Scavenging of 14-3-3 proteins reveals their involvement in the cell-surface transport of ATP-sensitive K^+ channels

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Summary

Arginine (Arg)-based endoplasmic reticulum (ER)-localization signals are involved in the quality control of different heteromultimeric membrane protein complexes. ATP-sensitive potassium (K_{ATP}) channels are unique because each subunit in the heterooctamer contains an Arg-based ER-localization signal. We have dissected the inactivation events that override the ER-localization activity of the eight peptide-sorting motifs. Employing a 14-3-3-scavenger construct to lower the availability of 14-3-3 proteins, we found that 14-3-3 proteins promote the cell-surface expression of heterologously expressed and native K_{ATP} channels. 14-3-3 proteins were detected in physical association with K_{ATP} channels in a pancreatic β-cell line. Our results suggest that the Arg-based signal present in Kir6.2 is sterically masked by the SUR1 subunit. By contrast, 14-3-3 proteins functionally antagonized the Arg-based signal present in SUR1. The last ten amino acids were required for efficient 14-3-3 recruitment to multimeric forms of the Kir6.2 C-terminus. Channels containing a pore-forming subunit lacking these residues reached the cell surface inefficiently but were functionally indistinguishable from channels formed by the full-length subunits. In conclusion, 14-3-3 proteins promote the cell-surface transport of correctly assembled complexes but do not regulate the activity of K_{ATP} channels at the cell surface.

Key words: Membrane protein assembly, Quality control, ER-localization signals, ABC proteins, Inward rectifier potassium channels, 14-3-3 proteins

Introduction

ATP-sensitive potassium (K_{ATP}) channels are metabolic sensors that couple the electrical excitability of many cell types to their metabolic status (Aguilar-Bryan and Bryan, 1999). They are hetero-octameric complexes formed by four inward rectifier K^+ channel subunits (Kir6.1 or Kir6.2) and four sulfonylurea receptor subunits (SUR1 or SUR2). Both subunits are involved in the metabolic regulation of the channel, so it is essential that partially assembled K_{ATP}-channel complexes are excluded from the plasma membrane. Heteromultimeric assembly is controlled by a checkpoint mechanism based on endoplasmic reticulum (ER) localization motifs present in both Kir6.2 and SUR subunits (Zerangue et al., 1999). These arginine (Arg)-based peptide retention signals prevent unassembled subunits or partially assembled complexes from reaching the cell surface. By employing an affinity purification approach, we previously showed that the Arg-based ER-localization signal (RKR) present in the cytoplasmic C-terminus of Kir6.2 is recognized by the coat protein I (COPI) vesicle coat, as well as by 14-3-3 proteins (Yuan et al., 2003). COPI binding can explain the ER-localization activity of the RKR motif. Experiments with multimeric reporter proteins suggest that recruitment of 14-3-3 proteins is involved in the forward transport of K_{ATP} channel multimers to the cell surface (Yuan et al., 2003). It is therefore possible that 14-3-3 proteins are involved in inactivating the RKR motifs during the course of channel assembly.

All membrane proteins for which 14-3-3-dependent forward transport has been described to date also contain a COPI-interacting motif (Kuwana et al., 1998; Mrowiec and Schwappach, 2006; O'Kelly et al., 2002; Rajan et al., 2002; Shikano et al., 2005; Shikano et al., 2006; Yuan et al., 2003). Recent results from yeast imply that 14-3-3-dependent forward transport might depend on specific isoforms of 14-3-3 (Michelsen et al., 2006). For some complexes, e.g. the MHC class II complex and the two-pore-domain K^+ channel KCNK3, recruitment of 14-3-3 required phosphorylation of a residue in the 14-3-3-binding motif (Anderson and Roche, 1998; Kuwana et al., 1998; O'Kelly et al., 2002; Rajan et al., 2002). Recruitment as a consequence of phosphorylation implies that 14-3-3-dependent forward transport will be regulated by activation of signal transduction cascades (Shikano et al., 2006). In other cases, such as Kir6.2, where 14-3-3 proteins are involved in monitoring the correct assembly of multimeric complexes, phosphorylation is not required.

In some proteins, such as KCNK3, the 14-3-3- and COPI-binding sites are physically distinct (O'Kelly et al., 2002). By contrast, the COPI-interacting motif and the 14-3-3-binding motif of Kir6.2 both lie in the C-terminus, and they physically

overlap (Yuan et al., 2003). Although neither binding site has been fully mapped, functional studies indicate that the RKR motif contributes to both. However, although the Arg-based signal of Kir6.2 is necessary to recruit 14-3-3 to a multimeric reporter protein (Yuan et al., 2003), it is not known whether it is sufficient for 14-3-3 binding. The K_{ATP}-channel is unique in that it has COPI-interacting motifs in both subunits (Kir6.2 and SUR1) of the heteromultimer. Binding of 14-3-3 requires multiple copies of the Kir6.2 tail presented in close proximity to each other, which suggests that the spatial arrangement of the two peptide-binding grooves in the 14-3-3 dimer acts as a template that checks the assembly state of the membrane protein complex (Yuan et al., 2003).

During assembly of the K_{ATP}-channel complex, the individual Kir6.2 and SUR1 subunits transiently expose an Arg-based ER-localization signal. These signals are inactivated as a consequence of heteromultimeric assembly. Consequently, the assembly state and forward transport are coupled, and only properly assembled hetero-octamers reach the cell surface. Steric masking is a simple way of explaining this coupling in molecular terms: co-assembly might render the Arg-based signals present in Kir6.2 and SUR1 inaccessible to the COPI-vesicle coat. Unfortunately, currently available homology models of the assembled K_{ATP} channel do not provide structural insights into how the regions containing the Arg-based signal of either subunit are oriented with respect to the rest of the proteins in the complex. However, the finding that 14-3-3 proteins could be involved in the forward transport of K_{ATP} channels (Yuan et al., 2003) implies that steric masking by the other subunit is not sufficient to explain inactivation of all the Arg-based signals present in the complex.

Previous studies using soluble protein-A fusion proteins and reporter membrane proteins have shown that the C-terminal tail of Kir6.2 can recruit 14-3-3 proteins when the 14-3-3-binding motif is present in multiple copies that lie in close proximity (Yuan et al., 2003). However, the role of 14-3-3 in the native K_{ATP}-channel complex, composed of full-length subunits, remains unclear. In particular, it is not known whether 14-3-3 proteins are involved in K_{ATP}-channel transport to the cell surface, whether they functionally inactivate the Arg-based signals present in Kir6.2 and SUR1, and whether recruitment of 14-3-3 alters the functional properties of K_{ATP} channels at the cell surface. To explore these questions, we have investigated the role of 14-3-3 proteins in the assembly and functional activity of native K_{ATP} channels.

It is difficult to manipulate cellular 14-3-3 proteins in order to assess their involvement in a given process of interest. Common approaches include the use of dominant-negative mutants (either incapable of ligand binding or of dimerization), downregulation by RNA-interference, and overexpression. There are seven isoforms of 14-3-3 expressed at high levels in most mammalian cell types. Applying any of the approaches listed above requires knowledge on the specific isoform involved and none of the manipulations will alter the total pool of 14-3-3 proteins. We therefore developed a novel 14-3-3-scavenger protein, which enabled us to show that 14-3-3 proteins promote the cell-surface expression of the native K_{ATP}-channel complex. Furthermore, our data offer mechanistic insight into 14-3-3 action, because they suggest that 14-3-3 proteins function by counteracting the Arg-based ER-localization signal present in the SUR1 subunit of the channel complex.

Results

A 14-3-3 scavenger interferes with 14-3-3-dependent surface expression of membrane proteins

To study the role of 14-3-3 proteins in the assembly and surface expression of K_{ATP} channels, we manipulated the availability of endogenous 14-3-3 proteins using an artificial 14-3-3-scavenger protein. This was constructed (Fig. 1A) by fusion of protein G, the tetramer-forming coiled-coil domain pLI (Harbury et al., 1993) and the R18 peptide that binds 14-3-3 proteins with high affinity (Wang et al., 1999). We refer to this fusion protein as pGpLI-R18. To assess the efficacy of pGpLI-R18 in scavenging 14-3-3 proteins, homogenates of *Xenopus* oocytes expressing pGpLI-R18 were incubated with IgG-Sepharose (Fig. 1B). This resulted in a cellular extract depleted of 14-3-3 proteins, and an eluate containing both pGpLI-R18 and 14-3-3 proteins. This indicates that a large portion of cellular 14-3-3 proteins are bound to the 14-3-3 scavenger: consequently, there will be less 14-3-3 available for binding to other interaction partners, particularly those that bind with lower affinity.

We next tested whether pGpLI-R18 was able to prevent surface expression of KCNK3, which is known to require 14-3-3 for cell-surface expression (O'Kelly et al., 2002; Rajan et al., 2002). The β2-adrenergic G-protein-coupled receptor and the inward rectifier K^+ channel Kir2.1 were used as negative controls because there is no evidence that they require 14-3-3 proteins to reach the cell surface. Surface expression was assessed by antibody labeling of extracellular epitopes combined with luminometry, as described previously (Zerangue et al., 1999). Surface expression of the β2-adrenergic receptor and Kir2.1 were unaffected by coexpression of pGpLI-R18, whereas that of KCNK3 was significantly reduced (Fig. 1C). We therefore conclude that the 14-3-3 scavenger can be used as a tool to probe the involvement of 14-3-3 proteins in the surface expression of membrane proteins.

14-3-3 proteins play a role in the cell-surface expression of K_{ATP} channels

We used pGpLI-R18 as a tool to assess whether the surface expression of K_{ATP} channels is sensitive to 14-3-3 proteins in *Xenopus* oocytes. Coexpression of pGpLI-R18 with the two K_{ATP}-channel subunits, Kir6.2 and SUR1, reduced surface expression of recombinant K_{ATP}-channels by more than 50% (Fig. 2A). Similar results were found for native K_{ATP} channels in the INS-1 pancreatic β cell line (Fig. 2B). INS-1 cells were infected with a retroviral construct encoding pGpLI-R18, and surface expression assessed by recording the magnitude of endogenous K_{ATP} (Kir6.2/SUR1) currents, using the standard whole-cell configuration of the patch-clamp method. Currents were initially small but increased with time after establishment of the whole-cell configuration, as ATP dialysed out of the cell (Trube et al., 1986). The amplitude of the steady-state washout current, which provides a measure of the maximal available K_{ATP} channels, was significantly smaller in cells expressing the 14-3-3 scavenger (49% of control; Fig. 2B). Steady-state currents were blocked by the K_{ATP}-channel inhibitor tolbutamide, indicating that they flow through K_{ATP} channels.

Thus, these data provide evidence that scavenging of 14-3-3 proteins leads to a decrease in the surface expression of native K_{ATP} channels.

The finding that 14-3-3 proteins promote transport of K_{ATP} channels to the cell surface predicts that 14-3-3 proteins should physically associate with K_{ATP} channels. To test this idea, INS-1 cells were infected with a retroviral vector encoding a variant

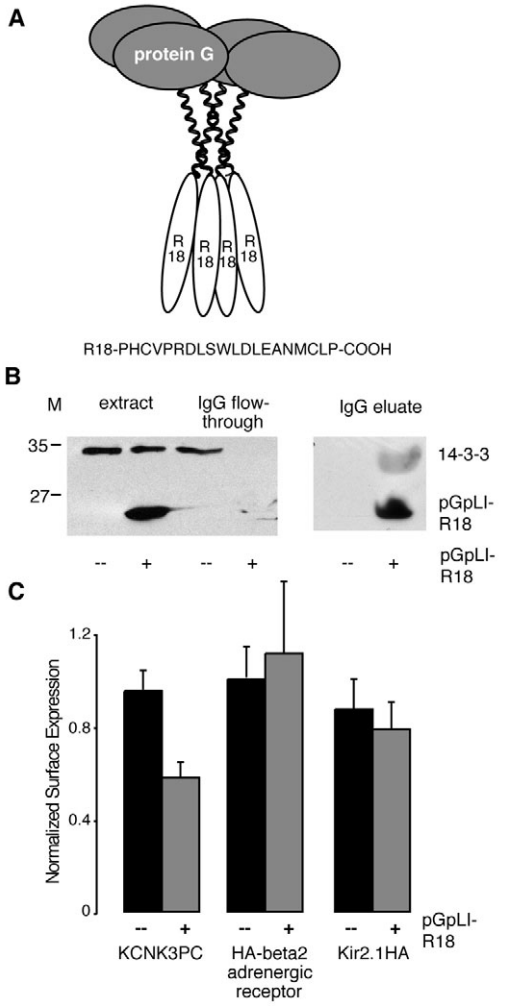

Fig. 1. Strategy for interfering with 14-3-3-dependent surface expression of membrane proteins. (A) Schematic diagram of the fusion protein employed to sequester 14-3-3 proteins and (below) sequence of the R18 peptide. (B) Endogenous 14-3-3 protein in *Xenopus* oocytes is associated with the scavenger protein pGpLI-R18. Western blots of 14-3-3 and pGpLI-R18 in cellular extracts (extract) from uninjected or pGpLI-R18-injected oocytes, and in the flow-through (IgG flow-through) and eluate (IgG eluate) after incubation of the extracts with IgG-Sepharose. Gels were probed with an anti-14-3-3 β antibody detecting all isoforms: the pGpLI-R18 protein is visible because of its protein-G moiety. M indicates molecular mass in kDa. (C) Surface expression of KCNK3, β2 adrenergic receptor and Kir2.1 measured in the absence (black bars) and presence (grey bars) of the 14-3-3-scavenger pGpLI-R18. Data are normalized to the values measured in the absence of pGpLI-R18. KCNK3PC, n=35 (six batches of oocytes); β2 adrenergic receptor, n=15 (three batches of oocytes); Kir2.1HA, n=13 (three batches of oocytes). Only for KCNK3 did 14-3-3 sequestration significantly reduce surface expression (P<0.0005).

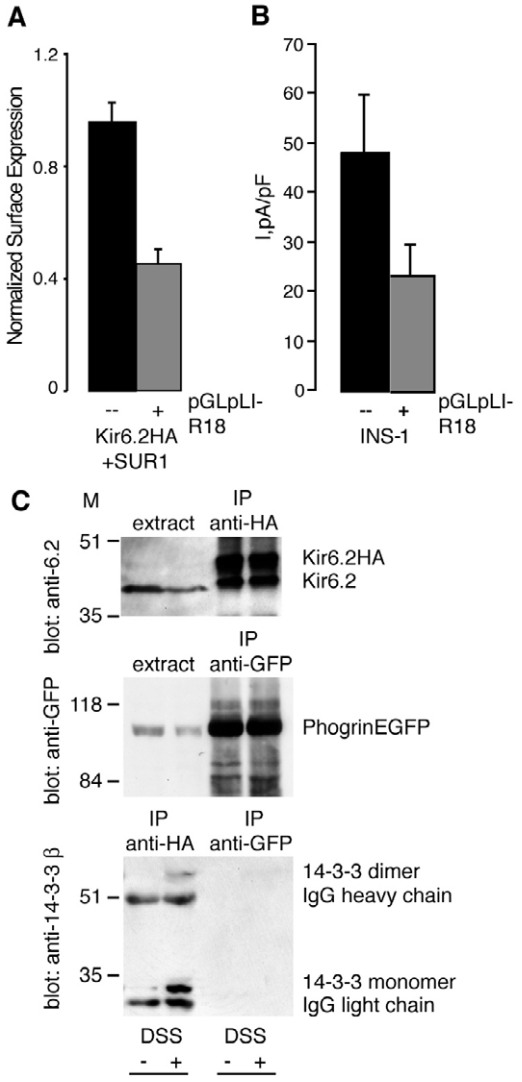

Fig. 2. Surface expression of K_{ATP} channels is impaired by 14-3-3 sequestration. (A) Surface expression in oocytes of K_{ATP} channels assembled from independently expressed Kir6.2HA and SUR1 in the absence (black bars) or presence (grey bars) of the 14-3-3-scavenger pGpLI-R18 (n=50, nine batches of oocytes; P<0.0001). (B) Mean steady-state whole-cell currents recorded from control INS-1 cells (n=6) and INS-1 cells expressing pGpLI-R18 (n=6; P<0.05). ATP concentration-inhibition relationships for K_{ATP} currents were almost identical for the two groups of cells (data not shown). (C) Coimmunoprecipitation of 14-3-3 proteins and K_{ATP} channels. Cells were treated with the crosslinker DSS where indicated. (Top and middle panels) Cell extracts (1-3%) and immunoprecipitates (IP, 40%) obtained with the indicated antibodies were resolved by SDS-PAGE. M indicates molecular mass in kDa. Western blots were probed with antibodies against Kir6.2 to detect Kir6.2 and Kir6.2-11HA (top panel), and against GFP to detect phogrin-EGFP (middle panel). (Bottom panel) Immunoprecipitates (40%) obtained with the indicated antibodies, probed with antibodies to 14-3-3 proteins. Note that the anti-14-3-3 β antibody recognizes all 14-3-3 isoforms. Monomeric and dimeric species of 14-3-3 proteins are indicated (the dimer was only observed after crosslinking). The presence of an IgG signal in lanes 1 and 2 is because both anti-HA and anti-14-3-3 antibodies were of the same species (mouse). For anti-GFP IP a rabbit antibody was used, hence there is no IgG signal in lanes 3 and 4.

of Kir6.2 containing an extracellular hemagglutinin (HA)-epitope tag (Zerangue et al., 1999), and phogrin fused to enhanced green fluorescent protein (EGFP) (Pouli et al., 1998). The HA-tag was used to facilitate immunoprecipitation of K_{ATP} channels in complex with cytosolic-interacting proteins. The membrane protein phogrin is a well-characterized marker of secretory granules in pancreatic β cells (Wasmeier and Hutton, 1996). Since most K_{ATP} channels appear to localize to the insulin secretory granules in pancreatic β cells (Geng et al., 2003; Varadi et al., 2006), we used phogrin as a control in our immunoprecipitation experiments. INS-1 cells were permeabilized, treated with the crosslinker disuccinimidyl suberate (DSS) and homogenized. Total membranes were solubilized and subjected to immunoprecipitation with anti-HA or anti-GFP antibody (Fig. 2C). The anti-HA immunoprecipitate was probed with an anti-Kir6.2 antiserum (top panel) and the anti-GFP immunoprecipitate with the anti-GFP serum (middle panel). Both immunoprecipitates were tested for the presence of 14-3-3 proteins (bottom panel). Coimmunoprecipitation of 14-3-3 proteins was detected for the K_{ATP}-channel complex but not for the phogrin-EGFP fusion protein (Fig. 2C). Thus, we conclude that 14-3-3 proteins are physically associated with K_{ATP} channels in INS-1 β-cells.

The Arg-based signals of K_{ATP} channels are crucial for 14-3-3 effects on forward transport

Several reports suggest that 14-3-3 proteins override ER localization mediated by Arg-based signals (Kuwana et al., 1998; Michelsen et al., 2006; Shikano and Li, 2003; Yuan et al., 2003). Thus, we next asked whether 14-3-3 scavenging only affected the cell-surface transport of K_{ATP} channels when Arg-based signals are present in the channel complex. For these experiments, we used a SUR1-Kir6.2 fusion protein, in which the C-terminus of SUR1 was linked to the N-terminus of Kir6.2 (Fig. 3). This was necessary because mutation of the Arg-based signal allows SUR1 and Kir6.2 to reach the cell surface independently of each other (Zerangue et al., 1999), which complicates interpretation of the experiments. Since they contain both partner subunits, when SUR1-Kir6.2 fusion proteins are heterologously expressed in *Xenopus* oocytes they reach the cell surface irrespective of the presence of an Arg-based signal (black bars in Fig. 3).

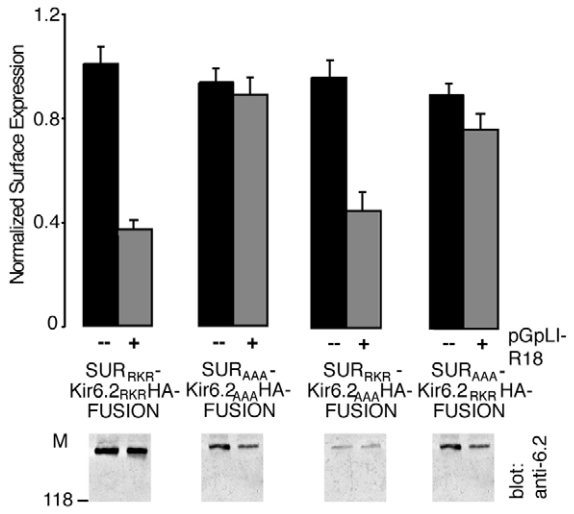

Fig. 3. Role of Arg-based signals in 14-3-3-dependent forward transport. Columns show surface expression of WT and mutant SUR1-Kir6.2-fusion proteins (as indicated) in the absence (black bars) or presence (grey bars) of the 14-3-3-scavenger pGpLI-R18. For each fusion protein, data were obtained from >90 oocytes (from 18 to 22 batches) and were normalized to the values measured in the absence of pGpLI-R18 (see Materials and Methods). Data for $SUR1_{RKR}$-$Kir6.2_{RKR}$ and $SUR1_{RKR}$-$Kir6.2_{AAA}$ were significantly different ($P<0.0001$) in the presence and absence of pGpLI-R18. Western blots of oocyte homogenates for the same constructs probed with an anti-Kir6.2 antibody are shown underneath the bar graph. For each construct, there was no difference in expression when pGpLI-R18 was present. M indicates molecular mass in kDa.

We tested the effect of pGpLI-R18 on the surface expression of K_{ATP}-channel complexes containing either wild-type (WT) or mutated Arg-based trafficking signals in *Xenopus* oocytes (grey bars in Fig. 3). K_{ATP} channels formed from the WT fusion protein were highly sensitive to scavenging of 14-3-3, surface expression being reduced to 30% of control (Fig. 3). By contrast, sequestration of 14-3-3 proteins by pGpLI-R18 had no effect on the surface expression of a SUR1-Kir6.2 fusion protein in which both Arg-based ER-localization signals were mutated (Fig. 3). This result is consistent with previous studies of K_{ATP}-reporter proteins (Yuan et al., 2003) and further demonstrates that 14-3-3 proteins are involved in inactivating Arg-based ER-localization signals present in K_{ATP}-channel complexes assembled from full-length subunits. It also indicates that the sole role of 14-3-3 proteins is to counteract the RKR signal directly or indirectly. They do not appear to have a positive effect on forward trafficking per se, because they have no effect on surface expression when all eight of the Arg-based ER-localization signals present in K_{ATP}-channel complexes are mutated.

When expressed alone, Kir6.2 and SUR1 are retained within the ER because of their Arg-based signals (Zerangue et al., 1999). In the absence of SUR1, Kir6.2 can still form tetramers, but these are retained inside the cell, indicating that co-assembly with other Kir6.2 subunits cannot mask the Arg-based signals. Only when SUR1 is present is Kir6.2 able to reach the membrane, which implies that inactivation of the Arg-based signal is a consequence of co-assembly with SUR1. The simplest explanation is that the RKR motif of Kir6.2 is sterically masked in the presence of SUR1 (Zerangue et al., 1999), thereby preventing COPI binding. However, this is difficult to reconcile with the fact that scavenging of 14-3-3 proteins prevents surface expression only when Arg-based signals are present in the K_{ATP}-channel complex (Fig. 3). This implies that, 14-3-3 binding contributes to forward transport and, thus, the RKR motif on either Kir6.2 or SUR1 must be accessible rather than sterically masked by the partner subunit.

Previous results using SUR1-Kir6.2 fusion proteins with a single mutated Arg-based signal suggested that the Arg-based motif in SUR1, but not that in Kir6.2, is partially exposed in the fully assembled octameric channel (Zerangue et al., 1999). To distinguish which of the two Arg-based signals (Kir6.2 or SUR1) is responsible for the observed 14-3-3-dependence, we used SUR1-Kir6.2 fusion proteins in which the Arg-based signal was mutated in either SUR1 or Kir6.2 (Fig. 3). Fig. 3 shows that mutation of the Arg-based signal in Kir6.2 alone has no effect on the ability of 14-3-3 scavenging to prevent surface expression. By contrast, when the RKR motif of SUR1

was mutated, the ability of pGpL1-R18 to reduce surface expression was abolished. This is consistent with the idea that the Arg-based signal in Kir6.2 is not accessible in the fully assembled K_{ATP}-channel complex (Zerangue et al., 1999) and argues that 14-3-3 proteins reduce the efficacy of the Arg-based ER-localization signal present on SUR1.

A simple explanation for this result could be that 14-3-3 binding to SUR1 sterically interferes with COPI binding. However, we were unable to consistently demonstrate direct binding of 14-3-3 proteins to the L1 loop of SUR1 in which the Arg-based signal is located (data not shown). The availability of 14-3-3 proteins clearly enhances the surface expression of the K_{ATP}-channel complex (Fig. 3), and we were able to detect binding of 14-3-3 to the K_{ATP}-channel complex (Fig. 2) but not the Kir6.2 tetramer in the absence of SUR1 (data not shown). In the context of the heteromeric complex, the Kir6.2-binding site for 14-3-3 must be distinct from the RKR motif because mutation of this motif does not affect the ability of the 14-3-3-scavenger pGpLI-R18 to prevent cell-surface expression (Fig. 3). This is not unreasonable, because some complexes between 14-3-3 proteins and ligand proteins have revealed an interplay between low-affinity and high-affinity sites as well as several low-affinity 14-3-3-binding sites (Giles et al., 2003; Wurtele et al., 2003).

14-3-3 binding to Kir6.2 is reduced when the distal C-terminus of Kir6.2 is truncated

We next tested this hypothesis by dissecting the roles of 14-3-3 and COPI in sorting of the K_{ATP} channel. To do this, we sought a mutation that abolishes 14-3-3 binding without disturbing the ER-localization activity of the Arg-based signal.

Binding of 14-3-3 to the C-terminal tail of Kir6.2 can only be detected in pull-down or coimmunoprecipitation assays when the tail is presented in multiple copies in close proximity (Michelsen et al., 2006; Yuan et al., 2003). For binding assays, we therefore employed tetrameric-affinity constructs consisting of protein A and the coiled-coil domain of pLI fused to the last 36 amino acid residues of Kir6.2, followed by a hexahistidine tag for purification of the full-length bait protein (Yuan et al., 2003). We then truncated the last ten (RKRΔC10), or 16 (RKRΔC16), residues of Kir6.2, which lie downstream of the Arg-based signal (Fig. 4A). The corresponding proteins were purified from *E. coli*, immobilized, and incubated with HeLa cytosol to pull-down 14-3-3 proteins (Fig. 4B). We probed the precipitates with an antibody recognizing all seven isoforms of 14-3-3 (anti-β antibody). Interestingly, both truncations reduced 14-3-3 binding to the same extent as mutation of the Arg-based signal in full-length Kir6.2 to KKK, despite the fact that neither truncation deleted the RKR motif (Fig. 4C). Mutation of the RKR signal to AAA abolished 14-3-3 binding to the WT and truncated variants of the distal Kir6.2 C-terminus. The data therefore suggest that C-terminal residues other than the RKR sequence contribute to 14-3-3 binding by Kir6.2. Since the last ten residues of Kir6.2 contain no known protein-interaction motif, we tested the sequence specificity of this effect by mutating the sequence KFSISPDSLS to KFSIS**A**DSLS (RKR_P-A), KFSIS**G**DS**G**S (RKR_PL-GG), or KF**A**IAPDA**L**A (RKR_S-A) (residues deviating from the WT sequence are indicated in bold). All three sequence variants show 14-3-3 binding very similar to the WT sequence (Fig. 4C), indicating that neither the Pro residue nor the Ser residues in this sequence were specifically required for 14-3-3 recruitment. Nevertheless, deleting these residues might decrease 14-3-3 recruitment without affecting the efficacy of the Arg-based signal as an ER-localization signal.

Reporter proteins distinguish between the effects of 14-3-3 and COPI binding

The Arg-based signal was present in both truncated Kir6.2 proteins; therefore, we first compared their ability to prevent a

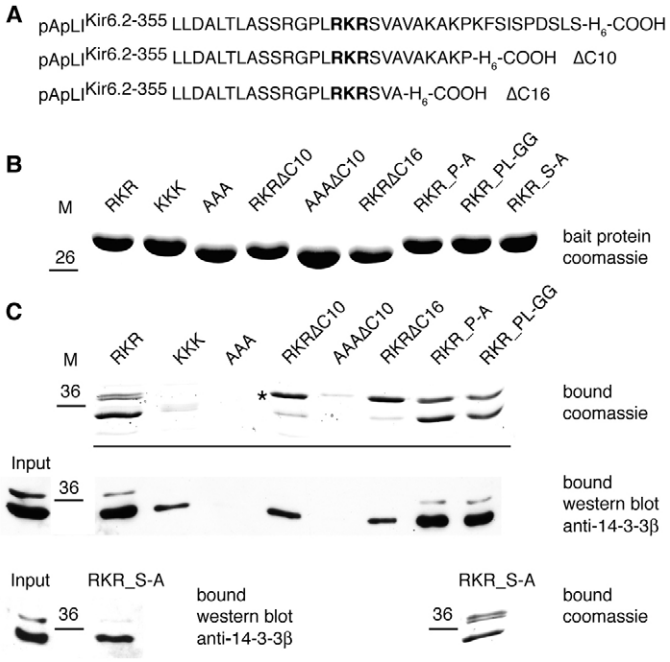

Fig. 4. C-terminal residues other than RKR contribute to 14-3-3 recruitment by the Kir6.2 tail. (A) Sequence of the WT, ΔC10 and ΔC16 Kir6.2 C-terminal tails used in the reporter constructs. (B) Coomassie-Blue-stained gels demonstrating equal loading of the Kir6.2 reporter proteins. The immobilized bait protein was eluted using SDS-PAGE sample buffer, resolved by electrophoresis and stained using Coomassie Blue. M indicates molecular mass in kDa. (C) HeLa cell cytosol (2% input) and eluates after incubation of cytosol with IgG-Sepharose preloaded with tetrameric protein A fusions (pApLI) of the indicated Kir6.2-derived tails (bait proteins, see B). RKR is the full-length WT sequence, KKK and AAA (Michelsen et al., 2006; Yuan et al., 2003; Zerangue et al., 1999) are inactive variants of the RKR motif (KKK preserves the charge), ΔC10 and ΔC16 are the C-terminal deletions (see A), RKR_P-A (KFSIS**A**DSLS), RKR_PL-GG (KFSIS**G**DS**G**S) and RKR_S-A (KF**A**IAPDA**L**A) are mutant variants of the last ten residues of Kir6.2 (pertinent sequence in parentheses, bold characters indicate mutated residues). (Top panel) Coomassie-Blue-stained gel of the eluates (80%). (Bottom panel) Western blot analysis of the same samples (10%). The membrane was probed with a pan-reacting antibody raised against the β isoform of 14-3-3 proteins. The lowest panel combines the results for the RKR_S-A variant. The asterisk indicates a band that was not detected by western blotting against 14-3-3. To confirm that the band did not contain 14-3-3, e.g. in a modified form, we analyzed the protein by mass spectrometry. This revealed that the band contains p32/C1QBP, the precursor of a strongly acidic protein destined for the mitochondrial matrix (Dedio et al., 1998; Muta et al., 1997). The results for all constructs were very similar in four independent experiments.

monomeric reporter protein (CD4) from reaching the plasma membrane. Since 14-3-3 proteins show no high-affinity interaction with monomeric Kir6.2 tails (Yuan et al., 2003), this provides an assay of COPI activity alone. We also tested whether the truncated Kir6.2 tails could support the forward transport of dimeric CD8-RKR and tetrameric CD4pLI-RKR to the cell surface (Fig. 5C,D) to assess 14-3-3-dependent forward transport.

As previously described (Yuan et al., 2003; Zerangue et al., 1999), monomeric CD4-RKR was unable to reach the plasma membrane because of the ER-localization activity of the Arg-based signal (Fig. 5B). By contrast, CD8-RKR and CD4pLI-RKR reached the cell surface efficiently (Fig. 5C,D). Mutation of the RKR motif resulted in marked surface expression of all proteins (Fig. 5B-D). These results are consistent with the earlier interpretation that 14-3-3 proteins interact with multimeric Kir6.2-reporter proteins and outcompete the activity of COPI (Michelsen et al., 2006; Yuan et al., 2003).

Deleting the last ten amino acids of the Kir6.2 tail had a minor effect on surface expression of the monomeric CD4-RKRΔC10 (Fig. 5B) but strongly reduced that of dimeric CD8-RKRΔC10 and tetrameric CD4pLI-RKRΔC10 (Fig. 5C,D), which suggests that the ability of 14-3-3 proteins to enhance forward trafficking of multimeric Kir6.2 reporters is dependent on the last ten amino acids of the protein. This effect is not sequence-specific because all three mutated C-termini (RKR_P-A, RKR_PL-GG, and RKR_S-A) supported forward transport of CD4pLI-RKR to the same extent as the WT sequence (data not shown). It also argues that COPI binding is unaffected, because intracellular retention of monomeric CD4-RKRΔC10 was unaffected. Thus deletion of the last ten amino acids of Kir6.2 appears to uncouple the effect of COPI binding (retention from the cell surface because of COPI-mediated ER retrieval) from the effect of 14-3-3 binding (expression at the cell surface).

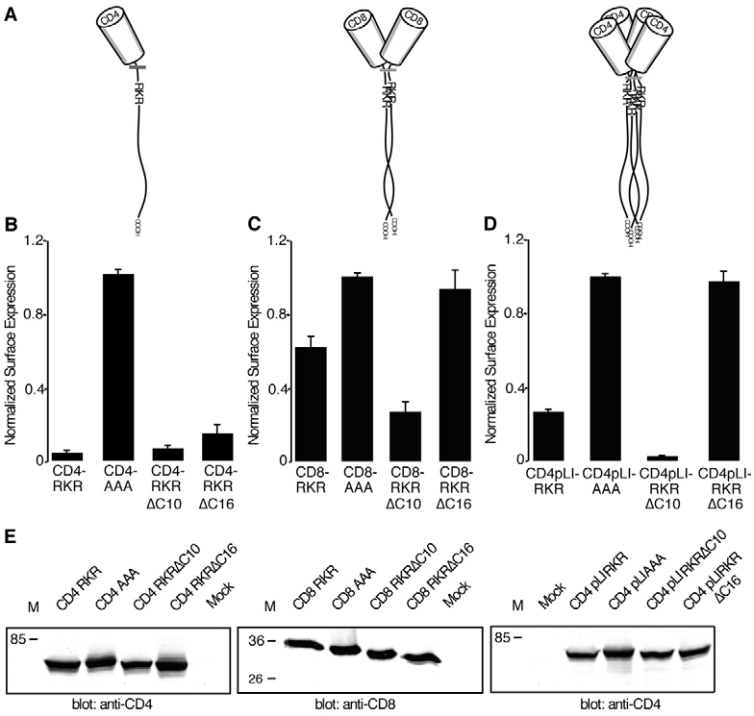

Fig. 5. Reporter proteins distinguish between the effects of 14-3-3 and COPI binding. (A) Schematic representation of the different reporter membrane proteins employed: monomeric CD4, dimeric CD8 and tetrameric CD4pLI. (B-D) Surface expression in COS1 cells of monomeric CD4 (B), dimeric CD8 (C) and tetrameric CD4pLI (D) fused to different C-terminal tails of Kir6.2 (as indicated below). Data are expressed as a fraction of the surface expression observed for the respective AAA construct and represent the mean ± s.e.m. of (B) eight to nine dishes, (C) nine dishes or (D) nine dishes of COS1 cells. (E) Western blots of transfected COS1 cells show similar expression levels for all constructs tested. Detection was performed using antibodies recognizing CD4 or CD8, as indicated. M indicates molecular mass in kDa.

Truncation of a further six residues (RKRΔC16) resulted in significantly higher expression of the monomeric CD4 fusion protein at the cell surface (Fig. 5B), indicating it impairs COPI binding. Very high levels of the corresponding dimer and tetramer were observed at the cell surface (Fig. 5C,D), probably because of a combination of impaired recognition by COPI and reduced 14-3-3 binding. To confirm the correlation between 14-3-3 binding and cell-surface expression for the CD4pLI membrane proteins, we tested their ability to coimmunoprecipitate 14-3-3 proteins (Fig. 6). This was important because the fusion proteins employed in the in vitro binding assay (Fig. 4) contained a hexahistidine tag at the distal C-terminus to allow purification of the full-length proteins, whereas the CD4pLI reporter proteins expressed in mammalian cells lacked the six histidines. As described previously (Yuan et al., 2003), mild crosslinking (that converted only a small fraction of 14-3-3 proteins into crosslinked dimers, Fig. 6A) was required to observe coimmunoprecipitation with CD4pLI-RKR (Fig. 6B). Under these conditions CD4pLI-AAA, CD4pLI-ΔC10 and CD4pLI-ΔC16 coimmunoprecipitated much less 14-3-3 protein. This result further supports the hypothesis that recruitment of 14-3-3 proteins can override the activity of the Arg-based signal when the distal C-terminus of Kir6.2 is present in multiple copies. We conclude that the RKRΔC10 variant, but not the RKRΔC16 variant, can be used to study the effect of 14-3-3 binding to the C-terminal tail of Kir6.2 independently of mutating the Arg-based signal. This enables us to assess the consequences of reducing 14-3-3 binding to Kir6.2 in K_{ATP} channels assembled from full-length subunits without disturbing the efficacy of the Arg-based signal as an ER retention and/or retrieval signal.

14-3-3 binding to Kir6.2 antagonizes the Arg-based signal present on SUR1

Introducing the truncated tail (ΔC10) into an SUR1-Kir6.2 fusion protein, while leaving the Arg-based signals of both subunits intact, reduced surface expression of the K_{ATP}-channel complex (Fig. 7A) to approximately the same extent as 14-3-3 scavenging (Fig. 3). This is consistent with the idea that recruitment of 14-3-3 proteins through the tail of Kir6.2 is impaired by the truncation. Mutation of the Arg-based signal of SUR1 in the truncated fusion protein restored cell-surface expression (Fig. 7A). Thus, we conclude that 14-3-3 binding

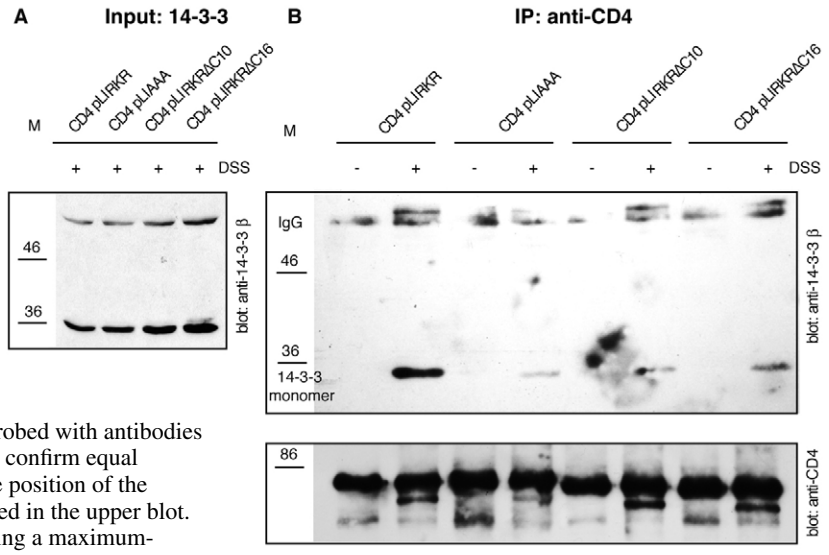

Fig. 6. 14-3-3 binding to the tetrameric CD4pLI reporter proteins correlates with their cell-surface expression. Co-immunoprecipitation of 14-3-3 proteins and indicated CD4pLI-fusion proteins. HEK293T cells transiently transfected with the specified constructs were treated with the crosslinker DSS where indicated. (A) Cell extracts (2% of input) treated with DSS are shown to demonstrate the degree of crosslinking as evident from the ratio between 14-3-3 monomer (lower band) and dimer (upper band). Note that the anti-14-3-3 β antibody recognizes all 14-3-3 isoforms. M indicates molecular mass in kDa. (B) Anti-CD4 immunoprecipitates (upper panel, 90%, lower panel 10%) were resolved by SDS-PAGE. Western blots were probed with antibodies to 14-3-3 proteins (upper panel) and CD4 (lower panel) to confirm equal precipitation of the membrane proteins in all samples. The position of the weakly cross-reacting IgG heavy chain (IgG hc) is indicated in the upper blot. Note that 14-3-3 co-immunoprecipitation was detected using a maximum-sensitivity substrate for HRP indicating that the 14-3-3-bound population of CD4pLI is small or difficult to solubilize. The relative efficiency of 14-3-3 co-immunoprecipitation with the indicated constructs was, however, identical in three independent experiments. The rabbit anti-14-3-3 β antibody employed for detection recognizes all 14-3-3 isoforms.

to Kir6.2 antagonizes the effect of the Arg-based signal present in SUR1.

Effects on channel activity

It is not known whether 14-3-3 proteins accompany KCNK3, Kir2.1-RKR-SWTY, or K_{ATP} channels to the cell surface (O'Kelly et al., 2002; Rajan et al., 2002; Shikano et al., 2005; Yuan et al., 2003). The fact that significant amounts of all K_{ATP} fusion proteins reached the plasma membrane allowed us to test whether impaired 14-3-3 recruitment would alter the functional properties of the K_{ATP} channel. Thus, we compared the ATP-sensitivity of the WT and ΔC10 fusion K_{ATP} channels in excised inside-out patches (Fig. 7B). Fusion of SUR1 to Kir6.2 produced a reduction in the ATP-sensitivity of the WT channel, as previously described (Shyng and Nichols, 1997): in the absence of Mg^{2+}, the IC_{50} was 115±29 μM (n=5), compared with ~10 μM for channels produced when Kir6.2 and SUR1 are expressed separately (Proks et al., 2004). Importantly, however, the ΔC10 deletion did not affect the ATP-sensitivity of the channel, either in the presence or absence of Mg^{2+} (Fig. 6B). In the absence of Mg^{2+}, IC_{50} values were 115±29 μM (n=5) and 156±46 μM (n=5) for SUR1-Kir6.2 and SUR1-Kir6.2ΔC10, respectively; in the presence of Mg^{2+}, IC_{50} values were 576±230 μM (n=6) and 491±133 μM

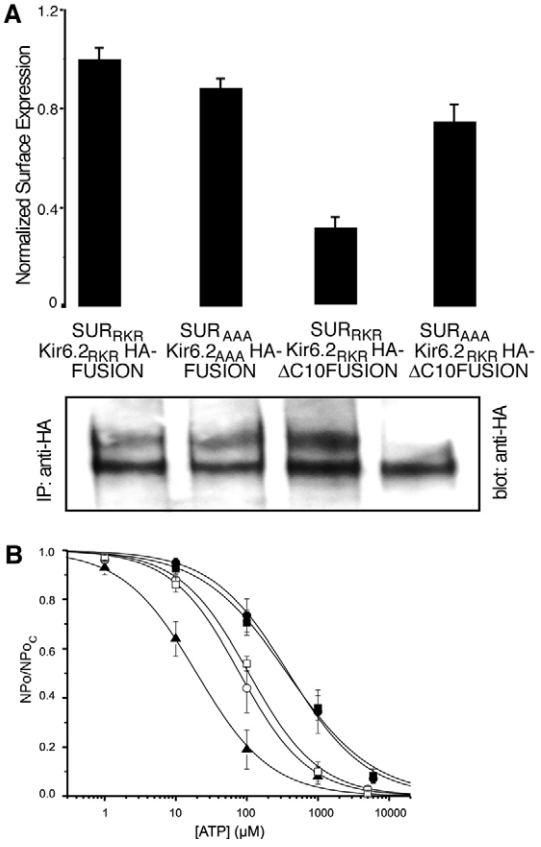

Fig. 7. Impairing 14-3-3 recruitment to the K_{ATP}-channel complex results in reduced surface expression but does not alter K_{ATP}-channel function. (A) (Top) Surface expression in COS1 cells of SUR1-Kir6.2 proteins containing WT or mutated RKR motifs, and the RKRΔC10 variant of Kir6.2, as indicated. Data are the mean ± s.e.m. of at least nine dishes of cells in each case. Data are normalized to the values measured for the $SUR1_{RKR}$-$Kir6.2_{RKR}$ construct. Data for $SUR1_{RKR}$-$Kir6.2_{RKR}$ΔC10 were significantly different to $SUR1_{RKR}$-$Kir6.2_{RKR}$ (P<0.0001) and $SUR1_{AAA}$-$Kir6.2_{RKR}$ (P<0.001). (Bottom) Western blots of anti-HA immunoprecipitates from cell extracts probed with an anti-HA antibody, showing similar expression levels for the fusion proteins tested. (B) Mean ATP concentration-inhibition relationships for K_{ATP} channels. Data were obtained from HEK293 cells expressing recombinant WT channels (triangles), the SUR1-Kir6.2 fusion (circles) or SUR1-Kir6.2ΔC10 (squares). Black symbols, Mg^{2+}-containing solution. White symbols, Mg^{2+}-free solution. The curves are the best fit of the Hill equation to the mean data, with IC_{50}=20 μM, h=0.8 (▲, n=6), IC_{50}=389 μM, h=0.8 (■, n=6), IC_{50}=370 μM, h=0.7 (●, n=6), IC_{50}=80 μM, h=0.9 (○, n=6), and IC_{50}=107 μM, h=0.9 (□, n=6).

(n=6) for SUR1-Kir6.2 and SUR1-Kir6.2ΔC10, respectively. Thus, 14-3-3 binding to K_{ATP} channels does not alter K_{ATP}-channel function.

Discussion

A 14-3-3 scavenger as a strategy to reduce cellular 14-3-3 availability

Because of their high abundance, their diverse functions, and the presence of seven closely related isoforms it is a daunting task to test the role of 14-3-3 proteins in the cellular context. Since it is not known whether specific mammalian isoforms of 14-3-3 are involved in the forward transport of membrane proteins, we devised a novel way to reduce the availability of all 14-3-3 isoforms simultaneously: we designed an artificial 14-3-3 scavenger, and used it to investigate the role of 14-3-3 proteins in the cell-surface transport of membrane proteins in *Xenopus* oocytes and in the INS-1 β-cell line.

Control experiments showed that titrating out 14-3-3 proteins does not cause a general impairment of the surface expression of membrane proteins (Fig. 1C). However, it reduced the cell-surface expression of KCNK3, which is known to reach the cell surface with very low efficiency unless 14-3-3 proteins can bind to its C-terminus (O'Kelly et al., 2002; Rajan et al., 2002). The fact that cell-surface expression was not abolished completely could be due to a leak of KCNK3 not in complex with 14-3-3, but presumably means that a significant population of KCNK3 proteins was still able to recruit 14-3-3, despite the presence of the scavenger. This might be attributed to the fact that binding of 14-3-3 to the C-terminus of KCNK3 must occur with rather high-affinity (Coblitz et al., 2005; Shikano et al., 2005).

14-3-3 interaction with Kir6.2

Experiments employing the 14-3-3 scavenger not only revealed that 14-3-3 proteins facilitate the surface expression of the K_{ATP} channel, but also imply that this role is mechanistically related to the inactivation of the Arg-based signals present in the complex (Fig. 8). There are eight of these signals in the fully assembled complex – four contributed by Kir6.2 and four by SUR1. It is important to bear in mind that although Kir6.2 can assemble into functional tetramers in the absence of SUR (Tucker et al., 1997), these tetramers do not reach the cell surface unless the Arg-based signal is mutated (Zerangue et al., 1999). We have been unable to coimmunoprecipitate 14-3-3 proteins with the Kir6.2 tetramer under any conditions (H.Y. and B.S., unpublished results). Similarly, the tail of Kir6.2 does not recruit 14-3-3 proteins to the homologous tetrameric Kir2.1 channel in the Kir2.1-RKR-reporter fusion (Shikano et al., 2005). However, 14-3-3 does interact with the tetrameric CD4pLI-RKR fusion protein (Fig. 6) (Yuan et al., 2003) and a very similar reporter membrane protein in yeast (Michelsen et al., 2006), presumably because the Kir6.2 tails are tethered in closer proximity than in a Kir6.2 tetramer lacking SUR1. Furthermore, we found that 14-3-3 proteins are recruited to the full K_{ATP}-channel complex (Fig. 2). Thus, we have to assume that the tails of Kir6.2 are brought into closer proximity to each other upon co-assembly with SUR1, or that SUR1 contributes to 14-3-3 recruitment by adding an additional low-affinity binding site (Fig. 8). This additional binding site could reside in the vicinity of the RKR signal of SUR1 or in a completely different region of the protein, but would have to be within 30 Å of the distal tail of Kir6.2 in the context of the assembled complex because this is the distance between the two ligand-binding grooves in the 14-3-3 dimer.

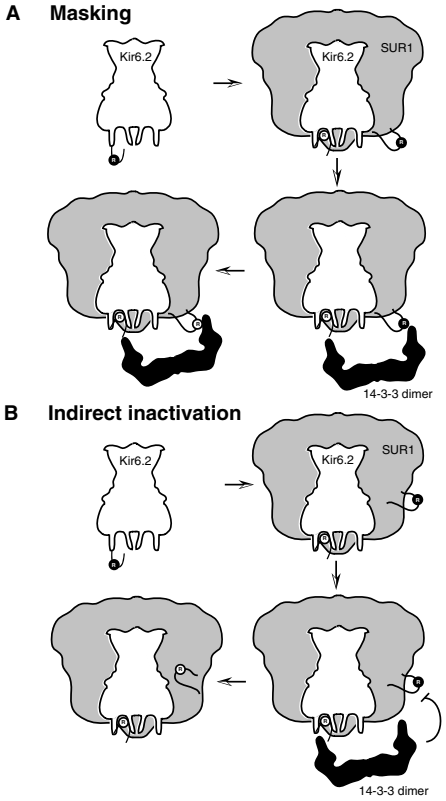

Fig. 8. Model for the sequence of events leading to the functional inactivation of the eight Arg-based signals present in the K_{ATP}-channel complex. The shapes illustrating the Kir6.2 tetramer (Antcliff et al., 2005), the heterooctameric K_{ATP}-channel complex (Mikhailov et al., 2005), or the 14-3-3 dimer (Petosa et al., 1998; Yaffe et al., 1997) are based on homology models or structural analysis by single-particle electron microscopy or X-ray crystallography. They are shown to scale in order to indicate the relative sizes. As 14-3-3-binding sites other than the one provided by the distal tail of Kir6.2 remain unknown, the position of the 14-3-3 dimer is entirely hypothetical. Filled circles with white Rs represent active Arg-based ER-localization signals, open circles symbolize inactiviation of the signal. (A) The Arg-based signal of SUR1 could be inactivated by direct binding of 14-3-3 to this region of the protein (masking). (B) The Arg-based signal of SUR1 could be indirectly inactivated by 14-3-3 recruitment to a binding site in the vicinity of the distal tail of Kir6.2.

14-3-3 and SUR1

Our data argue that it is not the Arg-based signal present in Kir6.2 but that present in SUR1 that requires 14-3-3 recruitment in order to be inactivated and thus relieve COPI-mediated retention (Fig. 8). First, inactivation of the Arg-based signal present in SUR1 rendered the whole complex insensitive to 14-3-3 scavenging, whereas a fusion protein in which only the Arg-based signal in Kir6.2 was mutated remained sensitive to 14-3-3 scavenging (Fig. 3). Second, a truncation of the Kir6.2 tail that decreased its ability to recruit 14-3-3 in the context of reporter proteins (Figs 4, 6) only resulted in reduced

surface expression of the K_{ATP}-channel complex when the Arg-based signal in SUR1 was present (Fig. 7). Therefore, steric masking by the partner subunit seems to apply to the Arg-based signal of Kir6.2, whereas 14-3-3 proteins appear to be required to inactivate the Arg-based signal of the SUR subunit (Fig. 8). This is entirely consistent with the earlier conclusion that the RKR motif in SUR1, but not in Kir6.2, is partially exposed in the fully assembled octamer (Zerangue et al., 1999).

We have not been able to obtain evidence for a 14-3-3-binding site on SUR1 but cannot exclude the possibility of a low-affinity site that contributes to 14-3-3 binding once 14-3-3 has been recruited to the complex through the tail of Kir6.2. Based on our results, it seems unlikely that 14-3-3 proteins have a scaffolding role in bringing or keeping the different subunits in close proximity to each other during primary assembly in the ER. Otherwise, surface expression of the independently expressed subunits would be expected to be more sensitive to 14-3-3 scavenging than surface expression of the physically linked subunits. Instead, 14-3-3 binding to Kir6.2 seems to impinge on the forward transport of the K_{ATP}-channel complex either by directly shielding the Arg-based signal of SUR1 from COPI (steric masking by 14-3-3) or by indirect activation, which could be because of the recruitment of additional proteins to the complex (Fig. 8). These could be proteins that affect the functional state of the COPI coat or that couple the protein to the forward-transport machinery, such as components of the COPII coat (Mrowiec and Schwappach, 2006; Shikano et al., 2006).

Effects on K_{ATP}-channel activity

It is not clear whether 14-3-3 proteins remain associated with K_{ATP} channels at the plasma membrane. Our results do, however, suggest that even if they remain associated, 14-3-3 proteins do not alter the ATP-sensitivity of K_{ATP} channels at the cell surface. A fusion protein of SUR1 and a truncated Kir6.2 subunit gave rise to K_{ATP} channels with an ATP-sensitivity indistinguishable from that of the corresponding WT channel. This was true both in the presence and absence of Mg^{2+}. In the absence of Mg^{2+}, ATP interacts only with the Kir6.2 subunit. In the presence of Mg^{2+}, however, ATP is also hydrolysed by the nucleotide-binding domains of SUR1, resulting in a stimulation of channel activity that produces an apparent reduction in ATP-sensitivity. Our data therefore indicate that neither the ATP-binding site on Kir6.2, nor the ability of MgATP binding and/or hydrolysis at SUR1 to influence channel activity, are affected by the ΔC10 truncation. This is consistent with the observation that truncations of up to 36 amino acids from the C-terminus of Kir6.2 do not influence K_{ATP}-channel function when Kir6.2 and SUR1 subunits are coexpressed (Tucker et al., 1997).

Conclusion

In conclusion, our data support the following model for trafficking of K_{ATP} channels to the surface membrane (Fig. 8). First, both Kir6.2 and SUR1 possess RKR motifs that bind COPI, thereby producing ER localization and preventing trafficking to the cell surface. This ensures that individual subunits, Kir6.2 tetramers or partially assembled complexes are prevented from reaching the plasma membrane. Second, the RKR motif on Kir6.2 is masked by the presence of SUR, preventing COPI binding. This is consistent with recent structural studies that show that the Kir6.2 tetramer is surrounded by SUR1 subunits in the channel complex (Mikhailov et al., 2005). Third, the RKR motif on SUR1 is inactivated directly (steric masking) or indirectly, by 14-3-3 initially recruited through the C-terminal tail of Kir6.2 and stabilized on the K_{ATP} channels by a combination of low-affinity binding sites. This model thus provides a mechanistic explanation for how both SUR1 and Kir6.2 partner subunits contribute to surface expression of K_{ATP} channels.

Finally, our results provide the first example of 14-3-3-dependent cell-surface expression in the context of a native, heteromultimeric membrane protein complex. They further dissect the signal-inactivation events that allow the forward transport of fully assembled K_{ATP} channels to the plasma membrane and thus contribute to our understanding of cellular quality-control mechanisms.

Materials and Methods

Molecular biology

For oocyte expression, constructs were in pGemHE (Liman et al., 1992). For retroviral gene transduction, constructs were engineered in the pBI-GFP vector (Liu et al., 2000) for Kir6.2-11HA (Zerangue et al., 1999) and in pBICD4 (Liu et al., 2000) for phogrin-EGFP (Pouli et al., 1998). The pGpLI-R18 fusion is analogous to the previously described pApLI-RKR fusion (Yuan et al., 2003). For KCNK3, an extracellular protein C (PC)-epitope tag (EDQVDPRLIDGK) was inserted at amino acid position 213, as described (O'Kelly et al., 2002). The SUR1-Kir6.2 fusion construct was made by inserting the following linker between the C-terminus of SUR1 and the N-terminus of Kir6.2: AAAGGGGGGGSA. All other constructs used to assess surface expression in *Xenopus* oocytes have been described previously (Zerangue et al., 1999). Fusion constructs of the last 36 amino acids of Kir6.2 to CD4, CD8, or CD4pLI, and the respective truncated variants (RKRΔC10 and RKRΔC16) were in pcDNA3. All protein-A fusion constructs used for recombinant protein expression in *E. coli* were in pQE60 (Yuan et al., 2003) and consist of two copies of the IgG-binding domain of protein A, the coiled-coil-forming domain pLI (Harbury et al., 1993) and the C-terminal residues from Kir6.2 as described in the text and Figure legends. A C-terminal hexahistidine tag ensured the purification of full-length proteins. For expression of SUR1-Kir6.2 fusion constructs (Zerangue et al., 1999) in mammalian cells, the cDNAs were engineered in pcDNA3.

Preparation and surface labeling of oocytes

Xenopus oocytes were prepared as described (Collins et al., 1997). Oocytes were injected with 0.1 ng cRNA for Kir6.2-11HA, 2 ng SUR1, 5 ng KCNK3-PC, 5 ng SUR1-Kir 6.2-11HA fusion constructs, 5 ng Kir 2.1-HA, 5 ng HA-β2 adrenergic receptor and 5 ng pGpLI-R18. Surface assays were performed 2-3 days after injection as described (Zerangue et al., 1999). Oocytes were homogenized in PBS (50 mM phosphate, 150 mM NaCl, pH 8.0) supplemented with a protease inhibitor cocktail (complete; Roche) by five passages through an 18-gauge and one passage through a 27-gauge needle (Tucker et al., 1996). Homogenates were cleared by repeated centrifugation at 1000 ***g***. The supernatants were supplemented with SDS-PAGE-loading buffer and subjected to gel electrophoresis.

INS-1 cell culture and retroviral gene transduction

INS-1 cells (Asfari et al., 1992) were cultured in RPMI 1640 medium containing L-glutamine (Gibco) supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 100 iu/ml penicillin, and 100 μg/ml streptomycin, at 37°C, 5% CO_2, absolute humidity. INS-1 cells were infected by incubation with retroviral particles produced by co-transfection of HEK-293T cells with pVPack-Eco and pVPack-GP (Stratagene) with either pBI-Kir6.2-11HA-IRES-GFP, pBI-phogrin-EGFP-IRES-CD4, or pBI pGpLI-R18-IRES-CD4. Populations of infected cells were purified by fluorescence-activated cell sorting. Expression of the epitope-tagged variant of Kir6.2 was low compared with the amount of endogenous channels expressed (Fig. 2C).

Electrophysiology

INS-1 cells expressing 14-3-3-scavenger protein were identified using phycoerythrin-conjugated anti-CD4 monoclonal antibody (EDU-2, Diatec). Whole-cell currents were recorded from $CD4^+$ cells using the standard whole-cell configuration of the patch-clamp method. Currents were elicited by ±10 mV voltage steps from a holding potential of –70 mV. Currents were normalized to cell capacitance to correct for differences in cell size. The pipette was filled with intracellular solution (in mM): 140 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 0.3 ATP, 10 HEPES (pH 7.2 with KOH). The bath contained extracellular solution (in mM): 137 NaCl, 5.6 KCl, 2.6 $CaCl_2$, 1.1 $MgCl_2$, 10 HEPES (pH 7.4 with NaOH). Tolbutamide

(0.5 mM) was added as indicated. All experiments were performed at 21-23°C. Data are expressed as the mean ± s.e.m.

Coimmunoprecipitation and binding assay

Permeabilization of retrovirally transduced INS-1 cells and transfected HEK293T cells was performed according to Jadot et al. (Jadot et al., 1995). The crosslinker DSS was then added to a final concentration of 200 μM from a 10 mM stock in dimethyl sulfoxide (DMSO) and the reaction allowed to proceed for 15 minutes at 25°C. Cells were homogenized in K-Hops buffer (130 mM KCl, 25 mM Tris-HCl; pH 7.5) by sequential repeated passage through 22- and 27-gauge needles. After clearing of the lysate, total membranes were prepared by centrifugation. Membranes were solubilized in lysis buffer (50 mM Tris/HCl, pH 8.5, 100 mM NaCl, 1% Triton X-100, 0.1% SDS) and up to 5 μg of the respective antibody was added overnight. Antibody–antigen complexes were immobilized on protein G-Sepharose (Roche), washed, and eluted in SDS sample buffer. Recombinant protein expression, purification through the C-terminal hexahistidine tag and the binding assay were performed as described (Yuan et al., 2003).

Western blot analysis

Blots were blocked in Tris-buffered saline (TBS) containing 5% milk powder and 0.02% NP-40. Primary antibodies were: anti-Kir6.2 guinea pig polyclonal, 1:2000; anti-GFP rabbit polyclonal, 1:1000; anti-CD4 rabbit polyclonal (H-370, Santa Cruz Biotechnology), 1:1000; anti-CD8 (H-160, Santa Cruz Biotechnology), 1:1000; anti-14-3-3 β mouse monoclonal (H-8, Santa Cruz Biotechnology), 1 μg/ml, anti-14-3-3 β rabbit polyclonal (K-19 and FL-246, Santa Cruz Biotechnology); 80 ng/ml for detection of coimmunoprecipitated 14-3-3 in Fig. 6. Secondary antibodies were: horseradish peroxidase (HRP)-conjugated anti-mouse, anti-guinea pig and anti-rabbit (Jackson ImmunoResearch Laboratories), at 1:4000 and 1:50,000 when using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology) diluted in TBS-blocking solution. Washes were in TBS-blocking solution and then in TBS, 0.02% NP-40. Detection was performed using the ECL system (Amersham) and with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) for the 14-3-3 coimmunoprecipitation shown in Fig. 6.

Luminometric surface-expression assay for mammalian cells

COS1 cells plated in 35-mm tissue-culture dishes were transfected and surface expression of all constructs was assayed 24 hours after transfection. Cells were fixed with 4% formaldehyde in PBS (20 minutes), blocked in PBS with 1% fetal bovine serum (30 minutes), and then labeled with primary antibody for 1 hour, and with an appropriate HRP-coupled secondary antibody for 20 minutes. Chemiluminescence of the whole 35-mm dish was quantitated in a TD-20/20 luminometer (Turner Designs) after 15 seconds of incubation in SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce). Extensive washing was performed between steps and before chemiluminescence measurement; all steps were performed at room temperature. Surface expression of CD4- or CD8-fusion proteins was detected by mouse monoclonal anti-CD4 antibody EDU-2 (Dianova) diluted 1:5000, or mouse monoclonal anti-CD8 antibody UCHT-4 (Dianova) diluted 1:5000 and goat anti-mouse HRP-conjugated IgG (Jackson) diluted 1:2000. The cell-surface expression of SUR1-Kir6.2 fusion constructs was detected by monoclonal mouse anti-HA antibody (clone 16B12; BAbCO) using an HA-epitope engineered into the Kir6.2 portion of the fusion protein (Kir6.2-11HA) (Zerangue et al., 1999). For each construct, surface expression was assayed in three 35-mm dishes, and each experiment was repeated several times (see figure legends for specific details), with nearly identical results when normalized to a given construct.

Statistics

Data are reported as the mean ± s.e.m. For the cell-surface expression assay in oocytes the values obtained for a given construct in the absence of the scavenger pGpLI-R18 were averaged in each batch and the value obtained for each oocyte in this batch was divided by the mean to normalize the data. Bar diagrams represent the mean values of these normalized values across all batches (see figure legends for pertinent numbers), and error bars depict the corresponding s.e.m. Statistical significance was determined using Student's *t*-test. The significance values are given in the relevant figure legend.

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References

Aguilar-Bryan, L. and Bryan, J. (1999). Molecular biology of adenosine triphosphate-sensitive potassium channels. *Endocr. Rev.* **20**, 101-135.

Anderson, H. A. and Roche, P. A. (1998). Phosphorylation regulates the delivery of MHC class II invariant chain complexes to antigen processing compartments. *J. Immunol.* **160**, 4850-4858.

Antcliff, J. F., Haider, S., Proks, P., Sansom, M. S. and Ashcroft, F. M. (2005). Functional analysis of a structural model of the ATP-binding site of the KATP channel Kir6.2 subunit. *EMBO J.* **24**, 229-239.

Asfari, M., Janjic, D., Meda, P., Li, G., Halban, P. A. and Wollheim, C. B. (1992). Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* **130**, 167-178.

Coblitz, B., Shikano, S., Wu, M., Gabelli, S. B., Cockrell, L. M., Spieker, M., Hanyu, Y., Fu, H., Amzel, L. M. and Li, M. (2005). C-terminal recognition by 14-3-3 proteins for surface expression of membrane receptors. *J. Biol. Chem.* **280**, 36263-36272.

Collins, A., Chuang, H., Jan, Y. N. and Jan, L. Y. (1997). Scanning mutagenesis of the putative transmembrane segments of Kir2.1, an inward rectifier potassium channel. *Proc. Natl. Acad. Sci. USA* **94**, 5456-5460.

Dedio, J., Jahnen-Dechent, W., Bachmann, M. and Muller-Esterl, W. (1998). The multiligand-binding protein gC1qR, putative C1q receptor, is a mitochondrial protein. *J. Immunol.* **160**, 3534-3542.

Geng, X., Li, L., Watkins, S., Robbins, P. D. and Drain, P. (2003). The insulin secretory granule is the major site of K(ATP) channels of the endocrine pancreas. *Diabetes* **52**, 767-776.

Giles, N., Forrest, A. and Gabrielli, B. (2003). 14-3-3 acts as an intramolecular bridge to regulate cdc25B localization and activity. *J. Biol. Chem.* **278**, 28580-28587.

Harbury, P. B., Zhang, T., Kim, P. S. and Alber, T. (1993). A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants. *Science* **262**, 1401-1407.

Jadot, M., Hofmann, M. W., Graf, R., Quader, H. and Martoglio, B. (1995). Protein insertion into the endoplasmic reticulum of permeabilized cells. *FEBS Lett.* **371**, 145-148.

Kuwana, T., Peterson, P. A. and Karlsson, L. (1998). Exit of major histocompatibility complex class II-invariant chain p35 complexes from the endoplasmic reticulum is modulated by phosphorylation. *Proc. Natl. Acad. Sci. USA* **95**, 1056-1061.

Liman, E. R., Tytgat, J. and Hess, P. (1992). Subunit stoichiometry of a mammalian K+ channel determined by construction of multimeric cDNAs. *Neuron* **9**, 861-871.

Liu, X., Constantinescu, S. N., Sun, Y., Bogan, J. S., Hirsch, D., Weinberg, R. A. and Lodish, H. F. (2000). Generation of mammalian cells stably expressing multiple genes at predetermined levels. *Anal. Biochem.* **280**, 20-28.

Michelsen, K., Mrowiec, T., Duderstadt, K. E., Frey, S., Minor, D. L., Mayer, M. P. and Schwappach, B. (2006). A multimeric membrane protein reveals 14-3-3 isoform specificity in forward transport in yeast. *Traffic* **7**, 903-916.

Mikhailov, M. V., Campbell, J. D., de Wet, H., Shimomura, K., Zadek, B., Collins, R. F., Sansom, M. S., Ford, R. C. and Ashcroft, F. M. (2005). 3-D structural and functional characterization of the purified KATP channel complex Kir6.2-SUR1. *EMBO J.* **24**, 4166-4175.

Mrowiec, T. and Schwappach, B. (2006). 14-3-3 proteins in membrane protein transport. *Biol. Chem.* **387**, 1227-1236.

Muta, T., Kang, D., Kitajima, S., Fujiwara, T. and Hamasaki, N. (1997). p32 protein, a splicing factor 2-associated protein, is localized in mitochondrial matrix and is functionally important in maintaining oxidative phosphorylation. *J. Biol. Chem.* **272**, 24363-24370.

O'Kelly, I., Butler, M. H., Zilberberg, N. and Goldstein, S. A. (2002). Forward transport. 14-3-3 binding overcomes retention in endoplasmic reticulum by dibasic signals. *Cell* **111**, 577-588.

Petosa, C., Masters, S. C., Bankston, L. A., Pohl, J., Wang, B., Fu, H. and Liddington, R. C. (1998). 14-3-3zeta binds a phosphorylated Raf peptide and an unphosphorylated peptide via its conserved amphipathic groove. *J. Biol. Chem.* **273**, 16305-16310.

Pouli, A. E., Emmanouilidou, E., Zhao, C., Wasmeier, C., Hutton, J. C. and Rutter, G. A. (1998). Secretory-granule dynamics visualized in vivo with a phogrin-green fluorescent protein chimaera. *Biochem. J.* **333**, 193-199.

Proks, P., Antcliff, J. F., Lippiat, J., Gloyn, A. L., Hattersley, A. T. and Ashcroft, F. M. (2004). Molecular basis of Kir6.2 mutations associated with neonatal diabetes or neonatal diabetes plus neurological features. *Proc. Natl. Acad. Sci. USA* **101**, 17539-17544.

Rajan, S., Preisig-Muller, R., Wischmeyer, E., Nehring, R., Hanley, P. J., Renigunta, V., Musset, B., Schlichthorl, G., Derst, C., Karschin, A. et al. (2002). Interaction with 14-3-3 proteins promotes functional expression of the potassium channels TASK-1 and TASK-3. *J. Physiol.* **545**, 13-26.

Shikano, S. and Li, M. (2003). Membrane receptor trafficking: evidence of proximal and distal zones conferred by two independent endoplasmic reticulum localization signals. *Proc. Natl. Acad. Sci. USA* **100**, 5783-5788.

Shikano, S., Coblitz, B., Sun, H. and Li, M. (2005). Genetic isolation of transport signals directing cell surface expression. *Nat. Cell Biol.* **7**, 985-992.

Shikano, S., Coblitz, B., Wu, M. and Li, M. (2006). 14-3-3 proteins: regulation of endoplasmic reticulum localization and surface expression of membrane proteins. *Trends Cell Biol.* **16**, 370-375.

Shyng, S. and Nichols, C. G. (1997). Octameric stoichiometry of the KATP channel complex. *J. Gen. Physiol.* **110**, 655-664.

Trube, G., Rorsman, P. and Ohno-Shosaku, T. (1986). Opposite effects of tolbutamide and diazoxide on the ATP-dependent K+ channel in mouse pancreatic beta-cells. *Pflugers Arch.* **407**, 493-499.

Tucker, S. J., Bond, C. T., Herson, P., Pessia, M. and Adelman, J. P. (1996). Inhibitory interactions between two inward rectifier K+ channel subunits mediated by the transmembrane domains. *J. Biol. Chem.* **271**, 5866-5870.

Tucker, S. J., Gribble, F. M., Zhao, C., Trapp, S. and Ashcroft, F. M. (1997). Truncation of Kir6.2 produces ATP-sensitive K+ channels in the absence of the sulphonylurea receptor. *Nature* **387**, 179-183.

Varadi, A., Grant, A., McCormack, M., Nicolson, T., Magistri, M., Mitchell, K. J., Halestrap, A. P., Yuan, H., Schwappach, B. and Rutter, G. A. (2006). Intracellular ATP-sensitive K+ channels in mouse pancreatic beta cells: against a role in organelle cation homeostasis. *Diabetologia* **49**, 1567-1577.

Wang, B., Yang, H., Liu, Y. C., Jelinek, T., Zhang, L., Ruoslahti, E. and Fu, H. (1999). Isolation of high-affinity peptide antagonists of 14-3-3 proteins by phage display. *Biochemistry* **38**, 12499-12504.

Wasmeier, C. and Hutton, J. C. (1996). Molecular cloning of phogrin, a protein-tyrosine phosphatase homologue localized to insulin secretory granule membranes. *J. Biol. Chem.* **271**, 18161-18170.

Wurtele, M., Jelich-Ottmann, C., Wittinghofer, A. and Oecking, C. (2003). Structural view of a fungal toxin acting on a 14-3-3 regulatory complex. *EMBO J.* **22**, 987-994.

Yaffe, M. B., Rittinger, K., Volinia, S., Caron, P. R., Aitken, A., Leffers, H., Gamblin, S. J., Smerdon, S. J. and Cantley, L. C. (1997). The structural basis for 14-3-3:phosphopeptide binding specificity. *Cell* **91**, 961-971.

Yuan, H., Michelsen, K. and Schwappach, B. (2003). 14-3-3 dimers probe the assembly status of multimeric membrane proteins. *Curr. Biol.* **13**, 638-646.

Zerangue, N., Schwappach, B., Jan, Y. N. and Jan, L. Y. (1999). A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K(ATP) channels. *Neuron* **22**, 537-548.