R7BP AUGMENTS THE FUNCTION OF RGS7/GB5 COMPLEXES BY A PLASMA-**MEMBRANE TARGETING MECHANISM**

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Running title: RGS7/Gβ5 function regulated by R7BP

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The RGS7 (R7) family of G protein regulators, **Gβ5** and **R**7**BP** form heterotrimeric complexes that potently regulate the kinetics of G proteincoupled receptor (GPCR) signaling. Reversible palmitoylation of R7BP regulates plasma membrane/nuclear shuttling of R7/G85/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 Cterminal 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/G**β**5/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/G**β**5/R7BP heterotrimers and RGS7/G**B5** heterodimers are equivalently inefficient regulators of GPCR 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.

INTRODUCTION

G protein-coupled receptor (GPCR¹) 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 GABA release. GPCRs transduce signals via G protein-dependent and -independent mechanisms (reviewed in (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 MAP kinase pathways (4), non-receptor tyrosine kinases (5,6), and phosphatidyl-inositol-3 phosphate kinase (PI3 kinase (7,8). GPCR signaling from the plasma membrane to the nucleus may also occur by nuclear shuttling of βarrestin1 (9-11).

GPCR signaling is regulated potently by the RGS (regulators of <u>G</u> protein signaling) protein family (reviewed in (12)). RGS proteins regulate the kinetics and amplitude of GPCR signaling by acting as GTPase-activating proteins (GAPs) for Ga subunits (13-15). Certain RGS proteins also function as Ga effectors or antagonists (reviewed in (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 $G\alpha$ subunits of the Gi/o family (20-22) that mediate phototransduction and the effects of modulatory neurotransmitters (reviewed in (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 GB subunit G_{β5} (16,29-32). Indeed, all R7 isoforms

are markedly destabilized when G β 5 is absent (33), and mice lacking G β 5 or RGS9 exhibit similar delay in phototransduction termination kinetics (34). RGS9-1/G β 5 complexes in retina also include a third subunit, R9AP (<u>RGS9</u> <u>anchoring protein</u>), 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 G65 throughout much of the nervous system (39.40). R7BP can bind any R7 family member complexed with G β 5 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 $R7/G\beta5/R7BP$ 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 depalmitoylationinduced 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 $R7/G\beta5/R7BP$ complexes. We have determined whether unpalmitoylated R7BP is trapped in the nucleus or undergoes nuclear/cytoplasmic shuttling, identified sequence motifs in R7BP that mediate both palmitoylation and nuclear localization, and used these R7BP mutants to determine whether cytoplasmic R7/G β 5/R7BP complexes can regulate GPCR signaling. The results provide new insight into the regulation of GPCR signaling by $R7/G\beta5/R7BP$ complexes, and the mechanisms by which palmitoylation regulates trafficking R7BP and a newly appreciated collection of signaling proteins that localize alternatively to the plasma membrane or nucleus.

EXPERIMENTAL PROCEDURES

Reagents and antibodies – Immnoblotting experiments used an anti-FLAG-M2 HRP conjugate (Sigma; 1:5000), an anti-MYC 9E10 HRP conjugate (Roche; 1:1000), an anti-HA 12CA5 HRP conjugate (Roche; 1:2000), anti-GFP (B34, Abcam; 1:2000), and HRP-conjugated goat anti-mouse secondary antibodies (Pierce; 1:10,000). Immunofluorescence experiments the anti-HA-11 antibody (BAbCo; 1:500) and goat anti-mouse Alexa 568 secondary (Molecular Probes: 1:100). Unless noted otherwise, all chemicals were obtained from Sigma. Cell culture and transfection - HEK293 cells (ATCC) were maintained and transfected in DME/F12 with 10% fetal bovine serum (Atlanta Biologicals) plus penicillin/streptomycin. 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 (Wash. Univ. School of Med. (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 PCR reactions/ligations introduced an NheI 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 DNA constructs were sequenced.

Cell lysis, immunoblotting and immunoprecipitation - Cells were washed once with ice cold PBS and lysed in lysis buffer (MCLB: 50mM Tris pH 8.0, 5mM EDTA, 0.5% Igepal, 100mM NaCl, 1mM sodium orthovanadate, 1mM PMSF) supplemented with protease inhibitor tablets (Roche). 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 (15,000xg) for 10 min at 4°C. Supernatant fractions were used directly for western blot analysis or for immunoprecipitation. For western blots, 50-100µg of total protein was electrophoresed through 10% SDS polyacrylamide gels followed by transfer to PVDF (Millipore) membranes. Membranes were blocked for 1 h in 5% milk/TBST followed by overnight incubation with primary antibodies. HRP-conjugated primary antibodies were removed, membranes were washed with TBST, and 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 50µl of FLAG M2agarose affinity gel (Sigma) for 90min followed by 3x5 min washes in MCLB. Immunoprecipitates were denatured with 1X SDSsample 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, cells were washed with media (DMEM/F12 with sodium pyruvate, nonessential amino acids and penicillin/streptomycin) and incubated with labeling media for 120 min (media plus 10% dialyzed FBS and [³H]palmitic acid (Perkin Elmer) at 1mCi per 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 - Cells were grown and transfected on glass coverslips prior to preparation for microscopy as follows. 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 (20mM HEPES pH 7.4, 0.5% Triton X-100, 50mM NaCl, 3mM MgCl2, 300mM sucrose). Cells were washed with PBS, blocked with 0.1% BSA (in PBS) for 10 min at 4°C and washed again with PBS. 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 followed by 10 washes in TBST. 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, cells grown on coverslips were transferred to glass-bottom imaging dishes (Bioptechs) and incubated in growth media without phenol-red and including 10µg/mL cycloheximide for 30 min to block de novo protein synthesis. Binucleate cells were identified and imaged prior to and after photobleaching at low laser intensity (1% output). For experiments using wild type GFP-R7BP, we treated cells 12h with the palmitoylation inhibitor 2-bromopalmitate $(100\mu M)$ before addition of cycloheximide. For bleaching, the cytoplasm and one of two nuclei were bleached at approximately 50% laser output for 50-75 iterations. Post-bleach images were taken every 5-10 minutes for 30 to 60 minutes after bleaching.

Electrophysiology – The effects of mutating the R7BP polybasic region (PBR-Q) on RGS7/G β 5 function were assayed using the *Xenopus* oocyte expression system as described previously (39). All 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 NIH guidelines.

Oocytes were injected with a mixture of 5' capped cRNA's synthesized in vitro from linearized cDNA vectors (mMessage mMachine, Ambion). Six experimental groups (~20 oocytes each) were injected with different cRNA mixtures (50 nl final volume) and incubated at 19°C in parallel for 48-60 h. To confer specific m2 receptor-Go-GIRK channel signaling, all groups received cRNA's 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 cRNA's for RGS7 (1 ng/oocyte) and G β 5 (1 ng/oocyte), with or without GFP-R7BP or GFP-R7BP PBR-Q at two different concentrations tested (1 ng and 5 ng/oocvte).

ACh-activated GIRK currents were recorded by two-electrode voltage clamp methods from a holding potential of -80 mV (GeneClamp 500, Axon Instruments). Oocytes were initially superfused with a minimal salt solution (98 mM NaCl, 1 mM MgCl2, and 5 mM HEPES at 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 at 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 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 ANOVA where p<0.05 was considered significant. 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/G β 5/R7BP complexes from the plasma membrane to the nucleus (39), suggesting the R7BP controls shuttling of the complex. Whether complexes containing unpalmitoylated R7BP undergo nuclear/cytoplasmic shuttling, however, had not been determined. We have addressed this question because it would suggest whether nuclear, unpalmitoylated R7BP/R7/G β 5 complexes have the potential to exit the nucleus in order 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 (fluorescence recovery after photobleaching) experiments (46). For reasons discussed below, these experiments used multinucleate HEK293 cells that appeared occasionally in culture. First, we transiently expressed a non-palmitoylated R7BP mutant (GFP-R7BP-C252S/C253S) with HA-tagged RGS9-1 and MYC-tagged G_{β5} in HEK293 cells, and treated cells with cycloheximide to block new protein synthesis. Non-palmitoylated 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 palmitovlated and plasma membrane-targeted. Once binucleate cells expressing mutant R7BP were identified, the cytoplasm and one nucleus per 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 (Figure 1A), indicating that $R7BP/R7/G\beta5$ 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 2bromopalmitate demonstrated that complexes containing wild type GFP-R7BP also undergo nuclear/cytoplasmic shuttling (Figure 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 (Figure 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-G65 with cycloheximide, 2-bromopalmitate and the Crm1 inhibitor leptomycin B. Under these conditions we found that GFP-R7BP exited the nucleus (Figure 1C), albeit with somewhat reduced rate or extent relative to what was observed in the absence of leptomycin B (Figure 1A). As a control, we demonstrated that leptomycin B efficiently inhibited Crm1-dependent export of GFP-CDC25C-S216A (Figure 1D (42)). Therefore, R7BP/R7/G65 complexes apparently are exported by Crm1-independent and -dependent mechanisms.

Identification of an NLS near the Cterminal palmitoylation sites of R7BP-R7/Gβ5/R7BP heterotrimers bearing unpalmitovlated R7BP must accumulate in the nucleus by active transport rather than by passive diffusion coupled with nuclear retention because these complexes exceed the ~60kDa 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 analyzing a series of GFP- or FLAG-tagged R7BP deletion mutants (Figure 2) for the ability to form complexes with HA-RGS9-1 and MYC-G_{β5} in coimmunoprecipitation experiments and to localize to the plasma membrane and/or nucleus in HEK293 cells. The following results were obtained.

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 G β 5 (Figure 3A). Efficient complex formation also

required additional flanking sequences (to residue 221; Figure 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 (Figure 3A).

Second, results suggested that the Cterminal region of R7BP is both an NLS and a domain required for palmitoylation and membrane anchoring (Figure 4). This conclusion was supported by evidence showing that R7BP lacking its C-terminal 15 residues ($\Delta 242-257$, which also removes the cysteine residues that are palmitoylated) was neither plasma membranebound nor nuclear-enriched when co-expressed with RGS9-1 and G β 5 (Figure 4). This was in contrast to the exclusively nuclear localization of an R7BP point mutant (C252S/C253) lacking just the palmitoylation acceptor sites (39), or the wild type localization pattern of mutants lacking the Nterminus (Δ 3-47) or the heptad repeat region $(\Delta 190-225)$ of R7BP (Figure 4). Therefore, the Cterminal domain containing residues 242-257 mediates nuclear and plasma membrane localization. This region proximal to the palmitoylated cysteine residues contains a cluster of basic residues (Figure 2B), similar to wellcharacterized nuclear localization sequences (reviewed in (47).

To confirm that R7BP contains an NLS near its C-terminus and to determine whether the R7 and G65 subunits of the complex are dispensable for nuclear localization, we analyzed additional mutant forms of GFP-R7BP without expressing an R7 protein and G_{β5}. 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/C253 mutant resulted in nuclear accumulation of the protein (Figure 5A). Because HEK293 cells do not significantly express R7 family members or G_{β5} (data not shown), these proteins are dispensable for palmitovlation-regulated localization of R7BP.

Analysis of R7BP mutants in the absence of R7 and G β 5 expression indicated that the Cterminal 31 residues of R7BP are sufficient both for NLS activity and palmitoylation-dependent plasma membrane targeting (Figure 5A and B). plasma membrane targeting of an R7BP mutant lacking the N-terminal and R7-binding domains (GFP-tagged R7BP- Δ 3-225), and depalmitoylation-dependent nuclear accumulation of this protein (Figure 5A). We also noted that Nterminal deletion mutants (Δ 3-12 and Δ 3-47) 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, Cterminal NLS of R7BP was mapped by analyzing small deletion mutants that retained the palmitoylated cysteine residues ($\Delta 222-231$, $\Delta 232$ -241 and Δ 242-247; Figures 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; Figures 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 $\Delta 242-247$ and PBR-Q mutants failed to concentrate in the nucleus, even when palmitoylation acceptor cysteines were changed to serine residues (Figure 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 $(\Delta 3-47/PBR-Q;$ Figure 5B), suggesting that the Nterminal domain lacks detectable NLS activity of its own. 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 (Figure 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.

This was shown by palmitoylation-dependent

The NLS of R7BP is required for plasma membrane localization and palmitoylation– By analyzing R7BP mutants lacking NLS activity (Δ 242-247 or PBR-Q), we made the unanticipated observation that plasma membrane localization was lost even though palmitoylation acceptor sites were present (Figure 5B). This result suggested that the polybasic domain of R7BP is both an NLS and a regulatory or recognition motif for palmitoylation.

To determine whether the C-terminal polybasic region of R7BP mediates palmitoylation, we expressed FLAG-tagged versions of R7BP (wild type; C252S/C253S; Δ 242-247 and PBR-Q) in HEK293 cells labeled with [³H]palmitate. FLAG-tagged proteins immunoprecipitated from cell extracts were analyzed by fluorography to determine which were palmitoylated (39). In contrast to wild type R7BP, neither the Δ 242-257 nor the PBR-Q mutant was palmitoylated (Figure 5D), although each was well expressed. These results demonstrated that the Cterminal polybasic region of R7BP is an NLS that also mediates palmitoylation.

GPCR signaling regulation by cytoplasmic R7BP/R7/G_{β5} complexes-We showed previously that R7/G65/R7BP complexes containing palmitoylated wild type R7BP are potent regulators of G protein signaling, whereas those containing unpalmitoylated mutant R7BP are weakly active because they are transported into the nucleus (39). These previous studies could not determine whether palmitoylation-dependent plasma membrane targeting of the complex is required for function because it was not possible to assess the activity of cytoplasmic R7/Gβ5/R7BP heterotrimers. However, because results of the present investigation identified R7BP mutants defective both in palmitoylation-dependent plasma membrane targeting and nuclear import, we had the means of determining whether cytoplasmic R7/Gβ5/R7BP complexes can regulate G proteincoupled receptor (GPCR) signaling at the plasma membrane.

To address this question, we used G protein-coupled receptor-mediated activation of inward rectifying K⁺-channels (GIRK channels) expressed in *Xenopus* 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 exquisitely 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/G65/R7BP complexes is required for full activity in this system. As shown in Figure 6, RGS7/GB5 heterodimers (i.e.-lacking R7BP) had moderate ability to accelerate the activation and deactivation time courses of GIRK currents evoked by Gocoupled muscarinic m2 receptors (Figure 6A-D). As we reported previously, these effects were significantly augmented upon co-expression with wild type GFP-R7BP (Figure 6A-D). 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 G β 5 were expressed (Figure 6A-D). This result was not due to poor expression of mutant R7BP or impaired ability to form complexes with RGS7 and G_{β5}, because results shown previously (Figure 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/G65/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, G β 5 and R7BP are regulated by palmitoylation of R7BP. Our results indicate that: 1) palmitovlation of R7BP regulates whether R7/G65/R7BP complexes localize to the plasma membrane or shuttle between the nucleus and cytoplasm; 2) the C-terminal polybasic region (PBR) of R7BP functions both 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 membranetargeting mechanism. The implications of these findings on our understanding of mechanisms regulating GPCR signaling and palmitoylationdependent shuttling of signaling proteins are discussed below.

 $R7/G\beta5/R7BP$ trafficking-We suggest that R7/G β 5/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 polybasic region (PBR) of R7BP is required both for nuclear localization and palmitoylation. The PBR apparently functions as the major nuclear localization sequence (NLS) in R7BP that binds nuclear import receptors, similar to short basic regions in many proteins that are bona fide NLSs (reviewed in (47)). Consistent with this hypothesis, inactivating just the palmitoylation acceptor sites (C252S/C253S mutant) of R7BP results in nuclear localization of R7/Gβ5/R7BP complexes (39), whereas removing both the PBR and the palmitoylation acceptor sites ($\Delta 242-257$ mutant) localizes R7/G65/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 development of new biochemical tools.

While our manuscript was in preparation, Song et al. reported several findings related to our studies (49). 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 plamitoylation-dependent manner, including estrogen receptor α , 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 non-genomic signaling events at the plasma membrane. Non-genomic, membraneinitiated actions of estrogen, including eNOS activation, are thought to be initiated by an estrogen receptor α (ER α) pool that is palmitoylated and localized to the plasma

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membrane (50,51). Likewise, membrane-tethered ER α exhibits augmented ability to trigger Erk1/2 and Akt activation and breast cancer cell proliferation in response to estradiol (52,53). Interestingly, estradiol stimulation reduces ER 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 IP3 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.

GPCR signaling regulated by R7/Gβ5/R7BP complexes-Compared to R7/Gβ5/R7BP heterotrimers bearing palmitoylated wild type R7BP, cytoplasmic R7/G65/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 palmitovlated proteins including the non-R7 family members RGS4 and RGS16 (reviewed in (61)), palmitoylation of R7BP could augment the function of $R7/G\beta5/R7BP$ 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 Ga subunits.

Our results suggest that cytoplasmic R7/G65/R7BP heterotrimers and R7/G65 heterodimers exhibit equivalently weak ability to regulate the kinetics of GPCR-mediated GIRK channel activity relative to R7/G65/R7BP heterotrimers bearing wild type, palmitoylated R7BP. Because the modulatory effects of RGS proteins on GIRK channel kinetics is an exquisitely sensitive measure of RGS protein GAP activity (62,63), these results suggested that complex formation between R7BP and RGS7/G65 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/GB5 heterodimers in vitro (36,37), mutant R9AP lacking its transmembrane 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 of the function of $R7/G\beta5$ complexes.

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FOOTNOTES

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¹Abbreviations used are: ACh, acetylcholine; G protein, guanine nucleotide-binding regulatory protein; GPCR, G protein-coupled receptor; GIRK, G protein-regulated inward-rectifying K⁺ channel; MAP kinase, mitogen-activated protein kinase; R7BP, RGS7-family binding protein; R9AP, RGS9-anchoring protein; RGS protein, regulator of G protein signaling protein.

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FIGURE LEGENDS

FIG. 1. R7/Gβ5/R7BP complexes containing unpalmitoylated R7BP undergo nuclear-cytoplasmic shuttling. A. Binucleate HEK293 cells expressing an unpalmitoylated GFP-R7BP mutant (SS) or wild type GFP-R7BP, HA-RGS9-1, and MYC-Gβ5 were subjected to fluorescence recovery after photobleaching (FRAP) experiments. After new protein synthesis was blocked with cycloheximide, the cytoplasm and/or one nucleus were bleached (dotted area). After bleaching, confocal scans were taken at the indicated time points to determine whether GFP-R7BP derived from the unbleached nucleus relocalized to the bleached nucleus and cytoplasm. Experiments using wild type GFP-R7BP also included the palmitoylation inhibitor 2-bromopalmitate. B. Rapid diffusion of GFP-R7BP within a bilobed nucleus. HEK293 cells expressing unpalmitoylated GFP-R7BP (SS), HA-RGS9-1, and MYC-Gβ5 were treated with cycloheximide and half of a bi-lobed nucleus was bleached. After bleaching, confocal images were acquired at the indicated time. C. Nuclear-cytoplasmic shuttling of R7BP is partially sensitive to leptomycin B (LMB). HEK293 cells expressing GFP-R7BP were treated with vehicle (DMSO) or LMB (10µg/ml) for 3h. D. GFP-CDC25C(S216A) localization was assayed as a positive control for the action of LMB.

FIG. 2. R7BP mutants used in this study. A. R7BP deletion mutants. Indicated are the R9AP-like domain, heptad repeats, polybasic domain and the palmitoylated cysteine residues (CC) of R7BP. The region of R7BP retained in each construct is indicated with a solid line. B. R7BP C-terminal mutants. The C-terminal sequence of R7BP containing the polybasic motif and palmitoylated cysteine residues (asterisks) is shown. The region of R7BP retained in each deletion mutant is indicated with a solid line. Residues changed to glutamine in the PBR-Q mutant are indicated. Unpalmitoylated forms of these mutants were constructed by changing cysteine-252 and -253 to serine (SS mutants).

FIG. 3. Identification of the R7/G β 5 binding domain of R7BP. A. The R9AP-like domain of R7BP is necessary for binding RGS9-1/G β 5 complexes. Transfected HEK293 cells expressing MYC-G β 5, the R7 protein HA-RGS9-1 and wild type or the indicated mutant forms of FLAG-R7BP were used for co-immunoprecipitation experiments. Additional R7 isoforms were not tested because they do not differ significantly in the ability to bind R7BP (39). FLAG immunoprecipitates (left panel) and total cell lysates (right panel) were probed with anti-FLAG, -HA and -MYC antibodies. B. The R9AP-like and heptad-repeat region of R7BP is sufficient for high affinity binding to RGS9-1 and G β 5. Transfected HEK293 cells expressing MYC-G β 5 and the R7 protein HA-RGS9-1 and wild type or the indicated mutant forms of FLAG-R7BP were used for co-immunoprecipitation experiments. FLAG immunoprecipitates (left panel) and total cell lysates (right panel) were probed with anti-FLAG, -HA and -MYC antibodies.

FIG. 4. The C-terminal domain of R7BP is required for nuclear and plasma membrane

localization. HEK293 cells expressing HA-RGS9-1, MYC-G β 5, and wild type or the indicated mutant forms of GFP-R7BP were fixed and stained with anti-HA antibodies. Localization of GFP-R7BP and HA-RGS9-1 was examined by using laser scanning confocal microscopy. Sites of co-localization in merged images are indicated in yellow.

FIG. 5. The C-terminal polybasic motif of R7BP is a nuclear localization sequence, plasma

membrane targeting domain and palmitoylation regulatory motif. A. The C-terminal 32 residues of R7BP are sufficient for palmitoylation-regulated plasma membrane and nuclear localization. HEK293 cells transfected with constructs expressing wild type or the indicated mutant forms of GFP-R7BP were imaged by confocal microscopy. Palmitoylation was inhibited by treating cells overnight with 2-bromopalmitate (+2Br-palmitate), or by expressing the indicated GFP-R7BP constructs in which the palmitoylated cysteine residues were changed to serines (+CC/SS mutation). B. The polybasic motif near the C-terminus of R7BP is required for palmitoylation-regulated nuclear and plasma membrane localization. HEK293 cells expressing wild type or the indicated mutant forms of GFP-R7BP were

analyzed by confocal microscopy. C. The polybasic motif of R7BP is required for plasma membrane and nuclear localization in neurons. Primary hippocampal neurons from neonatal rats were cultured 5d and transfected with cDNAs encoding wild type or the polybasic motif (PBR-Q) mutant form of GFP-R7BP. Cells were fixed and analyzed via confocal microscopy. Representative images for soma and dendrites are indicated. D. The polybasic motif near the C-terminus of R7BP is required for palmitoylation in vivo. HEK293 cells expressing wild type FLAG-R7BP (positive control), palmitoylation acceptor site mutant (SS; negative control), Δ 242-247, and PBR-Q were labeled with [³H]palmitate. FLAG-tagged R7BP in total cell lysates and anti-FLAG immunoprecipitates was detected by western blotting and fluorography (2d exposure).

FIG. 6. Cytoplasmic R7BP/RGS7/G^β5 complexes weakly regulate GPCR-mediated GIRK channel activity. A. Acceleration of GIRK current kinetics by RGS7/Gβ5 is potentiated by wild type GFP-R7BP. Superimposed ACh-activated GIRK currents were elicited by Gao-coupled muscarinic m2 receptors expressed in Xenopus oocytes in the absence (no RGS), presence of RGS7/GB5 heterodimers (+ RGS7/Gβ5), and the presence of wild type RGS7/Gβ5/GFP-R7BP heterotrimers (+ RGS7/Gβ5 + R7BP WT). Agonist (ACh, 1µM) 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/Gβ5/R7BP complexes are weakly active. Experiments like those in panel 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 G β 5 were expressed. Values are the mean <u>+</u> SEM from 6-9 oocytes for each condition, from 2 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 G β 5 were expressed. Values are the mean + SEM from 6-9 oocytes for each condition, from 2 separate oocyte preparations.

GFP-R7BP WT, 2Br-palmitate



GFP-R7BP SS



GFP-R7BP SS



GFP-R7BP WT, 2Br-palmitate, LMB



GFP-CDC25C (S216A)



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P-R7BP: WΤ ∆3-47 **∆190-225** △242-257 -RGS9-1: **ΙΥC-G**β5: GFP-R7BP HA-RGS9-1 Merge



