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Forward Transport of $K_{2P}3.1$: Mediation by 14-3-3 and COPI, Modulation by p11

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Surface expression of the $K_{2P}3.1$ two-pore domain potassium channel is regulated by phosphorylation-dependent binding of 14-3-3, leading to suppression of coatomer coat protein I (COPI)-mediated retention in endoplasmic reticulum (ER). Here, we investigate the nature of the macromolecular regulatory complexes that mediate forward and retrograde transport. We demonstrate that (i) the channel employs two separate but interacting COPI binding sites on the N- and C-termini; (ii) disrupting COPI binding to either site interferes with the ER retention; (iii) p11 and 14-3-3 do not interact on their own; (iv) p11 binding to the C-terminal retention motif is dependent on 14-3-3; and (v) p11 is coexpressed in only a subset of tissues with $K_{2P}3.1$, while 14-3-3 expression is ubiquitous. We conclude that $K_{2P}3.1$ forward transport requires 14-3-3 suppression of COPI binding, whereas p11 serves a modulatory role.

Key words: 14-3-3, annexin, background, COPI retention, leak, p11, potassium channel, resting potential, TASK-1

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$K_{2P}3.1$ channels pass background potassium currents that stabilize cells at negative resting potentials and return cells to baseline after excitatory events. The channels show widespread tissue distribution, have a variety of natural and medicinal regulators and have putative roles in responses to hormones, neurotransmitters, acidosis, oxygen tension and local and volatile anesthetics (1–7). Quality control mechanisms that ensure its controlled expression on the plasma membrane are considered here.

K_{2P} channel subunits have cytoplasmic N- and C-termini, four transmembrane domains and two pore-forming P loops, and two subunits appear both necessary and sufficient to form an ion conduction pore (8–11). In 2002, we observed that the $K_{2P}3.1$ N-terminus has a dibasic endoplasmic reticulum (ER) retrieval signal (KRQN) that binds β coat protein (COP) to inhibit forward transport (12). We demonstrated that phosphorylation-dependent binding of 14-3-3β, a cytoplasmic protein found in all cells (13), suppressed β-COP binding and enabled forward transport of the channel to the surface (12). Thus, two $K_{2P}3.1$ channel populations were observed *in vivo*; one with β-COP and no 14-3-3 and another with 14-3-3β and no β-COP. This forward transport mechanism also appears to be exploited by other proteins (12,14–16).

While we observed β-COP to bind on the $K_{2P}3.1$ N-terminus and 14-3-3β to bind on the C-terminus, Girard et al. found basic residues in the C-terminus that suppressed channel expression and observed C-terminal binding of the annexin protein p11 (S100A10) in association with forward transport (22). p11 has also been reported to enhance plasma membrane expression of Nav1.8 sodium channels (17), ASIC1a acid-sensing ion channels, transient receptor potential cation channels (TRPV5 and 6) and serotonin 1B receptor (18–20). In contrast, others found p11 to bind to the $K_{2P}3.1$ C-terminus, leading to ER retention through basic sites in p11 (21).

To examine these discordant observations regarding roles of 14-3-3, COPI and p11 in regulation of $K_{2P}3.1$ surface expression, we sought to further delineate mechanistic bases for their action. Here, we report that basic motifs on both the N- and C-termini of $K_{2P}3.1$ must be intact to achieve ER retention. Both motifs can bind β-COP, and the affinity of interaction is significantly reduced if either is altered by mutation or 14-3-3β is bound to the channel C-terminus. We find p11 in only some tissues that express $K_{2P}3.1$ (it is notably absent from heart) and show that p11 and 14-3-3 do not interact in the absence of the channel; rather, p11 binds to $K_{2P}3.1$ only after phosphorylation-dependent binding of 14-3-3.

These observations argue that 14-3-3 is required to suppress COPI-dependent retention of $K_{2P}3.1$ in ER and to enable p11 modulation of channel forward transport in a tissue-specific fashion.

Results

$K_{2P}3.1$ has COPI binding motifs on both termini

The N-terminal $K_{2P}3.1$ sequence M*KR*QN (but not the mutant sequence M*NQ*QN) plays a critical role in retention of the channel in the ER through COPI-mediated retrograde transport because of binding of the coatomer subunit β-COP (12). Girard et al. found $K_{2P}3.1$ to be

released from ER retention on mutation of residues in the C-terminus (e.g. K^{389}RRSSV to K389A or R390A or R391A) (22). Altering these residues might suppress COPI binding to the C-terminus and/or has the potential to suppress phosphorylation of S393, which is required for 14-3-3 binding and ablation of COPI-dependent ER retention through the N-terminus (12). We sought, therefore, to investigate if β-COP interacts with the K_{2P}3.1 C-terminus. As observed previously, peptides analogous to the N-terminus bound β-COP when the dibasic motif was intact, similarly. Indeed, peptides identical to the C-terminal 16 residues of K_{2P}3.1 (terminating -MKRRSSV) bound native β-COP from COS-7 cells, whereas peptides with the basic residues altered (-MN*QQ*SSV) failed to bind (Figure 1A). These data suggest that either the N- or C-terminal site alone might yield channel retention through the COPI pathway, however, this is not the case; both sites must be intact to achieve ER retention (12,22).

14-3-3 disrupts COPI binding on the C- but not the N-terminus

As 14-3-3 binding suppresses β-COP binding to intact K_{2P}3.1 channels (12), we sought to study if 14-3-3 and COPI competed for binding to the isolated N- or C-terminal motifs. Semi-quantitative analysis showed comparable amounts of β-COP bound to N- and C-terminal peptides with a lower level isolated when the C-terminal peptide was phosphorylated (Figure 1C). We hypothesized that the phosphopeptide bound 14-3-3 naturally present in the COS-7 cells interfered with β-COP association. To test this hypothesis, the peptides were preincubated with recombinant 14-3-3β. Again, the N- and C-terminal peptides bound comparable amounts of β-COP, whereas binding to phosphorylated C-terminal peptide was suppressed to undetectable levels (Figure 1B,C). This demonstrated a direct role for 14-3-3 on the C-terminal motif not seen with the N-terminal sequence.

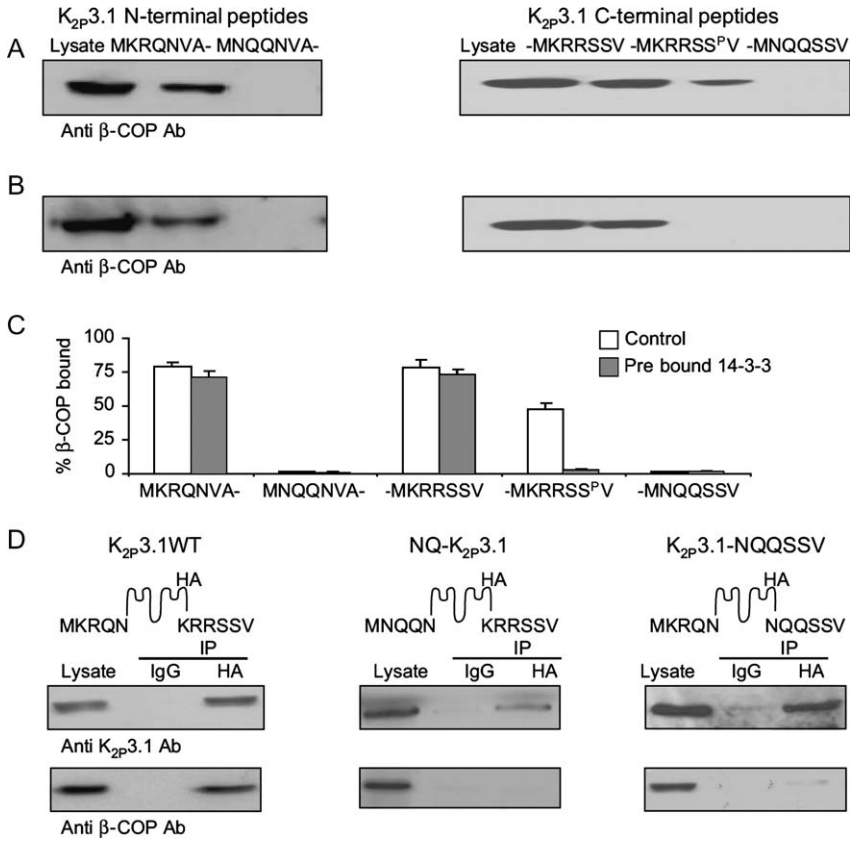

Figure 1: β-COP interacts with both K_{2P}3.1 N- and C-terminal motifs. A) Western blot analysis of native β-COP isolated from COS-7 cells on immobilized peptides analogous to the N- (MKRQNVA-) and C-termini (-MKRRSSV) of K_{2P}3.1. Mutation of basic sites to M*NQ*QNVA- and -M*NQQ*SSV results in failure to bind β-COP. Wild-type C-terminal peptide with phosphate (-MKRRSS^{P}V) binds β-COP less well because of binding of native 14-3-3 (12). B) Binding of recombinant 14-3-3β does not alter β-COP interaction with N-terminal or non-phosphorylated C-terminal peptides but ablates binding to the phosphorylated K_{2P}3.1 C-terminal peptide (-MKRRSS^{P}V). C) Bar chart shows semi-quantitative analysis of β-COP binding to the indicated peptides before and after incubation with recombinant 14-3-3β through computer-assisted densitometric scanning (IMAGE J software). Binding is presented as percentage of β-COP input based on total lysate lane. D) HA-tagged wild-type (WT) and mutant K_{2P}3.1 channels expressed in COS-7 cells purified by immunoprecipitation with HA antibodies and analyzed by Western blot with antibodies to K_{2P}3.1 (top) and β-COP (bottom) show WT channels affinity purified with β-COP, while channels lacking either N- or C-terminal basic motifs fail to copurify β-COP. Ab, antibody; Ha, hemagglutinin epitope tag.

N- and C-termini dibasic motifs are required in intact channels

To confirm β-COP interaction with intact channels was mediated through both the N- and C-termini, hemagglutinin epitope-tagged $K_{2P}3.1$ channels (HA-$K_{2P}3.1$) were expressed in COS-7 cells and their association with native β-COP evaluated. Figure 1D shows affinity purification through the tag with wild-type (WT) and mutant $K_{2P}3.1$ channels. Wild-type channels were purified in association with β-COP. Channels mutated from KR to NQ in the N-terminus (NQ-$K_{2P}3.1$) or those altered from KRR to NQQ in the C-terminus ($K_{2P}3.1$-NQQSSV) were isolated without β-COP (Figure 1D). Thus, mutation of either motif led to the loss of β-COP binding to intact channels. This demonstrates why each mutation alone could suppress ER retention, reconciling prior reports (12,22).

Previously (12), we found that the channels without the final channel residue ($K_{2P}3.1\Delta V^{395}$) failed to reach the surface because of disruption of 14-3-3 binding but that those also lacking their N-terminal basic site (NQ-$K_{2P}3.1\Delta V^{395}$) did not bind β-COP and so gained surface expression. Functional studies performed here in *Xenopus laevis* oocytes demonstrate that $K_{2P}3.1\Delta V^{395}$ channels also reach the surface to pass current if they lack the retention motif from C-terminus ($K_{2P}3.1$-NQQSSΔV^{395}) or both terminal motifs (NQ-$K_{2P}3.1$-NQQSSΔV^{395}), Figure 2 A. All channels reaching the surface demonstrated WT selectivity based on reversal potential studies (not shown) and inhibition by external acidification (Figure 2B) as found for NQ-$K_{2P}3.1\Delta V^{395}$ channels before suggesting that trafficking motifs do not alter biophysical function (12).

p11 binds to the C-terminus of $K_{2P}3.1$ through 14-3-3

Both p11 and 14-3-3 have been implicated in enabling forward transport of $K_{2P}3.1$ through the same C-terminal residues (22). We sought to define the basis for action of p11 by testing the hypothesis that it binds to the channel in a 14-3-3-dependent manner. Native 14-3-3 in COS-7 cells binds to peptides homologous to the phosphorylated C-terminus of $K_{2P}3.1$ (-RRSS^{P}V), $K_{2P}9.1$ (-RRKS^{P}V) and the classical motif found in Raf1 protein (-RSAS^{P}EP) but fails to bind to $K_{2P}3.1$ peptide without the final valine (-RRSS^{P}) or non-phosphorylated peptides Figure 3A). Native p11 in the COS-7 cells showed an identical pattern of interaction with the immobilized peptides, binding only in those cases where 14-3-3 also bound (Figure 3B). In the absence of native or recombinant 14-3-3β, recombinant p11 failed to bind to any of the immobilized peptides (Figure 3C). Conversely, recombinant 14-3-3β bound to peptides with the same pattern observed for native 14-3-3 (Figure 3D) and enabled interaction by recombinant p11 added subsequently (Figure 3E).

p11 is only in some tissues

p11 protein was found in only a subset of tissues examined (Figure 4A) in a manner consistent with its previously reported messenger RNA distribution (23).

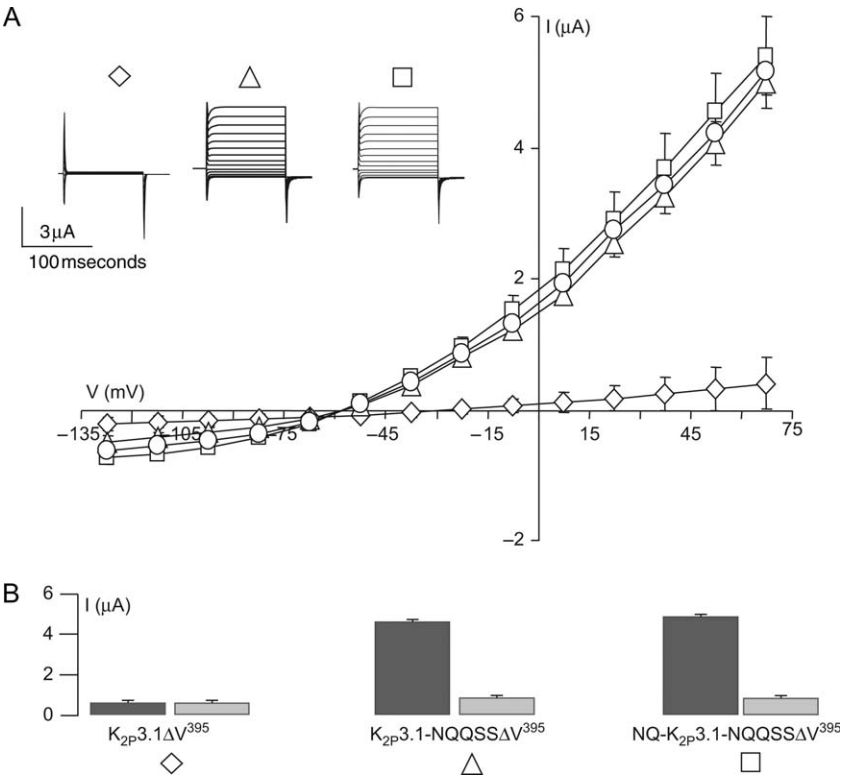

Figure 2: Retention motifs on both termini must be intact to suppress surface expression. A) Current–voltage relationship for $K_{2P}3.1\Delta V^{395}$ (◇), $K_{2P}3.1$-NQQSSΔV^{395} (Δ), NQ-$K_{2P}3.1$-NQQSSΔV^{395} □) and NQ-$K_{2P}3.1\Delta V^{395}$ (○) channels recorded by two-electrode voltage clamp in oocytes depolarized from −135 to +60 mV in 15-mV increments from a holding potential of −80 mV ($n = 6$). Inset shows representative raw currents. B) Mean (±SEM) whole-cell currents evoked by repeated step depolarizations from −80 to +30 mV at 10-second intervals, while bath solution was exchanged from pH 7.5 (dark bar) to pH 6.5 (light bar; $n = 7$–10 oocytes).

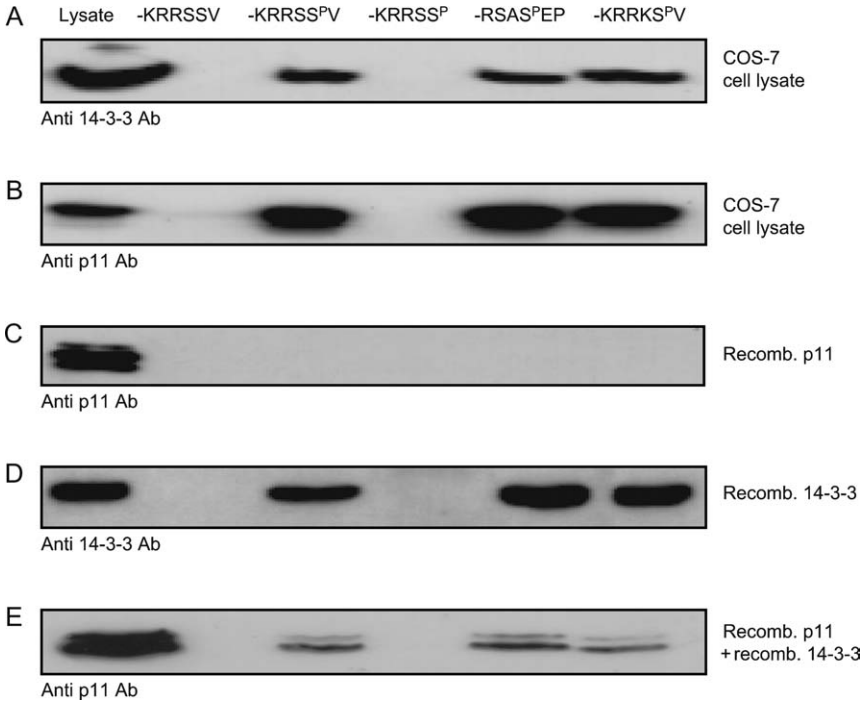

Figure 3: p11 fails to interact with K_{2P}3.1 in the absence of 14-3-3. A) Western blot with anti-14-3-3 antibody shows that native 14-3-3 from COS-7 cells binds to immobilized phosphopeptides homologous to WT K_{2P}3.1 C-terminus (-MKRRSS^{P}V), K_{2P}9.1 C-terminus (-KRRKS^{P}V) and Raf1 peptide (-RSAS^{P}EP). Non-phosphorylated peptides and phosphorylated K_{2P}3.1 peptide without the final valine (-MRRSS^{P}) fail to bind 14-3-3. B) Antibody for p11 shows native p11 to have an identical pattern of purification as for 14-3-3 in panel A. C) Recombinant p11 fails to interact with immobilized K_{2P}3.1 peptides in the absence of 14-3-3. D) Recombinant 14-3-3 binds like native 14-3-3 from COS-7 cells in panel A. E) Prior addition of recombinant 14-3-3 allows recombinant p11 to bind where it had not before (panel C). Ab, antibody; Recomb., recombinant.

Significantly, p11 was not seen in some tissues where K_{2P}3.1 and 14-3-3 were demonstrated (Figure 4B,C). Ubiquitous distribution of 14-3-3 (13), the requirement for phosphorylation-dependent binding of 14-3-3 to achieve forward transport of WT K_{2P}3.1 channels (12), and patchy tissue distribution of p11 argue that its role in trafficking is secondary.

p11 and 14-3-3 require K_{2P}3.1 to interact

Native 14-3-3β can be isolated from rat brain (Figure 5A) or human embryonic kidney (HEK 293) cell lysates that express K_{2P}3.1 (Figure 5B) on glutathione S-transferase (GST)–p11 fusion protein immobilized on beads. Conversely, GST–p11 beads do not isolate 14-3-3 from HEK 293 cells that do not express K_{2P}3.1 (Figure 5C) and do not bind pure

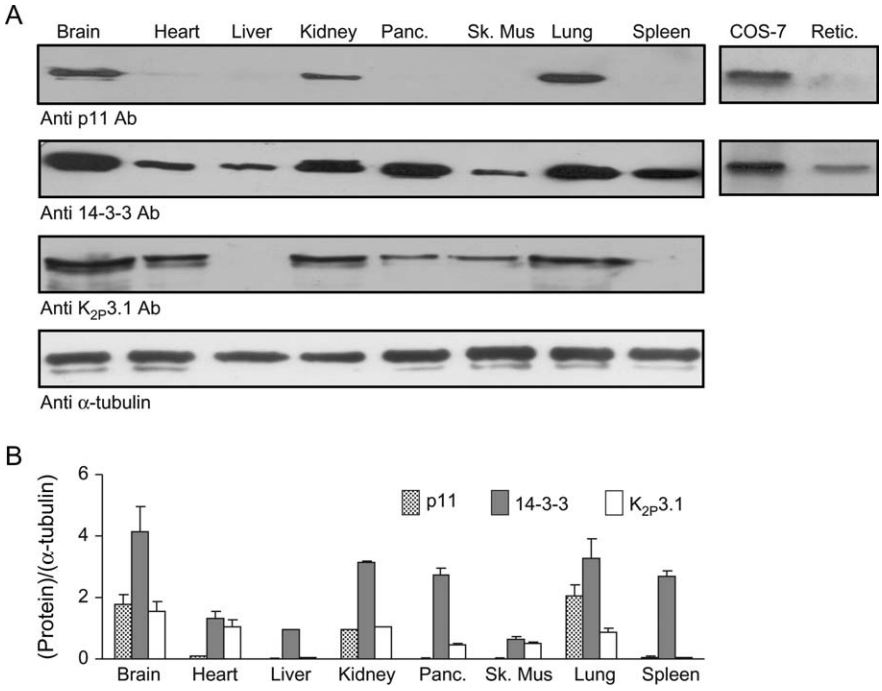

Figure 4: Comparative tissue distribution of p11, 14-3-3β and K_{2P}3.1. A) Western blot analyses from fresh tissue samples of p11 (top row), 14-3-3 (second row), K_{2P}3.1 (third row) and α-tubulin as a loading control (bottom row). B) Semi-quantitative evaluation of levels for each protein through computer-assisted densitometric scanning (IMAGE J software). Protein concentration was expressed relative to α-tubulin concentration in each tissue type studied. Ab, antibody; Panc., pancreas; Retic., rabbit reticulocyte; Sk. Mus, skeletal muscle.

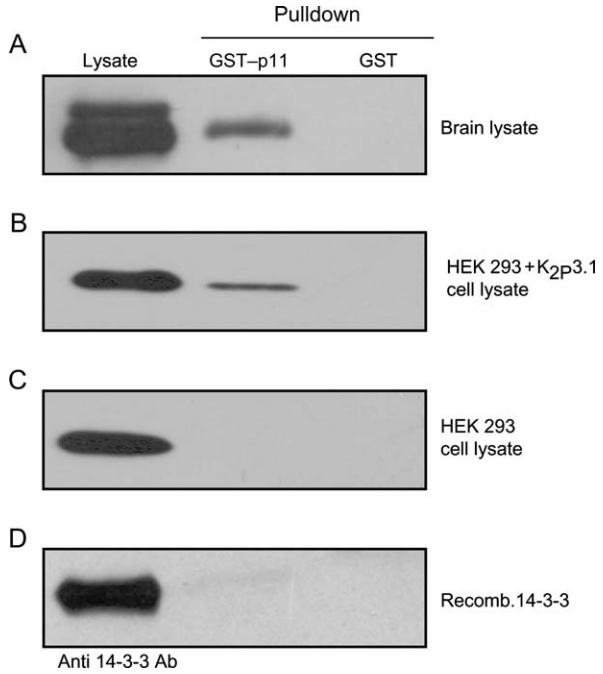

Figure 5: p11 and 14-3-3 only bind in the presence of K_{2P}3.1. Immobilized GST–p11 fusion protein isolates 14-3-3 from A) rat brain lysate and B) HEK 293 cells expressing K_{2P}3.1 but fails to isolate 14-3-3 from C) naïve HEK 293 cells and D) 10 μg added recombinant 14-3-3. Immobilized GST control protein (GST) failed to isolate 14-3-3 from all lysates tested. Ab, antibody; Recomb. 14-3-3, recombinant 14-3-3.

recombinant 14-3-3β (Figure 5D). These findings indicate that p11 and 14-3-3 do not interact on their own but require the channel to form a ternary complex.

Discussion

Quality and quantity control mechanisms are in place to ensure the emergence of correctly assembled membrane proteins. Previously, we found that 14-3-3 binding allows forward transport through inhibition of COPI binding to the N-termini of K_{2P}3.1 and K_{2P}9.1 or the intracellular domain of the immune molecule Iip35 (12). Here, we further explore the mechanism of action of COPI and the basis for binding of p11 to K_{2P}3.1. We find that β-COP is capable of binding to both the N- and C-terminal K_{2P}3.1 sites in isolation (Figure 1) but only assembles with the full channel when both sites are intact, and the C-terminus is free of 14-3-3 (Figure 2). The termini are therefore in direct contact or interact in allosteric fashion. As bound 14-3-3 is a direct steric impediment to β-COP binding on the C-terminus (the two sites overlap), 14-3-3 may also directly mask the N-terminus of the folded channel complex.

p11 has been implicated in targeting of vesicles to cytoskeleton and membrane–vesicle fusion (24) and, recently, in forward transport of an array of membrane proteins (17–20). While Girard et al. (22) suggest p11 binds directly to the C-terminus of K_{2P}3.1 to enhance surface expression, we find here that purified p11 cannot bind in the absence of added 14-3-3 (Figure 3). We submit that they observed binding of p11 through unappreciated 14-3-3 endogenous to the yeast cells (25) and rabbit reticulocyte lysates (Figure 4A). Here, purification of each protein and reconstitution allowed direct demonstration that phosphorylation-dependent binding of 14-3-3 to the channel C-terminus was required to enable subsequent association of p11 (Figures 3 and 5). Ubiquitous expression of 14-3-3 and selective distribution of p11 support the idea that the former plays a primary role in trafficking and the latter serves as a modulator. This thesis is validated by observation of K_{2P}3.1 in the heart, pancreas and skeletal muscle, where little or no p11 protein is found (Figure 4). While we suspect 14-3-3 will have a role with most proteins whose targeting is modified by p11, Renigunta et al. (21) find p11 to bind in a 14-3-3-independent manner to a distinct motif in K_{2P}3.1 (not present in K_{2P}9.1) that is approximately 100 residues upstream of the C-terminus; this indicates the need for more study of the roles of p11 in trafficking.

Release from ER is achieved through masking of retention signals (26,27) or powerful forward trafficking signals (15,28). We have proposed 14-3-3 to have an important role in forward transport through operation at sites that suppress COPI binding to retention motifs. Previously, we found the pathway to be phosphorylation dependent with K_{2P}3.1, K_{2P}9.1, nAChR and Iip35 (12). Recent studies extend the concept identifying 14-3-3 binding at release sites that do not require phosphorylation (14). The growing list of proteins recognized to require 14-3-3 to achieve membrane expression and those subject to the modifying influence of p11 reveals these trafficking regulators to be of emerging importance.

Materials and Methods

Tissue preparation

Fresh tissue samples were collected from Wistar rats, washed in PBS, weighed and diced. Further disruption and homogenization were performed in ice-cold Buffer A (150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton-X-100, 0.1% Na deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 10 mM Tris–HCl pH 7.2) plus protease inhibitors and incubated on ice for 30 min, followed by centrifugation to achieve whole cell lysate or crude membrane factions. Protein concentration was determined by bicinchoninic acid protein assay reagent (Pierce). Samples were diluted in 2× sample buffer, and 40 μg of each tissue sample was loaded on SDS–PAGE for analysis.

Antibodies and Western blotting analysis

Purchased polyclonal (p) and monoclonal (m) antibodies used included KCNK3 (p; Sigma-Aldrich), 14-3-3β (m, SC1657; Santa Cruz Biotechnology), β-COP (p, PA1601; Affinity Bioreagent), hemagglutinin tag (m, 1583816; Roche Molecular Biochemicals) and annexin II (light chain) clone LC148 (MP Biomedicals). SDS–PAGE (6, 12 or 18%) and Western blotting were preformed by standard methods using enhanced chemiluminescence (Pierce).

Molecular biology and production of recombinant protein

All K_{2P}3.1 and 14-3-3 constructs were created as previously described (12). Image clone #3889914 was subcloned in frame with GST into pGEX-6P-1 (Amersham Biosciences) between *Bam*H1 and *Eco*R1 to create a GST fusion of full-length p11. Cultures of *Escherichia coli* (BL21) were transformed with pGEX recombinant vectors. Fusion proteins were purified and coupled on Glutathione Sepharose 4B and released from the GST fusion protein with PreScission protease according to the manufacturer's protocol (Amersham Biosciences). GST–p11 was also used in binding studies following immobilization on Glutathione Sepharose 4B according to the manufacturer's instructions (Amersham Biosciences).

Peptide binding studies

All peptides and phosphopeptides were synthesized by the W.M. Keck Biotechnology Resource Center (New Haven, CT, USA) and coupled to Sulfolink Coupling Gel (Pierce) as previously described (12). COS-7 or HEK 293 whole cell lysate was prepared by solubilization of cells for 2 h at 4°C in 150 mM NaCl, 5 mM EDTA, 1% Triton-X-100, 0.1% Na deoxycholate and 10 mM Tris–HCl pH 8.0 and centrifuged at 100 000 × ***g*** for 30 min. The resultant supernatant, recombinant p11 or 14-3-3 or rat brain post-nuclear supernatant, was incubated with various immobilized peptides or GST fusion proteins at 4°C for 1 h. Unbound proteins were removed by three washing steps with a cold PBS with 1% Triton-X-100, and bound proteins were eluted with sample buffer for analysis by Western blotting.

Immunoprecipitation protocols

Affinity purification of HA-tagged K_{2P}3.1 WT and channel mutants from COS-7 cells was performed 24 h after transfection with Lipofectamine (Invitrogen) by incubation for 1 h in immuno-precipitation (IP) buffer (50 mM Tris, 100 mM NaCl and 1% Triton-X-100, pH 8.5) with 0.1% SDS and centrifuged at 13 000 × ***g*** for 20 min at 4°C. Resultant supernatants were immunoprecipitated with 5-μg HA monoclonal antibody or control IgG overnight at 4°C, purified on protein G–Sepharose gel (Amersham Biosciences), washed with IP buffer, eluted by boiling in sample buffer and loaded onto SDS–PAGE with 40 μg of lysate in control lane.

Electrophysiology

Oocytes were isolated from *X. laevis* frogs (Nasco), treated with collagenase to ease removal of the follicular layer and injected with 0.1–1 ng of K_{2P}3.1 WT or mutant channel complementary RNA in 46 nL of sterile water. Currents were measured 36–48 h after injection by two-electrode voltage clamp (Warner Instruments Corp.). Data were filtered at 1 kHz and sampled at 4 kHz. Electrodes of 1.5-mm borosilicate glass tubes (Garner Glass Co.) contained 3 M KCl and had resistances of 0.3–1 mOhm. Recordings were performed at room temperature with perfusion of 0.4–1 mL/min ND96 (in mM) 93 NaCl, 5 KCl, 1 $MgCl_2$, 0.3 $CaCl_2$ and 5 HEPES, pH 7.5 or 6.5 with NaOH or HCl. Holding potential was −80 mV. Currents were evoked by step depolarization from −135 to +60 mV in 15-mV increments. Acid sensitivity was determined by exchanging external bath solutions during repeated step depolarization from holding potential to +30 mV at 10-second intervals.

Acknowledgments

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