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Slob, a Novel Protein that Interacts with the Slowpoke Calcium-Dependent Potassium Channel

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Summary

Slob, a novel protein that binds to the carboxy-terminal domain of the *Drosophila* Slowpoke (dSlo) calcium-dependent potassium channel, was identified with a yeast two-hybrid screen. Slob and dSlo coimmunoprecipitate from *Drosophila* heads and heterologous host cells, suggesting that they interact in vivo. Slob also coimmunoprecipitates with the *Drosophila* EAG potassium channel but not with *Drosophila* Shaker, mouse Slowpoke, or rat K_V1.3. Confocal fluorescence microscopy demonstrates that Slob and dSlo redistribute in cotransfected cells and are colocalized in large intracellular structures. Direct application of Slob to the cytoplasmic face of detached membrane patches containing dSlo channels leads to an increase in channel activity. Slob may represent a new class of multi-functional channel-binding proteins.

Introduction

It has been evident since the first biochemical purifications of ion channel proteins that the pore-forming α subunits often copurify with one or several accessory proteins (reviewed by Hall and Sanes, 1993; Catterall, 1995). Although in many cases the functions of these accessory proteins are only poorly understood, it is now clear that at least some of them participate in channel clustering (e.g., Apel et al., 1995; Kornau et al., 1995; Gillespie et al., 1996; Müller et al., 1996; Niethammer et al., 1996; Brakeman et al., 1997; Dong et al., 1997) and modulation (e.g., Swope et al., 1995; Fuhrer and Hall, 1996; Ehlers et al., 1996). In the case of potassium channels, the lack of a sufficiently rich source of channel protein long precluded protein purification, but more recently several different kinds of β subunits that influence potassium channel kinetics and/or processing of the α subunit protein have been isolated by copurification or expression cloning strategies (Parcej and Dolly, 1989; Knaus et al., 1994; Rettig et al., 1994; McManus et al., 1995; Rhodes et al., 1995). In addition, a yeast two-hybrid screen using the carboxy-terminal region of the Shaker potassium channel as bait demonstrated a role for the major postsynaptic density protein, PSD-95, in potassium channel clustering (Kim et al., 1995; Tejedor et al., 1997).

Calcium-dependent potassium channels play critical roles in the regulation of neuronal excitability, and such channels from a variety of tissues can be modulated by several different protein kinases (reviewed by Levitan, 1994). In addition, calcium-dependent potassium channels from rat brain, reconstituted in artificial lipid bilayers, can be modulated by the addition of ATP alone without an exogenous protein kinase (Chung et al., 1991; Reinhart and Levitan, 1995), suggesting that there might be protein kinase activity intimately associated with the ion channel protein. It has been inferred from similar experiments that a phosphoprotein phosphatase activity might also be associated with the channel and kinase in a regulatory complex (Reinhart and Levitan, 1995). Because electrophysiological experiments to test these inferences with the cloned *Drosophila* Slowpoke (Atkinson et al., 1991; Adelman et al., 1992) calcium-dependent potassium channel (dSlo) have led to ambiguous results (Esguerra et al., 1994; Bowlby and Levitan, 1996), we have turned to genetic and biochemical approaches to investigate more directly the interaction of signaling and other proteins with calcium-dependent potassium channels.

We report here the cloning and characterization of Slob (for Slowpoke-binding protein), a novel protein isolated by a yeast two-hybrid screen based on its interaction with the carboxy-terminal domain of dSlo. Slob coimmunoprecipitates with the full-length dSlo channel, as well as with EAG, another *Drosophila* potassium channel. However, it does not coimmunoprecipitate with the *Drosophila* Shaker or rat K_V1.3 voltage-dependent potassium channels or with mouse Slowpoke (mSlo). Slob and dSlo redistribute and colocalize in discrete intracellular structures when they are coexpressed in heterologous host cells, and direct application of Slob to detached inside-out membrane patches can strongly activate dSlo (but not human Slowpoke [hSlo]) channels. Thus, Slob is a potassium channel–binding protein that influences multiple channel properties.

Results

Slob Sequence

The carboxy-terminal tail (804 amino acids) of dSlo (Adelman et al., 1992) was used as bait in a two-hybrid screen (Fields and Song, 1989) of a *Drosophila* head cDNA library. Approximately 400,000 transformed yeast colonies were screened for positive interactions with the dSlo tail. There were 30 positive colonies present on day 2 of the screen, all but three of which were eliminated as false positives. The library cDNA inserts of the three true positive colonies were sequenced and identified as different cDNA clones of the same gene, *Slob*. The organization of the three cDNA clones, designated *Slob1*, *Slob2*, and *Slob3*, is shown in Figure 1A. The overlapping regions of the corresponding Slob protein sequences (Figure 1B) are identical except for an

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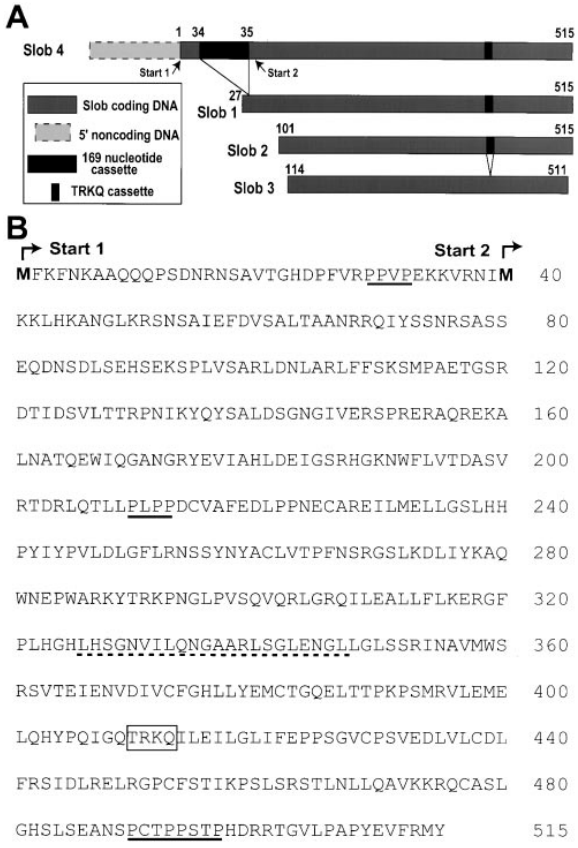

Figure 1. Cloning and Sequence of *Slob*

(A) The organization of the sequences isolated from the three positive yeast colonies in the two-hybrid screen, indicated as *Slob1*, *Slob2*, and *Slob3*. The TRKQ cassette (small black inserts) is not encoded in the *Slob3* cDNA. A *Slob4* cDNA was isolated from a *Drosophila* head phage library using *Slob1* as a probe. A putative start codon (indicated as Start 1) was identified in *Slob4* based on the presence of upstream stop codons in all three translation frames. Immediately downstream of where *Slob4* and *Slob1* overlap, *Slob4* contains a 169-nucleotide noncoding cassette (large black insert) that is not present in *Slob1*. When this cassette is present, the predicted protein open reading frame begins at the putative start codon indicated as Start 2.

(B) Predicted amino acid sequence of Slob. The two predicted amino-terminal start sites (without and with the 169-nucleotide cassette) are indicated. Proline-rich motifs that might bind to SH3 domains are underlined. The leucine zipper sequence is dash underlined, and the TRKQ cassette is boxed.

insert of four amino acids, TRKQ, which is indicated by the box in Figure 1B. These four amino acids are present in Slob1 and Slob2 but not in Slob3 (Figure 1A) and probably represent an alternatively spliced cassette. It is of interest that the threonine in this cassette is predicted (Kennelly and Krebs, 1991) to be a target for phosphorylation by protein kinase C (PKC).

To determine the translation start site of *Slob*, another *Drosophila* head cDNA library in bacteriophage λ was screened to find *Slob* cDNAs containing noncoding 5′ sequence. Using *Slob* cDNA as a probe, 300,000 phage plaques were screened and a 2 kb cDNA, named *Slob4* (Figure 1A), was isolated and sequenced. *Slob4* contains the entire *Slob1* sequence, including the TRKQ cassette. The sequence also includes a 460 nucleotide 5′ region, including an in-frame start methionine (Start 1 in Figure 1), upstream from the *Slob1* cDNA. This 5′ sequence contains stop codons in all three reading frames prior to the Start 1 methionine, indicating that a noncoding 5′ sequence has been identified. *Slob4* also has a 169-nucleotide noncoding cassette (Figure 1A) that is not present in the overlapping sequence of *Slob1*. This sequence in *Slob4* is inserted between the eighteenth and nineteenth nucleotides of the *Slob1* sequence. The insert sequence does not contain a long open reading frame, but does contain four AUG start sites, each having an in-frame downstream stop codon. Such start/stop sequences upstream from a coding start site are thought to be a mechanism for downregulating translation (Kozak, 1989). This noncoding cassette implies two amino-terminal splice variants of Slob. The Slob sequence shown in Figure 1B with the start methionine labeled Start 2 is the predicted amino acid sequence of Slob for the splice variant that contains the 169-nucleotide insert. This Slob protein would have 476 amino acids and a calculated molecular weight of ~53 kDa. The Slob sequence in Figure 1B with the start methionine labeled Start 1 is the predicted amino acid sequence of Slob for the splice variant that does not contain the 169-nucleotide insert. This Slob protein would have 515 amino acids and a calculated molecular weight of ~57 kDa. The two-hybrid clone designated *Slob1* was used for the heterologous expression experiments described here.

A Blast search (Altschul et al., 1990) of the Slob amino acid sequence revealed no homology matches with any known protein. A hydropathy map (Engelman et al., 1986) of Slob showed no stretches of hydrophobic amino acids that might serve as a transmembrane domain. The only readily identifiable domains in the Slob sequence, underlined in Figure 1B, are a leucine zipper motif (Landschulz et al., 1988) and several proline-rich motifs that might bind to Src homology 3 (SH3) domains (Feng et al., 1994; Rickles et al., 1994; Cohen et al., 1995). The first of these proline-rich motifs is not present in the shorter of the predicted amino-terminal splice variants of Slob (Figure 1B).

Slob in the Fly

The chromosomal location of the *Slob* gene was determined by hybridizing a biotinylated full-length *Slob* cDNA probe to polytene chromosomes from *Drosophila* salivary gland using standard techniques. The probe was visualized using streptavidin-conjugated alkaline phosphatase as described in the Experimental Procedures. A single locus, at 28B1–2 in the left arm of chromosome 2 (using the Bridges, 1942 mapping convention), was observed (Figure 2A).

A Slob antibody made in rabbits immunized with purified glutathione-S-transferase–Slob (GST–Slob) fusion protein was used to determine whether native Slob protein is present in *Drosophila*. As shown in Figure 2B, the Slob antibody (left) but not preimmune IgG (right) recognizes a ~57 kDa protein band (arrow) in a lysate

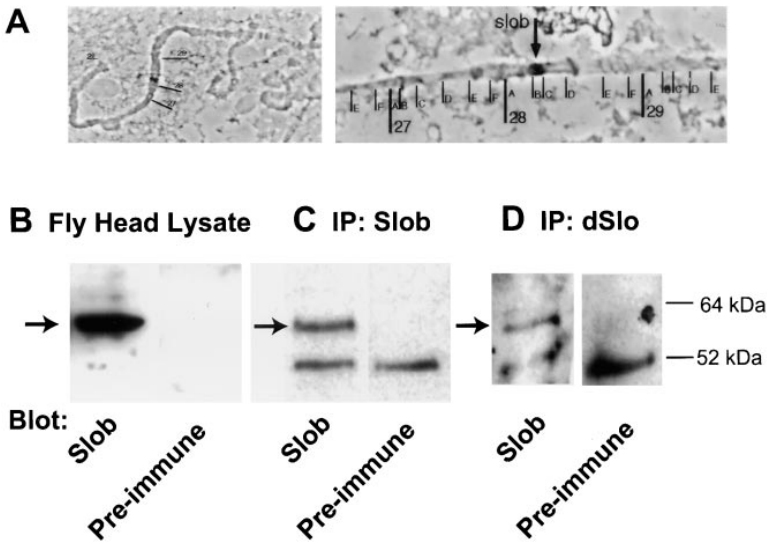

Figure 2. *Slob* in *Drosophila*

(A) Chromosomal localization of *Slob*. Polytene chromosomes from Canton S third instar larvae were prepared as described and hybridized with a biotinylated probe made from the full-length *Slob* cDNA. Bound probe was visualized using streptavidin-conjugated alkaline phosphatase. Landmarks indicating the boundaries of bands 27, 28, and 29 are indicated by bars. The two panels show this area at different magnifications, and the location of the Slob signal is marked by an arrow in the right panel.

(B) Western blots of fly head lysates, probed with Slob antibody (left) or preimmune IgG (right). A ~57 kDa protein (arrow) in the lysate is recognized specifically by the Slob antibody.

(C) Western blots of immunoprecipitates, prepared from fly head lysates with the Slob antibody, probed with Slob antibody (left panel) or preimmune IgG (right). A 57 kDa protein (arrow) is immunoprecipitated and recognized on the blot by the Slob antibody.

(D) Coimmunoprecipitation of native Slob and dSlo. Immunoprecipitates were prepared from fly head lysates with dSlo antibody and were probed on Western blots with Slob antibody (left) or preimmune IgG (right). A 57 kDa protein (arrow) that is recognized by the Slob antibody coimmunoprecipitates with dSlo. The ~53 kDa band present in all of the immunoprecipitate lanes in (C) and (D) is the heavy chain of IgG. The molecular weight markers on the right refer to all lanes in (B), (C), and (D).

prepared from *Drosophila* heads; this is the size predicted from the cDNA sequence for the larger amino-terminal splice variant of Slob. A band of this molecular weight is also immunoprecipitated from fly head lysates with the Slob antibody (Figure 2C). These results demonstrate the presence of native Slob in *Drosophila* heads.

To test whether native Slob and dSlo can be coimmunoprecipitated from *Drosophila*, a polyclonal rabbit antiserum specific for the last 58 amino acids of the dSlo channel (J. W. and I. B. L., unpublished data) was used to immunoprecipitate dSlo from a fly head lysate, and the immunoprecipitate was probed with the Slob antibody. As shown in Figure 2D (left), a 57 kDa band in the dSlo immunoprecipitate is recognized by the Slob antibody. No such band is present when the dSlo immunoprecipitate is probed with preimmune IgG (Figure 2D, right) or when the immunoprecipitate is prepared with control antiserum (data not shown). In the reciprocal experiment, dSlo can be detected in a Slob immunoprecipitate (data not shown). Thus, native Slob and dSlo can interact in the fly.

Heterologously Expressed Slob and dSlo Coimmunoprecipitate

To confirm these indications from the two-hybrid screen and the fly head experiments that Slob and dSlo form a complex, we expressed the two proteins together in tsa201 cells (a human embryonic kidney cell line) and tested for binding by coimmunoprecipitation. The level of expression of Slob (Figure 3A) is not altered by coexpression of dSlo, and similarly the level of expression of dSlo (Figure 3B) is the same in the absence and presence of Slob. When Slob is immunoprecipitated from the cell lysate, dSlo can be detected (by Western blot) in the immunoprecipitate (Figure 3C). Similarly, Slob is present in a dSlo immunoprecipitate from cotransfected cells (data not shown). Because the immunoprecipitation is carried out in a detergent-containing lysate, and coimmunoprecipitation is observed even in

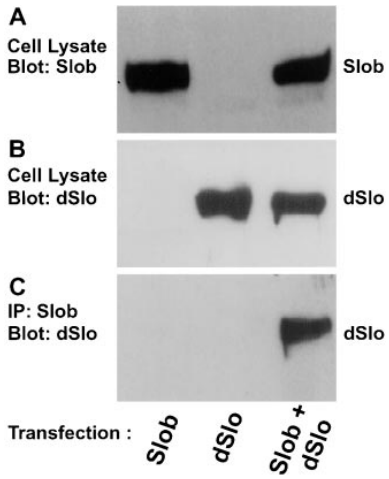

Figure 3. Slob and dSlo Coimmunoprecipitate from Cotransfected Cells

Cell lysates prepared from tsa201 cells transfected with Slob1–HA or dSlo, or cotransfected with both, were Western blotted with (A) anti-HA antibody to detect Slob or (B) anti-dSlo antibody.

(A) Slob is detected in lysates from Slob1–HA and Slob1–HA/dSlo transfected cells but not in cells transfected with dSlo alone.

(B) dSlo is detected in lysates from dSlo and Slob1–HA/dSlo transfected cells but not in cells transfected with Slob1–HA alone. Neither protein affects the level of expression of the other.

(C) Anti-HA immunoprecipitates from the lysates, Western blotted with anti-dSlo antibody. dSlo is detected only in immunoprecipitates from Slob/dSlo cotransfected cells.

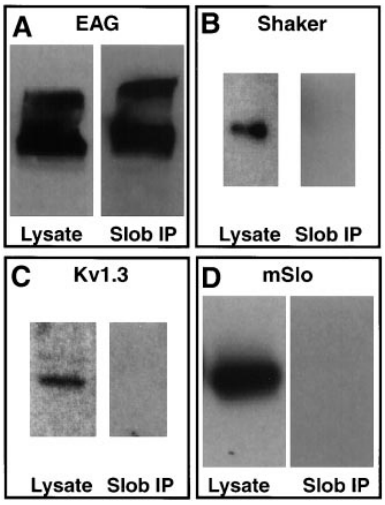

Figure 4. Specificity of Slob Channel Interactions

Cell lysates were prepared from tsa201 cells cotransfected with Slob1–HA and one of the following potassium channels: (A) EAG, (B) Shaker-IR, (C) $K_V1.3$, or (D) mSlo. Lysates were Western blotted with appropriate anti-channel antibodies to confirm channel expression (left panels). Anti-HA immunoprecipitates from each lysate were Western blotted with the appropriate channel antibody (right panels). EAG can be detected in the Slob1–HA immunoprecipitate, but there is no coimmunoprecipitation of Shaker-IR, $K_V1.3$, or mSlo.

the presence of 0.5 M NaCl (data not shown), these results provide strong evidence that Slob and dSlo interact in a complex.

Binding of Slob to Other Potassium Channels

To determine the specificity of Slob's interaction with dSlo, we coexpressed Slob with several other *Drosophila* and mammalian potassium channels and carried out coimmunoprecipitation/Western blot experiments identical to those described above. Expression of Slob protein is not altered by coexpression with any of the potassium channels tested (data not shown). When Slob is coexpressed with the EAG potassium channel from *Drosophila* (Warmke et al., 1991), EAG can be detected in a Slob immunoprecipitate (Figure 4A), and Slob is present in an EAG immunoprecipitate (data not shown). In contrast, the *Drosophila* Shaker potassium channel (Figure 4B), the rat $K_V1.3$ Shaker-like potassium channel (Figure 4C), and the mouse Slowpoke homolog mSlo (Figure 4D) fail to coimmunoprecipitate with Slob, although the expression of each of these channels is robust and can be detected readily (Figure 4, lysate panels). Thus, Slob is selective in the potassium channels with which it interacts.

Interaction of Slob and dSlo in Living Cells

To explore further the interaction of Slob and dSlo in mammalian cells, a *Slob1–Green Fluorescent Protein* (*GFP*; Marshall et al., 1995) DNA construct was made and transfected into tsa201 cells. When transfected alone, Slob–GFP exhibits a diffuse intracellular distribution (Figure 5A), consistent with the biochemical finding that Slob is a soluble protein (data not shown). In contrast, dSlo (detected immunocytochemically) exhibits the membrane distribution expected of an ion channel (Figure 5B). When Slob–GFP and dSlo are coexpressed, a dramatic redistribution of Slob–GFP is seen. As shown in Figures 5C and 5E1, Slob–GFP is predominantly in large intracellular doughnut-shaped structures under these conditions. In contrast, when Slob–GFP is cotransfected with $K_V1.3$, a channel with which it does not coimmunoprecipitate (Figure 4), the $K_V1.3$ immunofluorescence remains restricted to the plasma membrane (Figure 5D1) and there is no change in the distribution of Slob–GFP (Figure 5D2; compare with Figure 5A). As shown in Figure 5E2, much of the dSlo also is localized in doughnut-shaped structures in dSlo/Slob cotransfected cells, although some membrane staining of the channel is still observed. The overlay image in Figure 5E3 demonstrates the striking intracellular colocalization of Slob–GFP (Figure 5E1) and dSlo (Figure 5E2).

Activation of dSlo by Slob

To test the possibility that Slob might influence dSlo channel activity, patches were excised from cells expressing dSlo, and a preparation of purified GST–Slob fusion protein was applied to the cytoplasmic side of the patch. As shown in Figures 6A and 6B, GST–Slob application strongly increases the steady-state open probability of dSlo channels. This effect is rapid in onset and reverses readily upon washing (Figure 6B). GST–Slob also increases the peak dSlo current evoked by depolarizing voltage pulses (Figure 7A). GST alone, purified by the identical protocol used for GST–Slob and applied at an 8-fold higher concentration, has no detectable effect on dSlo (Figure 7B). Because dSlo is a calcium-dependent channel, calcium was buffered carefully to insure that the activation by GST–Slob is not due to a change in calcium concentration. Furthermore, GST–Slob has no effect on the activity of hSlo (Figure 7B), which is also calcium dependent. This lack of activation of hSlo is consistent with the finding that mSlo (which is virtually identical in amino acid sequence to hSlo) does not coimmunoprecipitate with Slob (Figure 4D). These results support the conclusion that Slob can interact specifically with the dSlo channel to cause a change in its functional properties.

Discussion

The dSlo channel protein contains the six putative transmembrane segments and the pore-forming loop seen in other voltage-sensitive potassium channels (Atkinson et al., 1991; Adelman et al., 1992). A second region of the channel, the dSlo tail, begins after the sixth transmembrane segment. It is $>$800 amino acids long, contains four hydrophobic segments (Butler et al., 1993), and is the site of numerous splice variants (Adelman et al., 1992). Although there exists some evidence that the tail serves as a calcium-sensing domain (Wei et al., 1994; Schreiber and Salkoff, 1997), its exact function remains elusive. The dSlo tail was used as bait against a fly head cDNA library in the yeast two-hybrid protein interaction assay, with the goal of identifying accessory proteins that are important for channel function. A novel cDNA

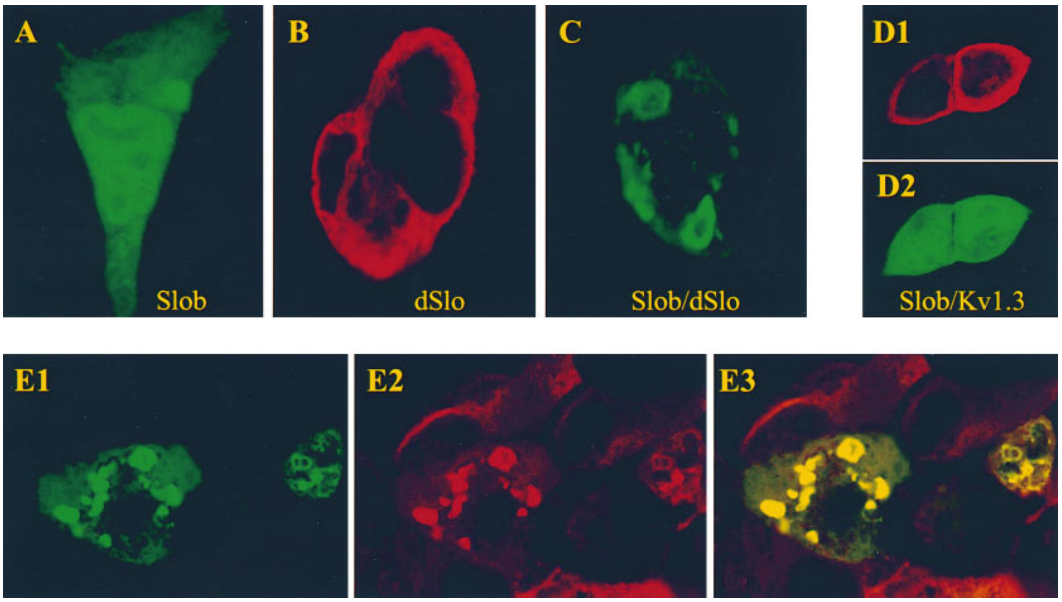

Figure 5. Slob and dSlo Colocalize in Living Cells

All panels are scanning confocal fluorescent images.

(A) Fluorescence of Slob–GFP expressed alone in tsa201 cells. Slob–GFP exhibits a diffuse distribution in the cytoplasm.

(B) dSlo channels, detected with rhodamine-conjugated secondary antibodies, are seen at the plasma membrane of transfected cells.

(C) Cotransfection of dSlo with Slob–GFP changes the cellular distribution of Slob–GFP. The Slob–GFP fluorescence is associated with large doughnut-shaped intracelluar structures.

(D) Cotransfection of $K_V1.3$ (immunofluorescence shown in D1) has no effect on the cellular distribution of Slob–GFP (D2).

(E) Slob–GFP (E1) and dSlo (E2) both redistribute in cotransfected cells and colocalize (E3). The yellow color signifies overlapping red and green pixels.

encoding a 515 amino acid protein was identified and named *Slob*.

dSlo and Slob can also interact outside the confines of the yeast cell. Four different lines of evidence confirm their specific interaction. First, dSlo and Slob can be coimmunoprecipitated from cotransfected mammalian cells. Not only does this confirm that Slob and dSlo can form a complex in vitro, it also reveals that Slob can interact with the full-length dSlo channel. This is an important observation, because it implies that the tail can exist as an independent domain (see also Wei et al., 1994), as defined by Slob binding, by itself or when tethered to the rest of the channel. Secondly, Slob and dSlo interact in living mammalian cells. In heterologous host cells, Slob normally exhibits a diffuse cytoplasmic distribution, and dSlo is largely associated with the plasma membrane. When Slob and dSlo are expressed together, there is a striking change in these subcellular distributions. Although some dSlo remains membrane associated, the two proteins colocalize in large doughnut-shaped structures that do not correspond obviously to known cellular organelles. The identity of these doughnut structures has not yet been determined, and it cannot be ruled out that they result from overexpression in a heterologous system. Time-lapse video microscopy shows that the structures are dynamic, moving within the cell and undergoing division into smaller structures (data not shown). Similar structures have been described in cells coexpressing Shaker potassium channels and SAP97, a postsynaptic density protein

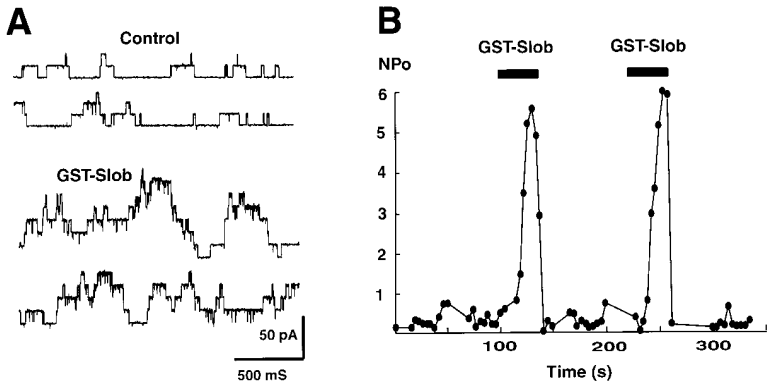

Figure 6. GST–Slob Fusion Protein Increases the Activity of dSlo

(A) Examples of steady-state dSlo channel activity at +100 mV in inside-out patches from HEK-293 cells transfected with dSlo, in the absence (top two traces) and presence (bottom two traces) of GST–Slob.

(B) Channel open probability (NP_O) as a function of time before, during, and after application of GST–Slob (indicated by the bar) with a gravity flow perfusion system. The patches were exposed to the same nominal zero calcium concentration solution before, during, and after the application of the fusion protein.

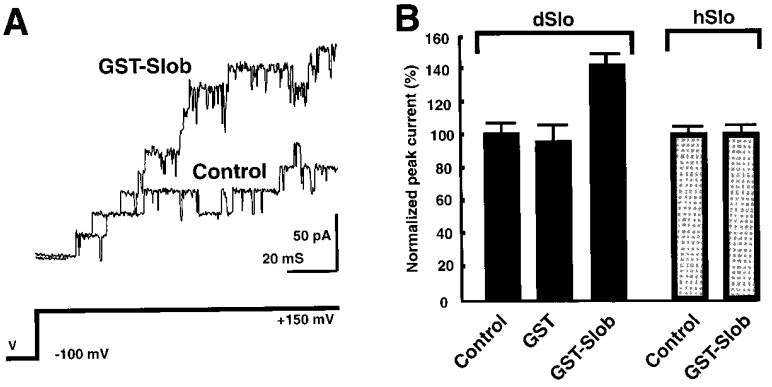

Figure 7. GST–Slob Increases the Peak Current of dSlo but Not hSlo

(A) Example traces of dSlo currents in inside-out patches, evoked by depolarizations from −100 mV to +150 mV in the absence or presence of GST–Slob.

(B) Normalized peak currents for patches containing dSlo channels (dark bars) or hSlo channels (gray bars). Each bar represents the mean normalized peak current, and the error bars represent SEM for each of the following conditions: nominal zero calcium solution (Control), GST alone, or GST–Slob.

(Kim and Sheng, 1996). One intriguing possibility is that Slob is involved in channel protein processing, targeting, and/or degradation, and that the unidentified structures contain dSlo channels on their way to or from the plasma membrane. In any event, these results demonstrate clearly an interaction between Slob and dSlo in the cotransfected cells. The third line of evidence for an interaction is the observation that Slob applied to the cytoplasmic side of excised patches causes an increase in the open probability of dSlo channels. The effect is reversed readily, which is curious considering the stable binding implied by the coimmunoprecipitation data. Finally, Slob and dSlo interact in the fly. Native Slob can be detected in and immunoprecipitated from *Drosophila* head lysates, and it coimmunoprecipitates from such lysates with native dSlo.

Slob also forms an immunoprecipitating complex with the *Drosophila* EAG potassium channel. The Slob–EAG interaction is surprising because there is almost no sequence similarity between dSlo and EAG in their amino- or carboxy-terminal regions (Warmke et al., 1991; Adelman et al., 1992). In contrast, Slob does not interact with the mammalian Slowpoke isoforms, mSlo and hSlo, even though dSlo shares over 60% sequence identity with these two channels. The Slob binding domains involved in its interaction with dSlo and EAG may not be identical. We do not know the number of channel-binding domains in Slob, but it is possible that Slob serves to cluster dSlo and EAG together in a complex. Indeed, an interaction between EAG and Slowpoke is predicted by the finding that *eag* mutants in *Drosophila* exhibit altered calcium-dependent potassium currents at the larval neuromuscular junction (Zhong and Wu, 1993).

What is the in vivo function of Slob? Analysis of the protein sequence provides no obvious answers. The sequence of Slob has not been reported previously, but it does contain several well-characterized sequence motifs. For example, it contains a leucine zipper region (Landschulz et al., 1988) that may allow it to form a complex with itself or other leucine zipper–containing proteins. Alternatively, Slob may be involved in signaling. The closest sequence matches to Slob are the signaling enzymes PKC and guanylate cyclase, but the matches are weak and the catalytic residues necessary for these enzymatic activities are not found in Slob. There is a PKC phosphorylation consensus site in the TRKQ cassette that can be removed by alternative splicing, suggesting possible regulation of Slob by phosphorylation. Another possibility is that Slob is an adaptor molecule. For example, there is an intriguing similarity of a stretch of 20 amino acids in Slob (residues 32–51) with a recently reported PDZ domain in the *Drosophila* signal transduction scaffolding protein, InaD (Tsunoda et al., 1997). PDZ domains are binding modules used for protein–protein interactions (Saras and Heldin, 1996). Finally, several proline-rich motifs that might bind SH3 domains (Ren et al., 1993; Rickles et al., 1994; Cohen et al., 1995), including a Src kinase binding motif present in only the larger of the predicted amino-terminal splice variants, can be identified in the Slob sequence. We have shown recently that the voltage-dependent potassium channel $K_V1.5$ binds directly to the Src tyrosine kinase via an interaction between a proline-rich motif in the channel and the SH3 domain of the kinase (Holmes et al., 1996b). Although many other ion channels also contain proline-rich motifs (Holmes et al., 1996b), dSlo does not. Hence, it is possible that Slob is required as an adaptor to bring signaling proteins in close proximity to dSlo, and we have in fact found that Slob and Src coimmunoprecipitate from cotransfected cells (data not shown). Taken together with the signaling protein binding domains within the Slob amino acid sequence, the effects on channel subcellular localization and gating properties described here suggest that Slob might link multiple channel properties to diverse upstream signals.

Experimental Procedures

Two-Hybrid Screen/Cloning

The two-hybrid screen was carried out according to a standard protocol (Ausubel et al., 1994). The yeast strain, EGY48, and the two-hybrid vectors were obtained from R. Brent (Harvard Medical School, Boston, MA). The last 804 amino acids of the dSlo channel (splice variant A1C2E1G3, Genbank accession number M96840) were subcloned into the bait vector, pEG202. The dSlo carboxy-terminal bait was screened against a *Drosophila* head cDNA library (provided by J. Huang and M. Rosbash, Brandeis University, Waltham, MA) subcloned into the activating domain vector, pJG45. Approximately 400,000 transformed yeast colonies were screened for dSlo-interacting clones. Three clones were identified as true-positive interactors, and the cDNA library insert of each positive clone was sequenced. Each clone contained overlapping cDNA sequence of the same gene, *Slob*. The clones were designated *Slob1*, *Slob2*, and *Slob3* (Figure 1A). A PCR-generated ^{32}P-DNA probe containing the last 500 nucleotides of *Slob1* was used to screen a second *Drosophila* head cDNA library in λ gt10 phage (Sambrook et al., 1989). A single positive phage clone was isolated, rescreened with a cDNA probe encoding the first 500 nucleotides of *Slob1*, and purified. The 2.0 kb insert was sequenced.

DNA Constructs

Slob was cloned into the two-hybrid activation plasmid pJG45 at EcoRI and XhoI sites. There is an additional internal XhoI site in the *Slob* sequence and a single EcoRI site in the 3′ noncoding region of *Slob*. The hemagglutinin (HA) epitope-tagged Slob construct (Slob–HA) was made by amplifying Slob from the *Slob1* clone by PCR with DNA primers that added a start methionine and cloning sites, BamHI and XhoI, to the amino terminus of Slob1. The HA epitope (amino acids YPYDVPDYA) is present in the two-hybrid vector pJG45 between the activating domain and the library cDNA insert. The Slob1–HA PCR product with the HA tag at the amino terminus of the Slob1 sequence was subcloned into the mammalian expression vector $pcDNA_3$ (InVitrogen). The *Slob1–GFP* construct was made by amplifying a PCR product of *Slob1* that contained BamHI and KpnI cloning sites and removing the stop codon of *Slob*. The *GFP* coding sequence was excised from the pGFP cDNA vector (Clontech Laboratories) at KpnI and EcoRI polylinker restriction sites, and *Slob1* and *GFP* were subcloned into the $pcDNA_3$ expression vector at BamHI and EcoRI sites with a triple ligation step. The GST–Slob construct was made by excising the *Slob1* sequence from the pJG45 vector by EcoRI digestion and subcloning the fragment into the pGEX4T *GST* fusion vector (Pharmacia). dSlo–GST and mSlo–GST constructs were made by amplifying a DNA fragment containing the last l00 amino acids of the dSlo or mSlo tail by PCR, and subcloning it into the pGEX2T fusion vector (Pharmacia). The rat $K_V1.3$ channel was subcloned into $pcDNA_3$ as described elsewhere (Holmes et al., 1996a), and EAG was cloned into pCS2+MT. The EAG–GST construct contains amino acid residues 44–210 of EAG. The Shaker–IR channel, subcloned into the expression vector pMT3 (Pharmacia) with the 1D4 epitope tag (ETSQVAPA), was provided by C. Miller (Brandeis University, Waltham, MA) (Goldstein et al., 1994). All constructs were propagated in *E. coli* DH1 cells and purified by Qiagen Maxi prep kits according to the manufacturer's instructions (Qiagen). Constructs were sequenced to verify the correct reading frame.

Chromosomal In Situ Hybridization

Canton S wild-type flies were grown at 18°C on cornmeal/dextrose/yeast food. Third instar larval salivary glands were squashed and pretreated as described (Engels et al., 1986). The biotinylated DNA probe was prepared from the *Slob1* cDNA using a BioNick kit (Gibco BRL). Hybridization was visualized by incubating the slide with 10 μg/ml streptavidin-alkaline phosphatase using a BlueGene kit following the manufacturer's instructions (Gibco BRL).

Preparation of Antibodies

GST–Slob, *dSlo–GST*, *mSlo–GST*, and *EAG–GST* constructs were transformed into DE-3 *E. coli*, and synthesis of fusion proteins was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside. Fusion proteins were affinity purified using glutathione-sepharose-4B beads. Approximately 100–250 μg of purified fusion protein was mixed with Freund's complete adjuvant (Sigma) and injected into rabbits. Two 50 μg boosts were given 2 and 4 weeks later, and the rabbits were bled at 6 weeks. Specific antibodies were affinity purified using CNBr-sepharose beads coupled to the respective purified fusion proteins according to the manufacturer's instructions (Sigma). $K_V1.3$ antibodies (Cai and Douglass, 1993) were provided by J. Douglass (Vollum Institute, Portland, OR), anti-1D4 monoclonal antibody (Oprian et al., 1991) was provided by D. Oprian (Brandeis University, Waltham, MA), and anti-HA antibody was purchased from Boehringer-Mannheim.

Lysates, Coimmunoprecipitation, and Western Blotting

Fly head lysates were made by isolating heads on a sizing sieve and homogenizing them in lysis buffer (1% Chaps [Sigma] in phosphate buffered saline [PBS] containing 1 mM phenylmethylsulfonyl fluoride, l μg/ml leupeptin, 1 μg/ml aprotinin, and l μg/ml pepstatin [Sigma]).

Lysates were centrifuged at 12,000 × g to remove insoluble material, and protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad). Immunoprecipitation of Slob or dSlo from centrifuged fly head lysates (~1 mg protein/ml) was done by incubating with purified antibodies coupled to CNBr sepharose beads (20 μl beads/ml lysate), with processing as described below. Cell lysates containing Slob1–HA, dSlo, and other potassium channels were made by homogenizing tsa201 cells that were transiently transfected with the appropriate cDNA constructs. Cells were maintained in modified Eagle medium containing 100 units/ml penicillin/streptomycin and 10% fetal bovine serum. The cells were transfected using a calcium phosphate protocol (Sambrook et al., 1989). Approximately 20–40 μg of purified cDNA was added to 0.5 ml of 0.25 mM $CaCl_2$ solution, which was mixed slowly with 0.5 ml of a HEPES-buffered saline solution (300 mM NaCl, 50 mM HEPES, and 40 mM Na_2HPO_4 [pH 7.05]). The 1.0 ml of DNA suspension was added to a 100 mm dish of tsa201 cells that were 20%–25% confluent. The transiently transfected cells were harvested 48 hr later by removing the medium and homogenizing the cells in lysis buffer (1 ml lysis buffer/100 mm dish). Immunoprecipitations were done by adding 10 μg of anti-HA antibody coupled to CNBr-sepharose beads to 0.5 ml of centrifuged cell lysate (~500 μg total protein), incubating for 2 hr at 4°C, and washing the beads three times with PBS. Alternatively, 1 μg of anti-HA antibody was incubated overnight with the cell lysate, and the complex was precipitated with Protein A coupled to sepharose beads followed by washing.

To identify coimmunoprecipitating proteins from fly heads or transfected cells, the washed beads were mixed with two volumes of 2% sodium dodecyl sulfate gel loading buffer. Alternatively, the washed beads were treated with citric acid (pH 2.5) to dissociate the antigen–antibody complexes; the acid eluates were then neutralized with 2 M Tris (pH 10.0) and mixed with an equal volume of two times concentrated gel loading buffer. Immunoprecipitated proteins were separated on 7.5% acrylamide gels and transfered to nitrocellulose for Western blotting. The blots were blocked with 5% nonfat milk in PBS, incubated with primary antibodies (diluted 1:1000 in PBS) overnight at 4°C, washed, and incubated with horseradish peroxidase–conjugated secondary antibody (diluted 1:3000 in PBS) (Amersham). Antibody binding was detected using the enhanced chemiluminescence (ECL) detection assay (Amersham).

Fluorescence Microscopy

DNA constructs for Slob1–GFP, dSlo, and $K_V1.3$ were calcium phosphate transfected into tsa201 cells grown on 12 mm cover slips coated with poly-D-lysine. After 48 hr, the cells were fixed with 4% paraformaldehyde and washed five times with PBS. To allow antibody access, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. The cell-bearing coverslips were incubated overnight at 4°C in anti-channel rabbit polyclonal antiserum, diluted 1:50 with 10% normal goat serum. Coverslips were washed five times with PBS and incubated in a 1:1000 dilution of secondary antibody (rhodamine-coupled goat anti-rabbit, Jackson Laboratories, diluted with 10% normal goat serum in PBS) for 30 min at 37°C. The coverslips were washed again and mounted on glass slides with Gel/Mount (Biomeda, Foster City, CA). Indirect immunofluorescence images were acquired by laser-scanning confocal microscopy (MRC-600, Bio-Rad, Hercules, CA) using equal gain and aperture setting for all images.

Electrophysiological Recordings

Human embryonic kidney (HEK-293) cells were maintained as described for the tsa201 cells. *dSlo* and *hSlo* were transfected using Lipofectamine (Gibco/BRL) following the manufacturer's instructions. Patch recording of cells was done 1–3 days after transfection. The pipette solution and nominal zero calcium solution both contained 150 mm KCl, 10 mM HEPES, 2 mM $MgCl_2$, and 0.5 mM BAPTA [pH 7.4]. Patch electrodes were pulled from borosilicate glass, and electrode resistances were 2–4 MΩ. Inside-out patches were voltage clamped with a List EPC-7 amplifier after obtaining a GΩ seal. The current signal was filtered at 5 kHz and then digitally acquired at 10 kHz. Steady-state recordings were done holding the membrane voltage at +100 mV. The average channel activity (NP_o) for each 2 s interval was calculated by summing the times the current record spent at current levels corresponding to N open channels. The inside-out patches were more stable when channel activity was observed using a pulse protocol with depolarizing pulses from a holding potential of −100 mV. The depolarizing pulses were 200 ms long to +50 mV (hSlo) or +150 mV (dSlo). GST and GST–Slob were

purified and dialyzed against the nominal zero calcium solution. The protein concentration in the gravity perfusion system was ~2.5 μM (GST–Slob) or 20 μM (GST).

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GenBank Accession Number

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