Intracellular Trafficking of KA2 Kainate Receptors Mediated by Interactions with Coatomer Protein Complex I (COPI) and 14-3-3 Chaperone Systems*

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Assembly and trafficking of neurotransmitter receptors are processes contingent upon interactions between intracellular chaperone systems and discrete determinants in the receptor proteins. Kainate receptor subunits, which form ionotropic glutamate receptors with diverse roles in the central nervous system, contain a variety of trafficking determinants that promote either membrane expression or intracellular sequestration. In this report, we identify the coatomer protein complex 1 (COPI) vesicle coat as a critical determinant in their cytoplasmic tail and therefore are highly expressed as homomeric receptors on the plasma membrane of heterologous cell lines (3), whereas the KA2 subunit has an endoplasmic reticulum (ER) retention/retrieval signal and consequently does not reach the plasma membrane in the absence of other KAR subunits (4). The KA2 ER retention/retrieval signal consists primarily of an arginine-rich domain similar to that characterized on several other ionotropic glutamate receptor subunits, including NR1 (5) and GluK5-2c (6), as well as other signaling proteins such as ATP-sensitive potassium channels (KATP) (7) and GABAergic receptors (8). Although a number of trafficking motifs in KAR subunits have been identified (9), little is known about how these trafficking signals control localization of receptors and which chaperone proteins are responsible for the modulation of receptor trafficking.

Kainate receptors (KARs) play a variety of roles in the mammalian central nervous system that include contributions to postsynaptic neurotransmission at a subset of excitatory synapses and presynaptic modulation of both excitatory and inhibitory transmission (1, 2). These diverse physiological roles of KARs require selective assembly, subcellular trafficking, and targeting of these proteins to their functional sites. Elucidating the cellular mechanisms that control these processes is important for understanding the full spectrum of KAR-mediated signaling in the brain.

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1 The abbreviations used are: KAR, kainate receptor; ER, endoplasmic reticulum; GluR, Glu receptor; IP, immunoprecipitate; COPI, coatomer protein complex I; VSVG, vesicular stomatitis virus glycoprotein; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; GFP, green fluorescent protein; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay.

Oligomerization and intracellular trafficking of KARs are controlled in part through interactions with chaperone proteins that bind to discrete cytoplasmic domains on the receptor proteins themselves. For example, GluR6a KAR subunits contain a forward trafficking determinant in their cytoplasmic tail and therefore are highly expressed as homomeric receptors on the plasma membrane of heterologous cell lines (3), whereas the KA2 subunit has an endoplasmic reticulum (ER) retention/retrieval signal and consequently does not reach the plasma membrane in the absence of other KAR subunits (4). The KA2 ER retention/retrieval signal consists primarily of an arginine-rich domain similar to that characterized on several other ionotropic glutamate receptor subunits, including NR1 (5) and GluK5-2c (6), as well as other signaling proteins such as ATP-sensitive potassium channels (KATP) (7) and GABAergic receptors (8). Although a number of trafficking motifs in KAR subunits have been identified (9), little is known about how these trafficking signals control localization of receptors and which chaperone proteins are responsible for the modulation of receptor trafficking.

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from Dr. Stephen Heinemann (Salk Institute, La Jolla, CA). Myc-GluR6(Q), myc-GluR5-2b, and myc-GluR5-2c were received from Dr. Christophe Muller (Université Bordeaux II, France). VSVG-14-3-3ζ was a gift from Dr. Melanie Darstine. All cDNAs were in pcDNA3 vectors. Other cDNA mutants were generated using the QuikChange site-directed mutagenesis protocol (Stratagene, La Jolla, CA). All mutants were subcloned back into the wild type vector using an EcoRI-XbaI site for myc-KA2 cDNAs. PCR-amplified sequences including the site mutations were verified by DNA sequencing.

Cerebellar Lysate Preparation—Cerebella were dissected from 129SvEv wild type mice or KA2−/− mice at 21–25 days old, homogenized in 50 mM Tris-HCl, 0.32 mM sucrose, pH 7.4, and centrifuged at 800 × g for 5 min at 4 °C. Supernatants were centrifuged again at 180,000 × g for 1 h at 4 °C. Pellets were resuspended in lysis buffer consisting of 50 mM Tris, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride-HCl, 0.8 μM aprotinin, 20 μM leupeptin, 40 μM bestatin, 15 μM pepstatin A, and 14 μM 1-trans-epoxysuccinyl-leucylamido(4-guanidino)butane.

Cell Culture, Transfection, and Protein Preparation—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. COS-7 cells were plated in 100-mm dishes at 1 × 10⁵ cells/dish and transfected with 1 μg of cDNA using FuGENE 6 reagent (Roche Applied Science) following the manufacturer’s recommended protocol. 48 h after transfection, cells were washed twice with cold phosphate-buffered saline (PBS) and lysed in 0.5 ml of lysis buffer. Crude protein lysates were obtained after centrifugation at 13,000 × g at 4 °C for 20 min. Wild type CHO and mutant CHO(IfdF) (clone 2) cells were a gift from Dr. Richard Anderson (University of Texas Southwestern Medical Center, Dallas, TX) and Dr. Monty Krieger (Massachusetts Institute of Technology, Cambridge, MA), and were maintained as described in Guo et al. (16). 48 h after transfection, cells were transferred to 39.5 °C for 12 h before biochemical analysis.

Immunoprecipitation and Immunoblotting—Immunoprecipitations were performed by first incubating membrane preparations with 50 μl of either 50% protein A/G-Septahose slurry (20422, Pierce), for COS-7 cell lysate, or TrueBlot anti-rabbit IgG beads (00-8800, eBioscience, San Diego, CA), for cerebellar preparations, overnight at 4 °C to eliminate nonspecific binding ("preclear" step). Preclear supernatants were incubated with 4 μg of anti-KA2 (06-315, Upstate Biotechnology, Waltham, MA), anti-GluR6/7 (06-309, Upstate Biotechnology), anti-β-COP (PA1–061, Affinity BioReagents, Golden, CO), anti-α-COP (PA1–067, Affinity BioReagents), anti-14-3-3β (K-19, sc-629, Santa Cruz Biotechnology, Santa Cruz, CA), anti-14-3-3γ (C-16, sc-731, Santa Cruz Biotechnology), anti-14-3-3ζ (C-16, sc-1019, Santa Cruz Biotechnology), or anti-VSVG glycoprotein antibody (clone PSD4, V5507, Sigma) at 4 °C for 2 h, followed by the incubation with 50 μl of either 50% protein A/G-Septahose beads or TrueBlot anti-rabbit IgG beads at 4 °C overnight. After five washes in lysis buffer, bound proteins were eluted from beads by boiling in 2× Laemmli sample buffer (Bio-Rad) for 5 min and then separated by electrophoresis on 8% SDS-PAGE gels. 2 μg of COS-7 proteins and 10 μg of cerebellar proteins were loaded into the lystate lane. Proteins were electrotransferred onto nitrocellulose membranes and probed with anti-β-COP (1:2000), anti-KA2 (1 μg/ml), anti-GluR6/7 (1 μg/ml), anti-myc (0.4 μg/ml, clone 9E10, Roche Applied Science), anti-GFP (1:500, 8371, JL-8, BD Biosciences, Palo Alto, CA), anti-14-3-3β (1:3000, H-8, Santa Cruz Biotechnology), anti-14-3-3ζ (1:10000), or anti-VSVG antibody (1:200,000). Immunoreactive bands were visualized using horseradish peroxidase (HRP)-conjugated sheep anti-mouse secondary antibody (1:5000, NA931V, Amersham Bio-science), HRP-conjugated donkey anti-rabbit secondary antibody (1:5000, NA934V, Amersham Biosciences), or HRP-conjugated anti-rabbit IgG TrueBlot (1:2000, 88-1688, eBioscience). The density of bands was quantitated using an Alphalager machine (model 5500, Alphalnageter, San Leandro, CA) and Chemilighter program (Alphalnageter). Quantitation of protein densities on Western blots was carried out by normalizing the immunoprecipitated band density to that of the lysate band (which was proportional to the expression levels of the protein). For each experiment, the density of the background preclear band was subtracted from the immunoprecipitate band density, and this value was divided by the density of the lysate band. The lysate lane in every Western blot was loaded with 2 μg, allowing us to compare density ratios between experiments. All experiments were performed at least three times. Statistical analyses were performed on the normalized values.

Immunolocalization of Receptors and Confocal Microscopy—Transfected COS-7 cells were fixed with 4% paraformaldehyde for 20 min and then permeabilized with 0.2% Triton X-100 for 5 min. Cells were incubated with anti-myc antibody (4 μg/ml in 10% goat serum) at room temperature for 2 h. Cells were washed 3 times with PBS and subsequently incubated with anti-β-COP antibody (1:500 in 10% goat serum) at room temperature for 2 h. After 3 washes, cells were incubated with appropriate fluorescence-conjugated secondary antibodies: Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes, Eugene, OR) at room temperature for 1 h. All images were taken from an inverted LSM 510 Meta confocal microscope at the Optical Imaging Laboratory using a Plan-Apochromat ×63/1.4 oil objective lens with excitation wavelength at 480 nm for Alexa Fluor 488 and at 543 nm for Alexa Fluor 594.

Peptide-binding Assay—Two peptides containing the KA2 cytoplasmic arginine-rich trafficking determinant and the associated alanine mutant were synthesized with amide terminal groups (KA2-Rpep, CRKTSRSRRRRP; KA2-Apep, CRKTSRSAAAP). These were linked to Sepharose beads at the initial cysteine residue (21st Century Biochemicals, Malboro, MA). Peptide beads were incubated with COS-7 cell lysate overnight. After five washes in lysis buffer, bound proteins were eluted and separated as described in the immunoblotting section. Band densities in Western blots were normalized to total β-COP expression in lysate. Experiments were performed four times.

Cell Surface Biotinylation Assay—COS-7 cells were plated in 6-well plates and transfected with 1 μg of cdNA. CHO cells were plated into 100-mm dishes and transfected with 5 μg of cdNA. Transfected cells were washed twice with cold PBS and incubated with 0.5 mg/ml EZ-link sulfo-NHS-SS-biotin (21331, Pierce) in PBS, pH 8.0, at 4 °C for 30 min with gentle agitation. Non-reacted biotin was quenched using quenching solution (50 μM glycine in PBS, pH 8.0) 3 times and then cells were washed 3 times with cold TBS (0.025 M Tris and 0.15 mM NaCl), pH 7.2. Cells were harvested in 0.5 ml of lysis buffer containing protease inhibitors. Lysates were incubated with 50 μl of 50% streptavidin-Sepharose high performance beads (17-5113-01, Amersham Biosciences) at 4 °C overnight. Beads were washed four times, and proteins were eluted and detected as co-immunoprecipitation experiments. In the experiments with CHO cells, the "total" lane was loaded with lysate equivalent to 1% of the protein loaded on the peptide-linked beads. Biotinylated proteins eluted from the beads (corresponding to subunits located in the plasma membrane) were normalized to total protein expression and reported in the term of percentage. All experiments were performed at least four times.

Enzyme-linked Immunosorbent Assay (ELISA)—COS-7 cells were inoculated at 4.5 × 10⁴ cells and transfected with 0.6 μg of cdNA using FuGENE 6 reagent in 12-well plates. After 48 h, cells were washed once with cold PBS and incubated with anti-myc antibody (4
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FIGURE 1. KA2 KAR subunits interact with β-COP vesicle coat protein. A, homomeric myc-tagged kainate receptor subunits expressed in COS-7 cells were immunoprecipitated (IP) by an anti-β-COP antibody and detected in Western blots (IB, immunoblot) by an anti-myc antibody (left panel). Three lanes are shown for each Western blot: a lysate lane (L) containing 2 μg of membrane protein lysate, an immunoprecipitation lane (IP) loaded with 30 μl of the immunoprecipitate, and a preclear lane (PC) showing the background binding of proteins to beads lacking antibody. Anti-β-COP antibody immunoprecipitated KA2 subunits but not GluR5-2b, GluR5-2c, or GluR6a subunits. Blots were stripped and re-probed with an anti-β-COP antibody (right panel) to verify precipitation of β-COP proteins. B, β-COP was weakly detected after immunoprecipitation with an anti-myc antibody (left panel). Blots were stripped and re-probed with an anti-myc antibody (right panel) to demonstrate precipitation of myc-KA2 receptors. C, cerebellar lysate prepared from 129SvEv or KA2−/− mice was used for immunoprecipitation with anti-KA2 antibody. β-COP protein co-precipitated with KA2 subunits in wild type cerebellar lysate but not lysate from KA2−/− cerebella. The right panel demonstrates the presence of KA2 protein in the immunoprecipitate from wild type mice. D, immunoprecipitation of β-COP from cerebellar lysate from 129SvEv mice co-precipitated KA2 (left blot) but not GluR6/7 (middle blot) subunits. E, immunoprecipitation of GluR6/7 subunits from cerebellar lysates did not co-precipitate β-COP protein (left blot), but did pull down GluR6/7 and KA2 protein (middle and right blots). F, co-localization of β-COP (green) with myc-KA2 subunits (red) in transfected COS-7 cells. COPI staining was most obviously localized to the Golgi apparatus and in small puncta likely representing transport vesicles. Homomeric myc-KA2 receptor staining showed the characteristic reticular pattern of endoplasmic reticulum. An overlay of β-COP and KA2 immunofluorescence at higher magnification shows that KA2 subunits were partly co-localized with β-COP (arrows).

RESULTS

Immunoprecipitation of KA2 and COPI Proteins—The COPI chaperone complex was shown recently to be a critical mediator of ER retrieval mediated by polyarginine signals in a subset of integral membrane proteins (11, 12, 14). The KA2 kainate receptor subunit is retained efficiently in the ER through a mechanism primarily mediated by a polyarginine signal in the cytoplasmic carboxyl-terminal domain. To determine whether COPI proteins subserve a retention/retrieval function for the KA2 kainate receptor subunit, we first tested for a biochemical interaction between KA2 subunits and members of the COPI complex in transfected COS-7 cells. NH2-terminal myc-tagged recombinant rat KA2, GluR5-2b, GluR5-2c, or GluR6a cDNAs were transfected into COS-7 cells before immunoprecipitation of cell lysates with an anti-β-COP antibody; KA2 or other subunit protein was then detected in Western immunoblots using an anti-myc antibody. We found that homomeric myc-KA2 receptors co-precipitated with endogenous β-COP in either COS-7 cells (IP lane, Fig. 1A) or in transfected HEK293 cells (data not shown). The PC lane in Fig. 1 showed the nonspecific binding of protein to the beads used for immunoprecipitation. In contrast to KA2, myc-GluR5-2b, myc-GluR5-2c, and myc-GluR6a subunits did not co-immunoprecipitate with β-COP (Fig. 1A), demonstrating that the interaction is relatively specific to KA2 subunits. We also tried

µg/ml in 10% goat serum) at 4 °C for 30 min to detect plasma membrane expression. Cells were then washed twice with cold PBS and fixed with 4% paraformaldehyde in PBS at room temperature for 20 min. For measuring total protein expression, cells were first fixed with paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS for 5 min, and incubated with anti-myc antibody at room temperature for 1 h. Both groups were washed 3 times with PBS and incubated with HRP-conjugated sheep anti-mouse secondary antibody (1:1000 in 10% goat serum, NA931V, Amersham Biosciences) at room temperature for 1 h. After the third washing with PBS, the HRP substrate o-phenylenediamine dihydrochloride (P9187, Sigma) was added and the color reaction was developed for 1 h. The optical density of 0.2 ml of supernatant was detected by spectrophotometer at 490 nm. All values were an average of four replicates in each experiment; the mean background absorbance of the negative controls (sham-transfected cells) were subtracted from surface and total absorbance values. Data are presented in the form of a ratio of plasma membrane expression to total cellular expression. At least four experiments were performed with each cDNA.

Statistical Analysis—Data were tested with a one-way analysis of variance and the post-hoc Tukey-Kramer multiple comparison test, or a one-tailed paired Student’s t test.

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the converse experiment, immunoprecipitation of myc-KA2 and immunoblotting for β-COP protein, and observed a very weak but detectable co-precipitation (Fig. 1B). We postulate from these data that the majority of KA2 subunits are resident in the ER and that a relatively small proportion of the receptor subunit transiently associates with COPI during retrieval to the ER.

We next tested if the interaction between KA2 subunits and COPI complex proteins could be detected in the central nervous system. The KA2 subunit is highly expressed in cerebellar granule cells, where they likely form heteromeric receptors with GluR6 subunits and modulate excitatory synaptic transmission at parallel fiber synapses (17). Membrane protein lysates were prepared from homogenized cerebella from 129SvEv mice as well as KA2−/− gene-targeted mice (as a control for nonspecific interactions with the precipitating antibody). KA2 protein was immunoprecipitated from the lysates with an anti-KA2 antibody and β-COP was detected in Western blots (Fig. 1C). As shown in Fig. 1, β-COP subunit co-immunoprecipitated with KA2 protein from wild type but not KA2−/− cerebella (Fig. 1C, left panels). Blots were stripped and re-probed with anti-KA2 antibody to verify that the immunoprecipitation of KA2 subunit protein was successful (Fig. 1C, right panel), and as expected immunoreactivity was not detected in the samples from KA2−/− mice. A reciprocal immunoprecipitation using an anti-β-COP antibody also supported a robust association between the KA2 subunit and β-COP (Fig. 1D). Because cerebellar KA2 subunits likely assemble with GluR6 subunits as heteromeric receptors, we also tested to see if β-COP co-precipitated GluR6 either directly or in association with KA2. As shown in Fig. 1D, GluR6/7 immunoreactivity did not precipitate with β-COP. In the reciprocal experiment, we immunoprecipitated GluR6/7 protein and Western blotted for β-COP. The vesicle protein was not detected in this assay (Fig. 1E). These results support the interpretation that KA2 subunits exist in at least two distinct pools in cerebellum: those assembled with GluR6 as heteromeric KARs and those associated with the COPI retrograde trafficking pathway fated for retrieval to the ER.

Subcellular Co-localization of KA2 and β-COP—Next, we determined if the subcellular localizations of KA2 subunits and COPI proteins were consistent with their interaction in our biochemical assays using confocal immunofluorescence staining of transfected COS-7 cells. Myc-KA2 subunits were detected with an anti-myc antibody (red in Fig. 1F) and β-COP was stained with an anti-β-COP antibody (green in Fig. 1F). In addition to COPI vesicle staining, anti-β-COP antibody strongly labels the Golgi apparatus, which is manifested as the intense green labeling in the image. The vast majority of KA2 receptor subunits are located in the ER and are unlikely to be actively associated with COPI, accounting for very weak β-COP bands when the KA2 subunit is the target of immunoprecipitation. As shown in the last row of images in Fig. 1F, the discrete vesicle-like structures (punctate dots) positively stained for both KA2 and β-COP likely represent COPI vesicles containing KA2 as cargo protein.

Elimination of COPI Function Promotes Plasma Expression of KA2 Receptors—If COPI association plays a central role in limiting forward trafficking of KA2 subunits beyond the cis-Golgi, homomeric KA2 receptors should exhibit higher plasma membrane expression when COPI functionality is disrupted. CHO(IdlF) cells contain a temperature-sensitive (ts) mutation in one subunit of the complex, e-COP (16). At a nonpermissive temperature (39.5 °C), e-COP is degraded and the cells lose the capability of forming COPI vesicle coats. We compared the plasma membrane expression of homomeric myc-KA2 receptors expressed in wild type CHO cells and CHO(IdlF) cells using a cell surface biotinylation assay (Fig. 2A). Cell surface proteins were labeled with biotin, purified on streptavidin columns, and eluted proteins were immunoblotted with anti-myc antibody. We included lanes with 1% of the input myc-KA2 protein to determine the relative amount of KA2 expressed at the plasma membrane, and detected actin immunoreactivity to verify equivalent protein loading (Fig. 2A). The relative plasma membrane to total protein expression of myc-KA2 receptors in CHO(IdlF) cells increased significantly by 3.6-fold (n = 4, p < 0.05) after a 12-h incubation at 39.5 °C compared with CHO(IdlF) cells kept at 34 °C for the same period of time (Fig. 2B). Transfected CHO cells kept at either 37 or 39.5 °C expressed the same relative level of myc-KA2 on the plasma membrane as CHO(IdlF) cells maintained at the permissive temperature. This data support the hypothesis that COPI-mediated retention/retrieval prevents plasma membrane expression KA2 receptors.

Dependence of COPI Association on the KA2 Trafficking Determinant—We hypothesized that COPI proteins interacted with KA2 subunits at the arginine-rich domain in the intracellular carboxyl terminus. To determine whether the critical amino acid residues responsible for COPI association site correlated with those required for ER sequestration, we examined COPI association with truncated and alanine-substituted KA2 subunits generated by site-directed mutagenesis of the receptor cDNA. The cytoplasmic tail of KA2 contains 155 amino acids; our numbering beginning at the initial methionine), which eliminated the cis-Golgi with COPI function, increased the relative level of plasma membrane KA2 subunits (while concurrently reducing total KA2 expression). β-d band density from A was measured by densitometry. After 12 h incubation at 39.5 °C, homomeric myc-KA2 receptors expressed in CHO(IdlF) cells had higher relative plasma membrane expression to total protein expression than cells incubated at 34 °C, representing a 3.6-fold increase. Data represent mean ± S.E. (n = 4, *p < 0.05).
FIGURE 3. The polyarginine trafficking determinant in carboxyl terminus of the KA2 subunit is a COPI association site. A, the diagram illustrates the truncation and substitution mutants made in the KA2 carboxyl terminus. KA2 mutants were generated with truncations at residues 827 and 856, and the polyarginine domain from residues 856 to 866 and the dileucine signal at residues 908–909 were substituted with alanines and valines, respectively. B, immunoprecipitation of β-COP co-precipitated significantly less myc-KA2(827-stop), myc-KA2(856-stop), myc-KA2(R862-6A), and myc-KA2(R862-6A,L908-9V) protein compared with unmodified myc-KA2 (left panel). Immunoprecipitation controls are shown from re-probed blots in the right panel. Abbreviations: L, lysate; PC, pre-clear background. C, quantitation of the IP band densities normalized to their corresponding lysate band density. Co-precipitation of the indicated receptor subunits was significantly reduced by 2.4–4.5-fold compared with unmodified receptors. (n = 3–6, **, p < 0.01). Data represent mean ± S.E.

The polyarginine trafficking determinant in carboxyl terminus of the KA2 subunit is a COPI association site. We tested the interaction of truncated KA2 subunits (Fig. 3, A and B), respectively (Fig. 3, A). Immunoprecipitation of β-COP co-precipitated significantly less myc-KA2(827-stop), myc-KA2(856-stop), myc-KA2(R862-6A), and myc-KA2(R862-6A,L908-9V) protein compared with unmodified myc-KA2 (left panel). Immunoprecipitation controls are shown from re-probed blots in the right panel. Abbreviations: L, lysate; PC, pre-clear background. C, quantitation of the IP band densities normalized to their corresponding lysate band density. Co-precipitation of the indicated receptor subunits was significantly reduced by 2.4–4.5-fold compared with unmodified receptors. (n = 3–6, **, p < 0.01). Data represent mean ± S.E.

β-COP is one of seven proteins that comprise the COPI chaperone system; to further validate the association between KA2 subunits and the COPI complex we tested for association between the receptor subunit and α-COP, another component of COPI vesicle coats. Immunoprecipitation of KA2-transfected cells with α-COP antibody resulted in co-precipitation of KA2 protein (Fig. 4A). As with β-COP, the truncation of receptor (myc-KA2(856-stop)) and mutation of the polyarginine motif (myc-KA2(R862–6A)) markedly reduced association with α-COP (by 88 ± 4 and 66 ± 15%, n = 3, respectively, p < 0.01) (Fig. 4, A and B). These results further support our hypothesis that the arginine-rich domain plays a role in COPI association.
Interaction between β-COP and Peptides Containing the KA2 Trafficking Determinant—We next tested if the association inferred from our immunoprecipitations likely resulted from a direct interaction between COPI proteins and the polyarginine domain in the KA2 subunit. This was tested by comparing the binding of β-COP protein in COS-7 cell lysates to two short peptides, KA2-Rpep and KA2-Apep, which were conjugated to Sepharose beads (Fig. 5A). KA2-Rpep contained the KA2 cytoplasmic arginine-rich trafficking determinant, whereas KA2-Apep had the five alanine substitutions used in previous immunoprecipitation experiments. The Lysate lane in the Fig. 5 shows expression of the β-COP protein in the COS-7 lysate, and the NS lane shows the nonspecific interaction between lysate and Sepharose beads. We found that β-COP was readily detected in Western blots after elution from KA2-Rpep beads. In contrast, the protein was only weakly detected after elution from the KA2-Apep peptide (Fig. 5A). To quantitate this result, we normalized the band densities of β-COP staining in the peptide lanes to the density of the lysate lane (each experiment was run with all four groups in parallel). We found that alannine substitution in the peptide reduced the interaction with β-COP by 90 ± 3% (n = 4, p < 0.01) (Fig. 5B). This result strongly supports the hypothesis that COPI proteins directly interact with the KA2 receptor at the arginine-rich sequence in the COOH terminus.

COPI Association Is Reduced in Heteromeric GluR6a/KA2 Receptors—Co-assembly of GluR6a with KA2 subunits leads to efficient plasma membrane expression of heteromeric GluR6a/KA2 receptors, suggesting that the interaction between COPI and KA2 subunits and consequent ER retention is occluded in the presence of GluR6a subunits. To test this hypothesis, COS-7 cells were co-transfected with myc-KA2 and GFP-GluR6a cDNAs, and cell lysate was immunoprecipitated with an anti-β-COP antibody. As predicted, we found a reduction in co-precipitated myc-KA2 (by 64 ± 6%, n = 5, p < 0.01, Fig. 6A and B). GFP-GluR6a was not detectable in the immunoprecipitated complex of β-COP and myc-KA2 (Fig. 6A), suggesting that heteromeric GFP-GluR6a/myc-KA2 receptors did not interact with COPI to a significant degree. It therefore is likely that myc-KA2 detected after immunoprecipitation in these experiments represented a pool of unassembled or homomeric subunits that were not associated with GFP-GluR6a subunits.

Inhibition of COPI association with KA2 by GluR6a subunits could occur through masking of the polyarginine retention motif similar to the mechanism proposed for K+ channels (7) and GABAB receptors (8), although our previous data suggested that this was not the case for ER retention of KA2 subunits (3). To test if COPI association with KA2 was masked by the GluR6a COOH terminal tail, we co-transfected myc-KA2 with GFP-GluR6a(850-stop) cDNA, a truncated mutant that lacks most of the GluR6a cytoplasmic tail. Previously, we showed that heteromeric GFP-GluR6a(850-stop)/myc-KA2 receptors were expressed at the plasma membrane (3), suggesting that retention signals in KA2 were suppressed despite the absence of the forward trafficking motif in GluR6a. We found that heteromeric assembly of myc-KA2 with GFP-GluR6a(850-stop) subunits decreased myc-KA2-βCOP interaction by 72 ± 9% (n = 5, p < 0.01) (Fig. 6B). These data support our earlier hypothesis that the retention signal in KA2 subunits were not masked by the GluR6a cytoplasmic tail, but rather became unavailable for COPI association as a result of conformational changes in the KA2 subunit induced by heteromeric assembly.

Association of 14-3-3 Proteins with Kainate Receptor Subunits—One of the many cellular functions of members of the protein 14-3-3 gene family is modulation of plasma membrane expression of several integral proteins by competing directly or indirectly with the COPI complex for binding (11, 12). We tested for interactions between KA2 subunits and three endogenous isoforms of 14-3-3: 14-3-3ε, 14-3-3γ, and 14-3-3ζ, in myc-KA2-transfected COS-7 cells. Using anti-14-3-3 antibodies specific to the β, γ, and ζ isoforms for immunoprecipitation, we found that homomeric myc-KA2 receptors associated with all three 14-3-3 proteins. The association with 14-3-3ζ protein increased (by 3.5-fold, n = 4, p < 0.001) when the ER retention/retrieval trafficking determinants (Arg662—Arg666 and Leu908—Leu909) were mutated to alamines and valines, respectively (Fig. 7, A and B); in contrast, there were no significant differences in the association with 14-3-3ε and 14-3-3γ.
no significant differences in association of 14-3-3β or 14-3-3γ with myc-KA2 and myc-KA2(R862–6A, L908–9V) receptors (Fig. 7, A and B). Western blot revealed were re-probed with anti-14-3-3β, anti-14-3-3γ, or anti-14-3-3ζ to verify immunoprecipitation of 14-3-3 proteins (Fig. 7A), which were successful despite the relatively low abundance of 14-3-3ζ and 14-3-3η (both were undetectable in the lysate lanes).

To further explore the interaction between KA2 and 14-3-3ζ proteins, we co-transfected myc-KA2 and VSVG-tagged 14-3-3ζ cDNAs and immunoprecipitated the complexes with anti-VSVG antibody. Homomeric KA2 receptors bound to exogenous 14-3-3ζ proteins and this interaction was clearly increased in all the alamine-substituted mutants we tested, including myc-KA2(R862–4A) and myc-KA2(R864–6A) (2.9- and 3.1-fold, respectively, n = 4, p < 0.05), the three alanine mutants that did not show altered interactions with COPI. The highest level of association with VSVG-14-3-3ζ interaction was observed with myc-KA2(R862–6A, L908–9V) (4.8-fold, n = 4, p < 0.001) (Fig. 7, C and D). Immunoprecipitation of 14-3-3ζ was confirmed in Western blots with the anti-VSVG antibody (Fig. 7C). These data demonstrate that residues critical to COPI interaction also influence receptor association with 14-3-3ζ proteins.

The interaction with 14-3-3ζ proteins was much weaker, but still detectable, with the GluR5-2b, and GluR6a but not with GluR5-2c subunits. Homomeric myc-GluR5-2b and myc-GluR6a receptors were weakly co-precipitated with endogenous 14-3-3ζ and GluR6a was more strongly associated with exogenous VSVG-14-3-3ζ (Fig. 8, A and C). In contrast, myc-GluR5-2c subunits did not exhibit detectable levels of immunoprecipitation with either endogenous or exogenous 14-3-3ζ (Fig. 8B). The association of each of these receptor subunits with endogenous 14-3-3ζ was significantly lower than that observed with myc-KA2 subunits (Fig. 7).

Plasma Membrane Expression of KA2 and Mutant Subunits—To correlate the competitive interaction of COPI and 14-3-3 proteins with localization of KA2 protein on the plasma membrane, we measured the surface expression of KA2 relative to total pools of the protein in cell ELISA. As is shown in Fig. 9A, only ~1% of myc-KA2 protein is transported to the plasma membrane, consistent with the efficient retention mechanism mediated by the cytoplasmic polyarginine signal. Truncation of the COOH-terminal tail at either residue 827 or 856 increased cell surface expression by 2.7- and 3.7-fold, respectively (n = 4, p < 0.05). Relative plasma membrane expression was enhanced further by partial alanine substitution of the polyarginine domain (myc-KA2(R862–4A), 3.9 ± 0.4%, n = 4, p < 0.05 and myc-KA2(R864–6A), 5.3 ± 1.4%, n = 4, p < 0.001), and complete substitution of polyarginine domain (myc-KA2(R862–6A), 5.4 ± 0.6%, n = 8, p < 0.001). Finally, substitution of both the polyarginine domain and the dileucine motif in the myc-KA2(R862–6A, L908–9V) mutant increased plasma membrane expression to 8.5 ± 1.6% (n = 4, p < 0.001).

To support these results, we also compared the relative plasma membrane expression of myc-KA2 receptor and mutants after labeling surface proteins with biotin. KA2 protein purified on streptavidin columns was immunoblotted in parallel with lysate lanes loaded with 1% of the input protein and actin loading controls (Fig. 9B). Truncation of the...
**Chaperone Proteins Involved in KA2 Intracellular Trafficking**

A. Interactions of endogenous 14-3-3 and exogenous VSVG-14-3-3 with GluR5-2b receptors were tested utilizing anti-14-3-3 and anti-VSVG antibodies, respectively. Western blotting with an anti-myc antibody showed that myc-GluR5-2b receptors weakly co-precipitated with endogenous 14-3-3 and exogenous VSVG-14-3-3. *Top* blots were stripped and re-probed with anti-14-3-3 and anti-VSVG antibodies for endogenous 14-3-3 and exogenous 14-3-3, respectively. Abbreviations: I, lysate; PC, preclear background. B, GluR5-2c receptors did not show detectable association with endogenous 14-3-3 or exogenous VSVG-14-3-3. C, similar to GluR5-2b receptor experiments, GluR6a receptors showed weak association with exogenous VSVG-14-3-3.

**FIGURE 8.** GluR5 and GluR6 KAR subunits only weakly interacted with 14-3-3 proteins. A, interactions of endogenous 14-3-3 and and exogenous VSVG-14-3-3 with GluR5-2b receptors were tested utilizing anti-14-3-3 and anti-VSVG antibodies, respectively. Western blotting with an anti-myc antibody showed that myc-GluR5-2b receptors weakly co-precipitated with endogenous 14-3-3 and exogenous VSVG-14-3-3. *Top* blots were stripped and re-probed with anti-14-3-3 and anti-VSVG antibodies for endogenous 14-3-3 and exogenous 14-3-3, respectively. Abbreviations: I, lysate; PC, preclear background. B, GluR5-2c receptors did not show detectable association with endogenous 14-3-3 or exogenous VSVG-14-3-3. C, similar to GluR5-2b receptor experiments, GluR6a receptors showed weak association with exogenous VSVG-14-3-3.

COOH terminus at either residue 827 or 856 and alanine substitution at residues 862–866 promoted the plasma membrane expression of myc-KA2 receptors as shown in the PM myc-KA2 lane (Fig. 9B). The relative plasma membrane to total protein expression of myc-KA2 receptors showed a significant increase by 3.6–4.1-fold (n = 5, p < 0.05) in myc-KA2(827-stop), myc-KA2(856-stop), myc-KA2(R862–4A), myc-KA2(R864–6A), and myc-KA2(R862–6A) receptors. Similar to the cell ELISA, the myc-KA2(826–6A, L908–9V) mutant had the highest plasma membrane expression with a 6.7-fold increase (n = 5, p < 0.01) compared with the wild type receptors (Fig. 9C). These data are consistent with our earlier results that measured plasma membrane expression of a chimeric Tac receptor containing the KA2 COOH-terminal tail (4).

**DISCUSSION**

The intracellular chaperone systems that control assembly and entry of kainate receptors into the secretory pathway are largely unknown. In this study, we elucidated the cellular mechanism for retention of homomeric KA2 kainate receptors in the ER. These receptors do not form functional glutamate receptors when expressed on the plasma membrane, and therefore retention of these subunits contributes to the assembly of appropriately functional heteromeric channels when other KAR subunits are expressed. Endogenous KA2 subunits in the cerebellum, where they are expressed at high levels in granule cells, and exogenous KA2 in transfected cells both associated with COPI proteins in immunoprecipitation assays. The association was dependent upon the arginine-rich determinant in the cytoplasmic tail of KA2 and results in altered plasma membrane expression of KA2 receptors. In summary, our results identify the first set of proteins associated with critical trafficking determinants in kainate receptor subunits.

**Regulation of ER Localization of Homomeric Kainate Receptor Subunits by COPI**—The major function of COPI in the intracellular trafficking of proteins is thought to be retrieval of ER-resident proteins from the Golgi back to the ER (13). Membrane proteins containing

**FIGURE 9.** Truncation and alanine substitution at the KA2 polyarginine signal increases plasma membrane expression of homomeric myc-KA2 receptors. A, cell ELISAs were used to determine the relative level of plasma membrane expression. Anti-myc antibody was used to label plasma membrane receptors (in live cells) or all the KA2 subunits (in permeabilized cells) in parallel sets of transfected COS-7 cells. The relative plasma membrane expression of KA2 subunits increased with truncation and alanine substitution from a basal level of 1.0 ± 0.3% (myc-KA2(827-stop), 2.7 ± 0.1%; myc-KA2, 856-stop), 1.7 ± 0.4%; myc-KA2(856-stop), 1.9 ± 0.4%; myc-KA2(R864-6A), 5.3 ± 1.4%, and myc-KA2(R862-6A,L908–9V) receptors showed the highest plasma membrane expression at 8.5 ± 1.6%. Data represent mean ± S.E. (n = 4–8, *p < 0.05, and ***, p < 0.001). B, equivalent experiments in which surface proteins were labeled with biotin and purified on streptavidin columns before immunoblotting. Total myc-KA2 expression was measured by loading 1% of the membrane protein loaded on the streptavidin column. Actin was detected as an additional loading control. C, densitometric quantitation showed the truncated and alanine-substituted mutants were expressed at higher levels on the plasma membrane compared with wild-type receptors (myc-KA2, 0.4 ± 0.2%; myc-KA2(827-stop), 1.5 ± 0.3% *, myc-KA2(856-stop), 1.5 ± 0.2% *; myc-KA2(R864-6A), 1.7 ± 0.3%; myc-KA2(R864-6A), 1.7 ± 0.3% *, myc-KA2(R862-6A), 1.5 ± 0.2%; and myc-KA2(R862-6A, L908–9V) receptors showed the highest plasma membrane expression at 1.6% ± 0.3%). Data represent mean ± S.E. (n = 5–8, *p < 0.05, and ***, p < 0.01). These changes in COPI and 14-3-3 association were correlated with altered plasma membrane expression of KA2 receptors. In summary, our results identify the first set of proteins associated with critical trafficking determinants in kainate receptor subunits.
arginine-based trafficking motifs, such as $K_{\text{ATP}}$ channels and GABA$_B$ receptors, exhibit an association with COPI inversely correlated with their level of plasma membrane expression (12, 18). We also found that either the disruption of COPI functionality, in the CHO(idf) cells, or a reduction in KA2-COPI association by mutagenesis was similarly correlated with increased cell surface expression (by 3–8-fold). We note that despite this significant increase, the large majority of KA2 protein remained in intracellular compartments (predominantly the ER). This suggests other mechanisms exist that limit the forward progress of homomorphic KA2 receptors through the biosynthetic pathway. Among the possibilities are inefficient folding or oligomerization processes. Indeed, our antibody-based detection assays would not differentiate between KA2 proteins in monomeric, dimeric, or tetrameric assemblies, and therefore much of the total KA2 protein we detect in these assays could be in immature forms. Finally, GluR5-2b and GluR5-2c receptors, both of which are largely retained in the ER via arginine-based determinants (6, 19), did not associate with COPI at a detectable level, suggesting that other retention/retrieval pathways exist for these receptors.

**The Arginine-rich Motif as a COPI Interaction Domain in Homomeric KA2 Receptors**—The classically defined COPI binding motif consists of a cytoplasmic dilyasine motif $K(X)KXX$ (20), but more recent research has demonstrated that the protein complex also mediates arginine-based retention/retrieval mechanisms in a variety of proteins that include lip35, KCNK3 channels, and $K_{\text{ATP}}$ Channels (21, 22). Importantly, Yuan and colleagues (12) demonstrated that this interaction occurred through a direct binding of the RKR sequence of $K_{\text{ATP}}$ channels by COPI proteins, rather than an association through intermediary proteins. We found that the string of arginines comprising residues 862–866 in the cytoplasmic tail of KA2 are critical for intracellular retention and immunoprecipitation with COPI vesicle coat proteins. As well, this determinant controlled interaction between $\beta$-COPI protein and immobilized KA2-Rpep peptide. Thus, the robust interaction between COPI and immobilized peptide and myc-KA2 receptors was significantly reduced upon alanine substitution of all five of the constituent arginines (by 90%), demonstrating that the occlusion of COPI binding and the resulting release of the heteromeric KAR units to form defined sets of heteromeric receptors that likely are differentially targeted to various membrane domains. We predicted that co-assembly of KA2 with other subunits would relieve COPI-mediated retention, because heteromeric KARs are trafficked to plasma membrane (in heterologous cell lines) and pre- and postsynaptic sites of action (in neurons). This was indeed the case; co-expression of KA2 with GluR6a subunits reduced association of $\beta$-COPI with KA2 subunits by 64 ± 6%, consistent with the robust plasma membrane expression of heteromeric GluR6a/KA2 receptors. It is likely that the residual interaction between COPI and KA2 occurred with subunits that had not formed heteromeric assemblies with GluR6a, because the latter subunit was not detectable in the immunoprecipitates isolated with the anti-$\beta$-COPI antibody. Thus, the arginine-based retention/retrieval in KA2 becomes occluded upon heteromeric assembly of receptors.

The mechanism of occlusion of the KA2 trafficking determinant upon assembly with GluR6a subunits is obscure. Similar motifs in $K_{\text{ATP}}$ channels, the major histocompatibility protein MHC II, N-methyl-D-aspartate receptors, and GABA$_A$ receptors are thought to be sterically masked by heterotypic cytoplasmic domains in other subunits or associated proteins (22). More recently, Gassmann and colleagues (23) proposed that inactivation of the GABA$_B1$ subunit RSRR retention signal upon heteromeric assembly with GABA$_B2$ subunits occurred because the determinant was removed from an "active zone" rather than by being masked through coiled-coiled domain interactions. Similarly, we previously reasoned that steric masking of the KA2 signal by cytoplasmic domains of co-assembled kinase receptor subunits (e.g. GluR6a) did not underlie inactivation of the KA2 arginine determinant because GluR6a subunits with truncated COOH-terminal domains assembled with KA2 subunits and promoted forward trafficking to the plasma membrane (3). Our current results further support this interpretation. Heteromeric assembly of KA2 with truncated GluR6a(850-stop) subunits, which contains only 9 amino acids in the COOH terminus, markedly decreased COPI binding by 72 ± 9%, demonstrating that the occlusion of COPI binding and the resulting release of the heteromeric KAR into the secretory pathway likely did not occur through direct masking by the COOH terminus of GluR6a subunits. These observations are most consistent with our hypothesis that structural alterations within the KA2 subunit itself upon assembly with appropriate partner subunits into hetero-oligomers are responsible for occlusion of COPI binding and inactivation of the retention/retrieval signal.

**Suppression of Arginine-based ER Retention/Retrieval Motif via Other Chaperone Proteins**—Inactivation of COPI-dependent arginine-based ER retention/retrieval signals in a number of integral membrane proteins results from competitive binding to cytoplasmic domains by other chaperone proteins, which permits egress from the ER into the secretory pathway. In particular, interactions with the 14-3-3 protein family were shown to counteract the COPI association through both phosphorylation-dependent and phosphorylation-independent mechanisms (21, 22). Several novel binding sites of 14-3-3 proteins overlap with arginine-based ER retention/retrieval motif (11, 12, 18). It has been proposed that the association to 14-3-3 proteins competes and prevents COPI binding, leading to the release of proteins from the ER retrieval pathway (11, 12), although this hypothesis was challenged recently (18). 14-3-3 proteins are a widely expressed family of chaperone proteins that subserve a variety of cellular functions including cell cycle and growth control, signal transduction, and apoptosis (24). Their participation in subcellular...
trafficking pathways and modulation of the signaling function of ion channels has only recently begun to be elucidated.

Our interest in the potential relevance of 14-3-3 proteins to KA2 subunit trafficking was initiated by low-stringency analysis of protein-protein interaction domains in the KA2 cytoplasmic tail, which identified a putative 14-3-3 binding site overlapping the arginine-based trafficking determinant (25). Consistent with this prediction, we found that KA2 subunits interacted with multiple 14-3-3 isoforms including those endogenous to the COS-7 heterologous cell line. However, mutagenesis of the polyarginine domain unexpectedly increased 14-3-3 binding, effectively eliminating the possibility that a putative association with the KA2 subunit occurs in a directly competitive fashion with COP1 (at least at this particular site). The domain in KA2 that mediates association with 14-3-3 proteins has yet to be elucidated. We found that all mutations to the KA2 COOH terminus that increased 14-3-3 binding correlated with a higher relative expression on the plasma membrane, even if the mutations did not significantly effect COP1 interactions. For example, mutation of the dileucine motif (Leu908–Leu909), which increases surface expression and 14-3-3 and GluR5-2c), in contrast to KA2 subunit, and therefore postulate that receptor biosynthesis through interactions with distinct subunits. This ER retention process is inactivated by conformational changes following heteromeric assembly with GluR6 subunits and is inversely correlated with interactions between KA2 and 14-3-3 chaperone proteins. The COP1 pathway therefore constitutes an important early pathway for cellular control of KA subunits and their availability for assembly into functional receptors.

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