C-terminus, an interaction between the two proteins has never been directly tested. To confirm the interaction specifically with Kv1.5, we assayed the binding of Kv1.5 fragments with the PDZ-containing core of PSD95.

GST-tagged fragments of Kv1.5 and a T7-tagged N-terminal fragment of PSD95 that contained all three of the protein's PDZ domains were expressed in *E. coli* using the pET system. After purification, the C-terminal (aa 535–613) and N-terminal (aa 1–240) fragments of Kv1.5 were tested for in vitro binding to the PSD95 fragment. Negative controls were included to ensure that the fragments were binding specifically to the tested partners and not to GST or to glutathione–Seph-  
 arosee. GST-tagged  $\alpha$ -actinin2, previously shown to bind the PSD95 fragment (Grace Lu, unpublished observations), was included as a positive control. Glutathione–Seph-  
 arosee beads were added and, after extensive washing, the protein complexes were pelleted and run on SDS–PAGE for Western analysis. As expected,  $\alpha$ -actinin2 bound the PDZ domains, as did the Kv1.5 C-terminus (Fig. 1A). GST or glutathione–

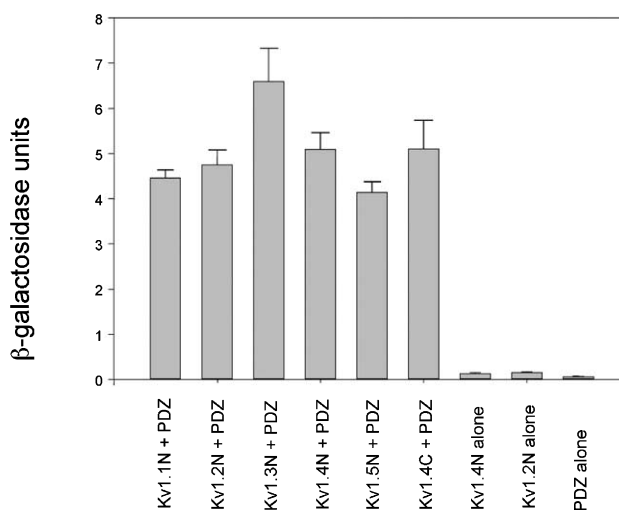


Fig. 2. Yeast two-hybrid experiments demonstrate PDZ binding to Kv channel N- and C-termini.  $\beta$ -Galactosidase activities from yeast strain Y190 initiated by the interaction of Kv1 channel N- or C-termini with the PSD domains of PSD95. The Kv channel fragments were expressed from the pGAD424 vector; PSD95 PDZ domains 1–3 were expressed from pGBD-C1. Representative examples of controls expressing Kv channel or PDZ channel constructs alone are shown at right. 1  $\beta$ -galactosidase unit =  $1000 \times OD_{420} / [OD_{600} \times \text{time (h)} \times \text{volume of initial culture used (ml)}]$ . All data represent means  $\pm$  S.E.M. for three experiments conducted with fresh transformants on separate experimental days. 'Alone' refers to yeast co-transformed with the reported construct plus its empty vector partner for the yeast two-hybrid assay.

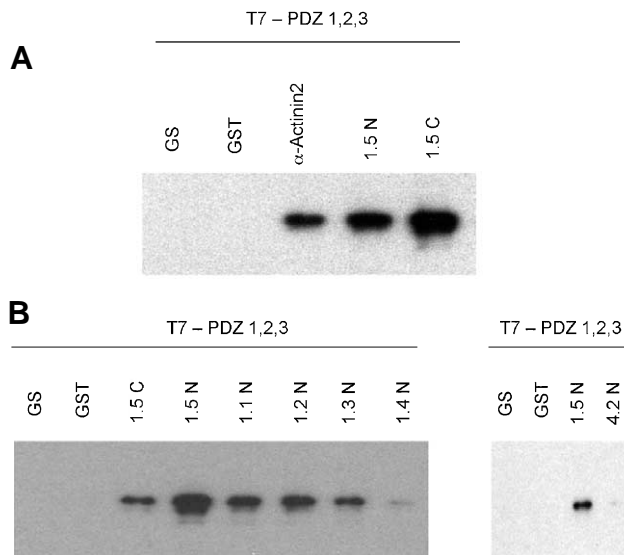


Fig. 1. PDZ domains bind N-termini of Kv1 channels. A: PDZ domains of PSD95 bind Kv1.5 C- and N-termini. Purified T7-tagged PSD95 N-terminus was incubated alone or with GST-tagged  $\alpha$ -actinin2, Kv1.5 N-terminus, Kv1.5 C-terminus, or GST-tag alone. Glutathione–Seph-  
 arosee beads were added and, following further incubation, the beads were pelleted, washed extensively, and subjected to SDS–PAGE. Western blots were performed using anti-T7 antibody to detect the PSD95 fragment. GS refers to PSD95 incubated with glutathione–Seph-  
 arosee in the absence of a GST-fusion protein. B: PSD95 binds other Kv1 channels but not Kv4.2. Experimental procedures were similar to A. T7-tagged PSD95 N-terminus was incubated with GST-tagged N-termini of Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5 and Kv4.2 prior to pelleting with glutathione–Seph-  
 arosee and visualization by Western blot.

Seph-  
 arosee alone failed to bring down the fragment. Surprisingly, however, the Kv1.5 N-terminus also bound the PDZ construct, and it did so with apparently similar avidity to that detected with the positive controls. This finding of N-terminal binding to the PDZ construct was in contrast to the many published reports that deletion or mutation of the extreme C-termini of Kv channels eliminates PDZ binding or its effects [5,13,24,25]. Most of these reports concern Kv1.4–PSD95 interactions, however, and only two [24,25] included N-termini in their test systems. As far as we are aware, there have been no specific tests of Kv1.5–PSD95 interactions.

To ascertain whether this N-terminal-binding property of Kv1.5 to PSD95 was shared by other *Shaker*-type channels, the N-termini of additional Kv channels were tested for binding to the PDZ construct. GST-tagged N-terminal fragments of Kv1.1, Kv1.2, Kv1.3, Kv1.4 [26] and Kv4.2 were expressed, purified and tested in our assay. Because of difficulty expressing the full N-terminus of Kv1.4, the fragment expressed of this channel lacked the first 89 amino acids. This deletion leaves the great majority of the N-terminus intact, including the entirety of its N-terminal homology with the other tested Kv channels.

As shown in Fig. 1B, all Kv1 N-termini were found to interact with the PDZ domains except Kv1.4, which bound very weakly if at all. This latter finding is consistent with the previous reports that failed to detect a Kv1.4–PSD95 interaction outside the C-terminus [5,13,24,25]. The N-terminus of Kv4.2 also did not bind the PDZ construct. Thus, at least as measured by our in vitro assay, N-terminal interactions with PDZ domains are common in Kv1 channels but are not general to all voltage-gated K<sup>+</sup> channels.

That an interaction occurs between two proteins in an in

in vitro system does not guarantee that the binding reflects an in vivo process. Misfolding of one or both of the tested proteins, (effectively) inappropriate compartmentalization or a lack of alternative binding partners, for example, could all allow binding where no interaction occurs in vivo. We therefore chose to test the putative interactions in a variety of living systems.

### 3.2. Yeast two-hybrid assays confirm interaction

While the yeast two-hybrid system is subject to many of the same caveats as the in vitro binding assays, the method is commonly used in protein–protein interaction studies and it provides a system in which the proteins are surrounded by a natural cellular milieu. It also provides a simple method to roughly compare the strengths of interactions between proteins [27]. We therefore used this system to further investigate the interactions of Kv1.5 N-terminus with our PDZ construct and to test also whether other Kv channel N-termini can also bind that construct.

With the exception of Kv1.4, the same fragments used in the in vitro binding assay were employed in vectors appropriate to the two-hybrid system (pGAD424 for the Kv channel N-termini, pGBD-C1 for the PDZ construct); for Kv1.4, the full N-terminus was used rather than the truncated piece necessary in the in vitro experiments. Yeast strain Y190 was co-transformed with the constructs and the  $\beta$ -galactosidase activities of the transformants were measured. Y190 co-transformed with the PDZ construct and the C-terminus of Kv1.4 served as positive control. Confirming the results of the in vitro studies, these experiments provided strong evidence of an interaction between all of the Kv1 N-termini and the PDZ construct. In every case, the Kv channel N-terminus was found to interact with the PDZ domains (Fig. 2). Strikingly, the apparent strengths of the PDZ interactions with each of the Kv1 channel N-terminal fragments were comparable to that with the Kv1.4 C-terminus. This was surprising in the case of Kv1.4 since it bound poorly to the PDZ domains in our in vitro assay. Further studies with this channel will be necessary to resolve this discrepancy.

### 3.3. PSD95 co-immunoprecipitates with C-terminally truncated Kv1.5

To determine whether PSD95 binds the N- and C-termini of Kv1.5 in vivo, co-immunoprecipitation experiments were performed in transfected HEK293 and COS cells (data not shown). Cells were transfected with GFP-tagged PSD95 and one of two T7-tagged Kv1.5 constructs. One, the Kv1.5 wild-type, included the C-terminal ETDL sequence reportedly necessary for Kv channel binding to PDZ domains [5,13,24,25]. In the other, Kv1.5 $\Delta$ ETDL, these four amino acids were specifically deleted.

As shown in Fig. 3A,B, antibody to GFP (which specifically immunoprecipitates the tagged PDZ protein) pulled down comparable amounts of both the wild-type and  $\Delta$ ETDL versions of Kv1.5 in HEK cells co-expressing PSD95 and the channel. Similarly, anti-T7 brought down PSD95 along with the Kv1.5 constructs to which the antibody was directed (data not shown). The Kv1.5 proteins were not pulled down by anti-GFP from cells expressing the channels and the GFP tag alone. Thus, the PDZ protein interacts with Kv1.5, irrespective of the presence of the canonical PDZ-binding motif at the channel's C-terminus. The N-terminal-binding region

identified in vitro very likely underlies this phenomenon. Interestingly, the antibody directed to the Kv1.5 protein consistently immunoprecipitated two isoforms of the channel, whether the channel expressed was the full length Kv1.5 or Kv1.5 $\Delta$ ETDL (Fig. 3C,D). The antibody directed to the PSD95 fusion protein also pulled down two Kv1.5 isoforms when the wild-type Kv1.5 channel was expressed with it. However, when the  $\Delta$ ETDL version of the channel was co-expressed with the PSD95 construct, the PSD95-specific GFP antibody brought down only the smaller Kv1.5 band (Fig. 3B). Similar results were observed in transfected COS-7 cells (data not shown), showing that this interaction is not a cell-specific phenomenon. Deglycosylation experiments using transfected HEK cells demonstrated that the migration difference between the two bands was due to differences in their

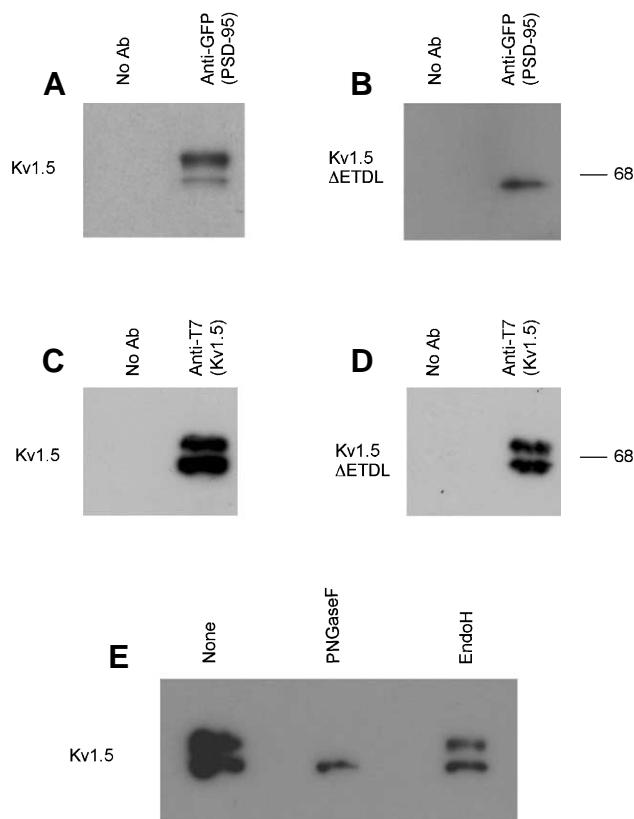


Fig. 3. Co-immunoprecipitation of Kv1.5 variants with PSD95 from HEK cells. A: Co-immunoprecipitation of Kv1.5 with PSD95. Extracts from T7-tagged Kv1.5, GFP-tagged PSD95 double-expressing HEK cells were mixed without antibody (lane 1) or with anti-GFP (lane 2). The protein–antibody complexes were precipitated with Protein A–Sepharose and subjected to Western analysis and probed with anti-T7 antibody. B: Co-immunoprecipitation of Kv1.5 $\Delta$ ETDL with PSD95. The blot is identical to that in A except that the HEK cells expressed Kv1.5 $\Delta$ ETDL rather than wild-type Kv1.5. The quantity of protein loaded, of antibody used and the exposure times were the same for both blots. C,D: Anti-T7 pulls down two forms of both Kv1.5 and Kv1.5 $\Delta$ ETDL. Extracts from T7-tagged Kv1.5, GFP-tagged PSD95 (lanes 1 and 2) or T7-tagged Kv1.5 $\Delta$ ETDL, GFP-tagged PSD95 (lanes 3 and 4) double-expressing HEK cells were mixed without antibody (lanes 1 and 3) or with anti-T7 (lanes 2 and 4). E: Glycosylation differences underlie Kv1.5 doublet. Kv1.5 was immunoprecipitated with anti-T7 and then denatured by boiling. Test aliquots were then treated with either PNGase F or EndoH glycosidase and subjected to Western analysis. The blot was probed with anti-T7 antibody.

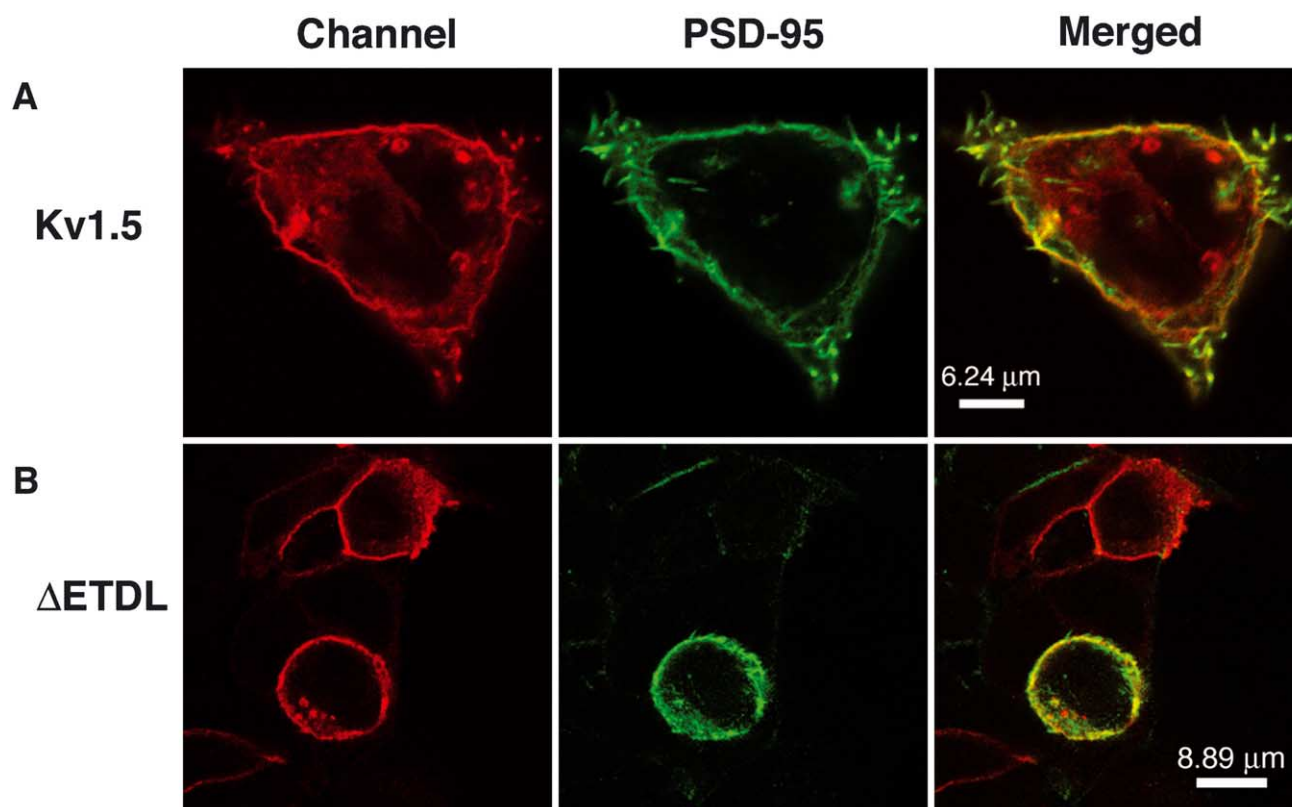


Fig. 4. PSD95 co-localizes with Kv1.5 and Kv1.5 $\Delta$ ETDL in transfected HEK293 cells. Confocal images of single optical slices of HEK293 cells stably expressing either T7-tagged hKv1.5 (A) or T7-tagged Kv1.5 $\Delta$ ETDL (B) also transfected with PSD95. Kv channels were detected with anti-T7 (red); PSD95 was detected with anti-PSD95 (green). Yellow indicates co-localization of the channel with PSD95 in the merged image.

degrees of glycosylation (Fig. 3E). PNGase F treatment of the proteins collapses the two bands to a single lower band, suggesting that the upper band represents a mature glycoprotein. Consistent with this interpretation, EndoH treatment had no effect. It is likely that the glycosylation differences reflect differences between cell surface and internal channel pools [28].

#### 3.4. PSD95 and Kv1.5 variants co-localize in cultured cells

In order to further confirm the interactions *in vivo*, confocal imaging experiments were conducted. As shown in Fig. 4A, when transfected into HEK293 cells both wild-type Kv1.5 and PSD95 localize to the cell surface, although a substantial portion of the Kv1.5 signal is found in the cytoplasm. When doubly transfected, the co-localization of the two proteins is substantial and restricted to the cell surface. Imaging experiments of PSD95 with Kv1.5 $\Delta$ ETDL, in which the channel's C-terminal PDZ-binding motif is deleted, yielded nearly identical results (Fig. 4B). As it did with the wild-type Kv1.5, PSD95 co-localized with Kv1.5 $\Delta$ ETDL at the membrane in doubly transfected HEK cells. Thus, the extreme C-terminal amino acids of Kv1.5 are not essential to its association with PSD95.

#### 3.5. Delineation of the N-terminal PDZ-binding region in hKv1.5

Binding of PDZ domains to internal sites in target proteins is not common [15]. Thus, delineation of the PSD95-binding site in the Kv1.5 N-terminus could add to our understanding of these rare interactions. To roughly locate the region in the Kv1.5 N-terminus where the PDZ domains are binding, a

number of deletion mutants were constructed. As illustrated in Fig. 5A, GST-tagged deletion constructs lacking amino acids 2–92, 2–162, 85–209, 135–240 and 150–208 of the Kv1.5 N-terminus were produced and tested for binding with the T7-tagged PSD95 PDZ construct. Fig. 5B illustrates that the PDZ domains bound separate Kv1.5 N-terminal deletion constructs lacking amino acids 2–92, and 150–208, but did not bind deletions of amino acids 2–161, 85–208, nor 135–240. This places the binding site in Kv1.5 between amino acids 92 and 149, immediately adjacent to or within the T1 domain of the channel.

There are a number of superficially canonical PDZ-binding motifs within the T1 domain of *Shaker*-type channels. Were the sequences located at the C-terminus of the channel, ETQL at positions 132–135 and ISGL spanning residues 126–129 would be excellent candidates for Type I PDZ-binding sites. An INI sequence at 124–126 would be a similarly good candidate binding site for Type II PDZ domains [15]. All three reside in a  $\beta$ -finger-like structure folded tightly into the T1 domain of the channel [29]. Hillier, et al. [18] have reported PDZ binding to a pseudocanonical domain at the end of a  $\beta$ -finger in nNOS. Only the INI sequence is located analogously to the nNOS motif in the T1  $\beta$ -finger-like structure, but it is oriented towards the interior of the domain. ETQL is oriented externally but is located at the base of the finger-like domain. The SGL sequence traverses the tip of the  $\beta$ -finger and, without significant divergence from the published crystal structure, could not interact with the PDZ groove. On the slight possibility that INI or ETQL might be involved in Kv1.5–PSD95 binding, they were modified by site-directed mutagenesis. Mu-

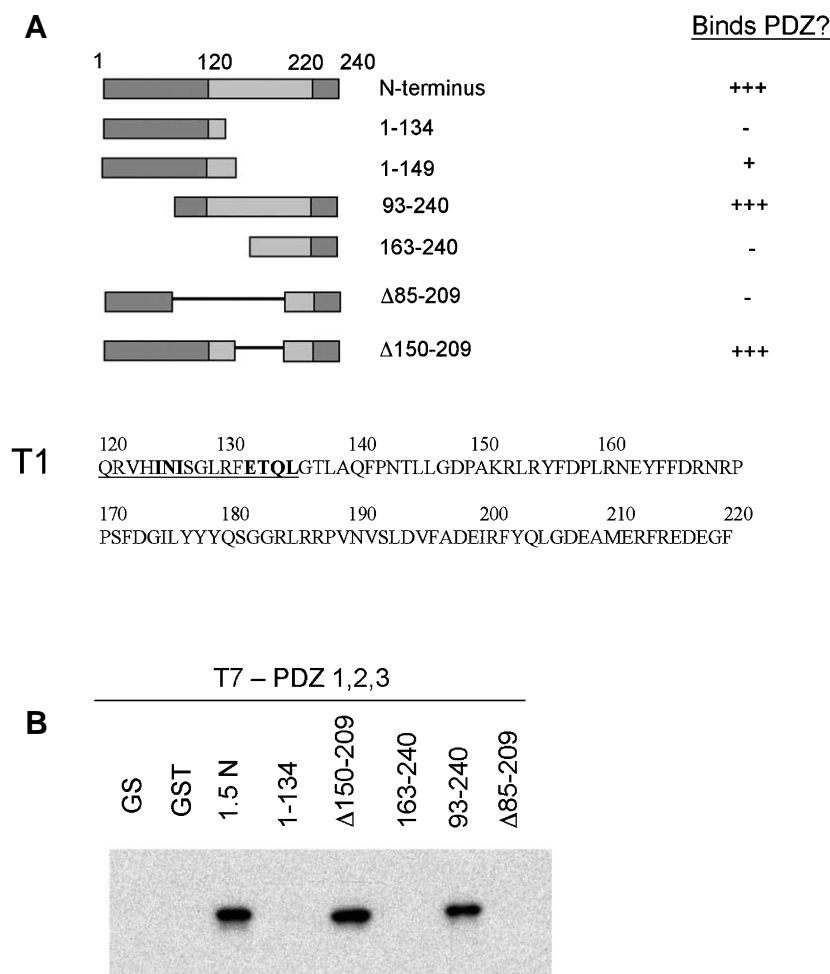


Fig. 5. Analysis of PDZ-domain binding to Kv1.5 N-terminal deletion constructs. A: Schematic diagram of the various Kv1.5 constructs. The binding activity of the constructs to PSD95 is indicated in the column on the right. B: Representative Western blots showing PDZ-domain binding by the various constructs. GST-tagged Kv1.5 N-terminal deletion constructs were incubated separately with PSD95 N-terminus and glutathione-Sepharose beads were added. Following incubation, the beads were pelleted, washed extensively, then subjected to SDS-PAGE. Western blots were performed using monoclonal anti-T7 (Novagen) to visualize the PSD95 fragment.

tation of INI to IND and ETQL to AAQA both failed, however, to affect the binding of the PSD95 fragment to the Kv1.5 N-terminus (data not shown).

### 3.6. PSD95 influences Kv1.5 $K^+$ currents

The effects of PSD95 co-expression with Kv1.5 on potassium current density were investigated in HEK cells. A PSD95–GFP fusion construct was transfected into HEK293 cell lines stably expressing Kv1.5 or one of two Kv1.5 deletion mutants in which either the N- or C-terminus of the channel was removed. As illustrated in Fig. 6A,D, PSD95 had no effect on Kv1.5 currents when the intact channel was expressed. However, PSD95 had profound effects on currents carried by the Kv1.5 deletion mutants. Currents in HEK cells expressing both PSD95 and an N-terminal deletion mutant of Kv1.5, Kv1.5ΔN209, were increased at least three-fold over those in cells expressing the Kv1.5 mutant alone (Fig. 6B,E). Currents from some cells co-expressing PSD95 and Kv1.5ΔN209 were so large that they could not be clamped. Thus, the average values obtained for the peak currents of these cells are underestimates of the actual peak values, because such large currents were omitted from the analysis. No cells expressing

Kv1.5ΔN209 without PSD95 exhibited currents that could not be clamped.

Similar experiments in which a Kv1.5 C-terminally deleted channel (lacking the C-terminal 51 amino acids) was co-expressed with PSD95 yielded very different results. Instead of increasing potassium currents, PSD95 substantially reduced those currents (Fig. 6 C,F). Potassium currents in the Kv1.5ΔC51 mutant cell line are unusually large and PSD95 attenuates those currents. Peak current measurements above  $-20$  mV could not be made since 67% of the Kv1.5ΔC51/PSD95 co-expressing cells and 100% of the cells expressing the Kv1.5ΔC51 mutant alone exhibited currents too large to clamp. Attempts to perform the same experiment using HEK cells transiently expressing the shorter C-terminal deletion mutant Kv1.5ΔETDL ± the PSD95–GFP fusion similarly yielded large currents although the general trend was similar (data not shown).

## 4. Discussion

The present study demonstrates that the prototypical PDZ domains of PSD95 bind to both C- and N-terminal fragments

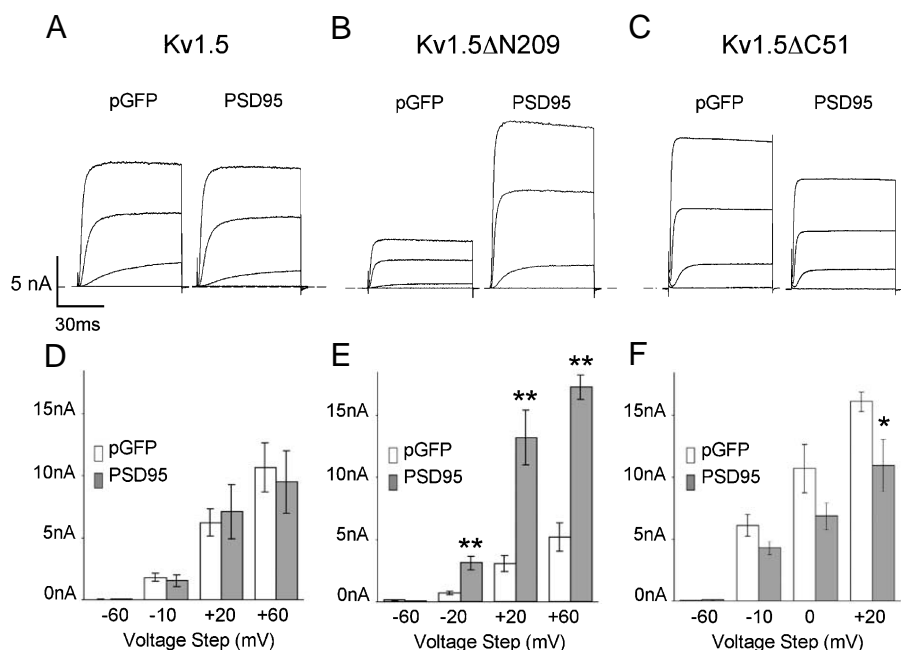


Fig. 6. Effect of PSD95 expression on peak currents on WT Kv1.5, and C- or N-terminal deletion mutants. HEK293 cells stably expressing Kv1.5, Kv1.5ΔC51, and Kv1.5ΔN209 were used to test the effect of PSD95 on peak currents when the N- and C-terminal PDZ-binding domains were removed. Cells were transiently transfected with either pGFP or PSD95:pGFP and peak currents at several voltages were measured under voltage-clamp in whole-cell configuration. The protocol consisted of four 75-ms pulses (sufficient to allow activating currents to reach steady-state) with an inter-sweep interval of 5 s. The voltage steps were as follows: the first below the activation threshold (−60 mV), the second near the  $V_{1/2}$ -activation of each channel (−10 mV), and the third and fourth above the maximum activation where the  $I$ - $V$  relationship is linear (+20, +60 mV). Representative current traces and mean data are shown for WT Kv1.5 (A,D), Kv1.5ΔN209 (B,E), and Kv1.5ΔC51 (C,F). Bar graph data are presented as mean data  $\pm$  S.E.M. from  $n=6$ –8 cells. The asterisks denote statistical significance with  $P < 0.1$  (\*) or  $P < 0.05$  (\*\*).

of Kv1.5 and other Kv1 channels and that these interactions affect Kv1.5 potassium currents. While, based on the sequence of the extreme C-terminus of the channel, the interaction of the two proteins was expected, the involvement of the N-terminus was wholly unpredicted. Nevertheless, the evidence for the N-terminal interaction is strong. The interaction is readily detected with *in vitro* binding assays, by yeast two-hybrid analysis, and by co-immunoprecipitation (Figs. 1–3). Imaging analysis shows that both wild-type Kv1.5 and the C-terminal mutant co-localize with PSD95 at or very near the cell surface (Fig. 4) and the relative amounts co-immunoprecipitated and the yeast two-hybrid results further show that the N- and C-terminal interactions occur with similar avidity. Finally, PSD95 has differential effects on Kv1.5 potassium currents, depending on the individual presence of the channel's two PDZ-binding sites (Fig. 6).

While PDZ-domain binding to internal motifs is uncommon, it is not unprecedented. Internal PDZ-binding sites have been demonstrated in phospholipase C [30,31], protein kinase C [30,31], and nNOS, among others [18]. The best characterized of these interactions occurs between a  $\beta$ -finger of nNOS and the PDZ domains of  $\alpha 1$ -syntrophin [18]. In this interaction, the first strand of the  $\beta$ -finger mimics a canonical COOH-peptide ligand. The sharp turn of the  $\beta$ -finger allows an internal ETTF sequence at the turn to slip into the binding groove of the syntrophin PDZ domain. Interestingly, this  $\beta$ -finger itself is part of a PDZ domain in nNOS. There are no PDZ domains in *Shaker*-type channels, and mutation of consensus PDZ-binding sequences within the one potential  $\beta$ -finger in T1 did not affect PSD95 binding. The interaction

described herein must, therefore, occur via some other mechanism. The mechanisms by which the *Drosophila* PDZ-protein InaD binds internally to protein kinase C and to phospholipase C- $\beta$  are also unknown [30,31], as is that between the mouse proteins PTP-BL and RIL [32]. The latter, like the Kv1.5-N-terminal–PSD95 interaction, does not involve PDZ–PDZ interactions. Neither does it appear to involve consensus PDZ-binding sequences in  $\beta$ - or zinc-fingers [32].

Previous work has established a role in subcellular localization for the C-terminal binding motif, and shown that the C-terminal site is in itself necessary and sufficient for that role. Deletion or mutation of the canonical C-terminal motifs abolishes PSD95-mediated clustering interactions with Kv1 channels [5,13,24,33]. A similar deletion in Kv4.2 eliminates clustering of that channel and prevents an apparent PSD95-mediated increase in its surface expression [34].

We were unable to detect any focal clustering of Kv1.5 by PSD95 for either the wild-type or  $\Delta$ ETDL forms of the channel (Fig. 4). This was true both in the HEK293 cells shown herein and in COS-7 cells (data not shown). While clustering was not evident, co-expression with PSD95 greatly increased Kv1.5 potassium currents in transfected HEK cells – but only when the N-terminus of the channel was deleted. Kv1.5ΔN209 is normally difficult to express and currents tend to be small with this mutant. So the increase in current magnitudes to greater than typically seen for the wild-type channel is quite striking. One possible explanation for this phenomenon is that the mutant traffics to the cell surface in much the same manner as does the wild-type channel. Lacking an N-terminus,

however, it is not stable there. Perhaps N-terminal interactions with actinin [23] or other cellular constituents are necessary to maintain the channel at the surface of the HEK cells. Addition of PSD95 to the system might allow its interaction with the Kv1.5 C-terminus to perform a similar function, stabilizing an otherwise temporary surface expression of the channel. This scenario would be consistent with the finding that PSD95 can stabilize but not promote cell surface expression of other Kv1 channels [33]. That the wild-type channel is not affected by co-expression with PSD95 suggests that wild-type surface retention is maximized via the N-terminal interactions, if this model is correct.

The discovery of a PDZ-binding region in the N-termini of Kv1 channels is more difficult to reconcile with previous work. It is highly unlikely that the PDZ–Kv channel N-terminal interaction can be important to clustering or to the promotion of cell surface expression. Too much data exists that the C-terminus alone is responsible for these effects [5,13,24,33,34]. The role of the N-terminal interaction must be quite different. We have two pieces of evidence for such a divergent role. Co-immunoprecipitation experiments give different results in the presence and absence of the Kv channel ETDL C-terminal binding motif (Fig. 3) and PSD95 co-expression has opposing effects on Kv1.5 currents, depending on whether the expressed channel retains the C- or the N-terminal PDZ-binding domain (Fig. 6). Two isoforms of Kv1.5 are routinely seen in Western blots of transfected HEK cell extracts, and antibody to the Kv1.5 fusion consistently pulls down both of these isoforms (Fig. 3). This is true whether the transfected channel is wild-type or  $\Delta$ ETDL. If the expressed channel is wild-type, the same two isoforms consistently co-immunoprecipitate with PSD95 when antibody against the PDZ protein is used. However, only the faster migrating isoform of Kv1.5 co-immunoprecipitates with PSD95 if the expressed channel lacks the C-terminal ETDL sequence. Deglycosylation experiments showed that this faster migrating Kv1.5 is a less glycosylated, possibly internal form of the channel (Fig. 3E). The differential binding of PSD95 to the two isoforms definitely suggests functional differences between N- and C-terminal PDZ binding. Perhaps PSD95 and/or other PDZ proteins might retain misfolded channels via the N-terminal interaction or influence the trafficking of the channels.

Effects on Kv1.5 potassium currents provide more evidence that PSD95 binding to the N-terminus of the channel has a very different role than does C-terminal binding. Whereas co-expression of PSD95 with an N-terminally deleted Kv1.5 mutant dramatically increased potassium currents, the effect of co-expression with a C-terminally deleted Kv1.5 mutant was quite different. Current levels were significantly reduced by this experimental manipulation (Fig. 6). It would seem that PSD95 interaction with the N-terminus somehow indeed interferes with surface expression of the Kv1.5 channel.

How could one protein, normally expressed at the cell membrane, have such divergent effects on the expression of another membrane protein? In this artificial system, both PSD95 and Kv1.5 are over-expressed. Both can be detected in the cytoplasm as well as at the cell surface (Fig. 4). Thus, there is at least opportunity for internal interactions that may or may not occur in neurons or other constitutively expressing cells. The N-termini of Kv channels interact with a large number of proteins.  $\beta$ -Subunits, kChIP, kChAP and  $\alpha$ -acti-

nin2 are among the proteins known to bind at or near the PDZ-binding site uncovered here. In such a busy area, it is quite possible that the PDZ-binding site is normally masked from PSD95 at the cell surface. It is even conceivable that PSD95 in our artificial system is taking on the role of some other PDZ protein(s) and interacting with an N-terminal Kv channel domain that PSD95 normally fails to see.

A plethora of PDZ proteins have been described (see Bezprozvanny and Maximov, [35], for a partial list) and, thus, there are many potential binding partners for these channel regions. Some obvious candidates in neurons include SAP102 and PSD93 [36]. ZASP [37] and ENH [38] are among many potential interactors in the heart. SAP97 is also expressed in the heart and has been shown to interact with various Kv1 channels, where it clusters the channels in the interior of the cell [24,25,33]. Mutations in the Kv1.4 C-terminal PDZ-binding motif eliminate this co-clustering phenotype, however [24,25,33].

The Kv channel N-terminal PDZ-binding site is within or very near the T1 domain, a region first identified as necessary for the assembly of the channel subunits themselves [39]. Binding to the T1 domain could thus affect channel assembly, intersubunit interactions, or even channel gating [40]. Alternatively, PDZ binding might compete with the binding of other cellular constituents, such as the  $\beta$ -subunits or  $\alpha$ -actinin2, perhaps affecting channel assembly, folding, trafficking or inactivation. It will be of great interest to learn which, if any, of these roles PDZ proteins may play.

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