The \( \zeta \) Isoform of 14-3-3 Proteins Interacts with the Third Intracellular Loop of Different \( \alpha_2 \)-Adrenergic Receptor Subtypes*

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The \( \alpha_2 \)-adrenergic receptors (\( \alpha_2 \)ARs) are localized to and function on the basolateral surface in polarized renal epithelial cells via a mechanism involving the third cytoplasmic loop. To identify proteins that contribute to this retention, \([^{35}S]\)Met-labeled Gen10 fusion proteins with the 3i loops of the \( \alpha_2 \)AR (Val \( ^{217} \)-Ala \( ^{377} \)), \( \alpha_2 \)BAR (Ly \( ^{210} \)-Trp \( ^{354} \)), and \( \alpha_2 \)CAR (Arg \( ^{248} \)-Val \( ^{363} \)) were used as ligands in gel overlay assays. A protein doublet of \( \sim 30 \) kDa in Madin-Darby canine kidney cells or pig brain cytosol (\( \alpha_2n \geq \alpha_2c \gg \alpha_2a \)) was identified. The interacting protein was purified by sequential DEAE and size exclusion chromatography, and subsequent microsequencing revealed that they are the \( \zeta \) isoform of 14-3-3 proteins. \([^{35}S]\)Met-14-3-3\( ^\zeta \) binds to all three native \( \alpha_2 \)AR subtypes, assessed using a solid phase binding assay (\( \alpha_2n \gg \alpha_2c \gg \alpha_2a \)), and this binding depends on the presence of the 3i loops. Attenuation of the \( \alpha_2 \)AR-14-3-3 interactions in the presence of a phosphorylated Raf-1 peptide corresponding to its 14-3-3 interacting domain (residues 251–266), but not by its non-phosphorylated counterpart, provides evidence for the functional specificity of these interactions and suggests one potential receptor interface for the \( \alpha_2 \)AR and 14-3-3 interactions. These studies represent the first evidence for G protein-coupled receptor interactions with 14-3-3 proteins and may provide a mechanism for receptor localization and/or coordination of signal transduction.

The three \( \alpha_2 \)-adrenergic receptor (\( \alpha_2 \)AR) subtypes, encoded by distinct genes (1), all couple via the \( \alpha_i \), \( \alpha_j \), and \( \alpha_k \) family of GTP-binding proteins to inhibition of adenylyl cyclase, suppression of voltage-sensitive calcium channels, and activation of receptor-operated potassium channels (2). These receptors also couple to activation of Ras (3, 4), the mitogen-activated protein kinase cascade (3, 5–7), and to activation of phospholipase D (8, 9).

Despite the qualitatively similar signaling properties of the three \( \alpha_2 \)AR subtypes, differences in trafficking of these receptors have been reported. For example, subtype-selective differences in agonist-elicted \( \alpha_2 \)AR redistribution occur (10–15). In addition, selective itineraries for the \( \alpha_2 \)AR subtypes are observed in polarized Madin-Darby canine kidney (MDCKII) renal epithelial cells. Thus, the \( \alpha_2 \)BAR subtype is targeted directly to the basolateral surface (16), whereas the \( \alpha_2 \)AR subtype is delivered randomly to both the apical and basolateral surfaces but is rapidly lost from the apical (\( t_{1/2} = 5–15 \) min) and selectively retained on the basolateral (\( t_{1/2} = 10–12 \) h) surface (17). These findings suggest that there is a molecular mechanism responsible for the selective retention of the \( \alpha_2 \)AR on the basolateral domain of MDCK cells that may be shared by all three \( \alpha_2 \)AR subtypes, as they manifest comparable half-lives on that surface (17).

Receptor retention on the lateral subdomain of MDCKII cells likely involves the third intracellular loop of the \( \alpha_2 \)AR, since deletion of this loop, creating the mutant \( \alpha_2 \)AR3iAR, results in accelerated basolateral turnover (\( t_{1/2} = 4.5 \) h) when compared with that for the wild-type receptor or with \( \alpha_2 \)AR structures that have been mutated in the N terminus or the C-terminal tail (all possessing a \( t_{1/2} \) of 10–12 h) (18). The accelerated turnover of the \( \alpha_2 \)AR3iAR when compared with the wild-type \( \alpha_2 \)AR structure suggests that the third intracellular loop interacts with proteins that either tether \( \alpha_2 \)AR to a particular surface domain or, alternatively, mask the \( \alpha_2 \)AR from interacting with endocytosis machinery.

Other functional roles have been attributed to the third intracellular loop of \( \alpha_2 \)AR. The N- and C-terminal 10–15 residues of the 3i loop, predicted to form amphipathic helices, are involved in coupling to G proteins (19–22). The C-terminal third of the 3i loop of the \( \alpha_2 \)AR subtype is implicated in the interaction with \( \beta \)-arrestin, a protein that preferentially associates with G protein-coupled receptor kinase-phosphorylated receptors sustaining agonist-elicted homologous desensitization (23). For the \( \alpha_2 \)AR subtypes, G protein-coupled receptor kinase phosphorylation sites are in the N-terminal region of the \( \alpha_2 \)AR 3i loop (24, 25), widely distributed throughout the \( \alpha_2n \)AR 3i loop, and presumed to be absent in the \( \alpha_2n \)AR 3i loop (13, 26).

The present studies were undertaken to identify interacting proteins for the intracellular 3i loops of the \( \alpha_2 \)AR subtypes. In vitro translation of Gen10-\( \alpha_2 \)AR 3i loop fusion proteins (Gen10-\( \alpha_2 \)AR3i) served as a means to create \([^{35}S]\)methionine-radiolabeled 3i loops as ligands for identifying interacting proteins via a gel overlay strategy. Our findings reveal that these loops, in a subtype-selective fashion, interact with the \( \zeta \) isoform of 14-3-3 proteins (14-3-3\( ^\zeta \)). Using a solid phase binding assay with \([^{35}S]\)Met-14-3-3\( ^\zeta \) as a probe and solubilized \( \alpha_2 \)AR as the target indicates that 14-3-3 proteins can bind to native \( \alpha_2 \)AR subtypes in a way that relies on the 3i loop in the receptor structure.
Further evidence for the functional relevance of these interactions is the ability of a Raf peptide, corresponding to a 14-3-3-interacting domain, to block Gen10-α3i loop interactions with 14-3-3ζ in its phosphorylated, but not in its non-phosphorylated, state.

EXPERIMENTAL PROCEDURES

Materials

The pGEMEX-2 vector and TNT in vitro translation kit were from Promega (Madison, WI). The [35S]methionine (1000 Ci/mmol, at 10 mCi/ml) was purchased from NEN Life Science Products. PVDF nylon membranes were from Millipore (Bedford, MA). The FPLC and DEAE-Sepharose columns were from Amersham Pharmacia Biotech. Dodecyl-β-maltoside and cholesterol hemisuccinate were purchased from Calbiochem and Sigma, respectively. Staph A immunoprecipitin was obtained from Life Technologies, Inc. 12CA5 monoclonal antibody was against the hemagglutinin epitope engineered into the α2AR structures was obtained from Babco; the M2 monoclonal antibody against theFLAG epitope engineered into the N terminus of 14-3-3ζ was from Eastman Kodak Co., and the rabbit anti-14-3-3ζ (or pan) and anti-ζ isoform antibodies were from Santa Cruz Laboratories (Santa Cruz, CA). Protein A beads were from Vector (Burlingame, CA). Centripron-10 concentrating filters were purchased from Amicon (Beverly, MA). The tube gel adapter kit was from Hoefer Scientific instruments (San Francisco, CA).

Subcloning and in Vitro Translation of the Gen10 Protein-α2AR 3i Loop Fusion Proteins

The residues corresponding to the 3i loops of the α2AR (amino acids 217–377) (27), the α2AR (amino acids 210–354) (28), and the α2AR (amino acids 248–363) (29) were subcloned into the pGEMEX-2 vector. The residues utilized are shown schematically in Fig. 2B. These 3i loop sequences were inserted in frame within the polylinker located downstream of the sequence encoding the Gen10 protein, a methionine-rich phage structural protein. The sequence encoding an epitope of the c-Myc protein was inserted 3′ to the sequence of the α2AR loop sequences.

The Gen10–3i loop fusion proteins and 14-3-3ζ were produced and [35S]Met-labeled using an in vitro T7 RNA polymerase-coupled translation system in reticulocyte lysates as follows: 25 μl of TNT lysate were added to 1 μl of amino acid mix (1 mM, minus methionine, TNT kit), 2 μl of TNT reaction buffer, 1 μl of TNT T7 RNA polymerase, 4 μl of [35S]methionine (1000 Ci/mmol, at 10 mCi/ml), 1 μl of RNasin ribonuclease inhibitor (40 units/μl). Then, 1 μg of the appropriate DNA template (presented as the circular plasmid DNA) was added, and the volume was adjusted to 50 μl with nuclease-free water. The mixture was incubated for 90 min at 30 °C. Products were analyzed and quantitated following each synthesis by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography, and the band representing each probe was cut out of the dried gel and counted in scintillation mixture. If smaller molecular weight species were generated during the translation reaction, they were eliminated by P30 size exclusion chromatography before use as probes. These [35S]-labeled Gen10-α3i loop fusion proteins were used as radioactive ligands in subsequent gel overlay assays as described previously (30) and detailed below.

Pig Brain Cortex and MDCK Cell Fractionation

Frozen pig brain cortex (2 g/preparation) was suspended in 20 ml of ice-cold lysis buffer (20 mM HEPES, 50 mM KCl, 2 mM MgCl2, 1 mM CaCl2, and the following protease inhibitors: 0.1 mM phenylmethylsulfonyl fluoride, 20 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, 1 mg/ml aprotinin, 1 mg/ml benzamidine) and homogenized using a Brinkmann Polytron (two 5-s bursts separated by 30 s on ice). The lysate was filtered through cheesecloth to remove debris and then centrifuged at 38,000 × g in an SS34 rotor (Sorvall RC 5B centrifuge) for 20 min. The supernatant of this centrifugation was removed and the pellet was resuspended and an aliquot saved to permit analysis of 3i loop binding activity using gel overlay analysis. The resolved proteins were transferred at 4 °C to PVDF nylon membranes (Millipore) by electrophoresis overnight at 30 V in Tris/glycine buffer (25 and 192 mM, respectively). The membranes were then cut in 2–4-mm strips for gel overlay and Western blot analysis.

For gel overlay analysis, PVDF membranes were blocked at least 1 h in blocking buffer: Tris-NCaCl (50 and 200 mM, respectively, referred to hereafter as TBS), containing Tween 20 (3% v/v) and non-fat powdered milk (5% w/v). The PVDF membranes were then washed for 30 min in rinsing buffer: TBS containing Tween 20 (0.1% v/v) and non-fat powdered milk (5% w/v). The PVDF membranes were then incubated with 300,000 cpm of the appropriate [35S]Met-labeled Gen10-α3i loop structure in a 1–ml incubation for 4 h (to overnight) at 4 °C with constant rocking in rinsing buffer. Based on the concentration of methionine contributed to the [35S]Met-labeling reaction by the rabbit reticulocyte lysate (5 μl) and the specific activity of the [35S]Met radiolabel, we estimated that this 300,000 cpm of Gen10-α3i loop represented 1–10 pmol of probe.

In experiments where the duration of the incubation or the amount of radioligand was varied, the times and concentrations evaluated are indicated in the figure legends. Following incubation with the various loop structures (or radiolabeled Gen10, as a control), membranes were washed 3 times with rinsing buffer, twice with cold TBS, and air-dried before autoradiography. Autoradiography was performed using a Molecular Dynamics PhosphorImager, and band intensities were calculated using the manufacturer's software, presented as arbitrary intensity units. Following quantitation, strips were exposed to x-ray film for 24–72 h.

For Raf competition experiments, phosphorylated and non-phosphorylated peptides corresponding to a 14-3-3 binding region of Raf-1 (LSQRQRSTSPO,TPNNVVM and LSQRQRSTSSTTPNVHVM, respectively (33)) were incubated with the membranes for 0.5 h prior to the addition of the [35S]Met-labeled Gen10-α3i loop probes, and the incubation was continued for 90 min followed by washing and detection, as described above.

Purification of the α2AR 3i Loop Interacting Proteins

DEAE Chromatography—For each purification protocol, 15 ml of a cytosolic protein fraction prepared from 2 g of frozen pig brain cortex were loaded onto a 2-ml DEAE-Sepharose column equilibrated overnight with ice-cold column equilibration buffer (20 mM HEPES (pH 7.0), 50 mM KCl, 2 mM MgCl2, 1 mM CaCl2, and protease inhibitors as utilized above). The column pass-through was saved for evaluation of 3i loop binding activity. The DEAE column was washed with 100 ml of 150 mM KCl-containing lysis buffer. The proteins were eluted using a gradient of KCl (from 150 to 500 mM KCl) in lysis buffer, at a rate of 6 ml/h. One-ml fractions were collected and subsequently evaluated for 3i loop binding activity using gel overlay analysis.

Gel Filtration—Preparative gel filtration using fast protein liquid chromatography (FPLC) was performed as follows: a 120-ml Superdex 200 column was equilibrated for 2 h with ice-cold buffer (20 mM HEPES (pH 7.0), 150 mM KCl, 2 mM MgCl2, 1 mM CaCl2, and protease inhibitors as utilized above). The column pass-through was saved for evaluation of 3i loop binding activity. A 2-ml sample, corresponding to peak fractions from the DEAE-Sepharose column, was loaded onto the Superdex 200 (2-ml fraction) were collected at a rate of 2 ml/min for 2 h. The 3i loop binding activity in individual fractions was determined by assaying aliquots using sequential SDS-PAGE and gel overlay analysis; in some studies, the proteins were concentrated and desalted using Centricon-10 concentrators before assaying 3i loop binding activity.

Two-dimensional Gel Electrophoresis—To be confident that the alpha2AR 3i Loop Interactions with 14-3-3ζ were stored at ~70 °C. The protein concentration in each fraction was estimated using the Bradford assay.

Cultured MDCK cells (two 100-mm dishes/preparation) were harvested at confluence by scraping into 1 ml of lysis buffer (see above) using a rubber policeman. MDCK cell lysates were disrupted further by 10–20 passes through a 23-gauge needle mounted onto a 5-ml syringe. The supernatant of the 15 min, 4 °C centrifugation (estimated at 30,000 × g in an Eppendorf centrifuge) was saved and defined as the cytosolic fraction.
bands on SDS-PAGE manifesting 3i loop binding activity were not “contaminated” by underlying bands, two-dimensional gel electrophoresis was performed using protocols and the tube gel adapter kit provided by Hoefer Scientific instruments.

**Microsequencing**—Microsequencing was performed at the Harvard Microsequencing Laboratory facility (Dr. William Lane, Director) by Edman degradation of tryptic digests of the ~30-kDa bands hydrolyzed in polyacrylamide gels, resolved by high pressure liquid chromatography, and assessed by mass spectrometry.

**Detergent Extraction of Functional α2AR Subtypes**

MDCKII cells, parental or stably transfected with the α2A-, α2B-, or α2C-AR subtype, were grown to confluence on 150-mm plates, serum-starved overnight, harvested in lysis buffer (15 mM HEPES, 5 mM EGTA, and 5 mM EDTA [pH 7.6]), containing 10 units/ml aprotinin and 100 μM phenylmethylsulfonyl fluoride, disrupted using a Teflon/glass homogenizer, split into 2 aliquots, and centrifuged at 30,000 × g. One aliquot was extracted with detergent, and the other was used to monitor receptor availability for extraction. For detergent extraction, one pellet was resuspended in 2.25 ml/150-mm plate Djm/CHS extraction buffer (4 mg/ml dodecyl-β-D-maltoside (Djm), 0.8 mg/ml cholesterol hemisuccinate (CHS), 25 mM glycine/cyline, 20 mM HEPES, 100 mM NaCl, 5 mM EGTA, 1 mg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, 10 units/ml aprotinin, and 100 μM phenylmethylsulfonyl fluoride), homogenized using a 27-gauge needle, and centrifuged at 100,000 × g at 4 °C for 1 h. The resulting supernatant was defined as the detergent-solubilized receptor. To assess the α2AR binding capacity of these preparations, [3H]rauwolscine was used as a radioligand, and Sephacel G-50 chromatography was used to separate bound from free ligand, as described previously (34). To assess the relative efficiency of the detergent to extract receptor from membranes, the results of the G-50 chromatography binding assays were compared with radioligand binding assays performed on the membrane pellet, derived from a fraction of the original preparation, with [3H]rauwolscine. Based on these determinations, we estimate that we extract >50% of the α2AR subtypes using this procedure. Equal concentrations of detergent-solubilized receptor were incubated with mouse anti-hemagglutinin antibodies for 1 h and then with 100 μl of protein A-agarose (1:1 slurry with Djm/CHS wash buffer) for a 2nd h. The protein A-agarose was rinsed twice with Djm/CHS wash buffer (1 mg/ml Djm, 0.2 mg/ml CHS, 25 mM glycine/cyline, 20 mM HEPES, 100 mM NaCl, 5 mM EGTA, 1 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, 10 units/ml aprotinin and 100 μM phenylmethylsulfonyl fluoride) and incubated for 16 h with [35S]Met-labeled 3i loops (corresponding to 5–10 pmol of [35S]Met-labeled Gen10-3i loop fusion proteins as described under “Experimental Procedures.” Incubations contained 300,000 dpm of each of the probes in 1 ml of buffer (corresponding to 5–10 pmol of [35S]Met-labeled 3i loop or Gen10 control) and were performed at 4 °C; the incubations were terminated by washing three times in rinse buffer (“Experimental Procedures”) and examined by autoradiography. The data shown are from a single experiment representative of at least six other experiments.

**RESULTS**

Because the 3i loops of the α2AR have been implicated in stabilization of these receptors on the basolateral surface of polarized renal epithelial cells, we sought to identify proteins that interact with these intracellular domains. We created fusion proteins of the 3i loops with the methionine-rich Gen10 protein. In vitro translation of these fusion proteins in the presence of [35S]methionine generated radiolabeled 3i loops that served as ligands for the identification of interacting proteins via gel overlay analysis. As can be seen in Fig. 1, the 3i loops of the α2A-, α2B-, and α2C-AR subtypes readily identified a doublet of apparent molecular mass of 30 kDa in cytosolic fractions of MDCKII cell lysate that was not detected by Gen10 protein or by the 3i loop of the α2AR under these incubation conditions (see Fig. 2, later, for delayed binding by α2A-AR 3i loop). The ~30-kDa doublet identified by the α2B-3i and α2C-3i loops is enriched in the cytosolic fraction and is barely detected in the membrane fractions of MDCKII cells. Similar binding profiles were seen in fractions from porcine brain cortex, albeit with greater membrane-associated binding activity, and from lysates of MDCKII cells that had been grown in Transwell® culture to foster polarization (data not shown), consistent with the published experience that confluent MDCKII cells grown in regular culture dishes manifest many of the properties characteristic of the polarized cellular phenotype (16, 35).

Fig. 2A demonstrates the time course for interaction of the α2AR 3i loops with the 30-kDa doublet in porcine brain cytosolic fractions. The 3i loops of the α2BAR and α2C-AR interacted more readily and to a significantly greater extent than the 3i loop of the α2A-AR subtype, whose binding to the 30-kDa doublet was detectable above background labeling only after longer (>3 h) incubations. When the ability of a 10× molar excess of unlabeled 3i loops to compete for binding of the 35S-labeled 3i loops for each subtype was evaluated after a 2- or 4-h incubation, it was evident that competition for the binding of the α2A-AR 3i loop was more facile than for the binding of the α2B-AR or the α2C-AR loop (data not shown), consistent with the apparent lower affinity of the α2AR 3i loop for the 30-kDa interacting proteins in the gel overlay assay (Fig. 2). The Gen10 fusion protein (control probe) did not compete for any of the 3i loop-specific binding nor did a Gen10 fusion protein encoding 58 amino acids of the distal C-terminal tail of the β2 adrenergic receptor (data not shown), a region previously implicated in β2AR stabilization on the cell surface (36, 37).

To reveal the molecular identity of the 30-kDa doublet, we undertook its purification from cytosolic fractions of porcine brain cortex. As shown in Fig. 3A, the interacting proteins were quantitatively adsorbed to DEAE-Sepharose and eluted, using a 50–500 mM KCl gradient, at approximately 250–300 mM KCl (fractions 82–95). Binding to these peak fractions showed a specificity of 3i loop binding characteristic of the unfractionated cytosol, as shown in Fig. 3B. The peak fractions were pooled and purified further using size exclusion FPLC, which
removed most of the proteins migrating on SDS-PAGE at >50 kDa and <20 kDa (Fig. 4). The elution position of the 3i loop interacting proteins on FPLC corresponded to an Mr of 50,000–80,000 (data not shown), suggesting that the ~30-kDa proteins may exist as a dimer, in a complex with other proteins, or both.

Material that had been purified by sequential chromatography on DEAE-Sepharose, FPLC, and concentrated by a second application to DEAE-Sepharose was subjected to two-dimensional isoelectric focusing and SDS-PAGE. As shown in Fig. 5A, the material migrating in the 30-kDa region on one-dimensional SDS-PAGE was resolved into three distinct spots upon two-dimensional gel analysis, as revealed by Zooni Coomassie staining. Gel overlay analysis indicated that two of the three spots, migrating at isoelectric points of 5 and 5.6, represented the a2AR 3i loop interacting proteins (Fig. 5B). In fact, the spot migrating at a pI of 5.0 has greater 35S-Gen10–3i loop binding on SDS-PAGE may exist as a dimer, in a complex with other proteins, or both.

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**Fig. 2.** Time course of the interaction of [35S]Met-labeled Gen10-α2i loop fusion proteins with the cytosolic fraction of porcine brain cortex. A, the [35S]Met-labeled Gen10-α2i loop fusion proteins for each of the α2AR receptor subtypes, or labeled Gen10 alone (control), were incubated with PVDF membranes, as described under “Experimental Procedures” and the legend to Fig. 1. The autoradiogram was used to identify the radioactive regions on the PVDF filter; these regions were cut and counted in NEN 965 scintillation fluor to obtain the CPM/gel overlay shown on the y axis. B, schematic representation of the α2AR with the portions of the 3i loop of each subtype (shown with bolder line and amino acid (a.a.) numbers) used as probes in the gel overlay assays.

**Fig. 3.** Isolation of the 30-kDa interacting proteins from porcine brain cortex using DEAE-Sepharose chromatography. A, a 7.5–20% gradient SDS-polyacrylamide gel of 20-μl aliquots of the cytosolic starting material (Cyt), DEAE-Sepharose column pass-through (PT), 50 mM KCl wash (W), and separation profile of proteins eluted in 1-ml fractions using a continuous 50–500 mM KCl gradient. The proteins in the samples were revealed with Zooni Coomassie Blue, as described under “Experimental Procedures.” B, gel overlay analysis of the cytosolic starting material (Cyt), column pass-through (PT), 50 mM KCl wash (W), and DEAE-Sepharose eluates from the fraction numbers indicated, corresponding to the samples shown in A. The probe used in the gel overlay assay is identified on each of the panels; for each incubation, the concentration of probe was as described in Fig. 2, and the incubation duration was 16 h.

**Fig. 4.** FPLC size exclusion chromatography of the 3i loop interacting proteins. A, silver-stained acrylamide gel showing the starting material (pooled DEAE eluate fractions), and FPLC eluate fractions. B, gel overlay analysis revealing the presence of the 3i loop interacting protein in the DEAE eluate starting material and in FPLC fraction 42. In this experiment, the 3i loop interacting proteins were identified using [35S]Met-labeled Gen10-α2AR 3i loop as the ligand.
Two-dimensional gel electrophoresis resolves the α2AR 3i loop interacting proteins from eIF2Bα. A, Zoon Coomassie-stained two-dimensional gel showing the presence of three proteins in the material purified by sequential DEAE-Sephacel, FPLC, and DEAE-Sephacel, the latter step used primarily to concentrate the purified proteins prior to two-dimensional gel analysis. B, gel overlay assay on a simultaneously prepared two-dimensional gel showing that only two of the three proteins interact with the 3i loop of the α2AR. C, Western blot showing that the third protein present in the purified fraction is eIF2Bα. The Western blot analysis was kindly performed by Scott R. Kimball and Leonard S. Jefferson, Penn State University College of Medicine.

relative to its Coomassie labeling intensity than the protein migrating with a pI \( \approx 5.6 \). Since the C-terminal tail of both the α2AR and the β2AR interacts with a ~30-kDa protein that corresponds to eIF2Bα (38), as revealed in yeast two-hybrid screens, we examined whether or not eIF2Bα represented any of the three spots detected on the two-dimensional gel. Indeed, the upper of the three spots on the two-dimensional gel analysis of the highly purified 3i loop interacting proteins, migrating at a pI of 4.8, corresponds to eIF2Bα, based on Western analysis with a polyclonal antibody directed against this protein (Fig. 5C).

Microsequencing of the α2AR 3i loop interacting proteins revealed the spots corresponding to the proteins of pI 5 and 5.6 on two-dimensional gel analysis both represented the ζ isoform of 14-3-3 proteins. As shown in Fig. 6, the 14-3-3 proteins represent a family of closely related proteins containing several highly homologous domains, as well as sequences unique to each of the isoforms. The name 14-3-3 derives from the partially denatured properties. A solid phase matrix was formed by incubating the epitope-tagged α2AR-subtype extracts with 12CA5 anti-hemagglutinin antibodies and protein A-Sepharose, as described under "Experimental Procedures." After washing to remove unbound protein, the resin-α2AR subtype matrix was incubated with \(^{[35]}\text{S}\)Met-labeled 14-3-3-ζ. Retained \(^{[35]}\text{S}\)Met-14-3-3-ζ, resistant to washing, was identified after separation by 12% SDS-PAGE and autoradiography and quantitated by cutting and counting the corresponding bands in scintillation fluor. Non-transfected (parental) MDCKII cells served as the negative control in these experiments. \(^{[35]}\text{S}\)Met-14-3-3-ζ was consistently retained on the solid phase pellet containing each of the α2AR subtypes. As in gel overlays, the α2AR subtype shows a prominent interaction with 14-3-3-ζ. Interactions with the α2AR subtype are more readily detectable for the native receptor in the solid phase binding assays than for the third intracellular loop alone in gel overlay assays. The precise reasons for these quantitative differences in receptor subtype-14-3-3 interactions in gel overlay versus native receptor solid phase binding assays are not known. This difference likely relates to the different state (native versus partially denatured) of 14-3-3-ζ in the two settings as well as that of the third intracellular loop versus the whole receptor in a native form. Nonetheless both methods demonstrate a reliably detectable interaction, especially for the α2AR subtype. Furthermore, receptor interaction with 14-3-3-ζ essentially was not detectable upon deletion of the third intracellular loop of either the α2AR or the α2β AR (α2βΔ3i (deletion of amino acids 217–377) or α2β3i (deletion of amino acids 214–357); Fig. 7B), further verifying that it is the third cytoplasmic loop of these receptors that promotes the interaction with 14-3-3-ζ.

In order to characterize the functional specificity of the α2AR-14-3-3ζ interaction, competition experiments were performed using Raf and Raf-PO4 peptides in gel overlay assays. These peptides represent a Raf-14-3-3ζ binding motif when the peptide is phosphorylated on serine 259 (see "Experimental Procedures" and Refs. 33 and 40). As can be seen in Fig. 8, co-incubation of membrane strips with Raf-PO4 attenuates the interaction of α2β3i \(^{[35]}\text{S}\)Met-labeled probe with pig brain cortex cytosolic fraction in gel overlay assays (45% of the intensity in the presence of probe alone), whereas the non-14-3-3ζ interacting, non-phosphorylated Raf peptide does not. Similarly, Raf-PO4 peptides attenuate the interaction of α2C3i \(^{[35]}\text{S}\)Met-labeled probe but to a lesser extent (80% of the intensity in the presence of probe alone), perhaps the reflection of a higher affinity of the α2C3i loop for 14-3-3-ζ (data not shown).

DISCUSSION

The third cytoplasmic loop of the α2AR has been implicated in the retention or stabilization of the receptors on the surface of cells (18, 41). Consequently we looked for proteins able to interact with the 3i loop of α2ARs which might implicate their involvement in receptor stabilization on the surface or perhaps receptor microcompartmentalization and signaling specificity. By using a gel overlay strategy, we demonstrated that proteins with an apparent mass of 30 kDa in brain and in MDCKII cell lysates were able to interact with the 3i loops of the α2AR subtypes. Purification and microsequencing revealed that these interacting proteins were the ζ isoform of 14-3-3 proteins. Subsequent analyses using \(^{[35]}\text{S}\)Met-14-3-3-ζ as the ligand demonstrate that this protein indeed interacts with the native
Peptide microsequencing reveals that the αγAR 3i loop interacting proteins represent the ζ isoform of 14-3-3 proteins. Bands excised from SDS-polyacrylamide gels corresponding to 3 μg (~100 pmol) of the purified 3i loop interacting proteins ("Experimental Procedures") were washed and stored in 50:50 water/acetonitrile and provided to the Harvard Microsequencing Core Facility. The bands were digested in situ with trypsin, further resolved by high pressure liquid chromatography, and evaluated by preliminary mass spectrophotometric analysis. Four of the peptides, three each derived from the higher molecular weight band and the fourth derived from the lower of the two 3i loop interacting bands/two-dimensional gel spots, were analyzed further by Edman degradation and peptide microsequencing. The aligned sequences obtained from the purified preparation of 3i loop interacting proteins, and the gray boxes indicate where this sequence is homologous to 14-3-3ζ.

Phosphorylated Raf peptide suppresses αγAR-3i loop interactions with 14-3-3 proteins in the cytosolic fraction of pig brain cortex. Gel overlay assays were performed as described under "Experimental Procedures" in the presence of an [35S]Met-labeled αγAR probe. Raf peptides that correspond to a 14-3-3ζ binding motif were co-incubated with the probe, in a native (Raf) or phosphorylated (Raf-PPO4) form, where indicated. A, [35S]Met Gen10-αγAR 3i loop labeling of 14-3-3 proteins in gel overlay assay. Shown is a representative autoradiograph of a gel overlay assay (bands correspond to the bars in the graph below). B, mean data from gel overlay assays (n = 5). Intensity of bands were calculated using a PhosphorImager and normalized to the values obtained in the presence of probe alone. Error bars represent standard error of the mean. * indicates p < 0.05 using Student's t test between the Raf and Raf-PPO4 treated samples.
development (51). It has been postulated that interaction of signaling molecules with 14-3-3 homo- or heterodimers serves as a scaffolding mechanism to facilitate interactions among molecular components of signaling cascades (42, 53).

The present experiments provide the first evidence for an interaction between 14-3-3 proteins and G protein-coupled receptors and lay the groundwork for establishing the molecular bases for the coordination of α2AR-containing, multicomponent signaling pathways. Since α2Rs are capable of activating the Ras/Raf cascade (3) via the βγ subunits of G proteins (54, 55), the interactions revealed in the present study suggest the possibility that a 14-3-3-based scaffold fosters coincident activation of these signaling components. One speculation that derives from our findings that phosphorylated Raf peptides compete for receptor-14-3-3 interactions is that 14-3-3 proteins could interact with inactive receptors and poise them for immediate coupling to the Ras/Raf cascade upon agonist-elicited conformational changes in the receptor which facilitate receptor interaction with G proteins. This would lead to the liberation of βγ subunits and simultaneously liberate the 14-3-dimer to coordinate activated Ras interactions with Raf in the receptor microenvironment.

It is of interest that the highly purified preparation of α2AR3i loop interacting proteins contained both a 14-3-3 protein doublet as well as eIF2Bα (cf. Fig. 5). Recent studies have demonstrated that the C-terminal tail of the β2- and α2-adrenergic receptors interact with eIF2Bα in two-hybrid screens (38). Unexpectedly, overexpression of eIF2Bα in HEK293 cells leads to an enrichment of this molecule in blebs on the surface membrane and co-localization of α2AR in those eIF2Bα-enriched blebs (38). If the co-purification of 14-3-3 proteins and eIF2Bα represents a molecular interaction that exists in the context of the cell and persists during the protein isolation steps, then it could be postulated that the cytoplasmic domains of these adrenergic receptors provide a surface for interaction of this 14-3-3-eIF2Bα complex, with the C-terminal tail interacting with the eIF2Bα member of the complex and the third intracellular loops interacting with the 14-3-3 dimer component of the complex. One potential strategy to address this possibility would be via co-immunoprecipitation studies; however, we have been unable to detect reliably the co-precipitation of the α2AR with 14-3-3, perhaps because the interaction is of too low affinity to persist during immunosolubilization procedures. This interpretation is consistent with the poor efficiency of α2AR-eIF2Bα co-immunoprecipitation noted by Von Zastrow and co-workers (38), only detectable in transient expression systems where both the receptor and eIF2Bα are simultaneously overexpressed. However, it is also possible that the detection of 14-3-3 and eIF2Bα in the highly purified preparations of α2AR 3i loop interacting proteins simply reflects the similar fractionation properties of these proteins on DEAE and size exclusion chromatography. Future studies will be necessary to distinguish between these possibilities.

A number of recent studies have suggested that the intracellular domains of G protein-coupled receptors represent a surface for association with proteins that may coordinate cellular signaling, beyond the well characterized interactions of these receptors with heterotrimeric G proteins. For example, phosphoinositide-linked metabotropic glutamate receptors have been shown to interact with a PDZ domain-containing protein, dubbed Homer, via interactions with their C-terminal tails (56); the functional consequence of these interactions for receptor-mediated signaling may play a role in long-term potentiation (57). The interaction of the C-terminal tail of G protein-coupled receptors with PDZ domain-containing proteins may represent a recurrent theme. For example, the β2-adrenergic receptor C-terminal tail recently has been shown to interact with the PDZ domain-containing Na+/H+ exchanger regulatory factor, resulting in sequestration of this regulatory protein and attenuation of its phosphorylation-dependent inhibition of ion translocation (58).

These studies represent the first report that α2AR subtypes can interact with 14-3-3 proteins in a manner mediated by the third intracellular loop. This interaction is suppressed by a phosphorylated Raf peptide but not by its non-phosphorylated counterpart. Genetic strategies have provided strong evidence that 14-3-3 proteins, via homo- and hetero-dimeric complexes, serve as scaffolds that facilitate interactions among molecular components of signaling cascades (43, 51, 53). The present findings suggest that 14-3-3 proteins may play a similar role in linking G protein-coupled receptors to diverse signaling pathways by coordinating these interactions in the receptor microenvironment.

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