14-3-3ζ Protein Regulates Anterograde Transport of the Human κ-Opioid Receptor (hKOPR)∗†§

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Background: Regulation of export of 7TMRs is not well understood.

Results: 14-3-3ζ binds hKOPR C-tail and facilitates hKOPR export by inhibiting COPI- and RVR motif-mediated ER localization.

Conclusion: 14-3-3ζ interaction with hKOPR C-tail promotes export of hKOPR.

Significance: This is among the first reports to show that interaction of a 7TMR with 14-3-3 regulates its anterograde transport. Potential mechanisms are delineated.

Opioid receptors belong to the rhodopsin subfamily of the seven-transmembrane domain receptor (7TMR) family. There are three subtypes of opioid receptors, μ, δ, and κ (MOPR, DOPR, and KOPR, respectively). Pharmacological effects of KOPR activation in vivo include analgesia (especially for visceral chemical pain), dysphoria/aversion, sedation, water diuresis, hypothermia, antipuritic effects, and modulation of immune responses (for review, see Ref. 1). The selective KOPR agonist nalfurafine is used clinically in Japan for treatment of uremic pruritus in kidney dialysis patients (2). KOPR antagonists produce axiolytic- and antidepressant-like effects in animal models (for review, see Ref. 3).

Studies have shown that 7TMRs interact with many proteins in addition to G proteins. These proteins directly participate in signaling of the receptor and act as part of a scaffolding complex to modulate receptor signaling or regulate receptor trafficking, localization, and pharmacological characteristics (for review, see Ref. 4). We have demonstrated that the human KOPR (hKOPR) interacts with NHERF-1/EBP50 (5, 6) and GEC1 (7) and that these interactions play important roles in signal transduction and trafficking of the KOPR in the internalization and export pathways, respectively. Using proteomic analyses, we found that 14-3-3ζ was one of the proteins co-immunoprecipitated with FLAG-tagged hKOPR from the extract of Neuro2a (N2A) cells stably expressing the FLAG-hKOPR (N2A-FLAG-hKOPR cells).

14-3-3ζ proteins are a group of abundant acidic proteins of 30 kDa in eukaryotic cells (for review, see Refs. 8–11). There are seven mammalian 14-3-3 isoforms (ζ, 1, 2, 3, 4, 5, and 6) with differences in their amino acid sequences mostly in the C-terminal region. 14-3-3 proteins form homodimers and heterodimers and are present in cytoplasm, chloroplasts, various membranes, and cytoskeletal and centrosome structures (for review, see Ref. 9). When they are recruited to associate with the seven-transmembrane domain receptor (7TMR), the 14-3-3 proteins bind to the receptor C-terminus and inhibit the receptor ER localization or ER export. There are several known receptor-14-3-3 binding motifs: COPI, RVR, and COPII. 

In our previous study, we demonstrated that 14-3-3ζ interacts with hKOPR (hKOPR) from extracts of solubilized Neuro2A cells stably expressing the FLAG-hKOPR (N2A-FLAG-hKOPR cells). 14-3-3ζ proteins co-immunoprecipitated with human hKOPR (hKOPR) from extracts of solubilized Neuro2A cells stably expressing the FLAG-hKOPR (N2A-FLAG-hKOPR cells). 14-3-3ζ proteins are a family of conserved regulatory molecules in eukaryotic cells, where they participate in signal transduction, metabolism, and membrane protein transport. 14-3-3ζ proteins co-localized with the hKOPR in N2A cells. The hKOPR C-tail interacted with 14-3-3ζ in rat brain extracts and bound directly to purified 14-3-3ζ as demonstrated by pulldown techniques. 14-3-3ζ siRNA decreased expression of the hKOPR in N2A-FLAG-hKOPR cells and cultured primary cortical neurons of E19 rats by ~25% as determined by immunoblotting, ligand binding, and flow cytometry. The effect of 14-3-3ζ siRNA was reversed by overexpression of 14-3-3ζ. Expression of the 14-3-3ζ scavenger protein GplLI-R18 also decreased hKOPR expression. 14-3-3ζ siRNA did not change expressions of the hDOPR and rMOPR in N2A cells. Pulse-chase study showed that 14-3-3ζ siRNA decreased the amount of mature hKOPR but did not change the rate of maturation or stability of hKOPR protein. Mutations of R354A/S358A in the putative 14-3-3ζ interaction motif KQQSTT368 in the hKOPR C-tail reduced interaction of the hKOPR with 14-3-3ζ and abolished the effect of 14-3-3ζ knockdown on hKOPR expression. Mutation of the endoplasmic reticulum retention motif RVR adjacent to the 14-3-3ζ interaction motif in the hKOPR C-tail decreased interaction of coatomer protein I (COPI) with the hKOPR and abolished 14-3-3ζ-mediated regulation of hKOPR expression. 14-3-3ζ knockdown increased association of COPI with the hKOPR. These results suggest that 14-3-3ζ promotes expression of the hKOPR by inhibiting COPI and RVR motif-mediated endoplasmic reticulum localization machinery.

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membranes, most of the 14-3-3 proteins are localized in the Golgi (12). 14-3-3 proteins interact with a variety of proteins and have many different functions including modulating surface protein expression and signal transduction pathways, acting as scaffolds for assembly of oligomeric complexes, serving as phosphoprotein adaptors, and regulating apoptosis and cell cycle (for review, see Refs. 8–11).

The capacity of agonists to modulate downstream signaling molecules depends on availability of the receptors on cell surface. The number of cell surface 7TMRs reflects a delicate balance between the biosynthesis export pathway and the endocytosis pathway. The post-activation endocytic events such as internalization, recycling, and degradation have been well documented, and most 7TMRs share similar mechanisms (1, 13); however, regulation along the export pathway is much less understood. Evidence is emerging showing that the transport process of 7TMRs along the endoplasmic reticulum (ER)-Golgi-plasma membranes is regulated and involves specific sorting motifs and proteins (7, 14–18). COP1 is an ADP-riboseylation factor-dependent adaptor protein that coats vesicles transporting proteins from cis-Golgi back to the ER, i.e. retrograde transport. Some membrane-bound proteins contain sorting motifs in their C-tails that interact with COP1 and direct the protein to exit the Golgi and return to the ER. In the C-tail of the hKOPR, there is such a motif, 359RVR361.

Deficiency in ER-Golgi-plasma membrane trafficking of several 7TMR mutants has been shown to be associated with human diseases. Mutations of vasopressin V2 receptor, gonadotropin-releasing hormone receptor, and rhodopsin are linked to nephrogenic diabetes insipidus, hypogonadotropic hypogonadism, and retinitis pigmentosa, respectively (19–21). In contrast, deletion in the HIV-1 co-receptor CCR5 (CCR5Δ32) decreases cell surface expression, which in turn reduces viral infection (22). Pharmaco-chaperones, which are lipophilic ligands of the receptors capable of penetrating plasma membranes, rescue cell surface expression of the mutant receptors by facilitating correct folding and stabilizing the receptor proteins (19–21, 23). In addition, pharmaco-chaperones also enhance cell surface expression of wild type 7TMRs (for review, see Ref. 24). Therefore, delineation of molecular mechanisms that regulate trafficking along the export pathway of 7TMRs will provide a better understanding of 7TMR biology and have implications for therapeutic intervention. In this study we investigated the interaction of 14-3-3ζ with the hKOPR and its functional significance with the focus on its role in membrane protein transport and possible mechanisms of effects of 14-3-3ζ.

**EXPERIMENTAL PROCEDURES**

**Materials**

[3H]Diprenorphine (58 Ci/mmol) and 1-[35S]methionine/cysteine (~1175 Ci/mmol) were purchased from PerkinElmer Life Sciences. Mouse 14-3-3ζ siRNA, rat 14-3-3ζ siRNA, and rabbit anti-14-3-3ζ antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse 14-3-3ζ siRNA (sc-29585) is a pool of three different siRNA duplexes. Their sense sequences are as follows: 5’-CUGCUGGAUGACAA-GAATT-3’; 5’-CCAUGUCUAAGCAAAGAAATT-3’; 5’-CCUCAGACUUACAGAAATT-3’.

Luciferase siRNA (targeting 5’-GCCATTCTATCCTCTAGGGATG-3’) was purchased from Dharmacon (Thermo Fisher Scientific, Lafayette, CO). Dynorphin A-(1–17) was purchased from Phoenix Pharmaceuticals (Belmont, CA). U50,488H and naloxone were provided by the National Institute on Drug Abuse, National Institutes of Health (Bethesda, MD). Purified rabbit anti-FLAG antibody (F7425), anti-FLAG M2 affinity gel, peroxidase-conjugated goat anti-rabbit IgG, poly-D-lysine, arabinosylcytosine, antibody (F7425), anti-FLAG M2 affinity gel, peroxidase-conjugated goat anti-rabbit IgG, poly-D-lysine, arabinosylcytosine, papain, and Ponceau S were obtained from Sigma. Anti-β COP polyclonal antibody was purchased from Thermo Scientific (Rockford, IL). Rabbit polyclonal anti-GAPDH antibody conjugated with horseradish peroxidase (ab9385) was obtained from Abcam (Cambridge, MA). Pansorbin cells were purchased from Calbiochem. SuperSignal West Pico chemiluminescence substrate kit was purchased from Pierce. Protease inhibitor mixture tablets were purchased from Roche Applied Science. Neurobasal medium, GlutaMax, and B27 were obtained from Invitrogen. Hanks’ balanced salt solution was obtained from Mediatech, Inc. (Herndon, VA). HA-14-3-3ζ in pcDNA3 was obtained from Addgene Inc. (Cambridge, MA). 14-3-3 scavenger protein (pGpL18-R18 in pcDNA3) was a gift from Dr. Blanche Schwappach of University of Manchester, Manchester, England. Glutathione-Sepharose 4B beads and pGEX-4T-1 bacterial expression plasmid were from GE Healthcare. pET-30a(+) bacterial expression vector and Bug-Buster protein extraction reagent were obtained from Novagen Co. (Madison, WI). Mouse neuro2A (N2A) cells were purchased from ATCC.

**Animals**

Timed pregnant Sprague-Dawley rats were purchased from Charles River, Inc. (Horsham, PA). Animals were allowed to acclimate to the animal facility for 2 days. The rats were housed one animal per cage on a 12:12-h light/dark cycle with lights on at 7 a.m. and with ad libitum access to food and water. Experimental protocols were approved by the Institutional Animal Care and Use Committee of Temple University School of Medicine. Animal care and experimental procedures were conducted according to the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

**Identification by Proteomic Analysis of Proteins Immunoprecipitated with the Human KOPR Expressed in N2A-FLAG-hKOPR Cells**

Mouse Neuro2A (N2A) neuroblastoma cells transfected with FLAG-hKOPR (N2A-FLAG-hKOPR cells) (clone 95) were used with untransfected N2A cells as the control. Cells (106 cells) were solubilized with TTSEC buffer (2% Triton X-100, 50 mM Tris HCl, 150 mM NaCl, 5 mM EDTA, and protease inhibitor mixture tablets (1 tablet/10 ml), pH 7.4) for 1 h at 4°C and centrifuged at 100,000 g for 30 min. Supernatant was filtered through a 0.22-μm filter, incubated overnight with M2 anti-FLAG antibody conjugated to agarose, washed with 25 mM Tris-HCl buffer/150 mM NaCl, pH 7.4 (TBS)/1% Triton X-100, 4 times, and eluted twice with 100 FLAG peptide in 0.1% Nonidet P-40/0.2 M NaCl/20 mM Tris-HCl buffer, pH 7.4. Immunoprecipitated proteins were then resolved with 8% SDS-PAGE.
14-3-3ζ Regulates Expression of KOPR

Twenty-nine protein bands that were immunoprecipitated from N2A-FLAG-hKOPR cells, but not from N2A cells, were excised for proteomic analyses, which were performed by Proteomics Core Facility, University of Pennsylvania School of Medicine. Microflow reverse-phase HPLC/MS/MS was used for the analyses. Among the proteins identified was 14-3-3ζ.

Generation of FLAG-hKOPR-AQSTA, FLAG-hKOPR-AVR, and FLAG-hKOPR-KVR cDNA Constructs

cDNA cloning and sequencing the hKOPR tagged with the FLAG epitope N-terminal to the first Met (FLAG-hKOR) in the vector pcDNA3 was generated previously (25). Site-directed mutagenesis and the overlap polymerase chain reaction method were used to mutate 354RQSTS358 to 354AQSTA358 (FLAG-hKOPR-AQSTA), 359RVR to 359AVR (FLAG-hKOPR-AVR) or 359RVR to 359KVR (FLAG-hKOPR-KVR) in the C-tail of the hKOPR with FLAG-hKOPR in pcDNA3 as the template. The mutated cDNAs were cloned into pcDNA3, and DNA sequence determination was performed to ensure correct generation of all mutations and no unwanted mutations.

Stable Expression of FLAG-hDOPR, FLAG-rMOPR, and FLAG-hKOPR Mutant in N2A Cells

Neuro2A cells were transfected with the FLAG-human δ-opioid receptor (N2A-FLAG-hDOPR), FLAG-rat μ-opioid receptor (N2A-FLAG-rMOPR), FLAG-hKOPR (N2A-FLAG-hKOPR), FLAG-hKOPR-AQSTA (N2A-FLAG-hKOPR-AQSTA), FLAG-hKOPR-KVR, FLAG-hKOPR-AVR, or 3HA-hKOPR, each in pcDNA3, and clonal cells stably expressing each receptor were established using Geneticin as the selection pressure. A clonal cell line for each receptor with a similar mutation was stably transfected with 10 μg of the indicated cDNA constructs or the vector (control) and 6 ml of Opti-MEM medium using 30 μl of Lipofectamine 2000 according to the manufacturer's instructions. N2A-FLAG-hKOPR cells (~80% confluence) in 6-well plates were transfected with 100 pmol/well 14-3-3ζ siRNA or luciferase siRNA (control) with 5 μl of Lipofectamine 2000. At 16 h after transfection, medium was replaced by 10 ml of Opti-MEM with 10% serum. About 40 h after transfection, cells were harvested for experiments.

14-3-3ζ Rescue Experiment

N2A-FLAG-hKOPR cells in 12-well plates were grown to ~70% confluence and transfected with 40 pmol of 14-3-3ζ siRNA or luciferase siRNA (control) using 2 μl of Lipofectamine 2000 in Opti-MEM medium according to the manufacturer's instructions. Twelve hours later, cells were transfected with 2.0 μg of HA-14-3-3ζ cDNA or the vector (control) using 5 μl of Lipofectamine 2000 in Opti-MEM medium. 4–6 h after the second transfection, medium was replaced with MEM medium + 10% serum. About 48 h after the second transfection, cells were harvested for immunoblotting.

Immunofluorescence of HA-hKOPR and 14-3-3ζ

N2A-HA-hKOPR cells were cultured on coverslips placed in a 12-well plate (2 × 10⁵ cells/well). Cells were fixed with methanol for 10 min at −20 °C. Immunofluorescence was performed by incubating cells with both mouse M1 anti-HA (1:1000) and rabbit anti-14-3-3ζ (1:62.5) primary antibodies overnight at 4 °C and followed by incubating with the secondary antibodies goat-anti-mouse IgG conjugated with Alexa-488 and Texas Red-conjugated goat anti-rabbit IgG (1:1000), respectively, at room temperature for 30 min. Images were acquired using a Nikon TE300 fluorescence microscope with a 40× objective lens and a Magnifire digital camera. NIH Image and Adobe Photoshop were used for image processing.

Immunoblotting of FLAG-hKOPR

Immunoblotting was performed to examine the expression of FLAG-hKOPR proteins as we described previously (7, 26). Cells were solubilized with 2× Laemmli sample buffer (4% SDS, 0.1 M DTT, 20% glycerol, 62.5 mM Tris, and bromphenol blue, pH6.8), subjected to 8% Tricine-SDS-PAGE, and transferred onto PDVF Immobilon membranes. Membranes were treated with blocking solution, incubated with polyclonal FLAG antibody (1:5000) followed by goat anti-rabbit polyclonal IgG conjugated with horseradish peroxidase (1:5000), and reacted with ECL Western-blotting detection reagents. GAPDH was used as the loading control, which was probed with rabbit polyclonal anti-GAPDH antibody conjugated with horseradish peroxidase (1:1000). Images were captured by use of a FUJIFILM LAS1000 plus system and quantitated using the ImageGauge software (Version 4.1, Fuji Photo Film Co. Ltd.).
Co-immunoprecipitation of the FLAG-hKOPR or Its Mutant with 14-3-3ζ or β-COP

Co-immunoprecipitation was performed according to our published procedure (5). Briefly, N2A-FLAG-hKOPR cells in a 100-mm plate were washed with cold PBS and solubilized with TTSEC buffer for 1 h at 4 °C and centrifuged at 100,000 × g for 1 h. The supernatant was incubated overnight at 4 °C with anti-FLAG M2-agarose to immunoprecipitate FLAG-hKOPR. Immunoprecipitated materials were then separated with SDS-PAGE, transferred, and immunoblotted with anti-14-3-3ζ (1:3000) or anti-β-COP (1:1000) antibodies as described above for the Western blot.

Pulldown Assays

Interaction of hKOPR-C-tail with 14-3-3ζ in Rat Brain Extracts—Rat brains were lysed and solubilized in TTSEC buffer containing 0.1 mM PMSF. After centrifugation at 100,000 × g for 40 min, the supernatant was filtered sequentially with 0.45 and 0.22 μm membranes. Supernatants were used for pulldown experiments as described previously (6) by use of glutathione-agarose beads preloaded with GST and GST-hKOPR-C-tail, respectively. 14-3-3ζ was detected by immunoblotting with anti-14-3-3ζ antibody (1:3000). The relative sizes and loading amounts of the GST fusion proteins were detected by Coomassie Blue staining.

Direct Interactions of hKOPR-C-tail with 14-3-3ζ—Experiments were performed as described previously (6, 7). GST and GST-hKOPR-C-tail bound to glutathione-Sepharose 4B beads at −5–10 μg of each protein/10 μl of resin with less loading of GST-hKOPR-C-tail due to its lower binding affinity. The beads (10 μl each) were incubated with 14-3-3ζ (3 μg) in 0.3 ml of 1× PBS buffer containing 1 mg/ml bovine serum albumin at 4 °C with end-over-end rotation overnight. The beads were washed 4 times with ice-cold 1× PBS buffer to remove nonspecific binding. To dissociate proteins from the Sepharose 4B beads, they were incubated with 40 μl of 2× SDS-PAGE sample buffer at 100 °C for 5–10 min. The solution was then centrifuged to remove the beads. The supernatants (20 μl each) were subjected to 8% SDS-PAGE, and the gel was transferred to Immobilon™-P PVDF membrane, which was rinsed 3 times with TBS-T buffer and blocked with 5% nonfat dry milk in TBS-T buffer. 14-3-3ζ was detected by blotting with a rabbit anti-14-3-3ζ antibody (1:2000–3000) followed by anti-rabbit-HRP and enhanced chemiluminescence reagents. The membranes were stained with 0.1% Ponceau S in 5% acetic acid to reveal the relative loading amounts of the GST (26 kDa) and GST-hKOR C-tail (34 kDa).

Receptor Ligand Binding on Intact Cells

The hKOPR binding on intact cells was conducted with [3H]diprenorphine as we described previously (27). Briefly, N2A-FLAG-hKOPR cells in 6-well plates were transiently transfected with 100 pmol of 14-3-3ζ or control siRNA with 5 μl of Lipofectamine 2000. Approximately 2 days later, cells were collected and binding of [3H]diprenorphine, to the intact cells was performed in Krebs buffer solution. Receptor binding was performed with 1 nM [3H]diprenorphine. Nonspecific binding was defined by 10 μM naloxone (a hydrophobic ligand) and 1 μM dynorphin A-(1–17) (a hydrophilic ligand) for total receptors and cell surface receptors, respectively.

Quantitation of Cell Surface Receptor Expression by Fluorescence Flow Cytometry Assay

The experiment was performed according to our published procedures (28, 29) with some modifications. About 48 h after transfection of 14-3-3ζ siRNA or control siRNA, cells were washed 3 times with ice-cold 1× PBS buffer and detached with PBS buffer containing 0.5 mM EDTA. Cells were incubated with 1× PBS buffer containing 10% serum and 1 mM CaCl2 for 20 min at 4 °C. After washing, cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (0.25 μg/ml) in 500 μl of buffer A (PBS buffer containing 10% serum and 1 mM CaCl2) for 20 min at 4 °C. Cells were washed 3 times and resuspended with 500 μl of buffer A. Mouse IgG was used as the isotype control. Immunoreactivity of cell surface receptors was quantitated with fluorescence flow cytometry (FACScan; BD Biosciences).

[35S]Met/Cys Labeling of the hKOPR and Pulse-Chase Experiments

Experiments were performed using our previously published procedure (7). Cells were washed with DMEM without L-Met and L-Cys and preincubated in the same medium for 1 h at 37 °C. Metabolic labeling was performed in fresh Met/Cys-free medium containing 150 μCi/ml of [35S]Met/Cys. After a 1-h incubation at 37 °C, cells were washed, detached, solubilized with TTSEC buffer, and centrifuged at 13,500 × g for 15 min. The supernatant was incubated with rabbit polyclonal anti-FLAG antibody followed by Pansorbin for immunoprecipitation of 35S-labeled FLAG-hKOR. The immunoprecipitation procedure was repeated once. The PANSORBIN pellet was suspended in 2× Laemmli buffer for SDS-PAGE. The dried gels were exposed to storage phosphor screens, and the autoradiograms were acquired using a Cyclone PhosphorImager (PerkinElmer Life Sciences). The intensities of radiolabeled receptor bands were quantitated with OptiQuant software. Background signal of each lane was subtracted before performing quantitative data analysis.

RESULTS

Proteomic Analysis of Proteins Co-immunoprecipitated with the hKOPR—Fig. 1A shows staining of a representative SDS-PAGE gel of proteins immunoprecipitated with anti-FLAG antibody from control N2A cells and N2A-FLAG-hKOPR cells. There were many more protein bands present in immunoprecipitated complex from N2A-FLAG-hKOPR cells than from N2A cells. Twenty-nine protein bands that were present in N2A-FLAG-hKOPR cells, but not in N2A cells, were excised and subjected to tryptic digestion and LC/MS/MS analysis.

More than 30 proteins were identified after digestion and analysis, including five isoforms of the 14-3-3 protein (Supplemental Table 1). These isoforms were immunoprecipitated from N2A-FLAG-hKOPR cells but not from control N2A cells (Fig. 1A). As 14-3-3ζ has the highest score among the five isoforms and this particular isoform is abundant in the
14-3-3ξ Regulates Expression of KOPR

The hKOPR Is Associated with Endogenous 14-3-3ξ and Transfected HA-14-3-3ξ in N2A-FLAG-hKOPR Cells—To confirm the interaction between hKOPR and 14-3-3ξ, we examined the association by immunoprecipitation of FLAG-hKOPR with anti-FLAG antibody and immunoblotting of 14-3-3ξ. We observed that endogenous 14-3-3ξ was co-immunoprecipitated with the hKOPR (Fig. 2A). In addition, the interaction of hKOPR with transfected 14-3-3ξ was also confirmed in N2A-FLAG-hKOPR cells transfected with HA-tagged 14-3-3ξ cDNA by similar co-immunoprecipitation procedure (Fig. 2B). When immunoprecipitation was performed on N2A-FLAG-hKOPR cells using anti-FLAG antibodies preincubated with the FLAG peptide (10 μg/ml) or normal rabbit serum, FLAG-KOPR or 14-3-3ξ was not detected in immunoprecipitated materials (data not shown). These results indicate that the hKOPR interacts with 14-3-3ξ.

Co-localization of hKOPR with 14-3-3ξ—We next examined if hKOPR and 14-3-3ξ co-localized in N2A cells. We used N2A-HA-hKOPR cells instead of N2A-FLAG-hKOPR cells for these experiments because methanol fixation was needed for 14-3-3ξ staining with rabbit anti-14-3-3ξ antibody per the manufacturer’s instructions. However, in our hands mouse M1 anti-FLAG antibodies did not work in methanol-fixed cells. As shown in Fig. 3, 14-3-3ξ (red) has diffuse intracellular distribution, consistent with its reported localization in cells (for review, see Ref. 9). On the other hand, the hKOPR (green) is present on plasma membranes and in intracellular compartments, forming a punctate staining pattern. There is extensive overlap in staining of the two proteins (yellow), indicating co-localization of the hKOPR with 14-3-3ξ.

Interaction of the hKOPR C-tail with 14-3-3ξ in Rat Brain Extracts by Pulldown Assay—The binding of 14-3-3 to client proteins occurs through short peptide motifs. 14-3-3 binding motifs in many proteins have been reported, and there is no definitive consensus motif (10). Many of the sequences start with Arg and are enriched in Arg and Ser. One possible sequence generalized from the interaction motifs defined to date is -RX1(2–5)SX1(3–5)S-. As the hKOPR C-tail contains a potential 14-3-3 protein binding site (354RQSTS358), we examined if the hKOPR C-tail binds 14-3-3ξ in rat brain extracts with pulldown techniques. Rat brains were homogenized and solubilized. After centrifugation, supernatants were filtered and used for pulldown experiments with GST or GST-hKOPR-C-tail. 14-3-3ξ was detected by immunoblotting. As shown in Fig. 4A, GST-hKOPR C-tail bound much more 14-3-3ξ than GST, indicating that hKOPR C-tail binds 14-3-3ξ.

Direct Interaction of KOPR C-tail with 14-3-3ξ—Because co-immunoprecipitation of hKOPR and 14-3-3ξ or interaction of hKOPR C-tail with 14-3-3ξ in brain extracts may be due to direct binding or through other proteins, we then examined if there is direct interaction between the two purified proteins using pulldown techniques. GST, GST-KOPR C-tail, and 14-3-3ξ were expressed in Escherichia coli and purified. GST and GST-KOPR C-tail were loaded onto agarose-glutathione beads, and 14-3-3ξ was added. Bound 14-3-3ξ was detected with immunoblotting. GST-KOPR C-tail bound much more 14-3-3ξ than GST (Fig. 4B), indicating that the KOPR C-tail binds to 14-3-3ξ directly.

Knockdown of 14-3-3ξ Decreased Expression of the hKOPR in N2A Cells and in Cultured Primary Cortical Neurons of E19 Rats—Studies have shown that 14-3-3 proteins bind to membrane-bound proteins and promote their cell surface expression (for review, see Ref. 9–11). We examined if 14-3-3ξ affected expression of the hKOPR in both mouse N2A-FLAG-hKOPR cells and cultured primary cortical neurons of E19 rats using mouse and rat 14-3-3ξ siRNAs, respectively.

In N2A-FLAG-hKOPR cells, transfection of a mouse 14-3-3ξ siRNA decreased the amount of 14-3-3ξ by ~60% and reduced the expression of mature hKOPR (55 kDa) by about

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FIGURE 1. A, SDS-PAGE separation of FLAG-hKOPR associated proteins. N2A-FLAG-hKOPR cells and untransfected N2A cells (control) were solubilized, and FLAG-hKOPR was immunoprecipitated with anti-FLAG antibodies as described under "Experimental Procedures." The immunoprecipitated complex was subjected to 10% SDS-PAGE separation. The gel was stained with ProteomIQ Blue. Twenty-nine gel slices were collected for mass spectrometry analysis. Five 14-3-3 isoforms were identified in bands 8 and 9 (see supplemental Table 1).

B, LC-MS identification of 14-3-3 peptides generated from tryptic digestion is shown. The five 14-3-3 isoforms were scored from 56 to 189 (underlined numbers), and the highest degree of confidence in identification. The m/z ratios observed were 454.5611 (M+2H)2, 427.0818 (M+3H)3, and 774.9987 (M+2H)2. The underlined numbers indicate the queries matched with 14-3-3ξ sequences.

| Query | Mass (Da) | Score | Peptide |
|-------|-----------|-------|---------|
| 143F | 454.5611 | 189   | ζ, β, γ, and α |
| 378Y | 427.0818 | 189   | |
25% compared with transfection of luciferase siRNA (Fig. 5, A and B). In contrast, 14-3-3ζ/H9256 siRNA did not affect the 45-kDa form of the hKOPR (Fig. 5, A and B). Transfection with luciferase siRNA, used as the control, did not affect the hKOPR level compared with no transfection (data not shown). Our previous data showed that FLAG-hKOR migrated as two bands of Mr 55 (mature receptor) and 45 (immature receptor) (7). Therefore, 14-3-3ζ/H9256 may be involved in regulation of export of hKOPR along the ER-Golgi-plasma membranes in N2A-FLAG-hKOPR cells.

Primary cortical neurons from E19 embryonic rats were transiently co-transfected with FLAG-hKOPR-pcDNA3 and rat 14-3-3ζ siRNA or control siRNA. As shown in Fig. 5, C and D, compared with the control siRNA, 14-3-3ζ siRNA decreased the amount of 14-3-3ζ significantly and reduced expression of the mature hKOPR by about 25% but did not affect expression of the immature form.

Effect of overexpression of 14-3-3ζ on KOPR expression was also examined. 14-3-3ζ expression did not change the KOPR level (data not shown), which is most likely due to high levels of endogenous 14-3-3 proteins.

14-3-3ζ siRNA Reduced Total and Cell Surface Expression of the hKOPR as Determined by Radioligand Binding—After N2A-FLAG-hKOPR cells were transfected with 14-3-3ζ siRNA or control siRNA, receptor binding was performed with [3H]diprenorphine (−1 nm) on intact cells. For detecting total hKOPR...
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(A) Rat Brain extracts

(B) Purified 14-3-3 zeta

FIGURE 4. A, interaction of the hKOPR C-tail with 14-3-3ζ in rat brain extracts by pulldown assay is shown. Rat brains were homogenized, solubilized, and centrifuged at 100,000 × g for 40 min, and the supernatants were filtered through 0.45-μm filter and then 0.22-μm membranes. The filtrate was used for pulldown experiments by incubating with glutathione-agarose beads preloaded with GST or GST-hKOPR C-tail overnight at 4 °C. IB, immunoblot. B, direct interaction between the hKOR C-tail and 14-3-3ζ is shown. Purified 14-3-3ζ was incubated with glutathione-Sepharose 4B beads preloaded with GST and GST-hKOPR C-tail overnight at 4 °C. A and B, the beads were washed extensively, and the bound proteins were eluted from the beads, resolved by 8% SDS-PAGE, and transferred onto Immobilon™-P PVDF membranes. A 1/250 supernatant was also loaded as an input control. Upper panel, 14-3-3ζ was detected by a rabbit anti-14-3-3ζ antibody. Lower panel, the same membrane was stained with Ponceau S, showing the relative sizes and the amount of the GST and GST-hKOPR C-tail loaded. The figure represents one of the three experiments with similar results.

and cell surface hKOPR, naloxone (10 μM) and dynorphin A (1–17) (1 μM) were used to define nonspecific binding and cell surface receptors, respectively (27). Knockdown of 14-3-3ζ decreased both total and cell surface receptor binding of hKOPR by about 25% (Fig. 6) without changing the % of the total KOPR present on the cell surface (control, 69.2 ± 1.8%; 14-3-3ζ siRNA, 72.1 ± 3.2%, n = 3, p > 0.05, by Student’s t test). In addition, cell surface receptors were quantified by using fluorescence flow cytometry assay. Cell surface FLAG-hKOPR was labeled with immunofluorescence without permeabilizing cells followed by flow cytometry. 14-3-3ζ siRNA decreased cell surface KOPR expression by 13 ± 1.4% (n = 3) compared with control siRNA. These results are consistent with immunoblotting data (see Fig. 5).

Overexpression of the 14-3-3 Scavenger Protein pGpLI-R18 Decreased Expression of the Mature hKOPR—The 14-3-3 scavenger protein pGpLI-R18 was constructed by fusion of the tetramer-forming coiled-coil domain pLI of protein G and the R18 peptide that is an unphosphorylated peptide identified in a phage display to bind 14-3-3 with high affinity (31). pGpLI-R18 has been shown to effectively bind to 14-3-3 proteins (32). Transient transfection of N2A-FLAG-hKOPR cells with pGpLI-R18 decreased expression of the mature hKOPR by 22.6% as determined by immunoblotting (Fig. 7).

14-3-3ζ siRNA Did Not Change Expression of the hDOPR and rMOPR in N2A Cells—To determine if the effect of 14-3-3ζ is specific to the hKOPR among opioid receptors, we examined if 14-3-3ζ siRNA affected the expression of the rMOPR and hDOPR. We generated stable N2A-FLAG-hDOPR cells and N2A-FLAG-rMOPR cells with similar receptor expression levels (~1.5 pmol/mg membrane protein) as N2A-FLAG-hKOPR cells. Transient transfection of 14-3-3ζ siRNA did not affect the expression of the hDOPR and the rMOPR, although it decreased the expression of the mature hKOPR by 26.4%, as revealed by immunoblotting with FLAG antibodies (Fig. 8, A and B). A comparison of the amino acid sequences of the C-tails of the MOPR, DOPR, and KOPR is shown in Fig. 8C.

Overexpression of 14-3-3ζ Reversed the Effect of 14-3-3ζ Knockdown on hKOPR Expression—To determine if decreased hKOPR expression by siRNA knockdown of 14-3-3ζ was due to off-target effects of the siRNA, we determined if the siRNA effects could be reversed by expression of 14-3-3ζ. We used HA-14-3-3ζ, therefore, 14-3-3ζ appears as two bands because HA-14-3-3ζ has higher a M, than does 14-3-3ζ.

Fig. 9, A and B, showed that the 14-3-3ζ siRNA decreased the amount of 14-3-3ζ by 55%, and HA-14-3-3ζ cDNA increased the amount of combined HA-14-3-3ζ and 14-3-3ζ by 114%, compared with transfection of luciferase siRNA (control). Cells transfected with both 14-3-3ζ siRNA and HA-14-3-3ζ exhibited increased 14-3-3ζ by 62%. Fig. 9, A and C, showed that 14-3-3ζ siRNA reduced the expression of the 55-kDa hKOPR by 26%, whereas overexpression of HA-14-3-3ζ did not significantly affect hKOPR expression, compared with the control siRNA. In contrast, expression of HA-14-3-3ζ after 14-3-3ζ siRNA restored the 55-kDa hKOPR to 108% that of the control siRNA group. These results indicate that the effects of 14-3-3ζ siRNA are due to its reduction of 14-3-3ζ but not off-target effects. Transfection with 14-3-3ζ siRNA or 14-3-3ζ cDNA did not significantly affect the 45-kDa form of the hKOPR (Fig. 9, A and D).

14-3-3ζ Knockdown Decreased the Amount of the Mature hKOPR (55 kDa band) but Did Not Change the Rate of Receptor Maturation—As shown in Fig. 5, 14-3-3ζ siRNAs decreased the level of the 55-kDa form of the hKOPR in N2A-FLAG-hKOPR cells without significantly affecting that of the 45-kDa form. Our previous report showed that the 45-kDa forms are glycosylated intermediates residing in the ER and cis-Golgi, and the 55-kDa band of the hKOPR represents fully glycosylated forms of the receptor, which are present in trans-Golgi and plasma membranes (26). We used the pulse-chase technique to investigate the effect of 14-3-3ζ siRNA on generation and turnover of the 55- and 45-kDa forms of the hKOPR. N2A-FLAG-hKOPR cells transiently transfected with 14-3-3ζ siRNA were pulse-labeled with [35S]Met/Cys for 30 min and chased for various intervals. FLAG-hKOPR was immunoprecipitated with anti-FLAG antibodies. 14-3-3ζ siRNA decreased the amount of the mature hKOPR (55 kDa) but did not change the rate of conversion from the 45-kDa form to the 55-kDa form or the turnover rates of the 55-kDa and 45-kDa forms of the hKOPR (Fig. 10). These data suggest that knockdown of 14-3-3ζ reduces the
amount of the mature hKOPR exported from ER and Golgi but does not change the rate of export or the stability of the receptor proteins.

R354A/S358A Mutations in the hKOPR C-tail Reduced Its Interaction with 14-3-3 and Abolished the Effect of 14-3-3 Knockdown on hKOPR Expression—As shown above, 14-3-3 binds directly to the hKOPR C-tail, which has a potential binding domain for 14-3-3 protein with the sequence 354RQSTS358. To test the role of this sequence in the interaction of hKOPR

**FIGURE 5.** Knockdown of 14-3-3 decreased the level of the hKOPR in N2A-FLAG-hKOPR cells and in primary cortical neurons of E19 rats by immunoblotting. A, N2A-FLAG-hKOPR cells in 6-well plates were transfected with 100 pmol/well 14-3-3 siRNA or luciferase siRNA (Control). C, primary cortical neurons from E19 embryonic rats were grown in 60-mm plates and transfected with 6 μg/plate FLAG-hKOPR-pcDNA3 with 200 pmol of rat 14-3-3 siRNA or luciferase siRNA (Control) using Lipofectamine 2000. A and C, approximately 2 days later cells were solubilized and subjected to SDS-PAGE. 14-3-3 and FLAG-hKOPR were detected by immunoblotting with the indicated antibodies. GADPH, the loading control, was detected with mouse HRP-conjugated anti-GAPDH antibody. Each immunoblot represents one of three independent experiments performed with similar results. B and D, protein band densities were quantitated using the ImageGauge program and normalized against that of GADPH. B and D are the results of A and C, respectively. Each value represents the mean ± S.E. of three independent experiments. * p < 0.05, compared with the control group by two-tailed Student’s t test.

**FIGURE 6.** 14-3-3 siRNA reduced total and cell surface expression of the hKOPR as determined by receptor ligand binding. N2A-FLAG-hKOPR cells were transfected with 14-3-3 siRNA or luciferase siRNA (Control) as described in the Fig. 5 legend. Receptor binding was performed with [3H]diprenorphine (1 nM) on intact cells. For total and cell surface hKOPR, naloxone (10 μM) and dynorphin A-(1–17) (1 μM) were used to define nonspecific binding, respectively. Each value represents the mean ± S.E. of three independent experiments. * p < 0.05, compared with the control group by two-tailed Student’s t test.

**FIGURE 7.** Overexpression of the 14-3-3 scavenger protein decreased the level of the hKOPR. A, N2A-FLAG-hKOPR cells in 6-well plates were transiently transfected with 4 μg/well 14-3-3 scavenger protein, pGpLI-R18, or the vector (control) with Lipofectamine 2000. Immunoblotting was performed with the indicated antibodies as described in Fig. 5. B, densities of receptor protein bands were quantitated and normalized against that of GAPDH. Each immunoblot represents one of three independent experiments performed with similar results. Each value represents the mean ± S.E. of three independent experiments. * p < 0.05, compared with the control group by two-tailed Student’s t test.
with 14-3-3ζ, we mutated 354RQSTS358 to 354AQSTA358 (hKOPR-AQSTA) and generated N2A cells stably expressing this mutant (N2A-FLAG-hKOPR-AQSTA). They had a comparable KOPR expression level as determined by [3H]diprenorphine binding and immunoblotting with anti-FLAG antibodies. The amount of 14-3-3ζ immunoprecipitated with hKOPR-AQSTA was significantly lower compared with the wild type, although the amounts of immunoprecipitated wild type and

FIGURE 8. 14-3-3ζ siRNA did not change expression of the hDOPR and rMOPR in N2A cells. A, N2A-FLAG-hKOPR cells, N2A-FLAG-hDOPR, and N2A-FLAG-rMOPR in 6-well plates were transiently transfected with 100 pmol/well 14-3-3ζ siRNA or luciferase siRNA (Control) with Lipofectamine 2000. Immunoblotting (IB) was performed with indicated antibodies as described in Fig. 5. B, densities of receptor protein bands were quantitated and normalized against that of GAPDH. Each immunoblot represents one of three independent experiments performed with similar results. Each value represents the mean ± S.E. of three independent experiments. *, p < 0.05, compared with the control group by two-tailed Student’s t test. C, shown is an amino acid sequence comparison of the C-tails of μ-, δ-, and κ- opioid receptors. – indicates identical amino acid to KOPR.

FIGURE 9. Expression of HA-14-3-3ζ reversed the effect of 14-3-3ζ knockdown on hKOPR expression. A, N2A-FLAG-hKOPR cells in 12-well plates were transfected with 14-3-3ζ siRNA or luciferase siRNA (Control) and 12 h later with HA-14-3-3ζ cDNA or vector (control). Forty-eight hours after the second transfection, cells were solubilized and subjected to SDS-PAGE. 14-3-3ζ and FLAG-hKOPR were detected by immunoblotting with the indicated antibodies. Note that 14-3-3ζ appears as two bands because HA-14-3-3ζ has higher a M, than 14-3-3ζ. Each immunoblot represents one of three independent experiments. B–D, densities of 14-3-3ζ, HA-14-3-3ζ, and receptor protein bands were quantitated. Densities of 14-3-3ζ and HA-14-3-3ζ were combined. Each bar represents the mean ± S.E. (n = 3). *, p < 0.05; **, p < 0.01, compared with the control siRNA group. #, p < 0.05; ##, p < 0.01 compared with 14-3-3ζ siRNA group by two-tailed Student’s t test.
mutant KOPRs were similar (Fig. 11A). In addition, knockdown of 14-3-3ζ by siRNA decreased expression of the hKOPR by 23.9% but did not change expression of FLAG-hKOPR-AQSTA in N2A cells (Fig. 11B). These results indicate that the RQSTS sequence in the hKOPR C-tail may be involved in 14-3-3ζ-mediated regulation of hKOPR expression. It is noteworthy that the RQSTS sequence is not present in the C-tails of the MOPR or DOPR (Fig. 8C), consistent with lack of effects of 14-3-3ζ on MOPR and DOPR expression.

Mutation of the ER Retention Motif RXR in the hKOPR C-tail
Decreased Association of COPI with the hKOPR and Blocked the Effect of 14-3-3ζ Knockdown on hKOPR Expression—ER localization motifs of membrane-bound proteins include one or more internal RXR sequence, where the middle position prefers a neutral or positively charged amino acid (33). In the C-terminal domain of the hKOPR, there is such a sequence, RVR361, that is immediately downstream of RQSTS358. We hypothesized that association of 14-3-3ζ with the hKOPR reduces the binding of COPI to RVR, thus decreasing retention of the hKOPR in the ER. To test this hypothesis, we generated two mutants, hKOPR-AVR and hKOPR-KVR, and established cell lines stably transfected with each mutant. We found that co-immunoprecipitation of β-COP, an essential COPI subunit, with hKOPR-AVR or hKOPR-KVR was greatly decreased compared with the wild type (Fig. 12, A and B). In addition, 14-3-3ζ knockdown did not change expression of hKOPR-AVR and hKOPR-KVR (Fig. 12, C and D). These results suggest that the RXR motif of the hKOPR C-tail plays an important role in 14-3-3ζ-mediated regulation of hKOPR expression.

14-3-3ζ Knockdown Increased Interaction of β-COP and hKOPR—To further examine the role of COPI in 14-3-3ζ regulation of hKOPR expression, we tested the effect of 14-3-3ζ knockdown on the interaction of β-COP with hKOPR. 14-3-3ζ knockdown increased the association of COPI with hKOPR (Fig. 13), suggesting that COPI-mediated trafficking is involved in the effect of 14-3-3ζ on hKOPR expression.

DISCUSSION

We have found that 14-3-3ζ interacts directly with the C-terminal domain of the hKOPR, and this association promotes expression of the hKOPR by facilitating trafficking to plasma membranes along the export pathway. In contrast, 14-3-3ζ does not affect expression of the MOPR or DOPR. The interaction site appears to be RQSTS358 in the hKOPR, which is adjacent to the ER localization motif RXR (RVR361). 14-3-3ζ inhibits binding of COPI to the RXR motif, which reduces ER retention.
of the hKOPR, allowing its anterograde transport to Golgi and plasma membranes. To the best of our knowledge this is among the first reports to demonstrate that 14-3-3 interacts with a 7TMRs and regulates cell surface expression of the receptor. This is also among the first to delineate the underlying mechanisms.

14-3-3/H9256 siRNA Effect Was Due to Reduction in 14-3-3/H9256 Level—The observation that 14-3-3/H9256 siRNA effect in reducing hKOPR was reversed by expression of 14-3-3/H9256 indicates that the siRNA effect is due to the reduction in 14-3-3 but not off-target effects.

14-3-3 Interacts with the hKOPR and Regulates Receptor Surface Expression—Our finding that interaction of 14-3-3 with the KOPR C-tail facilitates its cell surface expression is in accord with those on several voltage- or ligand-gated ion channels (for review, see Refs. 10 and 11). These channels include several types of potassium channels, nicotinic acetylcholine receptor 4 subunit, and the kainate receptor KA2. In addition, interactions with 14-3-3 proteins have recently been shown to be important for cell surface expression of the 7TMR GPR15, a co-receptor for HIV and simian immunodeficiency virus (15). As 14-3-3 proteins have many different potential binding motifs (10), it is likely that many 7TMRs contain one or more of these motifs, and some of them bind to 14-3-3 proteins.

The results of the pulse-chase experiments demonstrated that 14-3-3 siRNA decreased the amount of fully glycosylated hKOPR (the 55-kDa form) generated but did not alter the rates of turnover of the 55-kDa and the glycosylated intermediates (the 45-kDa form). N-Linked glycosylation of proteins occurs in the ER and Golgi in a series of enzymatic reactions. Initial glycosylation of proteins and trimming of glucoses and mannoses occur in the ER. In the Golgi complex, further trimming of mannose and the addition of N-acetylglucosamine, sialic acid, and galactose complete the glycosylation process. Fully glycosylated proteins are then exported to plasma membranes. Our results suggest that knockdown of 14-3-3/H9256 reduces the amount of hKOPR exported to the cell surface from the ER and Golgi but does not change the rate of export. In addition, 14-3-3 knockdown did not affect the half-lives of the 45- and 55-kDa bands of the hKOPR, indicating that it does not affect stability of the receptor proteins. In contrast, 14-3-3 binding was shown to stabilize the GPR15 protein (15). 14-3-3 siRNA did not change cell surface expressions of the DOPR or MOPR, indicating that 14-3-3/H9256 interacts specifically with the KOPR among the opioid receptors.

The 14-3-3 Binding Motif in the hKOPR C-tail and Possible Mechanisms of Effect of 14-3-3/H9256 on hKOPR Expression—We found that R354A/S358A mutations in the 354RQSTS358 sequence, a potential 14-3-3 binding motif, reduced interaction of the hKOPR with 14-3-3/H9256 and eliminated the effect of 14-3-3/H9256 knockdown on hKOPR expression. This indicates that this sequence is critical for interaction with 14-3-3/H9256.

Multiple mechanisms have been shown to be involved in regulating cell surface expression of receptors by 14-3-3 proteins, including inhibiting ER localization machinery and engaging forward transport machinery (for review, see Refs. 9–11). In the C-terminal domain of the hKOPR, there is an ER retention motif, 359RVR361, immediately downstream of the 14-3-3 interaction sequence 354RQSTS358. We found that R359A or R359K
mutation in \(359RVR^{361}\) decreased association of COPI with the hKOPR, indicating that the RVR sequence functions as an ER localization motif. In addition, R359A or R359K mutation eliminated 14-3-3\(\text{H}9256\) siRNA effect on hKOPR expression, suggesting that 14-3-3 acts by mechanisms that involve interaction of COPI with the RVR sequence. Knockdown of 14-3-3\(\text{H}9256\) by siRNA increased interaction of COPI and the hKOPR. Our results are consistent with the notion that binding of 14-3-3\(\text{H}9256\) to the \(354RQSTS^{358}\) sequence leads to decreased association of COPI to the hKOPR via \(359RVR^{361}\) and hence reduces ER retention of the hKOPR. This mode of action is termed “masking,” and it occurs either by steric hindrance or conformational change (10, 11). An alternative interpretation is that binding of 14-3-3 to the \(354RQSTS^{358}\) sequence leads to decreased association of COPI to the hKOPR via \(359RVR^{361}\) and hence reduces ER retention of the hKOPR. This mode of action is termed “masking,” and it occurs either by steric hindrance or conformational change (10, 11). An alternative interpretation is that binding of 14-3-3 to the \(354RQSTS^{358}\) sequence leads to decreased association of COPI to the hKOPR via \(359RVR^{361}\) and hence reduces ER retention of the hKOPR. This mode of action is termed “masking,” and it occurs either by steric hindrance or conformational change (10, 11).

This finding is in accord with the report on GPR15 (15). \(356RSVSL^{360}\) in the C-terminal domain of GPR15 was identified to be the 14-3-3 binding site, and there is an ER localization signal of the RXR type immediately upstream of the 14-3-3 binding site. The S359A mutation in GPR15 reduced interaction with 14-3-3 and GPR15 expression. In addition, such a masking function of 14-3-3 on the RXR motif has been demonstrated for non-7TMR membrane proteins as well (35–39).

14-3-3 proteins have also been shown to regulate protein transport by other mechanisms. Nakamura et al. (40) reported that 14-3-3 proteins bound PX-RICS, a \(\beta\)-catenin-interacting protein, and linked \(\beta\)-catenin/N-cadherin complex to the dynein/dynactin motor protein complex attached to microtubules to facilitate the export of \(\beta\)-catenin/N-cadherin from the ER. In addition, cell surface expression of the epithelial sodium channel is enhanced by 14-3-3. This enhancement is due to the binding of 14-3-3 to Nedd4-2, an E3 ubiquitin ligase, that results in reduction in ubiquitination and degradation of the epithelial sodium channel (41). Thus, 14-3-3 proteins enhance cell surface expression of proteins by various mechanisms. The mechanism changes depending on the proteins with which 14-3-3 interacts (9–11).

**The RXR Motif in 7TMRs**—Here we showed that \(\beta\)-COP, a component of COPI, co-immunoprecipitated with the hKOPR and that mutation of RVR to either AVR or KVR greatly reduced the amount of \(\beta\)-COP co-immunoprecipitated with the hKOPR. This indicates that RVR acts as an ER retention/retrieval motif, which is consistent with those of Brock et al. (42) and Pagano et al. (43) on GABA\(_A\) receptors and Okamoto and Shikano (15) on GPR15. A database search revealed that

**FIGURE 12.** Mutation of the ER retention motif RXR in the hKOPR C-tail decreased the association of COPI with the hKOPR and blocked the effect of 14-3-3\(\zeta\) knockdown on hKOPR expression. A, N2A cells in 60-mm plates were transfected with 6 \(\mu\)g/plate FLAG-hKOPR- pcDNA3, FLAG-hKOPR-AVR- pcDNA3, and FLAG-hKOPR-KVR-pcDNA3. Immunoprecipitation (IP) and immunoblotting (IB) were performed with indicated antibodies as described in Fig. 2. B, densities of immunoprecipitated \(\beta\)-COP bands were quantitated and normalized against FLAG-hKOPR or its mutants. C, N2A cells in 6-well plates were transiently transfected with 3 \(\mu\)g/plate FLAG-hKOPR or its AVR or KVR mutant and 100 pmol 14-3-3\(\zeta\) siRNA or luciferase siRNA (control). Immunoblotting was performed with the indicated antibodies as described in Fig. 5. D, densities of FLAG-hKOPR (55 kDa) were quantitated and normalized against that of GAPDH. Each immunoblot represents one of three independent experiments performed with similar results. Each value represents the mean \(\pm\) S.E. of three independent experiments. *, \(p < 0.05\), compared with the control group by two-tailed Student’s \(t\) test.
14-3-3ζ Regulates Expression of KOPR

A

hKOPR C-tail:

DENFKRCRDFCFPLKMRME\[354\]RQSTS\[358\]RVRNTVQDPAYLRLDI\[359\]GMNKPV

14-3-3 zeta binding site ER retention motif

B

N2A-FLAG-hKOPR cells

IB: anti-14-3-3 zeta

- +

14-3-3 zeta siRNA

14-3-3 zeta

IP: anti-FLAG

IB: beta-COP ab

IB: anti-FLAG

IB: beta-COP ab

C

[0x0]FIGURE 13. 14-3-3ζ knockdown increased the interaction between β-COP and hKOPR. A, the C-terminal domain of the hKOPR: the 14-3-3ζ binding site \[354\]RQSTS358 is adjacent to the ER retention motif \[359\]RVR. B, N2A-FLAG-hKOPR cells in 60-mm plates were transfected with 200 pmol/well 14-3-3ζ siRNA or luciferase siRNA (control). Immunoprecipitation (IP) and immunoblotting (IB) were performed with indicated antibodies (ab) as described in Fig. 2. Each immunoblot represents one of three independent experiments performed with similar results. C, densities of immunoprecipitated β-COP bands were quantitated using the ImageGauge program and normalized against FLAG-hKOPR. Each value represents the mean ± S.E. of three independent experiments. *, p < 0.05, compared with the control group by two-tailed Student’s t test.

>100 human 7TMRs have the RXR motif in intracellular regions; however, the function of this motif has been examined in only a few receptors (15, 42, 43). It is conceivable that this motif functions as an ER retention/retrieval signal in many 7TMRs.

Relationship between GEC1 and 14-3-3—We have reported previously that GEC1, a microtubule-associated protein, interacts with the hKOPR and promotes the export of the hKOPR to the trans-Golgi and plasma membranes (7). In pulse-chase experiments, like 14-3-3, GEC1 enhanced the amount of the mature form of the hKOPR without changing the rate of conversion from the precursor to the mature form. It is likely that 14-3-3 proteins and GEC1 are components of a larger protein complex. Based on the scheme of Nakamura et al. (40), we propose the following working hypothesis (Fig. 14). The hKOPR as a vesicular cargo interacts with both 14-3-3 proteins and GEC1. 14-3-3 proteins are associated with kinesin motor proteins, which move the hKOPR in vesicles along microtubules. GEC1 binds to microtubules and the hKOPR in vesicles and stabilizes the vesicle-microtubule complex as kinesins move the vesicular cargo forward. In addition, as vesicles dissociate from one kinesin complex, the interaction of hKOPR with GEC1 stabilizes the cargo on microtubules before being associated with another kinesin complex.

Phosphorylation of the hKOPR Is Not Required for 14-3-3 Binding—We found that the hKOPR under basal conditions co-immunoprecipitated with 14-3-3ζ and the non-phosphorylated hKOPR C-tail bound 14-3-3ζ in the pulldown assay, indicating that phosphorylation of the hKOPR is not required for 14-3-3 binding. Our finding is consistent with the observation that interaction of 14-3-3ζ with all three types of α2-adrenergic receptors does not require receptor phosphorylation (44). In addition, the parathyroid hormone receptor interacts with 14-3-3 in the C-tail, and the binding is reduced by phosphorylation of the C-tail by protein kinase A (45). In contrast, 14-3-3ζ binding to the GPR15 requires phosphorylation of the receptor in the 14-3-3 binding motif (15).

It is not clear if phosphorylation of the 14-3-3ζ binding site RQST in the hKOPR C-tail enhances association between the two. However, it is reasonable to assume that the hKOPR in the biogenesis export pathway is not phosphorylated to significant extents as phosphorylation occurs to cell surface receptors in response to agonist stimulation or activation of signal pathways by other receptors.

14-3-3 Isoforms May Have Similar Effects on hKOPR Expression—Transfection of siRNA against 14-3-3ζ into N2A cells reduced the ζ isoform by ~60% without affecting the levels of the γ isoforms (data not shown) but decreased hKOPR expression by only ~25%. The difference in the degrees of reduction in 14-3-3ζ and in hKOPR after 14-3-3ζ siRNA may stem in part from the high abundance of 14-3-3ζ in cells. The finding that overexpression of 14-3-3ζ in N2A cells did not affect hKOPR level (Fig. 9) supports this notion. In addition, it may be due to redundant functions of different 14-3-3 isoforms. By proteomic analyses we found that four isoforms of 14-3-3 (β, γ, σ, and ζ) co-immunoprecipitated with FLAG-hKOPR (supplemental Table 1). We focused on 14-3-3ζ because this iso-
form has the highest identification certainty in our proteomic analyses and is abundant in the brain (30). It is likely that other isoforms of 14-3-3-ζ may have a similar function; therefore, knockdown of 14-3-3-ζ has only a partial effect in the regulation of the KOPR level.

Binding of 14-3-3 Proteins to 7TMRs Has Different Biological Functions—Several 7TMRs have been shown to interact with 14-3-3 proteins. GABA<sub>B</sub>R1 interacts with 14-3-3-ζ and 14-3-3-η in the C-tail, and the 14-3-3 binding site overlaps partially with the coiled-coil domain and is close to the Arg-based ER localization motif of GABA<sub>B</sub>R1 (42, 46). In one study this interaction was demonstrated to be required for cell surface expression of GABA<sub>B</sub> receptors (46), but in another it was shown not to play a role (42). The follicle-stimulating hormone receptor interacts with 14-3-3-ζ in an agonist-dependent manner, and expression of 14-3-3-ζ decreased follitropin-induced cAMP accumulation (47). Thromboxane receptor binds 14-3-3-ζ in the third intracellular loop. Activation of the receptor recruits 14-3-3-ζ and Raf-1 to form a complex on the plasma membrane, which is involved in the activation of p42/p44 MAPKs (48). Both 14-3-3-ζ and 14-3-3-ζ bind directly to the C-tail of the Ca<sup>2+</sup>-sensing receptor (49). Expression of 14-3-3-ζ attenuated the Ca<sup>2+</sup>-sensing receptor-mediated Rho signaling but had no effect on ERK1/2 signaling, whereas expression of 14-3-3-ζ significantly reduced plasma membrane expression of the receptor (49). 14-3-3-ζ interacts with the third intracellular loops of all three types of α<sub>2</sub>-adrenergic receptors (44). The parathyroid hormone receptor interacts with 14-3-3 in the C-tail, which is reduced by treatment of the C-tail with protein kinase A (45). However, the functional significance of the 14-3-3 interaction with the α<sub>2</sub>- and parathyroid hormone receptors has not been delineated. It remains to be determined if interaction of 14-3-3 proteins with the hKOPR affects signaling of the receptor.

In conclusion, 14-3-3-ζ binds directly to the C-tail of the hKOPR and regulates trafficking of the receptor along the export pathway. It binds to <sup>35</sup>QTRGTS<sup>358</sup> which masks the RVR ER retention motif. By doing so, it inhibits the interaction of COPI with the RVR motif and releases the hKOPR from ER retrievalRETENTION pathway.

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