R7BP Augments the Function of RGS7·Gβ5 Complexes by a Plasma Membrane-targeting Mechanism*

Received for publication, May 9, 2006, and in revised form, July 20, 2006. Published, JBC Papers in Press, July 25, 2006, DOI 10.1074/jbc.M604428200

Ryan M. Drenan†‡, Craig A. Doupnik§, Muralidharan Jayaraman†, Abigail L. Buchwalter‡, Kevin M. Kaltenbronn‡, James E. Huettner‡, Maurine E. Linder‡, and Kendall J. Blumer†‡

From the †Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110 and the ‡Department of Physiology and Biophysics, University of South Florida College of Medicine, Tampa, Florida 33612

The R7GS (R7) family of G protein regulators, Gβ5, and R7BP form heterotrimeric complexes that potently regulate the kinetics of G protein-coupled receptor signaling. Reversible palmitoylation of R7BP regulates plasma membrane/nuclear shuttling of R7·Gβ5·R7BP heterotrimers. Here we have investigated mechanisms whereby R7BP controls the function of the R7 family. We show that unpalmitoylated R7BP undergoes nuclear/cytoplasmic shuttling and that a C-terminal polybasic motif proximal to the palmitoylation acceptor sites of R7BP mediates nuclear localization, palmitoylation, and plasma membrane targeting. These results suggest a novel mechanism whereby palmitoyltransferases and nuclear import receptors both utilize the C-terminal domain of R7BP to determine the trafficking fate of R7·Gβ5·R7BP heterotrimers. Analogous mechanisms may regulate other signaling proteins whose distribution between the plasma membrane and nucleus is controlled by palmitoylation. Lastly, we show that cytoplasmic RGS7·Gβ5·R7BP heterotrimers and RGS7·Gβ5 heterodimers are equivalently inefficient regulators of G protein-coupled receptor signaling relative to plasma membrane-bound heterotrimers bearing palmitoylated R7BP. Therefore, R7BP augments the function of the complex by a palmitoylation-regulated plasma membrane-targeting mechanism.

G protein-coupled receptor (GPCR)3 signal transduction regulates a wide variety of physiological processes. For example, in the nervous system, dopamine, serotonin, acetylcholine, and norepinephrine activate presynaptic GPCRs that regulate glutamate or γ-aminobutyric acid release. GPCRs transduce signals via G protein-dependent and -independent mechanisms (reviewed in Refs. 1–3). GPCR signaling via heterotrimeric G proteins regulates second messenger production and ion channel activity (1–3), whereas G protein-independent signaling can activate mitogen-activated protein kinase pathways (4), nonreceptor tyrosine kinases (5, 6), and phosphatidylinositol 3-phosphate kinase (7, 8). GPCR signaling from the plasma membrane to the nucleus may also occur by nuclear shuttling of β-arrestin 1 (9–11).

GPCR signaling is regulated potently by the RGS (regulator of G protein signaling) protein family (reviewed in Ref. 12). RGS proteins regulate the kinetics and amplitude of GPCR signaling by acting as GTPase-activating proteins (GAPs) for Gα subunits (13–15). Certain RGS proteins also function as Gα effectors or antagonists (reviewed in Ref. 12).

Members of the RGS7 (R7) subfamily of RGS proteins (RGS6, RGS7, RGS9-1, RGS9-2, and RGS11) are of particular interest because of their biologic importance and unusual subunit structures. R7 family members are highly expressed in the visual and nervous systems (16–19). They selectively deactivate Gα subunits of the Gi/o family (20–22) that mediate phototransduction and the effects of modulatory neurotransmitters (reviewed in Ref. 23). Best understood is RGS9, which regulates GPCR signaling in the basal ganglia (24–27) and phototransduction in retina (28). In contrast to other classes of RGS proteins, R7 family members form obligate complexes with the diverged Gβ subunit Gβ5 (16, 29–32). Indeed, all R7 isoforms are markedly destabilized when Gβ5 is absent (33), and mice lacking Gβ5 or RGS9 exhibit similar delays in phototransduction termination kinetics (34). RGS9-1·Gβ5 complexes in retina also include a third subunit, R9AP (RGS9 anchoring protein), a photoreceptor-specific transmembrane protein that augments the GAP activity of RGS9-1 and targets the trimeric complex to disk outer segment membranes (35–37). The importance of R9AP is underscored by studies showing that human R9AP mutants exhibit a visual syndrome termed bradyopsia characterized by impaired contrast detection and delayed adaptation to changes in light intensity (38).

We and others recently identified an R9AP-related protein called R7BP (R7 family binding protein) that is likely to serve as the membrane anchoring subunit for R7 and Gβ5 throughout much of the nervous system (39, 40). R7BP can bind any R7 family member complexed with Gβ5 and is widely expressed in brain (39, 40), in contrast to the restricted expression of R9AP in retinal photoreceptors (32). In further contrast to R9AP,
R7BP lacks a transmembrane domain; R7BP instead is palmitoylated at its C terminus (39). Palmitoylation of R7BP directs R7-Gβ5-R7BP complexes to the plasma membrane, whereas depalmitoylation of R7BP shunts the complex to the nucleus (39). Palmitoylation-regulated plasma membrane/nuclear shuttling therefore may provide a novel mechanism of modulating GPCR signaling at the plasma membrane or transducing signals from the plasma membrane to the nucleus (39, 41). Indeed, we have shown that depalmitoylation-induced nuclear sequestration of R7-Gβ5-R7BP complexes greatly slows the kinetics of GPCR signaling at the plasma membrane (39).

Here we have explored mechanisms whereby GPCR signaling is regulated by R7-Gβ5-R7BP complexes. We have determined whether unpalmitoylated R7BP is trapped in the nucleus or undergoes nuclear/cytosplasmic shuttling, identified sequence motifs in R7BP that mediate both palmitoylation and nuclear localization, and used these R7BP mutants to determine whether cytoplasmic R7-Gβ5-R7BP complexes can regulate GPCR signaling. The results provide new insight into the regulation of GPCR signaling by R7-Gβ5-R7BP complexes and the mechanisms by which palmitoylation regulates trafficking of R7BP and a newly appreciated collection of signaling proteins that localize alternatively to the plasma membrane or nucleus.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Immunoblotting experiments used an anti-FLAG-M2 HRP conjugate (Sigma; 1:5000), an anti-Myc 9E10 HRP conjugate (Roche Applied Science; 1:1000), an anti-HA 12CA5 HRP conjugate (Roche Applied Science; 1:2000), anti-GFP (B34, Abcam; 1:2000), and HRP-conjugated goat anti-mouse secondary antibodies (Pierce; 1:10,000). Immunofluorescence experiments used the anti-HA-11 antibody (BAbCo; 1:500) and goat anti-mouse Alexa 568 secondary antibodies (Molecular Probes; 1:2000). Unless noted otherwise, all of the chemicals were obtained from Sigma.

Cell Culture and Transfection—HEK293 cells (ATCC) were maintained and transfected in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium with 10% fetal bovine serum (Atlanta Biologicals) plus penicillin/streptomycin. The cells were grown at 37 °C and 5% CO2. Transfection of HEK293 cells was carried out using Effectene (Qiagen) according to the manufacturer’s instructions.

Plasmids and Mutagenesis—Cloning and expression of R7BP has been previously described (39), including GFP- and FLAG-tagged wild type R7BP and the R7BP-C252S/C253S double mutant. A GFP-CDC25C-S216A expression plasmid was a generous gift of Dr. Helen Piwnica-Worms (Washington University School of Medicine) (42). Deletion mutant forms of R7BP (GFP- or FLAG-tagged) were constructed using a one-step Vent PCR strategy (43, 44). Primers (sequences available upon request) were designed to delete the region of interest upon blunt end ligation of the PCR product. All of the PCRs/ligation introduced an Nhel restriction site in place of the deleted region, which was used for screening mutants during construction. FLAG-R7BP fragments 48–162 and 48–221 were constructed by amplifying the coding region of interest using primers containing HindIII/EcoRI sites. These PCR products were digested and cloned into the HindIII/EcoRI sites of p3FLAG-CMV10 (Sigma). Three rounds of mutagenesis (each changing two basic residues per round) using the QuikChange (Stratagene) procedure created the PBR-Q mutant in GFP-R7BP and FLAG-R7BP. All of the DNA constructs were sequenced.

Cell Lysis, Immunoblotting, and Immunoprecipitation—The cells were washed once with ice-cold PBS and lysed in mammalian cell lysis buffer (MCLB: 50 mM Tris, pH 8.0, 5 mM EDTA, 0.5% Igepal, 100 mM NaCl, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride) supplemented with protease inhibitor tablets (Roche Applied Science). The cells were incubated at 4 °C for 5 min with MCLB followed by further incubation by end-over-end rotation at 4 °C for 15 min. Cell debris and unbroken cells were pelleted by centrifugation (15,000 × g) for 10 min at 4 °C. Supernatant fractions were used directly for Western blot analysis or for immunoprecipitation. For Western blots, 50–100 µg of total protein was electrophoresed through 10% SDS-polyacrylamide gels followed by transfer to polyvinylidene difluoride (Millipore) membranes. The membranes were blocked for 1 h in 5% milk, TBST, followed by overnight incubation with primary antibodies. HRP-conjugated primary antibodies were removed, the membranes were washed with TBST, and the signal was detected with ECL reagent (Amersham Biosciences). For some Western blots, primary antibody was washed, and membranes were incubated with HRP-conjugated secondary antibodies for 30 min followed by processing as described above. For immunoprecipitation experiments, cleared cell lysates were incubated with 50 µl of FLAG M2-agarose affinity gel (Sigma) for 90 min followed by three 5-min washes in MCLB. Immunoprecipitates were denatured with 1 × SDS sample buffer for 5 min at 100 °C.

Metabolic Labeling—HEK293 cells were grown and transfected in 6-well plates for labeling experiments, as described previously (39). For [3H]palmitate labeling, the cells were washed with media (Dulbecco’s modified Eagle’s medium/ Ham’s F-12 medium with sodium pyruvate, nonessential amino acids, and penicillin/streptomycin) and incubated with labeling media for 120 min (media plus 10% dialyzed fetal bovine serum and [3H]palmitic acid (Perkin Elmer) at 1 mCi/well). Cell lysis, immunoprecipitation, and SDS-PAGE were carried out as described above. Gels containing [3H]palmitate-labeled proteins were treated with fluor solution (1M sodium salicylate in 15% methanol) for 30 min prior to drying and fluorography at −70 °C.

Confocal Immunofluorescence Microscopy—The cells were grown and transfected on glass coverslips prior to preparation for microscopy as follows. The cells were washed with PBS, fixed for 10 min in fixative (3% paraformaldehyde, 2% sucrose in PBS, pH 7.0) at room temperature, washed in PBS, and, if immunofluorescence was carried out, permeabilized in ice-cold buffer (20 mM HEPE, pH 7.4, 0.5% Triton X-100, 50 mM NaCl, 3 mM MgCl2, 300 mM sucrose). The cells were washed with PBS, blocked with 0.1% bovine serum albumin (in PBS) for 10 min at 4 °C, and washed again with PBS. The cells were incubated with primary antibodies (diluted in 5% goat serum, TBST) for 20 min at 37 °C in a humidified chamber followed by 10 washes in TBST. Secondary antibodies (diluted in 5% goat serum, TBST) were incubated with cells for 15 min at room temperature.
temperature followed by 10 washes in TBST. The cells were mounted on slides for confocal microscopy. Confocal microscopy was performed with a Zeiss LSM-510 laser scanning confocal microscope.

Fluorescence Recovery after Photobleaching (FRAP)—Twenty-four h after transfection, the cells grown on coverslips were transferred to glass-bottomed imaging dishes (Bioptech) and incubated in growth media without phenol red and including 10 μg/ml cycloheximide for 30 min to block de novo protein synthesis. Binucleate cells were identified and imaged prior to photobleaching at low laser intensity (1% output). For experiments using wild type GFP-R7BP, we treated cells for 12 h with the palmitoylation inhibitor 2-bromopalmitate (100 μM) before the addition of cycloheximide. For bleaching, the cytoplasm and one of two nuclei were bleached at ~50% laser output for 50–75 iterations. Post-bleach images were taken every 5–10 min for 30–60 min after bleaching.

Electrophysiology—The effects of mutating the R7BP polybasic region (PBR-Q) on RGS7-Gβ5 function were assayed using the Xenopus oocyte expression system as described previously (39). All of the procedures for using and handling Xenopus laevis (Xenopus One, Ann Arbor, MI) were approved by the University of South Florida Institutional Animal Care and Use Committee in accordance with National Institutes of Health guidelines.

The oocytes were injected with a mixture of 5′ capped cRNAs synthesized in vitro from linearized cDNA vectors (mMessage mMachine; Ambion). Six experimental groups (~20 oocytes each) were injected with different cRNA mixtures (final volume, 50 nl) and incubated at 19 °C in parallel for 48–60 h. To confer specific m2 receptor-Go-GIRK channel signaling, all groups received cRNAs for the human muscarinic m2 receptor (0.5 ng/oocyte), rat Kir3.1 subunit (0.5 ng/oocyte), mouse Kir3.2a subunit (0.5 ng/oocyte), mouse GoA(C351G) subunit (5 ng/oocyte), and PTX-S1 (1 ng/oocyte). Select groups then received cRNAs for RGS7 (1 ng/oocyte) and Gβ5 (1 ng/oocyte), with or without GFP-R7BP or GFP-R7BP(PBR-Q) at two different concentrations tested (1 ng and 5 ng/oocyte).

ACh-activated GIRK currents were recorded by two-electrode voltage clamp methods from a holding potential of −80 mV (GeneClamp 500; Axon Instruments). The oocytes were initially superfused with a minimal salt solution (98 mM NaCl, 1 mM MgCl2, and 5 mM HEPES, pH 7.5) and then switched to an isotonic high K+ solution (20 mM KCl, 78 mM NaCl, 1 mM MgCl2, and 5 mM HEPES, pH 7.5) to resolve the kinetics of ACh-activated inward GIRK currents. Rapid
application and washout of ACh in the high K⁺ solution was performed using a computer-triggered superfusion system (SF-77B; Warner Instruments) (45). To monitor inward rectification of the ACh-evoked GIRK currents, 1-s voltage ramps from -80 to +20 mV were evoked before and during agonist application. All of the recordings were performed at room temperature (21–23 °C).

The time course for ACh activation of GIRK currents and then deactivation associated with ACh washout were both analyzed using pCLAMP software. Time constants were derived from exponential fits of the current relaxations. Statistical comparisons between the various experimental groups were performed by one-way analysis of variance where p < 0.05 was considered significant. The experiments were each replicated in oocytes from two separate batches (dissections) of oocytes.

RESULTS

Nuclear/Cytoplasmic Shuttling of R7BP—We showed previously that depalmitoylation of R7BP triggers translocation of pre-existing R7-Gβ5-R7BP complexes from the plasma membrane to the nucleus (39), suggesting that R7BP controls shuttling of the complex. However, whether complexes containing unpalmitoylated R7BP undergo nuclear/cytoplasmic shuttling had not been determined. We have addressed this question because it would suggest whether nuclear, unpalmitoylated R7BP-R7-Gβ5 complexes have the potential to exit the nucleus to be repalmitoylated and shuttled back to the plasma membrane where they could regulate GPCR signaling.

We addressed whether R7BP can undergo nuclear/cytoplasmic shuttling by performing FRAP experiments (46). For reasons discussed below, these experiments used multinucleate HEK293 cells that appeared occasionally in culture. First, we transiently expressed a nonpalmitoylated R7BP mutant (GFP-R7BP-C252S/C253S) with HA-tagged RGS9-1 and Myc-tagged Gβ5 in HEK293 cells and treated cells with cycloheximide to block new protein synthesis. Nonpalmitoylated mutant R7BP was used to simplify the analysis because preliminary experiments with wild type R7BP were complicated by the ability of the protein to exit the nucleus and become palmitoylated and plasma membrane-targeted. Once binucleate cells expressing mutant R7BP were identified, the cytoplasm and one nucleus/cell were bleached, and GFP fluorescence confocal images were acquired over time after bleaching. Within 10–30 min after bleaching, GFP-R7BP was detected in the cytoplasm and the nucleus that had been bleached (Fig. 1A), indicating that R7BP-R7-Gβ5 complexes were exported from the unbleached nucleus into the cytoplasm and then imported into the bleached nucleus. Similar FRAP experiments performed under conditions where new protein synthesis and palmitoylation were blocked, respectively, with cycloheximide and 2-bromopalmitate demonstrated that complexes containing wild type GFP-R7BP also undergo nuclear/cytoplasmic shuttling (Fig. 1A). As a control to rule out that the results were due to diffusion of GFP-R7BP within a multi-lobed nucleus, we performed FRAP experiments using cells that had readily apparent multi-lobed nuclei. After bleaching half of a multi-lobed nucleus, we found that GFP-R7BP fluorescence equilibrated much more rapidly (<5 min) and completely (Fig. 1B) than was observed using binucleate cells. We therefore concluded that complexes containing unpalmitoylated R7BP, resulting either from inactivating the palmitoylation sites or by pharmacological blockade of palmitoylation, can shuttle between the cytoplasm and nucleus.

To determine whether the exportin Crm1 mediates nuclear export of complexes containing R7BP, we treated cells expressing wild type GFP-R7BP, HA-RGS9-1, and Myc-Gβ5 with cycloheximide, 2-bromopalmitate, and the Crm1 inhibitor leptomycin B. Under these conditions we found that GFP-R7BP exited the nucleus (Fig. 1C), albeit with a somewhat reduced rate or extent relative to what was observed in the absence of leptomycin B (Fig. 1A). As a control, we demonstrated that leptomycin B efficiently inhibited Crm1-dependent export of GFP-Cdc25C-S216A (Fig. 1D and Ref. 42). Therefore, R7BP-R7-Gβ5 complexes are apparently exported by Crm1-independent and -dependent mechanisms.

Identification of an NLS near the C-terminal Palmitoylation Sites of R7BP—R7-Gβ5-R7BP heterotrimers bearing unpalmitoylated R7BP must accumulate in the nucleus by active transport rather than by passive diffusion coupled with nuclear retention because these complexes exceed the ~60-kDa exclusion limit of nuclear pore complexes and because unpalmitoylated R7BP undergoes rapid nuclear/cytoplasmic shuttling. Therefore, R7BP may possess a nuclear localization sequence (NLS) that binds nuclear import receptors. We explored this possibility by
RGS7-Gβ5 Function Regulated by R7BP

First, we found that a region of R7BP (residues 48–149) homologous to its counterpart expressed in photoreceptors (R9AP) was required to form complexes with an R7 protein and Gβ5 (Fig. 3A). Efficient complex formation also required additional flanking sequences (to residue 221; Fig. 3B) containing a heptad repeat domain. In contrast, the N terminus (residues 3–47) and palmitoylated C-terminal domain (residues 242–257) of R7BP were dispensable for complex formation (Fig. 3A).

Second, results suggested that the C-terminal region of R7BP is both an NLS and a domain required for palmitoylation and membrane anchoring (Fig. 4). This conclusion was supported by evidence showing that R7BP lacking its C-terminal 15 residues (Δ242–257), which also removes the cysteine residues that are palmitoylated, was neither plasma membrane-bound nor nuclear-enriched when co-expressed with RGS9-1 and Gβ5 (Fig. 4). This was in contrast to the exclusively nuclear localization of an R7BP point mutant (C252S/C253S) lacking just the palmitoylation acceptor sites (39) or the wild type localization pattern of mutants lacking the N-terminal (Δ3–47) or the heptad repeat region (Δ190–225) of R7BP (Fig. 4). Therefore, the C-terminal domain-containing residues 242–257 mediate nuclear and plasma membrane localization. This region proximal to the palmitoylated cysteine residues contains a cluster of basic residues (Fig. 2B), similar to well characterized nuclear localization sequences (reviewed in Ref. 47).

To confirm that R7BP contains an NLS near its C terminus and to determine whether the R7 and Gβ5 subunits of the complex are dispensable for nuclear localization, we analyzed additional mutant forms of GFP-R7BP without expressing an R7 protein and Gβ5. Under these conditions, wild type R7BP localized to the plasma membrane and nucleus, and blocking R7BP palmitoylation by treating cells with the palmitoylation inhibitor 2-bromopalmitate or by expressing the R7BP-C252S/C253S mutant resulted in nuclear accumulation of the protein (Fig. 5A). Because HEK293 cells do not significantly express R7 family members or Gβ5 (data not shown), these proteins are dispensable for palmitoylation-regulated localization of R7BP.

Analysis of R7BP mutants in the absence of R7 and Gβ5 expression indicated that the C-terminal 31 residues of R7BP are sufficient both for NLS activity and palmitoylation-dependent plasma membrane targeting (Fig. 5, A and B). This was shown by palmitoylation-dependent plasma membrane targeting of an R7BP mutant lacking the N-terminal and R7-binding domains (GFP-tagged R7BP-Δ3–225) and depalmitoylation-dependent nuclear accumulation of this protein (Fig. 5A). We also noted that N-terminal deletion mutants (Δ3–12 and Δ3–47) blunted nuclear accumulation of R7BP under basal conditions but not when palmitoylation was blocked; the N terminus of R7BP therefore may have weak NLS activity that is dispensable when R7BP is unpalmitoylated. The dominant, C-terminal NLS of R7BP was mapped by analyzing small deletion mutants that retained the palmitoylated cysteine residues (Δ222–231, Δ232–241, and Δ242–247; Figs. 2B and 5B) and a point mutant form of the full-length protein in which all residues within the polybasic region proximal to the palmitoylated cysteine residues were changed to relatively isosteric glutamine residues (PBR-Q mutant; Figs. 2B and 5B), an approach commonly used to study the function of polybasic sequences without disrupting protein structure (48). The results indicated that only the Δ242–247 and PBR-Q mutants failed to concentrate in the nucleus, even when palmitoylation acceptor cysteines were changed to serine residues (Fig. 5B). In the absence of the strong C-terminal NLS, the N-terminal region of R7BP did not influence the localization of the protein as indicated by the localization of the PBR-Q mutant also lacking the N-terminal domain (Δ3–47-PBR-Q; Fig. 5B), suggesting that the N-terminal domain lacks detectable NLS activity of its own. The results obtained when the GFP-R7BP PBR-Q mutant was expressed transiently in primary hippocampal neurons where the protein is normally expressed confirmed the NLS activity of the C-terminal domain (Fig. 5C). Therefore, these results indicated that the polybasic region (PBR) near the C terminus of R7BP has the properties expected of an NLS that is recognized by nuclear import receptors.
To address this question, we used GIRK channels expressed in X. laevis oocytes as an assay of R7BP function, as we employed previously to characterize complexes containing wild type versus palmitoylation-defective mutant forms R7BP (39). This system provides a uniquely sensitive, high resolution live cell assay for RGS function at the plasma membrane because the kinetics of GPCR-regulated GIRK channel activity are accelerated greatly by the GAP activity of RGS proteins (45). Accordingly, we used this system to compare the activity of wild type R7BP versus the cytoplasmically localized PBR-Q mutant form of R7BP.

The following results indicated that plasma membrane targeting of R7-Gβ5-R7BP complexes is required for full activity in this system. As shown in Fig. 6, RGS7-Gβ5 heterotrimers (i.e. lacking R7BP) had moderate ability to accelerate the activation and deactivation time courses of GIRK currents evoked by Gαi3-coupled m2 receptors (Fig. 6). As we reported previously, these effects were significantly augmented upon co-expression with wild type GFP-R7BP (Fig. 6). In contrast, the PBR-Q mutant form of R7BP failed to augment GPCR-evoked GIRK activation and deactivation kinetics beyond what was observed when only RGS7 and Gβ5 were expressed (Fig. 6). This result was not due to poor expression of mutant R7BP or impaired ability to form complexes with RGS7 and Gβ5, because results shown previously (Fig. 3A) demonstrated that the C-terminal domain of R7BP including the PBR is dispensable for protein expression and efficient complex formation. Taken together, these results indicated that R7BP palmitoylation augments function of R7-Gβ5-R7BP complexes by a membrane-anchoring mechanism.

**DISCUSSION**

Here we have provided new insight into mechanisms whereby G protein regulatory complexes composed of an R7-class RGS protein, Gβ5, and R7BP are regulated by palmitoylation of R7BP. Our results indicate that: 1) palmitoylation of R7BP regulates whether R7-Gβ5-R7BP complexes localize to the plasma membrane or shuttle between the nucleus and cytoplasm; 2) the C-terminal PBR of R7BP functions as a nuclear localization sequence and a motif that promotes palmitoylation; and 3) R7BP augments the G protein regulatory function of R7-Gβ5-R7BP complexes by a membrane-targeting mechanism. The implications of these findings on our understanding of mechanisms regulating GPCR signaling and palmitoylation-dependent shuttling of signaling proteins are discussed below.
R7BP Trafficking—We suggest that R7BP trafficking is governed by a novel mechanism whereby palmitoyltransferases and nuclear import receptors potentially compete for R7BP. This hypothesis is suggested by our finding that the short C-terminal PBR of R7BP is required both for nuclear localization and palmitoylation. The PBR apparently functions as the major NLS in R7BP that binds nuclear import receptors, similar to short basic regions in many proteins that are bona fide NLSs (reviewed in Ref. 47). Consistent with this hypothesis, inactivating just the palmitoylation acceptor sites (C252S/C253S mutant) of R7BP results in nuclear localization of R7-G5-R7BP complexes (39), whereas removing both the PBR and the palmitoylation acceptor sites (Δ242–257 mutant) localizes R7-G5-R7BP heterotrimers in the cytoplasm. Whether nuclear import receptors and palmitoyltransferases bind directly and competitively to the C-terminal domain containing the PBR is not yet clear. Answering this question will require the development of new biochemical tools.

While our manuscript was in preparation, Song et al. (49) reported several findings related to our studies. They showed that the PBR of R7BP is a strong NLS and that the N-terminal region of R7BP affects nuclear localization. They also found that endogenous R7BP can be detected at relatively low levels in the nuclear fraction of brain lysates, whereas most of the protein associated with plasma membrane fractions. R7BP in brain therefore appears to be efficiently palmitoylated and membrane-targeted.

Palmitoylation-regulated Plasma Membrane/Nuclear Localization of Signaling Proteins—A diverse collection of proteins that localize alternatively to the nucleus or plasma membrane in a palmitoylation-dependent manner, including estrogen receptor, phospholipid scramblase 1, and the Cdc42-related GTPase Wrch-1, has recently become appreciated. Estrogen receptors exert their biological effects by binding promoters of specific target genes and by triggering nongenomic signaling events at the plasma membrane. Nongenomic, membrane-initiated actions of estrogen, including endothelial nitric-oxide synthase activation, are
thought to be initiated by an estrogen receptor α pool that is palmitoylated and localized to the plasma membrane (50, 51). Likewise, membrane-tethered estrogen receptor α exhibits augmented ability to trigger ERK1/2 (extracellular signal-regulated kinases 1/2) and Akt activation and breast cancer cell proliferation in response to estradiol (52, 53). Interestingly, estradiol stimulation reduces estrogen receptor α palmitoylation and interaction with caveolin-1 (52), which may turn off membrane-initiated responses to estrogen.

Phospholipid scramblase 1 (PLSCR1) was originally described as a protein that promotes Ca²⁺-dependent transbilayer movement of membrane phospholipids (54), as occurs during injury and apoptosis. However, PLSCR1 also functions as an interferon-induced transcription factor that regulates interferon-stimulated genes (55, 56) and the inositol 1,4,5-trisphosphate receptor gene (57). Prior to interferon stimulation, PLSCR1 is palmitoylated and membrane-bound, whereas upon interferon stimulation the protein is unpalmitoylated and localized to the nucleus (58).

Wrch-1 is Wnt-regulated GTPase related to Cdc42 (59). Wrch-1 transforms cells by a mechanism requiring palmitoylation and plasma membrane targeting (60). Unpalmitoylated Wrch-1 localizes to the cytoplasm and nucleus where it is unable to activate its effector PAK (60), suggesting that depalmitoylation could terminate Wrch-1 signaling.

Based on our studies of R7BP, we speculate that trafficking of palmitoylated proteins that localize alternatively to the plasma membrane and nucleus potentially is determined at least in part by competition between palmitoyltransferases and nuclear import receptors. Such competition could occur because the palmitoylation sites and NLS motifs are closely juxtaposed in the linear sequence of the substrate protein, as occurs in R7BP. Wrch-1 potentially uses this mechanism because it possesses a C-terminal polybasic region linked to a dicysteine motif that is palmitoylated (60). In other cases, palmitoyltransferases and nuclear import receptors may compete if their recognition sites are spatially clustered in the tertiary structure of the protein substrate. It therefore will be intriguing to determine whether such competition mechanisms regulate the trafficking and function of palmitoylated proteins such as estrogen receptor α, PLSCR1, and Wrch-1.

**FIGURE 6. Cytoplasmic R7BP-RGS7-Gß5 complexes weakly regulate GPCR-mediated GIRK channel activity.** A, acceleration of GIRK current kinetics by RGS7-Gß5 is potentiated by wild type (WT) GFP-R7BP. Superimposed Ach-activated GIRK currents were elicited by Gαo-coupled muscarinic m2 receptors expressed in Xenopus oocytes in the absence (no RGS), presence of RGS7-Gß5 heterodimers (+ RGS7/Gß5), and the presence of wild type RGS7-Gß5-GFP-R7BP heterotrimers (+ RGS7/Gß5 + R7BP WT). Agonist (ACh, 1 µM) was applied and removed as indicated by the bar. Current amplitudes were normalized for comparison and are representative of each experimental group. B, cytoplasmic RGS7-Gß5-R7BP complexes are weakly active. Experiments like those in A were conducted except that the PBR-Q mutant form of GFP-R7BP was substituted for wild type GFP-R7BP at the same expression level (5 ng cRNA/oocyte). C, quantification of deactivation time constants derived from the GIRK current time course following washout of agonist. R7BP accelerated the GIRK current deactivation rate in a concentration-dependent manner, whereas the R7BP PBR-Q had no significant effect beyond that observed when only RGS7 and Gß5 were expressed. The values are the means ± S.E. from 6–9 oocytes for each condition, from two separate oocyte cultures. D, quantification of the GIRK activation time constants associated with agonist application. Wild type R7BP accelerated the activation time course in a concentration-dependent manner, whereas the R7BP PBR-Q mutant had no significant effect beyond that observed when only RGS7 and Gß5 were expressed. The values are the means ± S.E. from 6 to 9 oocytes for each condition, from two separate oocyte preparations.

**GPCR Signaling Regulated by R7-Gß5-R7BP Complexes**—Compared with R7-Gß5-R7BP heterotrimers bearing palmitoylated wild type R7BP, cytoplasmic R7-Gß5-R7BP complexes containing palmitoylation- and nuclear import-defective R7BP have greatly impaired ability to regulate the kinetics of GPCR-mediated GIRK channel activity. By analogy with studies of other palmitoylated proteins including the non-R7 family members RGS4 and RGS16 (reviewed in Ref. 61), palmitoylation of R7BP could augment the function of R7-Gß5-R7BP heterotrimers by concentrating them on the plasma membrane where they diffuse in two rather than three dimensions to interact with substrate G protein α subunits, targeting them to specialized plasma membrane microdomains (lipid “rafts”) where G proteins and GIRK channels concentrate, or orienting the R7 subunit of the complex relative to the plane of the membrane in a manner that promotes interaction with Gα subunits.

Our results suggest that cytoplasmic R7-Gß5-R7BP heterotrimers and R7-Gß5 heterodimers exhibit equivalently weak ability to regulate the kinetics of GPCR-mediated GIRK channel activity relative to R7-Gß5-R7BP heterotrimers bearing wild type, palmitoylated R7BP. Because the modulatory effects of RGS proteins on GIRK channel kinetics is

---

*JOURNAL OF BIOLOGICAL CHEMISTRY* 28229
an exquisitely sensitive measure of RGS protein GAP activity (62, 63), these results suggested that complex formation between R7BP and RGS7-Gß5 heterodimers does not greatly augment the GAP activity of RGS7. The same apparently is true for the R7BP-related protein R9AP. Whereas membrane-bound R9AP greatly stimulates the GAP activity of RGS9-Gß5 heterodimers in vitro (36, 37), mutant R9AP lacking its transmembrane tail fails to do so (36, 37). Accordingly, it will be interesting to determine the precise mechanism by which membrane targeting by R7BP or R9AP augments the function of R7-Gß5 complexes.

Acknowledgments—We thank John Carvalho and Haiying Teng and members of the Blumer laboratory for helpful advice and discussion.

REFERENCES

1. Gilman, A. G. (1987) Annu. Rev. Biochem. 56, 615–649
2. Sheno, S. K., and Lekowitz, R. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 2449–2454
3. Luttrell, L. M., Roudabush, F. L., Choy, E. W., Miller, W. E., Field, M. E., Marullo, S., and Benmerah, A. (2002) Oncogene 21, 6965–6973
4. Scott, M. G., Le Rouzic, E., Perianin, A., Pierotti, V., Enslen, H., Benichou, K. H., Kovoor, A., Chen, C. K., DiLeone, R. J., Schwarz, S. C., Slepak, N. P., and Arshavsky, V. Y. (2005) J. Biol. Chem. 280, 5133–5139
5. Goel, R., Phillips-Mason, P. J., Raben, D. M., and Baldassare, J. J. (2002) J. Biol. Chem. 277, 18640–18648
6. Wang, P., Wu, Y., Ge, X., Ma, L., and Pei, G. (2003) J. Biol. Chem. 278, 11648–11653
7. Scott, M. G., Le Rouzic, E., Perianin, A., Pierotti, V., Enslen, H., Benichou, K. H., Kovoor, A., Chen, C. K., DiLeone, R. J., Schwarz, S. C., Slepak, N. P., and Arshavsky, V. Y. (2005) J. Biol. Chem. 280, 5133–5139
8. Hepler, R. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 2449–2454
9. Scott, M. G., Le Rouzic, E., Perianin, A., Pierotti, V., Enslen, H., Benichou, K. H., Kovoor, A., Chen, C. K., DiLeone, R. J., Schwarz, S. C., Slepak, N. P., and Arshavsky, V. Y. (2005) J. Biol. Chem. 280, 5133–5139
10. Goel, R., Phillips-Mason, P. J., Raben, D. M., and Baldassare, J. J. (2002) J. Biol. Chem. 277, 18640–18648
11. Wang, P., Wu, Y., Ge, X., Ma, L., and Pei, G. (2003) J. Biol. Chem. 278, 11648–11653
12. Scott, M. G., Le Rouzic, E., Perianin, A., Pierotti, V., Enslen, H., Benichou, K. H., Kovoor, A., Chen, C. K., DiLeone, R. J., Schwarz, S. C., Slepak, N. P., and Arshavsky, V. Y. (2005) J. Biol. Chem. 280, 5133–5139
13. Hepler, R. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 2449–2454
58. Wiedmer, T., Zhao, J., Nanjundan, M., and Sims, P. J. (2003) Biochemistry 42, 1227–1233
59. Tao, W., Pennica, D., Xu, L., Kalejta, R. F., and Levine, A. J. (2001) Genes Dev. 15, 1796–1807
60. Berzat, A. C., Buss, J. E., Chenette, E. J., Weinbaum, C. A., Shutes, A., Der,

C. J., Minden, A., and Cox, A. D. (2005) J. Biol. Chem. 280, 33055–33065
61. Jones, T. L. (2004) Methods Enzymol. 389, 33–55
62. Chuang, H. H., Yu, M., Jan, Y. N., and Jan, L. Y. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11727–11732
63. Doupnik, C. A., Davidson, N., Lester, H. A., and Kofuji, P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10461–10466