Spinophilin Stabilizes Cell Surface Expression of α2B-Adrenergic Receptors*

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Ashley E. Brady‡, Qin Wang†, Roger J. Colbran‡, Patrick B. Allen¶, Paul Greengard**, and Lee E. Limbird‡ ‡‡

From the Departments of ‡Pharmacology and ‡Molecular Physiology and Biophysics, Center for Molecular Neuroscience, Vanderbilt University Medical Center, Nashville, Tennessee 37232-6600, the †Department of Psychiatry, Yale University School of Medicine, New Haven, Connecticut 06520, and the **Laboratory of Molecular and Cellular Neuroscience, Rockefeller University, New York, New York 10021

α2-Adrenergic receptors (ARs)1 are members of the large superfamily of G protein-coupled receptors that contain seven putative transmembrane spanning regions. There are three α2-AR subtypes (α2A, α2B, and α2C), each of which is activated by the endogenous catecholamines, epinephrine and norepinephrine, and performs multiple physiological functions via pertussis toxin-sensitive G/γ proteins (1). Cellular signaling pathways regulated by α2A-AR in native cells include inhibition of adenyl cyclase, activation of receptor-operated K⁺ channels, inhibition of voltage-gated Ca²⁺ channels, and activation of the mitogen-activated protein kinase cascade (1–3). Many cells that express α2-ARs are polarized, including renal and intestinal epithelia, where the α2-AR serves to regulate sodium and water resorption (4, 5), as well as neurons, where these receptors act to suppress neurotransmitter release (6). The physiological functions mediated by α2-ARs in polarized cells are dependent upon precise localization of the receptor at the basolateral surface to gain access to neurally delivered and blood-delivered catecholamines.

The α2-AR subtypes demonstrate unique targeting and retention profiles in polarized renal epithelial Madin-Darby canine kidney (MDCKII) cells in culture (7). Previous work in our laboratory has shown that the α2A-AR subtype is directly targeted to the basolateral surface, where it exhibits a half-life of 10–12 h (8). Direct and exclusive basolateral targeting of α2A-AR was found to be dependent upon several noncontiguous regions within or near the bilayer, whereas retention of the receptor at the basolateral surface appears dependent upon the third intracellular (3i) loop (8). Deletion of the 3i loop results in accelerated surface turnover (t½ = −4.5 h) of the α2A-AR at the basolateral surface (9). Unlike the α2A-AR, the α2A-AR subtype is randomly targeted to both the apical and basolateral subdomains and then selectively retained at the basolateral surface of polarized MDCKII cells, where the receptor has a half-life comparable with that of the α2A-AR subtype (t½ = −10–12 h) (7). Like for the α2A-AR, the 3i loop of the α2A-AR also is critical for basolateral surface stabilization of this subtype (10). In contrast to stable retention of the α2B-AR on the basolateral surface, the half-life on the apical surface is estimated to be

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* The abbreviations used are: AR, adrenergic receptor; 3i loop, third intracellular loop; BSA, bovine serum albumin; CHS, cholesteryl hemisuccinate; DMEM, Dulbecco’s modified Eagle’s medium; DPBS/CM, Dulbecco’s phosphate-buffered saline supplemented with 1 mM CaCl₂ and 0.5 mM MgCl₂; ELISA, enzyme-linked immunosorbent assay; MESNA, 2-mercaptoethanesulfonic acid.

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‡‡ To whom correspondence should be addressed: Dept. of Pharmacology, Vanderbilt University Medical Center, 441 Robinson Research Bldg., Nashville, TN 37232-8600. Tel.: 615-343-3538; Fax: 615-343-7286; E-mail: lee.limbird@vanderbilt.edu.

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dramatically shorter, on the order of minutes (7). Taken together, these data suggest that the stabilization/retention of α2AR and α2β-AR at specific membrane domains is most likely mediated through interactions of the 3i loop with other proteins either within or underlying the basolateral membrane surface and not present (or expressed at much lower density) at the apical surface.

The α2-AR 3i loop has been used as a ligand to identify potential interacting proteins and has led to the identification of two α2-AR-interacting molecules: 14-3-3ε (11) and spinophilin (12). Spinophilin is an 818-amino acid, ubiquitously expressed, multidomain-containing protein with an apparent molecular mass on SDS-PAGE of ~130 kDa. It was originally identified both as a protein phosphatase 1 (PP1)-binding protein localized to dendritic spines, hence the name spinophilin (13), as well as an F-actin-binding protein (14). Spinophilin (also known as neurabin II) is highly homologous to the brain-specific protein, neurabin I (14). In addition to the domains described above, spinophilin contains a single PDZ (PSD-95, Discs large, ZO-1) domain and three coiled-coil domains at the C terminus, the latter of which mediate homo-multimerization in vitro (14) and may allow for the formation of multiprotein complexes in intact cells. Spinophilin previously was identified as a D2 dopamine receptor-interacting protein using the 3i loop of the D2 dopamine receptor as bait in a yeast two-hybrid screen (15). The D2 dopamine receptor-binding domain in spinophilin (residues 151–444), located between the F-actin-binding domain and the PP1 regulatory domain, also interacts with all three of the α2-AR subtypes (12) and will be referred to as the receptor-interacting domain. Because reports in the literature, as well as our own observations, indicate that spinophilin is specifically enriched at the basolateral surface of polarized epithelial cells (12, 15, 16), we postulate that spinophilin may be involved in tethering and/or stabilizing the receptor at the cell surface via interactions with the α2-AR 3i loops.

The present studies utilized two different biological systems to explore the role of spinophilin in α2-AR stabilization at the cell surface. First, the unique targeting profile of the α2-AR subtype in polarized MDCKII cells (random delivery with rapid turnover at the apical surface) was exploited to determine whether redirection of the receptor-interacting domain of spinophilin to the apical surface of polarized MDCKII cells would result in enhanced apical retention of randomly delivered α2AR. Second, the role of spinophilin in α2-AR surface turnover was addressed by studying the internalization profile of the α2AR in mouse embryonic fibroblasts (MEFs) derived from wild type (Sp+/−) or spinophilin knock-out (Sp−/−) mice (17). The findings from both lines of investigation implicate spinophilin in the stabilization of the α2AR at the cell surface.

EXPERIMENTAL PROCEDURES

Materials

Transwell culture chambers (0.4-μm pore size) were purchased from Costar (Cambridge, MA). Doxycycline hydrochloride was purchased from Sigma. NHS-SS-Biotin and Immunopure Immobilized Streptavidin were purchased from Pierce. Both [35S]EasyTag Express Protein Labeling mix (1200 Ci/mmol) and [3H]methoxy-inulin (126.5 mCi/g) were from PerkinElmer Life Sciences. Cysteine- and methionine-free DMEM was from Cellgro Mediatech. Dalbeco’s modified Eagle’s medium was prepared by the Cell Culture Core, a facility sponsored by the Diabetes Research and Training Center at Vanderbilt University Medical Center. Fetal calf serum was purchased from Sigma. Mouse monoclonal 12CA5 antibody against the HA epitope was obtained from BABCo. Affinity matrix-coupled high affinity rat monoclonal anti-HA antibody (clone 3F10), rat monoclonal anti-α2AR antibody (clone 3F10), and mouse monoclonal HA.11 antibody (clone 16B12) were purchased from Roche Applied Science. Mouse monoclonal anti-c-Myc (clone 9E10) ascites was purchased from Covance Research Products Inc. (Denver, PA). Both the mouse anti-gp135 and mouse anti-EGFR were gifts from Peter J. Dempsey (Department of Pathology, University of Washington, Harborview Medical Center, Seattle, WA). Rabbit anti-spinophilin antibody raised against spinophilin amino acids 286–390 (18) was purified in our lab (for details see Ref. 12). Alexa Fluor 488-conjugated fluorescent goat anti-mouse, goat anti-rat, and goat anti-rabbit IgG were purchased from Molecular Probes (Eugene, OR). Cy3-conjugated donkey anti-mouse IgG was purchased from Jackson Immunochemicals. goat anti-rabbit, donkey anti-mouse, and goat anti-rat horseradish peroxidase-conjugated IgG were purchased from Amersham Biosciences. The rat p75NTR cDNA was a generous gift from Dr. Bruce Carter (Department of Biochemistry, Vanderbilt University). The retroviral vector pBabe-HA-α2AR was kindly provided by Drs. Dan Gil and John Donnelly (Allergan, Irvine, CA).

MDCKII Cell Culture and Polarization

MDCKII cells were plated at a density of 1.2 × 106 cells/100-mm polycarbonate membrane filter (Transwell culture chambers, 0.4-μm pore size) and cultured in DMEM supplemented with 10% fetal calf serum (Sigma) and 100 units/ml penicillin and 10 μg/ml streptomycin at 37 °C and 5% CO2 with medium changes every other day for 5–7 days. Cells grown under these conditions achieve a morphologically and functionally polarized phenotype, as described previously (19). Leak assays of [3H]methoxy-inulin were performed as described previously (20) prior to each half-life experiment to verify that the MDCKII cells had developed tight junctions and that the apical and basolateral compartments were functionally separated.

Generation of cDNAs Encoding Myc-p75-Spinophilin Fusion Proteins

The cDNA encoding the pTRE-Myc-p75-Sp151–483 fusion protein was generated via overlapping PCR extension using Phu Turbo DNA polymerase (Stratagene). The Myc tag was inserted 5′ to the coding start site of full-length rat p75NTR and 3′ of the N-terminal cleavable signal sequence. Four glycine residues were engineered via PCR onto the C terminus of p75NTR with the intention of permitting independent folding of the spinophilin subdomain and decreased steric hindrance for interacting with other potential binding partners. The pTRE cDNA backbone (Clontech) has a tetracycline-inducible promoter that is intended to confer regulated expression of the fusion construct by treatment with the synthetic tetracycline analog, doxycycline. Two fusion proteins were generated. Myc-p75-Sp151–483 includes the receptor-binding domain and the PP1 regulatory domain of the full-length spinophilin, whereas Myc-p75-Sp151–586 also contains the PDZ-binding domain (cf. schematic of spinophilin domain structure in Fig. 2B). The cDNAs were sequenced in their entirety via 3′P-Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (U.S. Biochemical Corp.) to confirm that the sequences were correct.

Creation of Clonal MDCKII Cell Lines Expressing the Myc-p75-Spinophilin Fusion Protein

Permanent clonal cell lines were developed in MDCKII cells using FuGENE-6 (Roche Applied Science) transfection reagent, according to the manufacturer’s protocol; 6 𝜇g of linearized pTET-On plasmid (Clontech) was co-transfected with 6 𝜇g of pTRE-Myc-p75-Spinophilin construct and 1 𝜇g of a vector encoding a hygromycin resistance gene into MDCKII cells already stably expressing the HA-α2AR, generated as described previously (7). Stably transfected cells were selected through growth in 400 𝜇g/ml hygromycin and assayed for HA-α2AR expression via radioligand binding analysis using the antagonist [3H]rauwolscine (21) and for Myc-p75-spinophilin expression and inducibility via Western analysis and immunofluorescence using anti-c-Myc antibody. Despite the use of an inducible expression system, Myc-p75-spinophilin expression occurred even in the absence of doxycycline. Nonetheless, the cells were treated overnight with 1 μg/ml doxycycline before the day of the experiment to assure maximal Myc-p75-spinophilin expression.

Immunofluorescence in Polarized MDCKII Cells

Polarized MDCKII cells stably expressing HA-α2AR were grown on 12-mm Transwells for 5–7 days (as described above) and processed as described previously for immunolocalization of HA-α2AR and endogenous spinophilin (12) and for detection of endogenous apical (gp135) and basolateral (EGFR) marker proteins (22) (see Fig. 1). MDCKII cells expressing Myc-p75-Sp151–483 fusion protein in the HA-α2AR background were treated with 1 μg/ml doxycycline overnight to maximize the expression of Myc-p75-Sp151–483 prior to staining. All of the steps were performed essentially as described previously (22) except that a rat anti-HA (clone 3F10) antibody (Roche Applied Science) diluted
1:1000 in blocking buffer was used for the detection of HA-α2B-AR (see Fig. 2D).

**Immunoprecipitation of Apically Targeted Myc-p75-Spinophilin**

Fusion Proteins from Stable MDCKII Cell Lines

MDCKII cells stably expressing the HA-α2B-AR alone or Myc-p75-Sp151–483 or Myc-p75-Sp151–586 in the HA-α2B-AR background were grown to confluence in 100-mm tissue culture dishes. All of the dishes were treated with 1 μg/ml doxycycline for 16 h before harvesting the cells to maximize fusion protein expression. On the day of the assay, the Transwells were washed twice with Dulbecco’s phosphate-buffered saline supplemented with 1 mM MgCl₂ and 0.5 mM CaCl₂ (DPBS/CM) (4 °C) and scraped into 12 ml of lysis buffer (15 mM Tris-HCl, 5 mM EGTA, 5 mM EDTA, pH 7.6, with N-ethylmaleimide) containing protease inhibitors (1 μg/ml soybean trypsin inhibitor, 0.5 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride), triturated 10 times through a 20-gauge needle, and then centrifuged at 39,000 × g for 20 min. The pellet was resuspended in 1 ml of RIPA buffer plus protease inhibitors and incubated on ice 30 min before centrifugation for 1 h at 100,000 × g. Supernatants from this centrifugation (the solubilized preparation) were incubated with 25 μl of a 1:1 slurry of pre-equilibrated rat anti-HA affinity matrix overnight at 4 °C with rotation. The affinity matrix was pipetted and washed twice with 1 ml of ice-cold RIPA buffer plus protease inhibitors before elution of the immunosolated HA-α2B-AR from the affinity matrix by incubation twice for 10 min with 100 μl of SDS sample buffer (1.6% SDS, 8.3% glycerol, 160 mM Tris, pH 8.0) at 70 °C. The eluates were pooled and brought to 1.5 ml with RIPA (containing no SDS) plus protease inhibitors. The sample was allowed to sit for 10 min at room temperature to equilibrate the component detergents.

To isolate the apically biotinylated HA-α2B-AR from the entire immunosolated, streptavidin chromatography was performed as follows. The samples were incubated with a 1:1 slurry of streptavidin–agarose (50 μl) pre-equilibrated in RIPA buffer for 2 h at 4 °C with rotation. The precipitated streptavidin-agarose was washed three times with 1 ml of ice-cold RIPA buffer plus protease inhibitors, and biotinylated HA-α2B-AR was eluted by incubation twice for 20 min with 100 μl of SDS sample buffer containing 50 mM dithiothreitol at 90 °C. The eluted samples were then incubated 40 min at 50 °C. N-Ethylmaleimide was added to a final concentration of 15 mM, and the samples were incubated for an additional 40 min at 50 °C. The rationale for the high dithiothreitol/N-ethylmaleimide treatment is to alkylate all sulphydryl residues, thus better resolving the α2B-AR preparation on SDS-PAGE (24).

The samples were resolved overnight for a total of 160 μAmp-hr on a 7.5–12% gradient SDS-polyacrylamide gel. The gels were treated with ENHANCE Intensifying Solution (Perkin-Elmer Life Sciences) according to the manufacturer’s protocols, dried, and exposed to BioMax MR film. The bands on the film were quantitated using SCION image software, and/or bands were cut from the gel and counted directly in scintillation mixture. Equivalent findings were obtained from either quantitation procedure.

**Endogenous gp135**—Because endogenous gp135 is expressed at a relatively high concentration on the apical surface of polarized MDCKII cells, the surface half-life of this protein was determined by surface biotinylation, extraction into RIPA at various time points, resolution by SDS-PAGE, and identification of biotinylated gp135 via Western blot analysis for gp135, using methods described previously (29).

**Culturing of MEFs**

A 13.5-day pregnant female mouse (Sp+/− or Sp−/−) (17) was sacrificed, and the embryos were collected. The soft, dark colored tissues (i.e. heart, liver, and spleen) were dissected away from the embryo, and the head was removed. The remaining tissue was transferred to the barrel of a 5-ml syringe (five embryos/syringe) and passed through an 18-gauge needle into 3 ml of DPBS. The tissue was further dissociated by triturating five times, and the cell suspension was transferred to a 150-mm culture dish containing 25 ml of complete medium (DMEM with 10% fetal calf serum, 100 units/ml penicillin, and 10 μg/ml streptomycin supplemented with 2 mM glutamine). The cells were grown at 37 °C and 5% CO₂ until the plates reached confluence, at which point the cells were split 1:5, expanded to confluency, and frozen at 2 × 10⁶ cells/ml in freezing medium (50% fetal calf serum, 12% MeSO in DMEM).

**Transduction of MEFs with a Retroviral Vector Encoding HA-α2B-AR**

Primary cultures of MEFs (Sp+/− or Sp−/−) (17) were seeded at 1.4 × 10⁶ cells/100-mm dish the day before transduction with 4 ml of one part retroviral supernatant containing HA-α2B-AR-encoding virions harvested from BOSC2 cells (25) and one part complete DMEM containing a final concentration of 12 μg/ml polybrene. The viral application was repeated four times over the course of 8 h at 37 °C and 5% CO₂, empirically determined to yield optimal transduction. Afterward, the cells were returned to 9 ml of complete medium. Three days post-transduction, the cells were assayed for HA-α2B-AR expression via
radioligand binding analysis, essentially as described previously (9). For cell-surface immunofluorescent imaging, the transduced cells were selected for retroviral vector expression by treatment overnight with 4 μg/ml puromycin (the pBabe retroviral vector carries the resistance gene for puromycin).

Measuring Turnover of the HA-α2B-AR in MEFs

Intact Cell ELISA Assay—The day before the assay, Sp+/+ or Sp−/− MEFs (selected for HA-α2B-AR expression in 4 μg/ml puromycin) were plated on poly-L-lysine-coated 96-well culture plates at a density of 4 × 10⁵ cells/well in complete medium containing the α2B-AR antagonist propranolol (1 μM) to eliminate effects of catecholamines that might be present in the serum-containing DMEM. The day of assay, the cells were washed twice for 15 min at 37 °C in serum-free DMEM containing 0.1% BSA (200 μl/well) to wash away propranolol and twice for 10 min in serum-free DMEM containing no BSA (200 μl/well). The cells were returned to 37 °C with 90 μl of serum-free DMEM/well. The cells were stimulated at 37 °C for the indicated times by the addition of the agonist epinephrine (100 μM) and the β-antagonist propranolol (1 μM) to exclude the activation of endogenous β-adrenergic receptors. The cells were then fixed with 4% paraformaldehyde in 0.12 M sucrose in DPBS/CM (100 μl/well) for 20 min at room temperature and washed twice with DPBS/CM (200 μl/well). The cells were blocked for 30 min at 37 °C with 3% BSA in DPBS/CM (blocking buffer). Primary antibody (anti-HA) was diluted in blocking buffer and incubated with the cells (50 μl/well) for 1 h at 37 °C. Following labeling with primary antibody, the cells were washed three times for 5 min with DPBS/CM (200 μl/well). Incubation with secondary antibody (anti-rat horseradish peroxidase) diluted 1:1000 in blocking buffer was for 1 h at 37 °C (50 μl/well). Unbound secondary antibody was removed by three 5-min washes with DPBS/CM (200 μl/well). The colorimetric substrate o-phenylene-diamine dihydrochloride (1 mg/ml) was prepared according to the manufacturer's instructions (Pierce) and was incubated with cells for 10–20 min at room temperature (100 μl/well). Color development was stopped by the addition of 2.5 M sulfuric acid (100 μl/well).

Reversible Biotinylation—Surface HA-α2B-AR was labeled on ice with a disulfide cleavable biotin (sulfo-NHS-SS-Biotin), stimulated by agonist at 37 °C, and then treated with the cell-impermeant reducing agent, 2-mercaptoethanesulfonic acid (MESNA). Receptors that have been shown previously, the HA-α2B-AR expression in MDCKII cells (26). The spinophilin sequences were separated by SDS-PAGE (10% gels) and transferred to nitrocellulose for Western blot analysis as described previously (23).

RESULTS AND DISCUSSION

Co-localization of Endogenous Spinophilin with HA-α2B-AR in Polarized MDCKII Cells—Immunofluorescence staining and confocal laser scanning microscopy were used to confirm the localization of both the HA-α2B-AR and endogenous spinophilin in the MDCKII cell line used for these studies (Fig. 1). As has been shown previously, the HA-α2B-AR is localized to the basolateral surface in these polarized epithelial cells, albeit with a small pool of presumably endocytosed α2B-AR (7). Endogenous spinophilin also is enriched at the basolateral surface, as has been suggested in the literature (12, 14, 15). The co-localization of these two proteins at the basolateral surface is indicated by the yellow signal present in the overlay of the two images, particularly evident in the z scan. We postulated that the basolateral localization of spinophilin in MDCKII cells may contribute to retention of α2B-AR subtypes at that surface and that the lack of apical spinophilin may explain the rapid apical turnover of randomly delivered α2B-AR.

Redirection of a Spinophilin Subdomain to the Apical Surface of MDCKII Cells—To test the hypothesis that spinophilin contributes to cell surface α2B-AR retention, we explored whether redirection of the receptor-interacting domain of spinophilin to the apical surface of polarized MDCKII cells would lead to enhanced apical retention of randomly delivered α2B-AR. Fig. 2B provides a schematic diagram of the domain structure of the spinophilin protein (14). A region of spinophilin sequence (amino acids 151–483) including the receptor-interacting domain was fused-in frame to Myc epitope-tagged p75NTR, a single transmembrane spanning protein known to be expressed predominantly (80%) at the apical surface of polarized MDCKII cells (26). The spinophilin sequences were separated from p75NTR via a tetraglycine linker to permit independent folding and accessibility of the spinophilin domains. A cDNA encoding this fusion protein was then stably expressed in MDCKII cells already stably expressing HA-α2B-AR, as described under “Experimental Procedures.”

Expression of the Myc-p75-Sp151–483 and Myc-p75-Sp151–586 fusion proteins in these HA-α2B-AR-expressing MDCKII cell lines was confirmed via immunoprecipitation and Western blotting. As can be seen in Fig. 2C, the Myc-tagged fusion proteins can be specifically enriched by immunoprecipitation of
detergent-solubilized membrane fractions with anti-c-Myc antibody and then identified on Western blots with an antibody against an epitope within spinophilin (amino acids 286–390) (lanes 2 and 3). As expected, no Myc-spinophilin fusion protein is detected in the parental cell line expressing only heterologous HA-α2B-AR (lane 1).

As shown in Fig. 2D, immunolocalization studies using an antibody against the Myc epitope reveal that Myc-p75-Sp151–483 is expressed at the apical surface of polarized MDCKII cells (Fig. 2D, first panel) in a manner similar to the expression pattern of the known endogenous apical marker protein, gp135 (Fig. 2D, third panel). A similar apical expression pattern also
was detected for the clonal cell line expressing Myc-p75-Sp151–586 (data not shown). Fig. 2D (second panel) shows that the apical expression pattern of Myc-p75-Sp151–483 is in marked contrast to the basolateral expression pattern of endogenous spinophilin, heterologous HA-$\alpha_{2B}$-AR at steady state (Fig. 1), or the EGFR (Fig 2D, fourth panel), a marker protein for the basolateral surface. Thus, our fusion protein strategy successfully delivered the receptor-interacting domain of spinophilin to the apical surface and did so without altering expression of endogenous markers for the polarized phenotype, gp135, and the EGFR.

An Apically Targeted Spinophilin Subdomain Extends the Apical Half-life of Randomly Delivered $\alpha_{2B}$-AR—To determine whether apical expression of the receptor-interacting domain of spinophilin would extend the apical surface half-life of randomly delivered $\alpha_{2B}$-AR, a cell surface biotinylation strategy in metabolically labeled, polarized MDCKII cells was used to quantify the loss of apical HA-$\alpha_{2B}$-AR over time (see Refs. 7 and 19 and “Experimental Procedures”). The autoradiogram in Fig. 3A shows that the rapid loss of apical HA-$\alpha_{2B}$-AR over the initial 60 min is attenuated in cells expressing Myc-p75-Sp151–483. Fig. 3B summarizes data from multiple experiments and shows a time course extended over 6 h. These data reveal a biphasic loss of HA-$\alpha_{2B}$-AR from the apical surface for cells not expressing exogenous apical spinophilin, such that ~50% of the apical HA-$\alpha_{2B}$-AR was lost with a $t_{1/2}$ = ~50 min. By contrast, in cells expressing the apically targeted spinophilin fusion protein, the half-life of the HA-$\alpha_{2B}$-AR at the apical surface is extended to ~3.6 h. Coincidently, in cells expressing Myc-p75-Sp151–483, HA-$\alpha_{2B}$-AR loss from the surface occurred at a constant rate, similar to that of the slower phase observed in cells not expressing exogenous apical spinophilin. The initial phase on the apical surface was comparably lengthened in a cell line expressing a p75 fusion protein that also contains the PDZ domain of spinophilin, Myc-p75-Sp151–586 (data not shown), indicating that protein interactions fostered by the PDZ domain do not contribute to $\alpha_{2B}$-AR retention, at least on the apical surface. Moreover, because these spinophilin fusion constructs did not contain coiled-coil or F-actin-binding domains, neither multimerization nor F-actin-binding of spinophilin appears to be necessary to stabilize $\alpha_{2B}$-AR expression at the cell surface. In addition, the extended apical half-life of the HA-$\alpha_{2B}$-AR does not appear to be a consequence of generalized changes in apical membrane turnover, because the turnover of the endogenous apical marker protein, gp135, was not significantly altered in cells expressing Myc-p75-Sp151–483 (Fig. 3C).

Previous studies of apical retention of the randomly delivered HA-$\alpha_{2B}$-AR had only revealed a single population of rapidly ($t_{1/2}$ = ~15–45 min) turning over HA-$\alpha_{2B}$-AR (7). The second, slower phase of the HA-$\alpha_{2B}$-AR loss from the apical surface detected in the present studies is not due to leak of the biotinylating reagent to the basolateral compartment, because the slower phase of HA-$\alpha_{2B}$-AR loss from the apical surface ($t_{1/2}$ = ~3.6 h) is entirely different from the half-life of the HA-$\alpha_{2B}$-AR on the basolateral surface ($t_{1/2}$ = ~10–12 h). Our lack of detection of the second, extended apical expression ($t_{1/2}$ = ~3.6 h) of a subfraction of the apically delivered $\alpha_{2B}$-AR in these earlier studies may have been due to the lesser amount of biological material evaluated (the present studies examined cells grown in 100-mm rather than 24-mm Transwell cultures). It is not entirely surprising that the apical half-life of the HA-$\alpha_{2B}$-AR in cells engineered to express an apically targeted receptor-interacting domain of spinophilin is extended beyond 60 min to ~3.6 h but is not restored to that of the half-life of the HA-$\alpha_{2B}$-AR at the basolateral surface ($t_{1/2}$ = ~10–12 h); it is likely that interactions of spinophilin and/or the receptor with additional proteins underlying the basolateral surface also contribute to $\alpha_{2B}$-AR residence time. For example, although the F-actin-binding and coiled-coil domains are not necessary for stabilization of $\alpha_{2B}$-AR expression on the apical surface (see above), multimerization of endogenous spinophilin and/or its inter-
action with F-actin may contribute to the more extended half-life on the basolateral surface. Despite the enhanced apical retention of HA-α2B-AR in cells harboring the apically targeted spinophilin fusion protein observed in biochemical studies, we were unable to detect an apical pool of HA-α2B-AR via immunofluorescence studies, perhaps because metabolic labeling and biotinylation are intrinsically more sensitive for examining receptor turnover at the cell surface than is immunofluorescence.

The most straightforward interpretation of our findings is that the receptor-interacting domain of spinophilin itself is stabilizing the α2B-AR at the apical surface. However, we cannot rule out a role for the PP1 interacting domain, which is inherent in each of the fusion proteins examined.

Agonist-induced Internalization Is Enhanced in Spinophilin-deficient (Sp^{-/}) MEFs—As a complementary strategy to explore whether spinophilin plays a role in stabilizing the α2B-AR at the cell surface, we took advantage of primary MEFs derived from wild type (Sp^{+/+}) or spinophilin knock-out (Sp^{-/-}) mice (17). Western blot analysis of whole cell lysate using an antibody against endogenous spinophilin reveals that spinophilin is absent from the Sp^{-/-} MEFs, whereas it is readily detected in the wild type (Sp^{+/+}) cells (Fig. 4A).

Wild type (Sp^{+/+}) and spinophilin knock-out (Sp^{-/-}) MEFs were transfected with HA-α2B-AR using a retroviral expression system, and receptor expression was verified via radioligand binding analysis. In contrast to the lack of effect of agonists on short term α2A-AR turnover in MDCKII (8) or other (27, 28) target cells, the α2B-AR subtype has previously been documented to rapidly internalize in response to agonist treatment (28–31). We postulated that if spinophilin is important for stabilizing the receptor at the cell surface, then internalization of the α2B-AR may be accelerated in a cell background lacking spinophilin. We evaluated HA-α2B-AR internalization using two independent strategies in Sp^{+/+} versus Sp^{-/-} MEFs: 1) cell surface ELISA, which measured the loss of receptor from the cell surface, and 2) reversible biotinylation, which examined...
HA-α2B-AR that is internalized and protected from MESNA-evoked removal of the biotin moiety from the cell surface.

For the intact cell ELISA, Sp+/− versus Sp−/− MEFs were treated with an α2B-AR agonist for the indicated times and then labeled with primary antibody directed against the HA epitope. Consistent with previous findings in cultured cell lines (all of which express endogenous spinophilin) (28, 31), ~30% of the HA-α2B-AR was lost from the cell surface of Sp−/− MEFs in response to agonist treatment for 60 min (Fig. 4B). In the absence of agonist, the HA-α2B-AR remained at the cell surface for the entire time course of the incubation (data not shown). Importantly, however, ~55% of the HA-α2B-AR was lost from the cell surface of Sp−/− MEFs over the same 60-min incubation duration (Fig. 4B). These data provide additional evidence that the presence of spinophilin stabilizes the α2B-AR at the cell surface.

A reversible biotinylation strategy, which measures the amount of internalized HA-α2B-AR that occurred over time following agonist exposure, was also exploited. For these studies, HA-α2B-AR expressing MEFs (Sp+/− and Sp−/−) were labeled at 4 °C with the membrane-impermeant, cleavable biotinylating reagent, sulfo-NHS-SS-biotin, before incubation with agonist for varying amounts of time. At the end of each incubation period, the cells were placed at 4 °C and treated with a membrane-impermeant reducing agent, MESNA, to cleave disulfide-linked biotin remaining on the surface. Receptors isolated via streptavidin-agarose from detergent-solubilized cells represent the receptors that remain biotinylated at each incubation point after MESNA treatment, which reveals the fraction of the receptor pool that was protected from reversal of biotinylation (i.e. internalized) during the course of the experiment. As shown in Fig. 5, this experimental strategy also reveals increased internalization of the HA-α2B-AR following agonist treatment in Sp−/− MEFs as compared with Sp+/− MEFs.

Conclusion—The present studies demonstrate a role for spinophilin in the stabilization/retention of the α2B-AR at the cell surface both in a cultured cell system and in cells derived from spinophilin knock-out mice. An apically targeted spinophilin subdomain containing the receptor-interacting domain extends from residues 319 to 32412 of the receptor and regulates endocytic processes. This region of spinophilin also interacts with the 3i loop of the D2 dopamine receptor (15). Future studies can establish whether the multimeric structure of spinophilin may serve to bring other proteins into the receptor microcompartment that participate not only in receptor localization but also in coordination of receptor-elicted signal transduction.

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