Ezrin Directly Interacts with the α1b-Adrenergic Receptor and Plays a Role in Receptor Recycling*

Laura Stanasila, Liliane Abuin, Dario Diviani, and Susanna Cotecchia

From the Département de Pharmacologie et de Toxicologie, Faculté de Biologie et de Médecine, 1005 Lausanne, Switzerland

Using the yeast two-hybrid system, we identified ezrin as a protein interacting with the C-tail of the α1b-adrenergic receptor (AR). The interaction was shown to occur in vitro between the receptor C-tail and the N-terminal portion of ezrin, or Four-point-one ERM (FERM) domain. The α1b-AR/ezrin interaction occurred inside the cells as shown by the finding that the transfected α1b-AR and FERM domain or ezrin could be communoprecipitated from human embryonic kidney 293 cell extracts. Mutational analysis of the α1b-AR revealed that the binding site for ezrin involves a stretch of at least four arginines on the receptor C-tail. The results from both receptor biotinylation and immunofluorescence experiments indicated that the FERM domain impaired α1b-AR recycling to the plasma membrane without affecting receptor internalization. The dominant negative effect of the FERM domain, which relies on its ability to mask the ezrin binding site for actin, was mimicked by treatment of cells with cytochalasin D, an actin depolymerizing agent. A receptor mutant (∆R8) lacking its binding site in the C-tail for ezrin displayed delayed receptor recycling. These findings identify ezrin as a new protein directly interacting with a G protein-coupled receptor and demonstrate the direct implication of ezrin in GPCR trafficking via an actin-dependent mechanism.

G protein-coupled receptors (GPCRs) can act as scaffolds binding a variety of proteins involved in receptor regulation and signaling. Beyond G protein-coupled receptor kinases, β-arrestins, and heterotrimeric G protein subunits, a number of proteins have been found to interact with GPCRs using the yeast 2-hybrid system and other biochemical methods (for review, see Ref. 1–3). The functional implications of these interactions are not fully understood; however, they add an increasing complexity to the signaling mechanisms mediated by GPCRs and their regulation. We have previously reported that the α1b-adrenergic receptor (AR) undergoes rapid desensitization and endocytosis upon exposure to the agonist (4) and that the agonist-dependent receptor regulation involves both G protein-coupled receptor kinases and β-arrestins (5). The molecular determinants involved in agonist-induced regulation of the α1b-AR reside within the C-tail of the receptor, as demonstrated by the fact that truncation of this region almost completely abolished receptor desensitization and internalization (4). The α1b-AR can internalize in clathrin-coated vesicles, and this phenomenon depends at least in part on the direct binding to the receptor C-tail of the μ2 subunit of the AP2 clathrin-adaptor complex (6).

To identify new proteins that interact with the α1b-AR which could be involved in regulating receptor function, we have used the yeast two-hybrid system and identified ezrin, a member of the ERM (Ezrin, Radixin, Moesin) family of proteins. ERM proteins were originally characterized as structural components of the cell cortex but were soon shown also to participate in signaling pathways (for review, see Ref. 7). ERM proteins share a conserved structure including two main domains: the N-terminal portion, or Four-point-one-ERM (FERM) domain, which binds to protein targets at the plasma membrane, and the C-terminal portion, containing the binding site for F-actin. In the closed, or inactive, conformation, the C terminus of ERM proteins tightly binds to the FERM domain, thus preventing both interaction with membrane targets and actin. Upon activation of ERM proteins by phosphatidylinositol 4,5-bisphosphate binding and phosphorylation, this intramolecular interaction is released, and the FERM domain is free to bind to its protein targets, whereas the C terminus binds to actin filaments (8).

An indirect link between ERM proteins and GPCRs has been suggested by previous findings showing that some GPCRs can interact with the ERM adaptor EBP50 (also known as NHERF1) (9–11). This interaction seems to play a role in GPCR trafficking, but the involvement of ezrin has not been directly demonstrated. In this study we demonstrate that ezrin can directly interact with the α1b-AR through a polyarginine motif located on the C-tail of the receptor and that this interaction contributes to receptor recycling to the plasma membrane. This is to our knowledge the first report demonstrating a direct interaction of an ERM protein with a GPCR and might provide some generalities about the link between GPCRs and events associated with cytoskeletal reorganization including receptor trafficking.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**

Full-length human ezrin was PCR-amplified from an EST clone and subcloned in pCMV-Script, pCMV-FLAG, pEGFP-N3, pET30a, and pAct2 expression vectors. The FERM domain (FERM*) of ezrin, including amino acids (aa) 1–308 and its fragments F1 (aa 1–95), F2 (aa 89–202), F3 (aa 200–308), F1/2 (aa 1–202), and F2/3 (aa 89–308) were PCR-cloned and subcloned in pEGFP-N3. The cDNA coding for FERM* was subcloned in pET30a and pAct2 vectors. A cDNA encoding the hamster α1b-AR was PCR-amplified to introduce in-frame the HA tag (YPYDVPDYA) at its C terminus and subcloned into pRK5. A cDNA fragment encoding the receptor C-tail (351–515) or its fragments (amino acids 351–380, 351–395, 351–425, 351–449, and 351–477) was PCR-amplified and subcloned in...
pGEX-4T1 and pLexA plasmids to obtain fusion proteins with GST and LexA at the N terminus of the fragments. The deletion of 2, 4, 6, 7, or 8 consecutive arginines at positions 371–378 in the receptor C-tail was achieved by PCR, and the mutated cDNA fragments were subcloned in pGEX-4T1 or pLexA vectors. The construct pEGFP-N1-α2 was described previously (6).

**Yeast Two-hybrid Screening**

The yeast strain L40 was transformed with the C-tail-pLexA plasmid encoding the α1b-AR C-tail fused to LexA, and clones were selected and subsequently transformed with 250 μg of a human brain Matchmaker cDNA library in the pAct2 vector (Clontech). Of 13 million double transformants, 40 exhibited moderate to strong growth on histidine-deficient plates. The plasmids isolated from the positive clones were used to co-transform the L40 strain with either the C-tail-pLexA or the empty pLexA plasmid, and the specificity of the interaction was confirmed by growth on histidine-deficient plates as well as by β-galactosidase activity measures (Yeast Protocols Handbook; Clontech).

**Cell Culture and Transfections**

HEK-293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum and gentamycin (100 μg/ml) and transfected at 40–60% confluence in 100-mm dishes using the calcium phosphate method. After transfection, cells were grown for 48 h before harvesting. The total amount of transfected DNA was 10 μg/100-mm dish.

**Expression and Purification of Recombinant Proteins in Bacteria**

GST-tagged fusion proteins of α1b-AR C-tail fragments were expressed using the bacterial expression vector pGEX4T1 in the BL21DE3 strain of *Escherichia coli* and affinity-purified on glutathione-Sepharose beads (Amersham Biosciences) as described before (6). Briefly, *E. coli* lysates were prepared by centrifugation of the bacterial culture followed by sonication in buffer A (20 mM Tris, pH 7.4, 100 mM NaCl, 0.5% (v/v) Triton X-100, 1 mM benzamidine, 2 μg/ml leupeptin, 2 μg/ml pepstatin) and centrifugation at 38,000 × g. After overnight incubation of the supernatants with glutathione-Sepharose beads (Amersham Biosciences) at 4 °C, the resin was washed, resuspended in 1/100× culture volume of the same buffer, and stored at 4 °C. Histagged fusion of the full-length ezrin, its N-terminal, or C-terminal domains was expressed using the bacterial expression vector pET30a in the BL21DE3 strain and purified. Lysates of bacterial cultures expressing His-tagged proteins were prepared by centrifugation and sonication of the bacterial pellet in lysis buffer (50 mM NaH2PO4, pH 8, 300 mM NaCl, 10 mM imidazole, 1 mM benzamidine, 2 μg/ml leupeptin, 2 μg/ml pepstatin). The lysates were centrifuged at 38,000 × g, and the supernatants were incubated with nickel nitritotriacetic acid chelating resin (Amersham Biosciences) for 1 h at 4 °C. The resin was then washed 5 times and stored at 4 °C. His-tagged fusion proteins were eluted from the resin with elution buffer (50 mM NaH2PO4, pH 8, 300 mM NaCl, 300 mM imidazole) for 1 h at room temperature, dialyzed, and stored at −20 °C after the addition of 10% glycerol. The protein concentration was assessed by Coomassie staining of SDS-PAGE gels.

**GST Pull-down and Immunoprecipitation**

For GST pull-down experiments, HEK-293 cells expressing the various constructs grown on 100-mm dishes were lysed in 1 ml of buffer B (20 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% digitonin (Sigma), 5 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride). After 2 h of solubilization at 4 °C followed by centrifugation at 18,000 × g for 20 min, the protein content in the supernatants was measured using the Bradford reagent (Sigma). Glutathione-Sepharose beads coupled to the different GST fusion proteins were incubated with 1.5 mg of proteins from cell lysates in a total volume of 1 ml (buffer B) overnight at 4 °C. The beads were then washed 4 times with 1 ml of buffer C (20 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.2% digitonin) and resuspended in SDS-PAGE sample buffer. Eluted proteins were analyzed by SDS-PAGE and Western blotting. The amount of cell extract loaded on the gel was 5% that used for the immunoprecipitation.

For in vitro pull-down experiments, glutathione-Sepharose beads coupled to the different GST-fused fragments of the α1b-AR C-tail were incubated with different quantities of purified Hisα2-tagged N-terminal or C-terminal ezrin domains in a total volume of 1 ml of buffer D (20 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride) overnight at 4 °C. The beads were then washed 4 times and resuspended in SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE and Western blotting. For immunoprecipitation experiments, HEK-293 cells expressing the various constructs were grown in 100-mm dishes and harvested 48 h post-transfection. Cells were incubated for 1 h in serum-free DMEM, treated for various times with 10−4 M epinephrine (Sigma), and then washed twice with PBS. For cross-linking experiments, 3.5 ml of Hepes buffer (20 mM Hepes, pH 7.4, 150 mM NaCl) containing 1 mM DSP (3,3′-dithiobis(propioninic acid N-hydroxysuccinimid) ester) were added to each plate. Cross-linking was allowed to proceed for 30 min at room temperature with gentle rocking. Cells were then washed twice with ice-cold DMEM supplemented with 10% fetal calf serum, then twice with PBS and lysed in 1 ml of buffer B for 2 h at 4 °C. Cell lysates were centrifuged at 18,000 × g for 20 min, and the protein content in the supernatants was measured using the Bradford assay. Equal amounts of total protein were then added to 20 μl of protein A-Sepharose beads (Amersham Biosciences) in a final volume of 1 ml (buffer C). The beads were incubated at 4 °C for 2 h, washed 4 times with 1 ml of buffer C, and resuspended in SDS-PAGE sample buffer. Eluted proteins were analyzed by SDS-PAGE and Western blotting. The amount of extract loaded on the gel was 5% that used for the immunoprecipitation.

**SDS-PAGE and Western Blotting**

Samples were denatured in SDS-PAGE sample buffer (65 mM Tris, pH 6.8, 2% SDS, 5% glycerol, 5% β-mercaptoethanol, bromphenol blue) for 1 h at 37 °C or 5 min at 95 °C, separated on 10% acrylamide gels, and electroblotted onto nitrocellulose membranes. The samples from pull-down experiments (see Figs. 1 and 3) were heated for 5 min at 95 °C before loading on the gel. In contrast, because the α1b-AR aggregates if heated at 95 °C, the samples from co-immunoprecipitation experiments with the α1b-AR were incubated for 1 h at 37 °C (see Figs. 2, 4, 5A, and 7). After heating at 95 °C, the FERM fusion proteins appeared as single bands, whereas in most of the samples incubated at 37 °C they migrated as multiple bands, probably because of incomplete denaturation of the fusion proteins. The blots were incubated for 1 h in TBS-Tween (100 mM Tris, pH 7.4, 140 mM NaCl, 0.05% Tween 20) containing 5% (v/v) nonfat dry milk and then incubated with the specific primary antibody diluted in TBS-Tween for 2 h at room temperature. After washing, the membranes were probed with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) diluted 1:10,000 in TBS-Tween for 1 h, washed 3 times with TBS-Tween, and developed using the Enhanced Chemiluminescence detection system (Amersham Biosciences). The following primary antibodies were used for immunoblotting:
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ing: rabbit polyclonal anti-HA (1:2,000; Sigma); mouse monoclonal anti-HA (1:10,000; Sigma); mouse monoclonal anti-GFP (1:1,000; Roche Applied Science); mouse monoclonal anti-ezrin (1:4,000; Sigma); mouse monoclonal anti-FLAG (1:2,000; Roche Applied Science).

Receptor Biotinylation

Protocol A—To measure cell surface-biotinylated receptors, HEK-293 cells, grown in 100-mm dishes, were transfected with the cDNAs encoding the HA-tagged $\alpha_{1b}$-AR alone or in combination of FERM*-GFP. 48 h after transfection cells were incubated for 1 h in serum-free DMEM containing 10 $\mu$M cycloheximide, then treated with $10^{-4}$ M epinephrine (Sigma) and $10^{-6}$ M propranolol (to block endogenous $\beta_2$-adrenergic receptors) at 37 °C for various times. To measure receptor recycling to the cell surface, after incubation with $10^{-4}$ M epinephrine for 60 min the medium was removed, the cells were washed, fresh medium containing $10^{-7}$ M prazosin was added, and cells were incubated again at 37 °C for various times. Cells were then placed on ice and washed twice with ice-cold PBS. Surface proteins were biotinylated by incubating the cells with 300 $\mu$L/ml membrane-impermeable biotin analog sulfo-NHS-SS-biotin (EZ-link biotin, Pierce) in PBS for 30 min at 4 °C with gentle shaking. Unreacted biotin was quenched and removed by 3 washes with ice-cold TBS (50 mM Tris, pH 8, 150 mM NaCl). Cells were then lysed in buffer C for 3 h at 4 °C, and the cellular homogenate was centrifuged at 18,000 $\times$ g for 20 min. The supernatants were incubated overnight with 30 $\mu$L of streptavidin-Sepharose beads (Amersham Biosciences), pelleted by brief centrifugation, and washed 4 times with 1 ml of buffer D. Biotinylated proteins were eluted from the beads by incubation with 40 $\mu$L of 100 mM dithiothreitol (Sigma) for 1 h at 37 °C. The samples were briefly centrifuged, and SDS-PAGE sample buffer was added to the supernatants. Samples were analyzed by SDS-PAGE and Western blotting. Biotinylated HA-tagged $\alpha_{1b}$-AR was detected using the mouse monoclonal anti-HA antibody (Sigma) as described above.

Protocol B, Adapted from Cao et al. (12)—To measure internalized biotinylated receptors, HEK-293 cells were transfected as in protocol A. After transfection, cells were incubated for 1 h in serum-free DMEM containing 10 $\mu$M cycloheximide, then washed twice with cold PBS and biotinylated using 4 $\mu$L of PBS containing 300 $\mu$g/ml sulfo-NHS-SS-biotin, for 30 min at 4 °C. The dishes were washed three times with cold TBS to quench unreacted biotin, and fresh medium was added. After biotinylation, cells were treated as in protocol A to induce receptor internalization and recycling. When appropriate, 1 $\mu$L cytochalasin D (Sigma) was added 10 min before epinephrine, and cytochalasin D was kept throughout the experiment. After incubations, cells were washed twice with cold PBS, and biotin molecules covalently bound to cell surface proteins were cleaved off by incubation with 5 $\mu$L of stripping solution (50 $\mu$L glutathione, 300 $\mu$L NaCl, 75 $\mu$L NaOH, 1% fetal calf serum) for 30 min at 4 °C. The remaining glutathione was then quenched using 5 $\mu$L of quenching solution (50 $\mu$L iodoaceticamide, 1% bovine serum albumin) for 20 min at 4 °C. Cells were then washed twice with PBS and lysed in buffer C for 3 h at 4 °C.

The intensity of each band was quantified by densitometry of films exposed in the linear range and analyzed using NIH Image software (National Institutes of Health). The statistical significance of the data was assessed using Student’s $t$ test.

Immunofluorescence

HEK-293 cells were grown on glass coverslips. DNA transfections and measurements of receptor internalization and recycling were done as described for the biotinylation experiments. After the treatment cells were fixed in PBS containing 3.7% formaldehyde for 10 min and permeabilized for 5 min with PBS plus 0.2% (w/v) Triton X-100. After incubation in PBS plus 1% bovine serum albumin for 1 h, the primary antibody in PBS plus 0.1% bovine serum albumin was added for 1 h. Texas Red-phalloidin (2 $\mu$L/coverslip) was added together with the primary antibody. The coverslips were washed in PBS and incubated with the Texas Red- or FITC-conjugated secondary antibody (Jackson ImmunoResearch) diluted 1:100 in PBS plus 0.1% bovine serum albumin for 1 h. After three washes in PBS and one wash in H$_2$O, coverslips were mounted on glass slides using Prolong (Molecular Probes). The fluorescent staining was visualized on laser-scanning confocal microscope (Zeiss LSM 510 Meta).

RESULTS

Identification of Ezrin as a Protein Directly Interacting with the $\alpha_{1b}$-AR C-tail—Using the C-tail of the $\alpha_{1b}$-AR ($\alpha_{1b}$-Ct) (including amino acids 351–515 as shown in Fig. 3) as bait in a yeast two-hybrid screening of a human brain cDNA library, we identified one positive clone encoding amino acids 18–364 of human ezrin (also known as villin-2 (7)). The L40 yeast strain was transformed with the bait plasmid expressing the $\alpha_{1b}$-Ct in combination with empty pACT2 vector or with pACT2 encoding either the N-terminal (amino acids 1–308), the C-terminal half (amino acids 285–586) of ezrin, or the full-length ezrin. Yeast transformed with $\alpha_{1b}$-Ct and the N-terminal half of ezrin were able to grow in the absence of histidine and to produce $\beta_2$-galactosidase, whereas those transformed with the receptor C-tail and either the C-terminal portion or full-length ezrin did not (results not shown).

Pull-down experiments were performed by incubating the GST-fused $\alpha_{1b}$-Ct immobilized on Sepharose beads with lysates of cells expressing either GFP alone or ezrin, its N-terminal or C-terminal half fused to GFP (Fig. 1A). The results of these experiments indicated that the ezrin N-terminal half, but neither its C-terminal portion nor the full-length ezrin, specifically bound the $\alpha_{1b}$-Ct. Within the ERM family of proteins, their N-terminal portion is generally indicated as the FERM domain (FERM*), which is involved in the interaction with membrane-bound proteins and phosphatidylinositol 4,5-bisphosphate (7).

To check whether the ezrin/$\alpha_{1b}$-AR interaction was direct, i.e. not mediated by another protein, we incubated GST-fused $\alpha_{1b}$-Ct immobilized on Sepharose beads with increasing amounts of the His-tagged FERM* or C-terminal portion of ezrin purified from bacteria. In addi-
tion to the His tag, the fragments carried the S tag, which could be detected using S protein-horseradish peroxidase. As shown in Fig. 1B, the FERM* was able to directly bind the α1b-Ct with an estimated affinity of ≈200 nM. In contrast, the C-terminal portion of ezrin did not bind even at concentrations as high as 1 μM (data not shown).

The α1b-AR Interacts with Ezrin in Intact Cells—To demonstrate that the α1b-AR and ezrin can form a complex inside the cells, we performed coimmunoprecipitation experiments from HEK-293 cells transiently co-expressing the HA-tagged α1b-AR with ezrin, its FERM* or C-terminal portion fused to GFP. After immunoprecipitating the receptor using anti-HA antibodies, anti-GFP antibodies were used to immunoblot the immunoprecipitated samples. As shown in Fig. 2, the fusion proteins were expressed at similar levels in the cell extracts and ran as a doublet on SDS-PAGE, as has been often observed. Whereas the FERM* co-immunoprecipitated with the receptor, the C-terminal portion of ezrin did not (Fig. 2). A small amount of full-length ezrin-GFP could also co-immunoprecipitate with the receptor, thus suggesting that the α1b-AR and ezrin can associate inside the cells and that this interaction is mediated by the FERM domain.

To assess whether the α1b-AR/ezrin interaction was regulated by receptor agonists, we overexpressed both the α1b-AR and wild type ezrin and co-immunoprecipitated them in the presence or absence of 1 μM DSP (3,3′-dithio-bis(propionic acid N-hydroxy succinimide ester) cross-linker from cells stimulated with epinephrine for different times as well as after removal of the agonist (results not shown). The amount of ezrin co-immunoprecipitated with the receptor was similar in cells stimulated or not with the agonist, and it was hard to detect in the absence of the cross-linker. Co-immunoprecipitation of the receptor with endogenous ezrin did not result in any signal probably because the amount of co-immunoprecipitated endogenous ezrin is too low to be detected. Altogether these findings suggest that despite the fact that the α1b-AR and ezrin can form a complex in intact cells, the α1b-AR/ezrin interaction is rather weak, in agreement with the apparent affinity of ≈200 nM estimated in the pull-down experiments (Fig. 1B).

Identification of the Binding Site for Ezrin on the α1b-AR—To map the binding site of ezrin on the α1b-1Ct, we fused to GST different fragments of the C-tail carrying progressive truncations (Fig. 3A) and assessed their ability to bind the purified His-tagged FERM* (Fig. 2B). All the fusion constructs truncated up to residue 380 could interact with the FERM*, thus suggesting that binding site for ezrin is located upstream of residue 380. Interestingly, the C-tail fragment lacking the eight arginines at positions 371–378 (ΔR8) was unable to bind the FERM*, suggesting that this stretch of arginines represents the binding site of the α1b-Ct for ezrin (Fig. 3). In fact, deletions of additional amino acids upstream of the polyarginine motif did not impair the binding of the α1b-Ct to FERM* (results not shown).

It is important to notice that this polyarginine motif was recently shown to be involved in the direct binding of the α1b-AR with the Δ2 subunit of the AP2 complex (6). Therefore, to assess whether the same number of arginines was involved in binding both ezrin and μ2, we generated α1b-Ct fragments containing 1 (ΔR7), 2 (ΔR6), 4 (ΔR4), or 6 (ΔR2) of the eight arginines at positions 371–378. These fragments fused to GST were tested for their ability to pull down either the FERM-GFP or the μ2 subunit of the AP2 complex fused to GFP (Fig. 3B). As shown in Fig. 3 (C), the ΔR2 and ΔR4 fragments behaved like the wild type α1b-Ct, whereas the ΔR6 and ΔR7 were profoundly impaired in their ability to bind both the FERM* and μ2. This indicates that the same number of positive charges on the α1b-Ct is required to bind ezrin and the μ2 subunit of the AP2 complex.

Identification of the Binding Site for the α1b-AR on Ezrin—FERM domains of the ERM family of proteins have a highly conserved tridimensional structure consisting of three lobes or subdomains, F1, F2, F3 (13). To identify which portions of FERM* bind the receptor, we fused to GFP each of the three subdomains (F1, amino acids 1–95; F2, amino acids 89–202; F3, amino acids 200–308) or fragments including two consecutive subdomains (F1/2, amino acids 1–202; F2/3, amino acids 89–308) (Fig. 4A). These FGP-fused proteins expressed in cells were tested in pull-down experiments for their capacity to bind the GST-fused α1b-Ct. All the fusion proteins were highly expressed even if the appearance of multiple bands on Western blots in the cell extracts sug-

![FIGURE 2. Ezrin interacts with the α1b-AR in intact cells. Ezrin (Ez), FERM*, or its C-terminal portion fused to GFP were expressed in HEK-293 cells alone or with the HA-tagged α1b-AR. Cell lysates were immunoprecipitated (IP) using polyclonal anti-HA antibodies. Western blots (WB) of cell extracts or immunoprecipitates (IP) were revealed using monoclonal anti-GFP and monoclonal (for immunoprecipitates) or polyclonal anti-HA antibodies (for cell extracts). Results are representative of three independent experiments.](image-url)

![FIGURE 3. Mapping of the binding site for ezrin on the α1b-AR C-tail. A, schematic drawing of the various fragments derived from the α1b-AR C-tail (α1b-Ct). B, His-tagged FERM* was incubated with beads coupled to GST alone or to GST fused with different fragments of the α1b-AR. C-terminal FERM* contained the S tag, detected using horse-radish peroxidase-conjugated S protein. C, extracts from HEK-293 cells expressing either FERM-GFP or the μ2 subunit of the AP2 complex fused to GFP (μ2-GFP) were incubated with beads coupled to GST alone or to GST fused with fragments of the α1b-AR C-tail carrying mutation of the polyarginine stretch (371–378). Ext, cell extracts. Results are representative of at least three independent experiments. WB, Western blot.](image-url)
It has been established that in the closed or inactive conformation of ERM proteins, the distal 30 amino acids of their C terminus bind with high affinity to the FERM*, thus preventing its interaction with other determinants at the boundary between F1 and F2. This suggests that F1 and F3 contain the most important structural determinants involved in binding the FERM*. F1/2 and F3 displayed the strongest signal, whereas no binding was detected for F2. This suggests that F1 and F3 in the tridimensional structure of FERM* (Fig. 4A). The fact that F1/2 binds better than F1 suggests that the conformation of F1 alone is perturbed compared with that in the context of the whole protein or that some determinants are at the boundary between F1 and F2.

It has been established that in the closed or inactive conformation of ERM proteins, the distal 30 amino acids of their C terminus bind with high affinity to the FERM*, thus preventing its interaction with membrane bound proteins (7, 13). Therefore, we checked whether the binding of α1b-Ct and of the ezrin C terminus on FERM* were mutually exclusive. The GST-fused α1b-Ct immobilized on Sepharose beads was incubated with cell extracts expressing GFP-tagged FERM* in the absence or presence of increasing amounts of purified His-tagged C-terminal portion of ezrin (His-EzCt). Ext, cell extracts. Results are representative of at least three independent experiments.

Assessing the Mechanism Underlying the Dominant Negative Role of the FERM Domain—Previous studies reported that the FERM domain of ezrin has a dominant negative effect on ezrin function (14, 15), but no clear mechanism was demonstrated for its action. Therefore, we investigated whether overexpression of FERM* could interfere with α1b-AR/ezrin interaction. For this purpose, the α1b-AR and wild type ezrin were co-expressed in HEK-293 cells in the absence or presence of the FERM* fused to GFP (Fig. 5A). The immunoprecipitated samples were revealed using antibodies against ezrin or anti-GFP antibodies to detect the FERM*-GFP.

As shown in Fig. 5 (A, left upper panel, lanes 5 and 6), a much larger amount of overexpressed ezrin could be co-immunoprecipitated with the receptor in the presence of FERM*-GFP compared with its absence. Considering the significantly higher affinity of the FERM* for ezrin C terminus (≈20 nM) than for the α1b-Ct (≈200 nM), it is possible that the overexpressed FERM* bound the C terminus of endogenous ezrin, thus inducing ezrin to adopt its open conformation. This should result in an increased amount of open, or activated, endogenous ezrin able to bind the receptor. This hypothesis is supported by the findings that the FERM*-GFP could efficiently coimmunoprecipitate with FLAG-tagged ezrin, thus suggesting that overexpressed FERM* and full-length ezrin tightly interact in intact cells (Fig. 5B). As summarized in Fig. 5C, altogether these findings indicate that the FERM*, instead of competing with endogenous ezrin for its binding to the α1b-AR, binds to the C terminus of ezrin, thus blocking its interaction with α1b-AR. This would result at same time in a dominant negative effect of the FERM* on α1b-AR/ezrin interaction.
The α1b-AR/Ezrin Interaction Is Involved in the Regulation of Receptor Recycling—It is well established that actin filaments participate to endocytosis in organisms ranging from yeast to mammals. However, the role of actin on GPRC trafficking in mammalian cells has not been extensively studied (16). Because ezrin binds to actin and regulates dynamic actin rearrangement, we investigated the potential role of α1b-AR/ezrin interaction in receptor endocytosis and recycling.

To quantify receptor endocytosis, we used a biotinylation assay to selectively label the receptors expressed at the cell surface (Protocol A). Cells expressing HA-tagged α1b-AR in the absence or presence of FERM* fused to GFP were exposed to 10^{-4} \text{M} epinephrine for various times and subsequently incubated with a membrane-impermeant biotinylation reagent (sulfo-NHS-biotin). Cells were treated with cycloheximide 1 h before the experiment to block protein synthesis. Biotinylated surface receptors were precipitated using streptavidin-Sepharose beads and detected by Western blotting using anti-HA antibodies. The internalized receptors carried the biotin moieties. Biotinylated internalized receptors were covalently bound at the cell surface were cleaved off so that only internalized receptors were detected by Western blotting using anti-HA antibodies. As shown in Fig. 6, A and B, cell surface biotinylated receptors were measured according to protocol A, whereas in C–D, the internalized biotinylated receptors were assessed following protocol B (“Experimental Procedures”). Western blots (WB) of biotinylated receptors in the precipitate (Pr) were revealed using anti-HA monoclonal antibodies, whereas blots of cell extracts (Ext) were revealed with monoclonal anti-GFP and polyclonal anti-HA antibodies. Results are the mean ± S.E. of five independent experiments. * p < 0.05 Student’s t test compared with GFP in B and D; ** p < 0.05 Student’s t test compared with −CCD in F.

actin-dependent ezrin function and in increased binding of ezrin to the receptor.

To confirm these results, we used an alternative protocol for receptor biotinylation to selectively label internalized receptors (Protocol B). Cells pretreated with cycloheximide were incubated with biotin before treatment with epinephrine. After treatment, the biotin molecules covalently bound at the cell surface were cleaved off so that only internalized receptors carried the biotin moieties. Biotinylated internalized receptors were precipitated using streptavidin-Sepharose beads and detected by Western blotting using anti-HA antibodies. The internalized receptors were normalized to the total amount of biotinylated α1b-AR in cells in which biotin molecules were not cleaved off. As shown in Fig. 6, C and D, before the treatment with epinephrine (time 0) about 25% of the total amount of biotinylated receptors were internalized probably because of constitutive endocytosis. After 60 min of exposure to 10^{-4} \text{M} epinephrine, about 60% of the receptors were internalized and returned to the surface 90 min after removal of the agonist. Co-expression of the FERM*–GFP profoundly impaired receptor recycling but not internalization. These results indicate that the FERM domain had a dominant negative effect on α1b-AR recycling without affecting its endocytosis.

The dominant negative effect of FERM* might be explained by the fact that its binding to the C terminus of ezrin blocks ezrin interaction with actin, which might play a role in α1b-AR recycling. To test this hypothesis, we performed biotinylation experiments in cells expressing the HA-tagged α1b-AR in the absence or presence of cytochalasin D, an
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actin depolymerizing agent. As shown in Fig. 6, E and F, the effect of cytochalasin D mimicked that of FERM*, impairing receptor recycling but not endocytosis. Altogether, these findings support the hypothesis that ezrin binding to the α1b-AR plays a role in receptor recycling via its action on actin.

A Receptor Mutant Impaired in the Ezrin Binding Site Displays Delayed Recycling—Because the deletion of the eight arginines in the α1b-CT entirely abolished ezrin binding, we explored the ability of the ΔR8 receptor mutant (lacking the eight arginines in the C-tail) to co-immunoprecipitate with FERM* in intact cells and to recycle back to the surface after internalization. As shown in Fig. 7A, the ΔR8 mutant was markedly, but not entirely, impaired in its ability to co-immunoprecipitate FERM*. This finding was somewhat surprising and raises the hypothesis that the FERM* might also interact with other portions of the receptor or that some interaction might occur indirectly via a yet unknown protein.

We have previously reported that the ΔR8 mutant is partially impaired in internalization probably because it does not bind the μ2 subunit of the AP2 complex (6). The impaired internalization was mainly evident at 15 and 30 min after exposure to the agonist. As shown in Fig. 7B, 60 min after exposure to epinephrine, about 40% of the ΔR8 mutant was internalized. Interestingly, 60 min after removal of epinephrine (+60 min), when the wild type α1b-AR had recycled back to the surface, the ΔR8 mutant had not. However, at longer times (>90 min) after removal of the agonist, the ΔR8 mutant did recycle back to the plasma membrane (results not shown).

Confocal Microscopy to Investigate the Localization of α1b-AR and Ezrin—To assess whether the α1b-AR colocalizes with endogenous ezrin in living cells, we performed confocal microscopy on HEK-293 cells expressing the HA-tagged α1b-AR and followed receptor internalization as well as recycling. Endogenous ezrin was labeled in green using a monoclonal anti-ezrin antibody and a secondary FITC-labeled antibody. The receptor was labeled in red using a secondary rhodamine-labeled antibody. As shown in Fig. 8, in unstimulated cells (time 0'), the receptor and ezrin colocalize at the plasma membrane, with ezrin accentuated at the membrane protrusions and anchor points to the coverslip. After 15 min of stimulation with epinephrine, roughly 50% of the receptor fluorescence was already internalized, but no ezrin signal colocalized with the receptor in endocytic vesicles. After 60 min with epinephrine, the internalization of the receptor was almost complete, whereas ezrin was still mostly localized at the plasma membrane. Only in a few intracellular vesicles was colocalization of ezrin and the receptor observed. Fifteen minutes after removal of epinephrine, most of the ezrin signal was distributed in intracellular vesicles, many of which contained also the α1b-AR. Sixty minutes after the agonist removal, part of the receptors had returned to the plasma membrane, where it colocalized with ezrin, whereas part remained in intracellular vesicles that showed little ezrin labeling. In conclusion, the results from confocal imaging suggest that the α1b-AR and ezrin colocalize both at the plasma membrane and in recycling vesicles after removal of the agonist.

We also compared the endocytosis and recycling of the α1b-AR in the absence or presence of the FERM* tagged to GFP (Fig. 9). The receptor

FIGURE 7. The ΔR8 receptor mutant displays impaired interaction with ezrin in intact cells. A, the GFP-fused FERM* was expressed in HEK-293 cells alone or with the HA-tagged α1b-AR or ΔR8 receptor mutant, lacking arginines 371–378. Cell lysates were immunoprecipitated using polyclonal anti-HA antibodies. Western blots (WB) of cell extracts or immunoprecipitates (IP) were revealed using monoclonal anti-GFP, monoclonal (for immunoprecipitates) or polyclonal anti-HA antibodies (for cell extracts). Results are representative of three independent experiments. B, quantification of the amount of FERM*-GFP co-immunoprecipitated with HA-tagged ΔR8. C, in HEK-293 cells expressing the HA-tagged α1b-AR or ΔR8 receptor mutant, cell surface biotinylated receptors were measured according to protocol A (“Experimental Procedures”). Results are the mean ± S.E. of three independent experiments. *, p < 0.05 Student’s t test compared with α1bHA.

FIGURE 8. Cellular localization of the α1b-AR and ezrin monitored by confocal microscopy. Confocal microscopy of HEK-293 cells transfected with HA-tagged α1b-AR. Cells pretreated for 1 h with cycloheximide were incubated with 10−4 M epinephrine for 15 or 60 min. To monitor receptor recycling, after a treatment of 60 min with epinephrine, the agonist was removed (wash), and the cells were incubated for another 15 or 60 min. Cells were then fixed, permeabilized, and stained using polyclonal anti-HA and secondary rhodamine (rhod)-coupled antibodies to detect the receptor and monoclonal anti-ezrin and secondary FITC-coupled antibodies to detect endogenous ezrin. The images are representative of four independent experiments.
was labeled in red using a secondary rhodamine-labeled antibody. In unstimulated cells the FERM*-GFP fluorescence was distributed throughout the cytosol and in the nucleus as well as at the plasma membrane where it colocalized with the receptor. During receptor internalization, the distribution of FERM*-GFP did not differ significantly as compared with unstimulated cells. A striking difference was observed 60 min after agonist removal between cells expressing or not the FERM*. In fact, as can be observed for the two cells shown in Fig. 9, in the cell on the left expressing both the receptor and FERM* and the one on the right expressing only the receptor. The images are representative of three independent experiments.

Confocal microscopy was also performed on cells expressing the HA-tagged α1b-AR in the presence (+ CCD) or absence (− CCD) of cytochalasin D (Fig. 10). The HA-tagged α1b-AR was labeled in green using a secondary antibody coupled to FITC, whereas actin was labeled in red using Texas Red-phalloidin. In − CCD cells, the receptor was mostly found at the plasma membrane, and actin showed a subcortical localization. In + CCD cells, actin was disorganized, and the cell shape was distorted. After 15 min of stimulation with epinephrine, actin formed stress fibers in − CCD but not in + CCD cells. The receptor was internalized after the stimulation with the agonist in both − CCD and + CCD cells. However, the shape and size of the FITC-positive vesicles containing the receptor were different between the − CCD and + CCD cells, the vesicles being larger and more irregular in shape in the + CCD. After removal of the agonist, recycling of the HA-tagged α1b-AR was detected in − CCD cells but to a much lesser extent in + CCD cells.

**DISCUSSION**

In this study we provide evidence that ezrin, a member of the ERM family of proteins, can directly interact with the α1b-AR. The interaction was shown to occur in vitro between the purified ezrin FERM domain and the receptor C-tail as well as in intact cells between the α1b-AR and either full-length ezrin or its FERM domain. To our knowledge this is the first report of a direct interaction between a GPCR and an ERM protein. ERM proteins can directly interact with monotopic membrane proteins such as CD43, CD44, and ICAM-1 and -2 (17, 18) or axonal CAM L1 (19). With respect to polytopic membrane proteins, ezrin interacts with NHE1 (20) and podocalyxin (21) both directly and through the adaptors EBP50 or E3KARP.

Our results strongly suggest that the binding site for ezrin on the α1b-AR C-tail is represented by a cluster of at least four arginines. This is consistent with data on other ERM substrates, which in most cases contained three or more positively charged amino acids (7). The affinity of the ezrin FERM domain for the α1b-AR C-tail (−200 nM) was similar to the affinity of ERM proteins for other targets such as CD43 or VCAM-1 (22). The same arginine cluster was identified as being the AP50 binding site on the α1b-AR C-tail (23). The affinity of the ezrin FERM domain for the α1b-AR C-tail (−200 nM) was similar to the affinity of ERM proteins for other targets such as CD43 or VCAM-1 (22). The same arginine cluster was identified as being the AP50 binding site on the α1b-AR C-tail (23). The affinity of the ezrin FERM domain for the α1b-AR C-tail (−200 nM) was similar to the affinity of ERM proteins for other targets such as CD43 or VCAM-1 (22). The same arginine cluster was identified as being the AP50 binding site on the α1b-AR C-tail (23). The affinity of the ezrin FERM domain for the α1b-AR C-tail (−200 nM) was similar to the affinity of ERM proteins for other targets such as CD43 or VCAM-1 (22). The same arginine cluster was identified as being the AP50 binding site on the α1b-AR C-tail (23). The affinity of the ezrin FERM domain for the α1b-AR C-tail (−200 nM) was similar to the affinity of ERM proteins for other targets such as CD43 or VCAM-1 (22). The same arginine cluster was identified as being the AP50 binding site on the α1b-AR C-tail (23). The affinity of the ezrin FERM domain for the α1b-AR C-tail (−200 nM) was similar to the affinity of ERM proteins for other targets such as CD43 or VCAM-1 (22). The same arginine cluster was identified as being the AP50 binding site on the α1b-AR C-tail (23). The affinity of the ezrin FERM domain for the α1b-AR C-tail (−200 nM) was similar to the affinity of ERM proteins for other targets such as CD43 or VCAM-1 (22). The same arginine cluster was identified as being the AP50 binding site on the α1b-AR C-tail (23). The affinity of the ezrin FERM domain for the α1b-AR C-tail (−200 nM) was similar to the affinity of ERM proteins for other targets such as CD43 or VCAM-1 (22). The same arginine cluster was identified as being the AP50 binding site on the α1b-AR C-tail (23).
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C-tail even though less efficiently than the entire FERM domain (Fig. 4). Thus, ezrin seems to bind the α1b-AR C-tail in a similar fashion to other ERM substrates, such as ICAM-2. In fact, in the crystal structure of the ICAM-2 C-tail bound to radixin FERM domain, ICAM-2 bound through a stretch of 28 amino acids lying in a cleft between the subdomains 1 and 3 of the FERM (22).

We took advantage of the dominant negative effect of FERM domain to explore the functional implications of α1b-AR/ezrin interaction. The results from both biotinylation (Fig. 6) and immunofluorescence (Fig. 9) experiments indicated that the FERM domain impaired α1b-AR recycling to the plasma membrane without affecting receptor internalization. Overexpression of ezrin failed to produce any significant effect on receptor recycling probably because endogenous ezrin is not limiting (results not shown).

It was previously reported that some GPCRs carrying a PDZ sequence in their C-tail can interact with the ezrin adaptor EBP50 (also known as NHERF1) and that this interaction plays a role in the regulation of receptor trafficking (for review, see Ref. 8). In our experiments, endogenous EBP50 did not co-immunoprecipitate with the α1b-AR, which does not possess a PDZ motif in its C-tail, thus ruling out that the α1b-AR/ezrin interaction is mediated by EBP50 (results not shown).

Different effects have been attributed to EBP50 in regulating GPCR trafficking despite the fact that in these studies the direct implication of ezrin was not demonstrated. For example, for the β2-AR, the interaction with EBP50 seems to be required for receptor recycling (10). Overexpression of EBP50 was shown to impair agonist-induced down-regulation of the κ opioid receptor by enhancing its recycling rate (11). Thus, the most common effects reported for EBP50 are increased recycling and decreased internalization of GPCRs, the second potentially being a consequence of the first. Altogether our findings on the α1b-AR/ezrin interaction together with the results on the interaction of EBP50 with other GPCRs support the hypothesis that ezrin itself is involved in the regulation of GPCR recycling. However, whereas for some GPCRs the role for ezrin is mediated by an adaptor protein like EBP50, for the α1b-AR the effect of ezrin might rely on its direct interaction with the receptor.

The effect of the FERM domain on α1b-AR recycling might be linked to the property of ezrin to bind filamentous actin. This hypothesis is supported by two lines of evidence. First, the FERM domain binds to the C terminus of ezrin, thus presumably masking the actin binding site (Fig. 5). Second, the dominant negative effect of the FERM domain on receptor recycling was similar to that induced by cytochalasin D, an actin depolymerizing agent (Fig. 6).

Actin is known to play a role in clathrin-dependent endocytosis in yeast as well as in mammalian cells (for review, see Ref. 16). In particular, this role relies on a number of protein–protein interactions between components of the endocytic machinery and actin filaments. However, the role of actin in GPCR trafficking has not been extensively investigated. An active remodeling of the actin cytoskeleton was shown to be necessary for thromboxane A2 receptor endocytosis (23). Cytochalasin D inhibited both internalization and recycling of the chemokine receptor CCR5 (24) and of the adenosine A2 receptor (25).

The findings of this study indicate that actin depolymerization induced by cytochalasin D inhibited recycling, but not internalization, of the α1b-AR. This is in agreement with the results of a previous study showing that actin depolymerization caused missorting of the β2-AR to lysosomes instead of recycling vesicles (10). In contrast with our findings, a previous study reported that cytochalasin D could inhibit the internalization of the α1b-AR in Chinese hamster ovary cells (26). However, several differences exist between the two studies, including the fact that in Chinese hamster ovary cells the internalization of the α1b-AR seemed to be clathrin-independent, whereas we have recently reported that in HEK-293 cells it involves clathrin-coated pits (6).

Proposing general paradigms for the role of actin in GPCR trafficking would be inappropriate at this stage because the few studies existing differ both in their experimental approaches and cell systems investigated. However, in the case of the α1b-AR the results of our study provide two clear lines of evidence, that actin plays a role in recycling of the α1b-AR and that ezrin might be a mediator between actin and the receptor. ERM proteins are known to regulate actin assembly after their activation by Rho (27). The activation of ERM proteins results in their interaction with WASp, which stimulates actin polymerization, thus promoting endosome motility (28, 29).

Does the role of ezrin in α1b-AR recycling involve its direct interaction with the receptor, and how is this interaction regulated? Our findings provide some answers to these questions, which concern, however, a quite complex problem. The observation that the ΔR8 receptor mutant, lacking most of its interaction with ezrin, is delayed in recycling to the cell surface (Fig. 7B) suggests that the α1b-AR/ezrin interaction is directly involved in receptor recycling. However, the behavior of the ΔR8 receptor mutant is difficult to interpret since this mutant is also delayed in its internalization, as it is unable to directly interact with the μ2 subunit of the AP2 clathrin adaptor complex (6).

The results from confocal imaging suggest that the dynamics of the α1b-AR/ezrin interaction is quite complex. In fact, as shown in Fig. 8, at 60 min of agonist-induced receptor internalization, ezrin was mainly at the plasma membrane, whereas most of the α1b-AR was localized in endocytic vesicles. Only after removal of the agonist, ezrin partly colocalized with the receptor in intracellular vesicles. This raises the hypothesis that α1b-AR/ezrin interaction might occur during the late events of receptor endocytosis rather than immediately after the stimulation of the receptor with the agonist. How the agonist regulates these events remains to be further investigated. Under the experimental conditions used in our study, the coinmunoprecipitation experiments were not able to detect any agonist-induced regulation of the α1b-AR/ezrin interaction, probably because this interaction is weak, localized in specific cell compartments, or highly dynamically regulated.

Our findings strongly suggest that ezrin interacts with the α1b-AR in its open or active conformation after the release of the intramolecular interaction between the FERM domain and the C terminus of ezrin (Fig. 4E). However, at which step of α1b-AR trafficking ezrin activation occurs, the mechanisms involved, and whether it is mediated by receptor activation or by the interaction of ezrin with other proteins of the endocytic machinery are all questions which remain to be answered.

A recent study by Cant and Pitcher (30) has reported that G protein-coupled receptor kinase 2 can phosphorylate ezrin in vitro, suggesting that kinases of the G protein-coupled receptor kinase family can represent a link between GPCRs and ERM proteins. In this study phosphorylation of ERM proteins in intact cells was found to correlate with the internalization of the β2-AR and muscarinic M1 receptor for which a direct interaction with any ERM protein has not been shown. At the moment, it is difficult to interpret these findings at a mechanistic level and to provide a conclusive hypothesis on the role of ERM proteins in GPCR trafficking since the receptor behavior might differ depending on the nature of its interactions (direct or indirect) with ERM proteins as well as with other proteins involved in the endocytic process.

In conclusion, the main implications of our study are 2-fold. First, they identify ezrin as a new protein directly interacting with a GPCR. Second, they demonstrate the direct implication of ezrin in GPCR trafficking via an actin-dependent mechanism. The interaction of ezrin
with a GPCR might be, at least in part, involved in receptor trafficking but could also play a role in other effects mediated by ezrin, for example, its ability to activate the Rho signaling pathway. Future studies will aim at using the RNA interference strategy to further investigate the functional implications of α1b-AR/ezrin interaction. They will also aim at answering some of the following open questions. Which mechanisms underlie receptor-mediated activation of ezrin? In which cellular compartment might the α1b-AR/ezrin interaction occur? Which other portions of the receptor might bind ezrin? What is the interplay between binding of the AP2 complex and ezrin to the α1b-AR? It will also be important to assess whether direct binding of ERM proteins is a new paradigm for other GPCRs.

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REFERENCES
1. Hall, R. A., and Lefkowitz, R. J. (2002) Circ. Res. 91, 672–680
2. Lefkowitz, R. J., and Shenoy, S. K. (2005) Science 308, 512–517
3. Bockaert, J., Fagni, L., Dumuis, A., and Marin, P. (2004) Pharmacol. Ther. 103, 203–221
4. Lattion, A. L., Diviani, D., and Cotecchia, S. (1994) J. Biol. Chem. 269, 22887–22893
5. Diviani, D., Lattion, A. L., Larbi, N., Kunapuli, P., Pronin, A., Benovic, J. L., and Cotecchia, S. (1996) J. Biol. Chem. 271, 5049–5058
6. Diviani, D., Lattion, A. L., Abuin, L., Staub, O., and Cotecchia, S. (2003) J. Biol. Chem. 278, 19331–19340
7. Bretscher, A., Edwards, K., and Fehon, R. G. (2002) Nat. Rev. Mol. Cell Biol. 3, 586–599
8. Gary, R., and Bretscher, A. (1995) Mol. Biol. Cell 6, 1061–1075
9. Weinman, E. J., Hall, R. A., Friedman, P. A., Liu-Chen, L.-Y., and Shenolikar, S. (2005) Annu. Rev. Physiol. 68, 1.1–1.15
10. Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A., and von Zastrow, M. (1999) Nature 401, 286–290
11. Li, J. G., Chen, C., and Liu-Chen, L. Y. (2002) J. Biol. Chem. 277, 27545–27552
12. Cao, T. T., Naya, R. W., and von Zastrow, M. (1998) J. Biol. Chem. 273, 24592–24602
13. Pearson, M. A., Reczek, D., Bretscher, A., and Karplus, P. A. (2000) Cell 101, 259–270
14. Crepaldi, T., Gauthreau, A., Comoglio, P. M., Louvard, D., and Arpin, M. (1997) J. Cell Biol. 138, 423–434
15. Zhao, H., Shiue, H., Palkon, S., Wang, Y., Cullinan, P., Burkhardt, J. K., Musch, M. W., Chang, E. B., and Turner, J. R. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 9485–9490
16. Engvist-Goldstein, A. E., and Drubin, D. G. (2003) Annu. Rev. Cell Dev. Biol. 19, 287–332
17. Yonemura, S., Hirao, M., Doi, Y., Takahashi, N., Kondo, T., and Tsukita, S. (1998) J. Cell Biol. 140, 885–895
18. Heiska, L., Alfthan, K., Gronholm, M., Vilja, P., Valheri, A., and Carpen, O. (1998) J. Biol. Chem. 273, 21893–21900
19. Dickson, T. C., Mintz, C. D., Benson, D. L., and Salton, S. R. (2002) J. Cell Biol. 157, 1105–1112
20. Denker, S. P., Huang, D. C., Orlowiski, J., Furthmayr, H., and Barber, D. L. (2000) Mol. Cell 6, 1425–1436
21. Schmieder, S., Nagai, M., Orlando, R. A., Takeda, T., and Farquhar, M. G. (2004) J. Am. Soc. Nephrol. 15, 2289–2298
22. Hamada, K., Shimizu, T., Yonemura, S., Tsukita, S., and Hakoshima, T. (2003) EMBO J. 22, 502–514
23. Laroche, G., Rochdi, M. D., Laporte, S. A., and Parent, J. L. (2005) J. Biol. Chem. 280, 23215–23224
24. Mueller, A., and Strange, P. G. (2004) Eur. J. Biochem. 271, 243–252
25. Burgueno, J., Blake, D. J., Benson, M. A., Tinley, C. L., Esapa, C. T., Canela, E. L., Penela, P., Mallol, J., Mayor, F., Jr., Lluis, C., Franco, R., and Ciruela, F. (2003) J. Biol. Chem. 278, 37545–37552
26. Hirasawa, A., Awaji, T., Sugawara, T., Tsujimoto, A., and Tsujimoto, G. (1998) Br. J. Pharmacol. 124, 55–62
27. Mackay, D. J., Esch, F., Furthmayr, H., and Hall, A. (1997) J. Cell Biol. 138, 927–938
28. Manchanda, N., Lyubimova, A., Ho, H. Y., James, M. F., Gusella, J. F., Ramesh, N., Snapper, S. B., and Ramesh, V. (2005) J. Biol. Chem. 280, 12517–12522
29. Chang, F. S., Stefan, C. J., and Blumer, K. J. (2003) Curr. Biol. 13, 455–463
30. Cant, S. H., and Pitcher, J. A. (2005) Mol. Biol. Cell 16, 3088–3099