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14-3-3 Dimers Probe the Assembly Status of Multimeric Membrane Proteins

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Summary

Background: Arginine-based endoplasmic reticulum (ER) localization signals are involved in the heteromultimeric assembly of membrane protein complexes like ATP-sensitive potassium channels (K_{ATP}) or $GABA_B$ G protein-coupled receptors. They constitute a trafficking checkpoint that prevents ER exit of unassembled subunits or partially assembled complexes. For K_{ATP} channels, the mechanism that leads to masking of the ER localization signals in the fully assembled octameric complex is unknown.
Results: By employing a tetrameric affinity construct of the C terminus of the K_{ATP} channel α subunit, Kir6.2, we found that 14-3-3 isoforms epsilon and zeta specifically recognize the arginine-based ER localization signal present in this cytosolic tail. The interaction was reconstituted by using purified 14-3-3 proteins. Competition with a nonphosphorylated 14-3-3 high-affinity binding peptide implies that the canonical substrate binding groove of 14-3-3 is involved. Comparison of monomeric CD4, dimeric CD8, and artificially tetramerized CD4 fusions correlates the copy number of the tail containing the arginine-based signal with 14-3-3 binding, resulting in the surface expression of the membrane protein. Binding experiments revealed that the COPI vesicle coat can specifically recognize the arginine-based ER localization signal and competes with 14-3-3 for the binding site.
Conclusions: The COPI vesicle coat and proteins of the 14-3-3 family recognize arginine-based ER localization signals on multimeric membrane proteins. The equilibrium between these two competing reactions depends on the valency and spatial arrangement of the signal-containing tails. We propose a mechanism in which 14-3-3 bound to the correctly assembled multimer mediates release of the complex from the ER.

Introduction

Higher eukaryotic cells possess a variety of heteromultimeric membrane protein complexes, particularly those involved in signal transduction from the cell surface, e.g., neurotransmitter receptors, ion channels, and immune receptor complexes. This adds a specific problem to the general issue of membrane protein quality control: in addition to those mechanisms that ensure the proper folding of membrane proteins, there have to be others directed at the correct stoichiometry of the complex [1]. One such mechanism of quality control is based on peptide trafficking signals whose exposure reflects the assembly state of the complex. For example, assembly-dependent masking of arginine-based ER localization signals ensures proper assembly of heteromultimeric membrane protein complexes like the ATP-sensitive potassium channel (K_{ATP}) or the $GABA_B$ G protein-coupled receptor [2, 3].

Arginine-based ER localization signals can function at the N or C terminus of a membrane protein as well as in an intracellular loop, and no specific spacing to the distal termini of the protein is required [3, 4]. Due to their position independence, these signals are potentially very common, and more and more examples are being characterized [5–11]. There are currently three open questions with regard to arginine-based ER localization signals. First, how flexible is the consensus, e.g., does any basic cluster suffice to cause ER localization if it is accessible to the machinery? Second, what cellular machinery is responsible for mediating ER retention/retrieval specified by these signals? Third, how is the masking of the ER localization signal and therefore ER exit coupled to the assembly of heteromultimeric complexes?

K_{ATP} channels link the metabolic state of the cell to membrane excitability. They are important in many tissues and regulate insulin secretion in the pancreas, control vascular tone, and protect neurons and muscles from ischemia. K_{ATP} channels have an unusual octameric stoichiometry consisting of four pore-lining, inward rectifier α subunits (Kir6.1/6.2; 2 transmembrane segments) like other $K+$ channels, but they also contain 4 regulatory sulphonylurea binding β subunits (SUR1/2A/2B; 17 transmembrane segments) that belong to the ATP binding cassette (ABC) family of proteins [12–14].

We have previously found that only octameric K_{ATP} channel complexes were capable of expressing on the cell surface; this finding implies that quality control mechanisms must exist to prevent monomers and partial complexes from expressing on the cell surface [3]. It turned out that the primary quality control mechanism during K_{ATP} assembly involved the exposure of an ER retention/retrieval signal (RKR) present in cytosolic domains of each subunit. We have now employed an affinity purification strategy by using the distal Kir6.2 C terminus to gain further insight into the recognition and masking of the arginine-based ER localization signal during K_{ATP} assembly.

Results

14-3-3 Proteins Specifically Recognize the RKR Motif of Kir6.2

To address the question of which cellular proteins recognize the arginine-based ER localization signal on the inward rectifier subunit of the K_{ATP} channel, Kir6.2, we constructed a tetrameric affinity construct displaying

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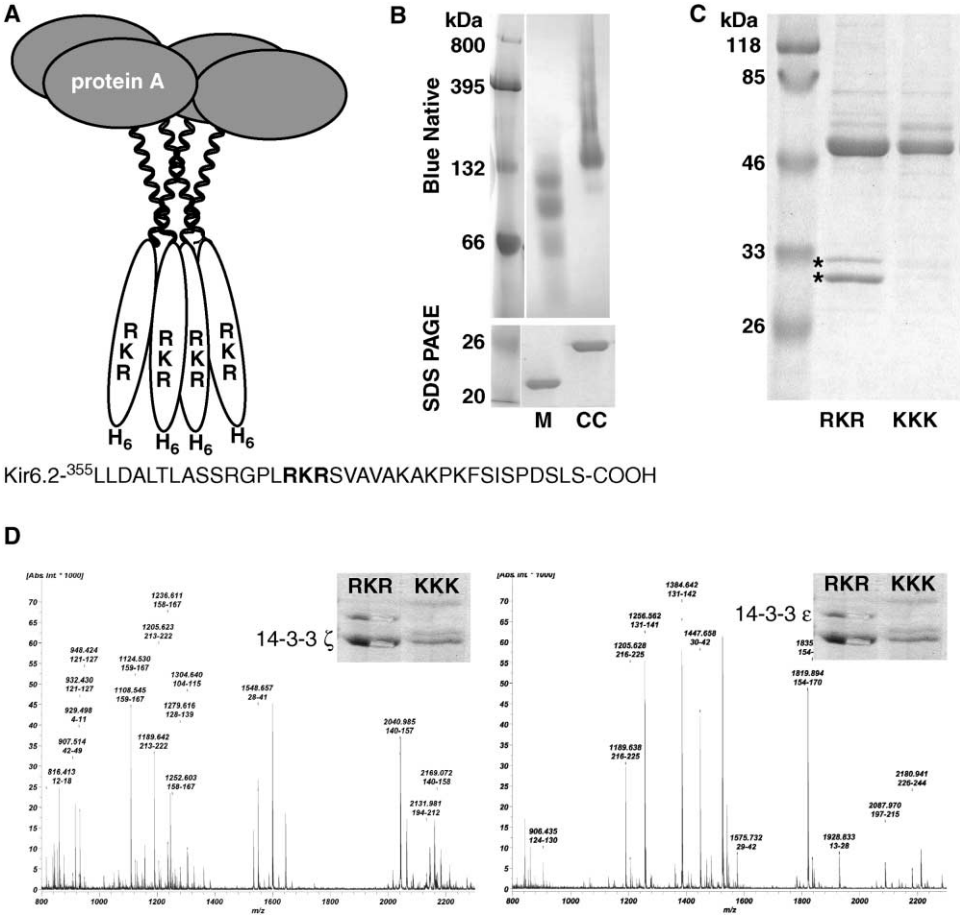

Figure 1. The C-Terminal Tail of Kir6.2 Is Recognized by 14-3-3 Zeta and Epsilon Isoforms

(A) A schematic diagram of the affinity construct employed in the purification strategy and the sequence of the distal Kir6.2 C terminus.

(B) Blue native gel electrophoresis demonstrates multimeric assembly of the affinity construct mediated by the artificial coiled-coil domain. A total of 40 μg purified affinity construct without (M) or with (CC) the tetramerization domain was loaded on the blue native gel. Ferritin (800 and 395 kDa) and BSA (132 and 66 kDa) were used as marker proteins. A total of 4 μg of the corresponding proteins was resolved on a standard SDS-PAGE gel. The gels were stained with Coomassie to visualize proteins.

(C) A binding assay using HeLa cytosol as input material. Coomassie staining of the gel reveals two strongly enriched bands with a mobility close to 30 kDa (asterisks).

(D) Identification of the enriched proteins by mass spectrometry. Bands excised from Coomassie-stained gels and spectra from the MALDI analysis are shown.

the last 36 amino acids of Kir6.2 (Figure 1A). To this end, a mutated version of the *GCN4* leucine zipper, pLI [15], was fused between the affinity tag protein A and the Kir6.2 C terminus. The rationale was to increase the avidity of a presumably transient interaction due to the multimeric presentation of the peptide tails. Multimeric assembly of the affinity construct was verified by blue native gel electrophoresis (Figure 1B; [16]). This technique revealed a protein complex migrating much higher than the corresponding protein without the 35 amino acid tetramerization domain, whereas the two proteins migrate close to each other on a standard SDS-PAGE gel (Figure 1B). The affinity construct was immobilized on IgG sepharose by means of the protein A moiety and was incubated with cytosol from HeLa cells. Proteins bound to the Kir6.2 distal C terminus were compared to the eluate from a control construct with the RKR motif mutated to lysines (Figure 1C). The strategy was to distinguish proteins binding to the basic cluster in an unspecific fashion from those specifically recognizing the arginine-based motif, since it is known that mutation of the RKR signal to KKK allows surface expression of Kir6.2 in the absence of SUR, whereas the wild-type form of Kir6.2 is retained in the ER when expressed alone [3]. Two proteins migrating close to 30 kDa were specifically enriched on the RKR-containing tail and were, therefore, further analyzed by mass spectrometry (Figure 1D), identifying them as the 14-3-3 epsilon and zeta isoforms.

The Interaction Can Be Competed by the Nonphosphorylated 14-3-3 Ligand R18

To further characterize the interaction between the Kir6.2 tail containing the RKR signal and the 14-3-3 proteins, we expressed and purified 14-3-3 epsilon fused to the bacterial maltose binding protein (MBP).

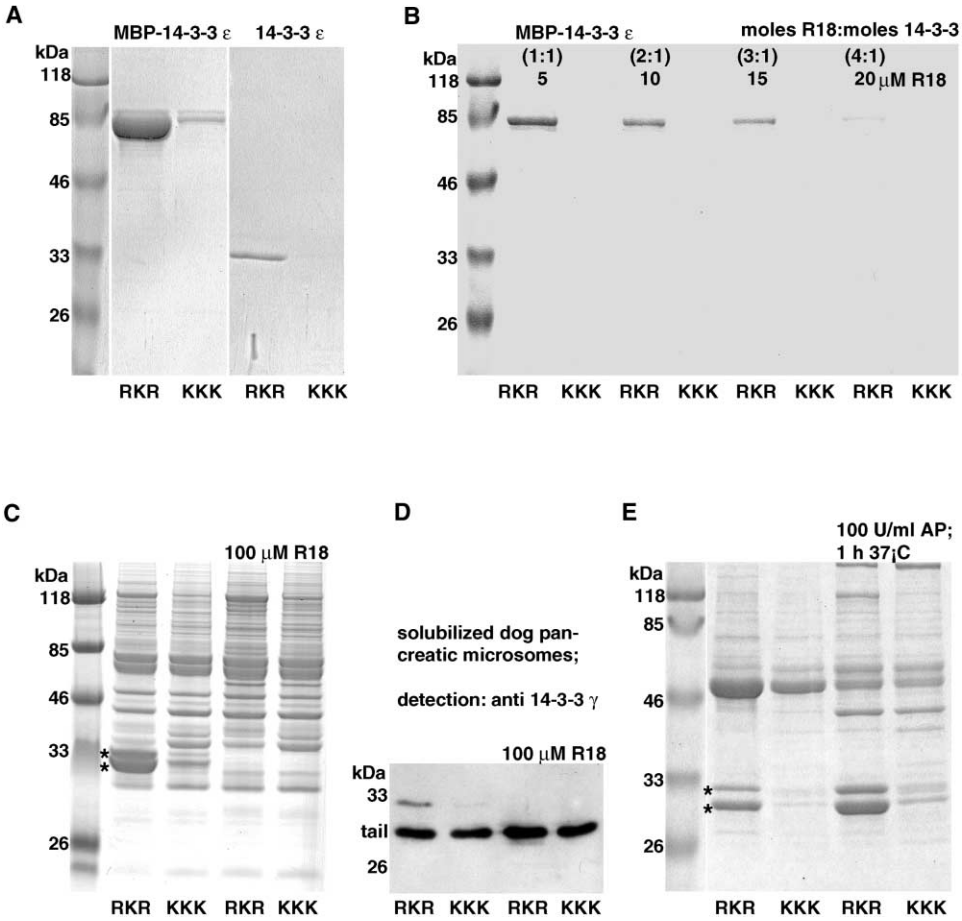

Figure 2. Reconstitution of the Interaction Employing Proteins Purified from *E. coli*

(A) The binding assay was performed identically as described in Figure 1C, except for the use of purified 14-3-3 epsilon as input instead of HeLa cytosol (the immobilized tail protein was in 2-fold excess). Specific recognition of the RKR-containing tail was assessed before and after cleavage of the maltose binding protein (MBP) affinity tag employed for purification of the 14-3-3 protein. Coomassie-stained SDS-PAGE gels are shown.

(B) Competition of the high-affinity 14-3-3 ligand R18 (K_D 7–9 $\times$ 10^{-8} M; [17]) and the Kir6.2 tail for the binding of 14-3-3. Identical amounts of 14-3-3 fusion protein were used in the binding assay, while the concentration of R18 was increased between 5 and 20 μM.

(C) The R18 peptide can displace 14-3-3 from the Kir6.2 tail in HeLa cytosol. The last two lanes show the protein eluted from the affinity column in the presence of 100 μM R18 peptide. Asterisks mark Coomassie-stained bands containing 14-3-3 proteins, as identified by Western blotting (not shown).

(D) 14-3-3 proteins can be recruited to the Kir6.2 tail from solubilized dog pancreatic microsomes. This specific interaction is abolished in the presence of the R18 peptide. The eluates from the binding assay were probed by Western blotting with an antiserum recognizing the 14-3-3 gamma isoform.

(E) Phosphorylation of the affinity construct is not required for the enrichment of 14-3-3 proteins from HeLa cytosol. Binding was performed under identical conditions as those described in Figure 1C. The last two lanes show the protein eluted from the affinity column when the input was treated with 100 U/ml alkaline phosphatase at 37°C for 1 hr. Changes in the pattern of unspecifically bound proteins suggest that dephosphorylation was taking place. However, the intensity of the Coomassie-stained bands containing 14-3-3 proteins (asterisks) was increased rather than decreased, possibly indicating the liberation of 14-3-3 protein from phosphorylation-dependent binding sites on proteins present in the cellular extract.

Binding assays analogous to those performed in Figure 1C revealed specific binding of the maltose binding protein fusion as well as the cleaved 14-3-3 portion (Figure 2A). To assess whether the canonical 14-3-3 binding groove is involved, we used the R18 peptide in competition experiments with the MBP-14-3-3 fusion protein (Figure 2B) and with 14-3-3 proteins present in HeLa cytosol (Figure 2C) or solubilized dog pancreatic microsomes (Figure 2D). This peptide was originally identified as a high-affinity 14-3-3 ligand by phage display [17]. While many interactions between the 14-3-3 proteins and their protein ligands involve phosphorylated motifs on the ligand, there are examples for interactions involving nonphosphorylated binding motifs [18]. The crystal structure of the nonphosphorylated R18 peptide in complex with the 14-3-3 zeta isoform [19] reveals that the same amphipathic binding groove responsible for the binding of phosphorylated peptides accommodates R18. We conclude from the ability of R18 to compete with the Kir6.2 tail for the 14-3-3 protein that the recognition of the RKR motif involves the canonical 14-3-3 binding groove.

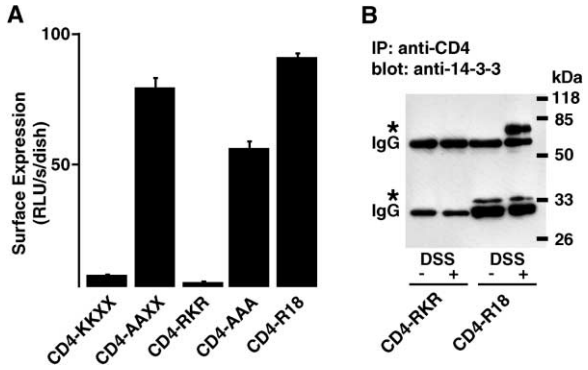

Figure 3. Binding of 14-3-3 Proteins to a Membrane Protein Does Not Suppress Surface Expression

(A) Quantitative assessment of surface expression of CD4 fused to different cytosolic tails. COS1 cells were transfected with the indicated constructs. Surface expression was measured by luminometry by using a luminogenic substrate of HRP. Results for two dishes per construct are shown with error bars and were nearly identical in several independent experiments.

(B) Coimmunoprecipitation and crosslinking of 14-3-3 to CD4 fusion constructs. HEK293T cells were transiently transfected with the indicated CD4 fusion proteins. The plasma membrane was permeabilized with saponin [49], and cells were treated with the crosslinker DSS where indicated. Anti-CD4 immunoprecipitates were resolved by SDS-PAGE and were probed by anti-14-3-3 Western blotting. Asterisks indicate monomeric and dimeric species of 14-3-3 proteins.

The RKR motif in the distal C terminus of Kir6.2 is part of a PKA consensus site (see Figure 1A), raising the issue of whether the 14-3-3 proteins recognize the motif in the phosphorylated state. The interaction is unlikely to require phosphorylation of S372 on Kir6.2, since it could be reconstituted by using two proteins expressed in and purified from *E. coli* (Figure 2A). This was further supported by the fact that the binding assay performed with HeLa cytosol enriched 14-3-3 to the same extent or more when alkaline phosphatase was included in the reaction instead of the nucleotide regeneration system (Figure 2E). Taken together, this data suggests that 14-3-3 recognizes the Kir6.2 tail in the unphosphorylated state.

Binding of 14-3-3 to the Tail of CD4 Does Not Cause ER Localization

Given the specific interaction of 14-3-3 proteins with the RKR-containing tail of Kir6.2 and the fact that the RKR signal mediates ER localization [3, 4], we wanted to assess the consequence of 14-3-3 binding to a reporter membrane protein. To this end, we created a variant of CD4 with the R18 peptide sequence fused to the cytosolic side of CD4 and compared surface expression of this fusion protein to CD4 fusions with the tail of the yeast protein Wbp1 (containing the well-characterized C-terminal dilysine ER localization motif), a mutant version of this tail (-AAXX-COOH instead of -KKXX-COOH), the last 36 amino acids of Kir6.2 that we also employed in our affinity construct (Figure 1A), as well as the corresponding alanine mutant of that tail [3]. The different CD4 fusion proteins were transiently expressed in COS1 cells, and surface expression was quantified according to previously published protocols (Figure 3A; [2, 20]). While both tails containing ER localization signals (namely the -KKXX-COOH signal and the RKR signal) suppressed surface expression of CD4, CD4-R18 was expressed at the cell surface to a similar extent as CD4-AAXX and CD4-AAA. These differences were not due to differences in total protein levels, as assessed by immunofluorescence or analysis of the respective GFP fusion proteins by flow cytometry ([3, 4] and data not shown).

In order to verify 14-3-3 binding to CD4-R18, we performed chemical crosslinking in combination with immunoprecipitation of the CD4 fusion protein (Figure 3B). 14-3-3 proteins could be coimmunoprecipitated with CD4-R18 even in the absence of the chemical crosslinker, as evidenced by the 14-3-3 immunoreactive band migrating just above the IgG light chain. In the presence of the crosslinker disuccinimidyl suberate (DSS), 14-3-3 was also coimmunoprecipitated as a dimeric species migrating above the IgG heavy chain (also visible in the input extract, not shown). In contrast, no 14-3-3 was coimmunoprecipitated with CD4-RKR irrespective of the presence or absence of crosslinker (Figure 3B). This implies that 14-3-3 binding cannot account for the ER retention of CD4 by an arginine signal-containing tail.

Dimeric Presentation of the Kir6.2 Tail Allows Exit of the Reporter Protein

To our surprise and in contrast to the tail containing the C-terminal dilysine signal, the capacity of the RKR-containing tail to localize the membrane protein to the ER was strongly reduced when the tail was presented on CD8 instead of CD4. Figure 4A shows the quantitative assessment of CD8 surface expression. ER exit of CD4-RKR is also reflected in the appearance of a glycosylated species (marked "G" in Figure 4B) that could barely be detected for CD8-KKXX (not shown). While CD4 exists as a monomer, CD8 is known to form dimers [21]. Since 14-3-3 proteins exist as stable dimers within cells [18], we reasoned that dimeric presentation of the Kir6.2 tail on the CD8 protein complex might increase the avidity of the interaction. Thus, we performed crosslinking of cellular extracts, followed by immunoprecipitation of the CD8 protein for the CD8-RKR and CD8-AAA fusions (Figure 4B). Indeed, 14-3-3 protein could be specifically detected in the anti-CD8 immunoprecipitate from cells expressing CD8-RKR; this finding suggests that 14-3-3 protein binding reduces the efficacy of the Kir6.2 tail in retaining CD8 from the cell surface.

Artificial Tetramerization of CD4-RKR Causes Efficient ER Exit

So far, our results may be taken to imply that the valency and spacing of the RKR-containing tail determines the avidity of 14-3-3 binding and thus the balance between retention from the cell surface and forward trafficking. To take this idea one step further, we used the same artificial tetramerization domain employed in the affinity construct to cluster CD4-RKR and the relevant control proteins. This domain has also been used to assemble Shaker potassium channels into functional channel tetramers in the absence of the T1 domain that normally mediates tetrameric assembly of this channel [22]. When

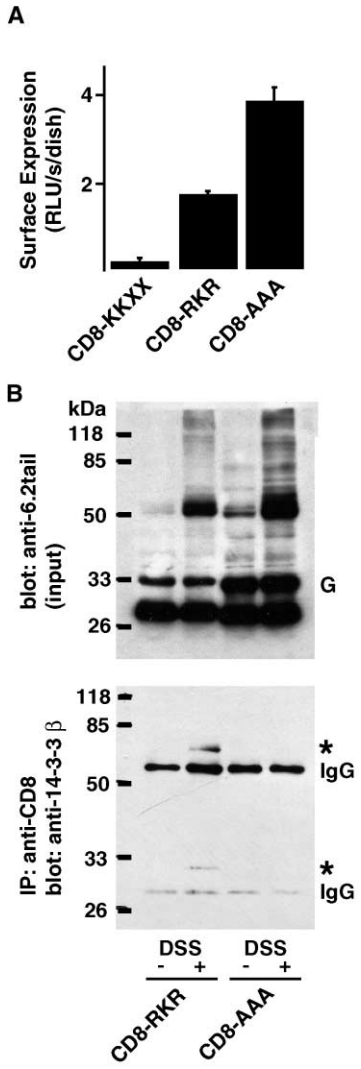

Figure 4. The Kir6.2 Tail Is Ineffective in Retaining CD8 from the Cell Surface

(A) Luminometric assessment of CD8 surface expression reveals strong surface expression of CD8-RKR. COS1 cells were transiently transfected with the indicated constructs, and the quantitative surface assay was performed as described in Figure 3.

(B) Crosslinking and coimmunoprecipitation of 14-3-3 with CD8 fusion constructs was performed as described in Figure 3B. Detection of the CD8 fusion proteins in the input is shown to demonstrate similar expression levels ("G" indicates the glycosylated form of CD8; [21]). The migration positions for the dimeric species of 14-3-3 proteins are marked by an asterisk.

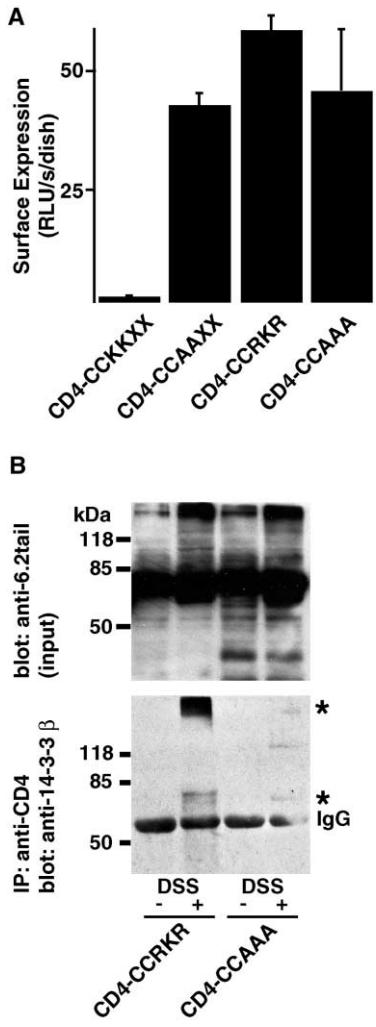

Figure 5. Tetramerization of CD4-RKR Abolishes ER Localization

(A) Luminometric assessment of CD4 surface expression reveals strong surface expression of CD4-CCRKR. COS1 cells were transiently transfected with the indicated constructs, and the quantitative surface assay was performed as described in Figure 3.

(B) Crosslinking and immunoprecipitation demonstrates binding of 14-3-3 to CD4-CCRKR. HEK293T cells were transiently transfected with the indicated CD4 fusion proteins. The experiment was performed as described in Figure 3B. Detection of the CD4-CC fusion proteins in the input is shown to demonstrate similar expression levels. Asterisks indicate monomeric and dimeric species of 14-3-3 proteins.

the quantitative surface expression assay was employed to compare surface expression levels of CD4-CCKKXX, CD4-CCAAXX, CD4-CCRKR, and CD4-CCAAA, surface levels of CD4-CCRKR were found to exceed those of CD4-CCAAXX and CD4-CCAAA (Figure 5A). Interestingly, the ability of the -KKXX tail to retain CD4 from the cell surface was not affected by the artificial tetramerization. Since CD4-RKR and CD4-CCRKR are identical except for their different stoichiometry, we assessed whether 14-3-3 proteins would bind to the tetramerized tail in the context of full-length CD4 (Figure 5B). Crosslinking followed by immunoprecipitation revealed a dimeric species of 14-3-3 in the anti-CD4 immunoprecipitate from cells expressing CD4-CCRKR. Furthermore, higher-molecular weight species, which presumably represent 14-3-3 protein crosslinked to CD4 tetramers, could be detected by the anti-14-3-3 antibody. Taken together, the immunoprecipitation experiments with CD4-RKR, CD8-RKR, and CD4-CCRKR establish a correlation between the valency of the tail on the membrane protein, 14-3-3 binding, and cell surface expression. This raises the question of how the RKR-containing tail brings about ER localization of monomeric CD4-RKR or Kir6.2 when not assembled with SUR subunits.

The Kir6.2 Tail Is Specifically Recognized by the COPI Vesicle Coat

As the COPI coat is responsible for recognizing C-terminal dilysine signals, it may be a good candidate for the recognition of arginine-based ER localization signals. Thus, we repeated the same binding assay presented in Figure 1 with a 200,000 × g pellet fraction obtained from HeLa extract under low-salt conditions. This fraction contains microsomes and large protein complexes like ribosomes and the COPI coat. In parallel, solubilized dog pancreatic microsomes were used as input material for the binding assay. Binding of the COPI coat complex was assessed by Western blotting employing antibodies directed against six of the seven coat subunits (Figure 6A). Estimating from the signals for the input and the eluate, COPI proteins were enriched 40-fold or more in the eluate. This enrichment was not observed for the KKK mutant control tail. These results suggest that the COPI coat recognizes the RKR-containing tail in a specific manner. They make it likely that recognition by the COPI coat can explain the ER localization of membrane proteins bearing these signals.

To further test this hypothesis, we performed the binding assay with COPI complex purified from rabbit liver. Again, specific binding of the coat complex to the affinity matrix presenting the arginine-based signal was observed (Figure 6B, first two lanes). This suggests that the interaction between the Kir6.2 tail and the COPI complex is direct. Our previous results imply that the COPI coat and 14-3-3 proteins may compete for the presented affinity construct. We therefore titrated recombinantly expressed 14-3-3 protein into the binding reaction between the Kir6.2 tail and the purified COPI coat (Figure 6B). 14-3-3 levels exceeding the tail protein molecules 2-fold were able to displace a significant amount of COPI coat complex (as evidenced by the disappearance of the α-COP immunoreactive band), and binding was completely abolished when the 14-3-3 protein was added in greater excess. This indicates that the 14-3-3 proteins and the COPI vesicle coat compete for an RKR-containing binding site on the Kir6.2 cytosolic tail.

The Monomeric Kir6.2 Tail Has a Drastically Reduced Affinity for 14-3-3 Proteins

The idea that the valency of an RKR-containing tail would determine the equilibrium between COPI coat and 14-3-3 protein binding predicts that the apparent affinity of the monomeric version of Kir6.2 tail (Figure 1B) for 14-3-3 proteins should be drastically reduced. We tested this in the binding assay and found that binding of 14-3-3 isoforms epsilon and zeta to the monomeric form of the tail was reduced to the detection limit for epsilon and was strongly reduced for zeta (Figure 6C). At the same time, binding of COPI coat proteins to the monomeric tail was still well detectable (Figure 6D). We conclude that the spatial arrangement and copy number of the Kir6.2 tail determine the relative avidity of interaction with the COPI coat and 14-3-3 proteins.

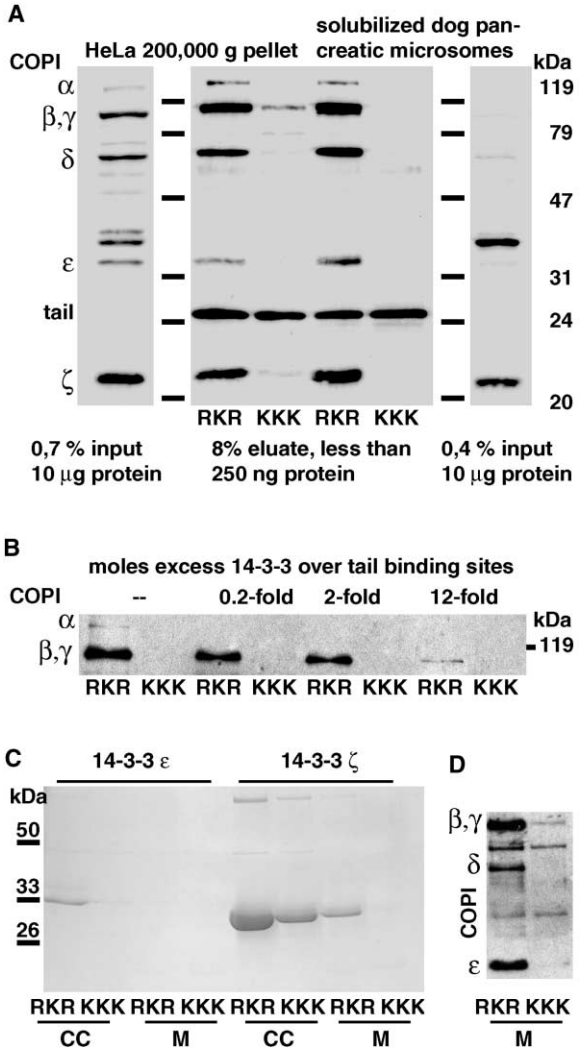

Figure 6. The COPI Coat Complex Directly Recognizes the Kir6.2 Tail

(A) Binding of the COPI coat from a high-speed pellet prepared from HeLa extract under low-salt conditions and from solubilized dog pancreatic microsomes. The protein amounts that were loaded are indicated. COPI subunits were detected by Western blotting with a cocktail of several anti-COPI antibodies.

(B) Reconstitution of the interaction with purified COPI complex and competition between COPI coat proteins and 14-3-3 protein. When 14-3-3 was added to the binding assay in amounts exceeding the available tail binding sites, COPI was displaced from the affinity construct.

(C) Binding assays comparing the avidity of interaction between purified 14-3-3 epsilon and zeta isoforms and the monomeric (M) and tetrameric (CC) version of the tail, respectively. Proteins are visualized by Coomassie staining.

(D) Binding of the COPI vesicle coat to the monomeric variant of the Kir6.2 tail. Individual COPI subunits were detected as described in (A) and (B).

Discussion

By employing a biochemical affinity purification strategy, we have identified proteins of the 14-3-3 family as interaction partners that can specifically recognize the arginine-based ER localization motif present in the distal C terminus of the K_{ATP} pore-forming subunit Kir6.2.

14-3-3 proteins are abundant cytosolic, dimeric proteins that can interact with a host of cellular proteins [18, 23–25]. The current concepts of 14-3-3 action on ligand proteins include the induction of conformational

changes or the masking of peptide signals, e.g., nuclear localization signals. A third concept draws from the fact that each 14-3-3 dimer has two ligand binding domains, thus enabling the dimer to serve as a scaffold that brings the two ligand proteins in close proximity.

Since the arginine-based motif functions as an ER localization signal on a variety of reporter proteins [3], the first question was whether binding of the 14-3-3 protein would mediate ER localization. We tested this by fusing a well-characterized 14-3-3 binding peptide to the cytosolic C terminus of the CD4 reporter protein and found that 14-3-3 binding is compatible with efficient membrane protein exit from the ER. This result is consistent with two recent studies addressing 14-3-3 binding to the two-pore potassium channel KCNK3 [5, 26]. This membrane protein has a 14-3-3 interaction motif in the distal C terminus that is only recognized with high affinity when phosphorylated. The study by O'Kelly et al. demonstrates that 14-3-3 binding to the C terminus of KCNK3 is required to overcome ER localization mediated by a dibasic motif in the N terminus of the protein. These results corroborate a scenario that had previously emerged from studies of the li35p major histocompatibility antigen class II-associated invariant chain: li35p is retained in the ER by an arginine-based retention motif [27], and li35p phosphorylation resulting in 14-3-3 binding has been correlated with forward trafficking from the ER [28–30].

In contrast to the C-terminal dilysine motif in which the consensus is well defined and restricted to the distal C terminus of membrane proteins, arginine-based signals have a more flexible consensus and are position independent. Strong evidence from the yeast and higher eukaryotic systems has established the COPI vesicle coat as the machinery recognizing the C-terminal dilysine signals [31–36]. Initially, Schutze et al. defined the so-called N-terminal diarginine signal by using the li35p invariant chain as a model protein and concluded that the spacing requirements for this signal were similarly strict as those observed for the C-terminal dilysine signal [27]. Our analysis of arginine-based ER localization signals in the K_{ATP} channel complex [3] revealed the surprising position independence of this class of signals and established a requirement for at least two arginines in the cluster (as opposed to lysine residues). Up to now, there is no evidence from any system that arginine-based signals are recognized by the COPI coat. O'Kelly et al. provide evidence that the sequence KR at the distal N terminus of KCNK3 is recognized by the COPI coat (as monitored by coimmunoprecipitation of the β-COP subunit). The results from our binding assay demonstrate the specific enrichment of purified COPI coat complex on the RKR-containing tail. This specificity in comparison to the KKK mutant is consistent with our in vivo analysis of signal efficacy [3, 4] and, thus, further supports the involvement of the COPI vesicle coat.

Because the recognition of the KCNK3 14-3-3 binding motif requires phosphorylation of a serine in the motif, O'Kelly et al. focused their attention on the role of this interaction in regulated forward trafficking from the ER. Our results argue against phosphorylation of S372 (part of a PKA consensus phosphorylation site also comprising the RKR signal in the Kir6.2 C-terminal tail) as a prerequisite for 14-3-3 binding to the multimeric construct. It is also clear that the phosphorylation of Kir6.2 on S372A is not required for forward trafficking of K_{ATP} channels, since two studies demonstrate normal surface expression of a K_{ATP} channel complex containing Kir6.2 S372A [37, 38]. The transient or permanent association of the full K_{ATP} channel complex with 14-3-3 proteins has yet to be demonstrated. The generally ubiquitous expression pattern of the 14-3-3 genes [39], the high abundance of 14-3-3 proteins in the brain, and the specific expression analysis of the 14-3-3 epsilon gene [40] make it likely that 14-3-3 proteins are present in the cell types expressing K_{ATP} channels, like neurons, cardiac myocytes, and pancreatic β-cells.

Our results show that low-affinity 14-3-3 ligands can be recognized with high avidity when presented in a multimeric fashion. This effect is based on the fact that 14-3-3 proteins exist as dimeric proteins that can bind two ligands simultaneously. Thus, the geometry of the 14-3-3 dimer may serve to probe the valency and spatial arrangement of the cytosolic tails of membrane proteins during assembly and thereby fulfill a checkpoint function for forward trafficking. For K_{ATP} channels, such a mechanism may consist of a specific spatial arrangement of the Kir6.2 tails in the Kir6.2/SUR hetero-octameric complex or the recognition of closely juxtaposed RKR signals from the Kir6.2 and SUR subunits.

It will be important to directly demonstrate binding of the 14-3-3 proteins to membrane proteins at the level of the ER. Furthermore, it will be interesting to establish whether the interaction with 14-3-3 proteins persists at the cell surface. Previously described interactions of 14-3-3 proteins with ion transport proteins like the plant plasma membrane H^+-ATPase, the *Drosophila* Slowpoke potassium channel, and the human ether-a-go-go-related gene (HERG) channel [41–44] have delineated a role for 14-3-3 binding in the regulation of these proteins at the cell surface. In addition to regulatory effects on protein function, it is conceivable that 14-3-3 proteins may control the accessibility of additional peptide trafficking signals and thus determine the steady-state levels of a given membrane protein at multiple cellular locations.

Conclusions

We propose fine tuning the avidity of 14-3-3 proteins for the cytosolic domains of membrane proteins as a general mechanism that can serve to control the subcellular localization of multimeric membrane protein complexes.

Experimental Procedures

Molecular Biology

Standard molecular biology protocols were adapted from [45]. For recombinant protein expression, cDNAs were cloned into a pQE60 (Qiagen) derivative containing two Z (protein A) domains [46] or into pMAL-c2X (NEB). For expression in mammalian cells, all constructs were in pcDNA3 (Invitrogen). Fusions were created by the polymerase chain reaction and were verified by sequencing. The exact amino acid sequence of the coiled-coil domain pLI employed for artificial tetramerization and surrounding linkers reads: GGGSGS**RMKQIED KLEEILSKLYHIENELARIKKLLGER**GGSGSAA (the bold text indicates the coiled-coil domain) [15]. COSI and HEK293T cells were maintained under standard tissue culture conditions and were transfected by using Fugene 6 (Roche).

Recombinant Expression of Proteins and Purification from *E. coli*

Tail constructs used as bait in binding assays were expressed in an *ompT* protease-deficient strain of *E. coli* and were purified on nickel-NTA agarose. MBP fusion of 14-3-3 epsilon was expressed in DH5α and was purified on amylose resin. The R18 peptide (PHCVPRDLSWLDLEANMCLP) was synthesized by Peptide Specialty Laboratories (Heidelberg).

Antibodies

Antibodies against 14-3-3 beta (mouse monoclonal H-8; recognizes all isoforms) and 14-3-3 gamma (rabbit polyclonal; partial crossreactivity with 14-3-3 eta) were from Santa Cruz Biotechnology. Mouse monoclonal antibodies against CD4 (EDU-2) and CD8 (UCH-T4; RFT8γ) were from Diatec and Cymbus Biotechnology, respectively. An antibody recognizing the last 36 amino acids of Kir6.2 was raised in guinea pig. A cocktail containing rabbit polyclonal and mouse monoclonal antibodies against COPI proteins was a gift of Felix Wieland [34].

Preparation of Protein Extracts from HeLa Cells

A total of 15 g frozen HeLa cells were lysed hypotonically in buffer L (5 mM Tris/HCl [pH 7.5], 0.5 mM $Mg(OAc)_2$, 0.5 mM EGTA, 3 mM β-mercaptoethanol). Sucrose was added to 250 mM and Tris/HCl (pH 7.5) to 20 mM, and the extract was precleared from cell remnants by low-speed centrifugation, cleared from organelles at 48,000 $\times$ g, and centrifuged at 200,000 $\times$ g for 2 hr to sediment particles $>$40 S. The pellet containing these particles was resuspended in 50 mM HEPES-KOH (pH 7.5), 250 mM sucrose.

Binding Assays

Purified bait proteins were immobilized on IgG sepharose (Pharmacia) in immobilization buffer (50 mM Tris/HCl [pH 7.5], 5 mM $Mg(OAc)_2$, 300 mM NaCl) at 0.6 mg/ml, except for the experiments shown in Figure 6B in which 0.1 mg/ml bait protein was used. A total of 500 μl HeLa cytosol substituted with 150 mM NaCl and energy mix (10 mM phosphocreatine, 0.5 mM ATP, 0.5 mM GTP, 50 μg/ml creatine phosphokinase) was incubated with 40 μl of the affinity matrix for at least 4 hr. After four washes with binding buffer (25 mM Tris/HCl [pH 7.5], 0.5 mM $Mg(OAc)_2$, 150 mM NaCl), bound proteins were eluted with 1.5 M magnesium chloride and precipitated with isopropanol. Binding assays with recombinantly expressed 14-3-3 epsilon as a MBP fusion protein were performed in the same buffer by using a molar ratio of 0.5 of 14-3-3 with respect to the immobilized bait protein, except for the experiments shown in Figure 6B in which the ratio between the 14-3-3 and tail proteins was 0.25. Dog pancreatic microsomes were prepared as described [47] and were solubilized with 10 mM CHAPS. COPI coat was purified from rabbit liver as described [48]. For the 200,000 $\times$ g pellet fraction from HeLa cell extracts and the solubilized microsomes, binding and washing steps were performed in 50 mM HEPES-KOH (pH 7.5), 150 mM NaCl, 250 mM sucrose. Excised Coomassie-stained protein bands were identified by MALDI mass spectrometry on a Bruker Reflex III employing α-cyano-hydroxy-cinnamic acid as matrix.

Luminometric Surface Expression Assay

COSI cells plated in 35 mm tissue culture dishes were transfected, and surface expression of all constructs was assayed 25 hr after transfection. Cells were fixed with 4% formaldehyde in PBS (20 min), blocked in PBS with 1% fetal bovine serum (30 min), and then labeled with primary antibody for 1 hr and with an appropriate HRP-coupled secondary antibody for 20 min. Chemiluminescence of the entire 35 mm dish was quantitated in a TD-20/20 luminometer (Turner Designs) after 15 s 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 diluted 1:1000, mouse monoclonal anti-CD8 antibody UCH-T4 diluted 1:1000, and goat anti-mouse HRP-conjugated IgG (Jackson) diluted 1:2000. For each construct, surface expression was assayed in two 35 mm dishes, and each experiment was repeated several times with nearly identical results when normalized to a given construct.

Crosslinking and Immunoprecipitation

Permeabilization of transfected HEK293T cells 40 hr after transfection was performed according to [49]. Briefly, cells were resuspended in buffer H (165 mM KOAc, 2 mM $Mg(OAc)_2$, 50 mM HEPES-KOH [pH 7.4]) and were treated with saponin at a concentration of 0.01% for 10 min. Then, the crosslinker DSS was added from a 10 mM stock in DMSO, and the reaction was allowed to proceed for 15 min at 25°C. Cells were then 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.

Western Blotting

Blots were blocked in TBS containing 5% milk powder and 0.02% NP-40. Primary antibodies (anti-14-3-3 beta mouse monoclonal H-8, 0.4 μg/ml; anti-14-3-3 gamma rabbit polyclonal, 1 μg/ml; anti-Kir6.2 guinea pig polyclonal) and secondary antibodies (HRP-conjugated anti-mouse, anti-guinea pig, and anti-rabbit antibodies, Jackson, 1:1,000) were diluted in TBS-blocking solution. Blots were washed in TBS-blocking solution and then in TBS, 0.02% NP-40. Detection was performed by using the ECL system (Amersham).

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