Palmitoylation regulates plasma membrane–nuclear shuttling of R7BP, a novel membrane anchor for the RGS7 family

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The RGS7 (R7) family of RGS proteins bound to the divergent Gβ subunit Gβ5 is a crucial regulator of G protein–coupled receptor (GPCR) signaling in the visual and nervous systems. Here, we identify R7BP, a novel neuronally expressed protein that binds R7–Gβ5 complexes and shuttles them between the plasma membrane and nucleus. Regional expression of R7BP, Gβ5, and R7 isoforms in brain is highly coincident. R7BP is palmitoylated near its COOH terminus, which targets the protein to the plasma membrane. Depalmitoylation of R7BP translocates R7BP–R7–Gβ5 complexes from the plasma membrane to the nucleus. Compared with non-palmitoylated R7BP, palmitoylated R7BP greatly augments the ability of RGS7 to attenuate GPCR-mediated G protein–regulated inward rectifying potassium channel activation. Thus, by controlling plasma membrane nuclear–shuttling of R7BP–R7–Gβ5 complexes, reversible palmitoylation of R7BP provides a novel mechanism that regulates GPCR signaling and potentially transduces signals directly from the plasma membrane to the nucleus.

Introduction

Hundreds of neurotransmitters regulate neuronal development and function by signaling through G protein–coupled receptors (GPCRs; for review see Gainetdinov et al., 2004). GPCRs exert their effects by regulating ion channels, second messenger production, and protein kinase cascades, which in turn control neuronal activity, gene expression, plasticity, differentiation, morphogenesis, and migration.

GPCR signaling is tightly regulated to determine the sensitivity, kinetics, and fidelity of neuronal activity. Disruption of GPCR regulatory mechanisms, such as the GPCR phosphorylation–arrestin pathway, dramatically affects processes such as nociception and addiction (for reviews see Chao and Nestler, 2004; Gainetdinov et al., 2004). GPCR signaling also is potently regulated by RGS proteins (regulators of G protein signaling; for review see Hollinger and Hepler, 2002). RGS proteins attenuate signaling by functioning as GTPase-activating proteins (GAPs) for Gα subunits (Berman et al., 1996; Hunt et al., 1996; Watson et al., 1996). Certain RGS proteins, such as the RGS protein p115RhoGEF (Hart et al., 1998), also function as Gα effectors.

Among more than 20 RGS family members in vertebrates, the R7 subfamily consisting of RGS6, RGS7, RGS9-1, RGS9-2, and RGS11 is emerging as an important set of neuronal GPCR-signaling regulators. The R7 family is highly expressed in the central and peripheral nervous systems (Gold et al., 1997; Zhang et al., 2000; Witherow et al., 2003; Larminie et al., 2004). R7 proteins selectively deactivate the Gi/o-class of Gα subunits that mediate the action of GPCRs for many modulatory neurotransmitters (Posner et al., 1999; Rose et al., 2000; Hooks et al., 2003). RGS9 is the best understood R7 family member (Cowan et al., 2001; Witherow and Slepak, 2003; Jones et al., 2004). RGS9 potently regulates GPCR-mediated Ca2+ channel inhibition in striatal neurons (Cabrera-Vera et al., 2004). RGS9 knockout mice exhibit augmentation of the antinociceptive and rewarding effects of μ-opioid receptors and enhancement of the locomotor and rewarding effects mediated by D2 dopamine receptors (Rahman et al., 2003; Zachariou et al., 2003). Human or mouse RGS9 mutants exhibit bradyopsia, a visual defect caused by prolonged activation of the Gα subunit transducin, resulting in impaired light adaptation and contrast detection (Chen et al., 2000; Nishiguchi et al., 2004).
R7 proteins function as complexes with Gβ5, a divergent member of the Gβ family (Watson et al., 1994; Jones et al., 2004). Mice lacking Gβ5 exhibit visual defects indistinguishable from RGS9 knockout mice (Krispel et al., 2003) and high mortality and slow growth due to destabilization of the entire R7 family (Chen et al., 2003). In the retina, RGS9-1–Gβ5L complexes are anchored to disk membranes by binding the retina-specific membrane protein R9AP (RGS9-anchoring protein; Hu and Wensel, 2002). R9AP also stimulates the GAP activity of RGS9-1 (Lishko et al., 2002; Hu et al., 2003). R9AP knockout mice recapitulate the visual phenotypes of RGS9 or Gβ5 knockout mice (Keresztes et al., 2004). Likewise, humans with R9AP or RGS9 mutations exhibit similar defects in visual perception (Nishiguchi et al., 2004).

Because R9AP is expressed insignificantly in nonretinal tissues (Hu and Wensel, 2002), the mechanisms that control subcellular localization and function of R7–Gβ5 complexes in the central and peripheral nervous systems are unknown. However, R7–Gβ5 localization and function probably are regulated tightly because these proteins localize both to membranes and nuclei in neurons where they regulate GPCR signaling at the plasma membrane and gene expression in the nucleus (Zhang and Simonds, 2000; Bouhamdan et al., 2004; Cabrera-Vera et al., 2004; Krumins et al., 2004; Liu and Fisher, 2004). Mechanisms that regulate R7–Gβ5 localization and function may be neuron-specific because R7 isoforms associate poorly with the plasma membrane when expressed in nonneuronal cells (Posner et al., 1999; Chatterjee et al., 2003; Witherow et al., 2003; Bouhamdan et al., 2004; Liu and Fisher, 2004; Takida et al., 2005).

Here, we report the identification and characterization of R7BP, a novel palmitoylated R9AP-related protein that is highly expressed in the nervous system. We present evidence indicating that reversible palmitoylation of R7BP controls the shuttling of R7–Gβ5 complexes between the plasma membrane and nucleus and the ability of an R7 protein to regulate GPCR signaling.

**Results**

Identification, cloning, and expression of R7BP

We identified R9AP-like proteins by performing PSI-BLAST searches of the mouse genome. This identified a novel 257-residue protein that for reasons presented in subsequent paragraphs was named R7BP (RGS7 family binding protein; Fig. 1A and B). Analysis of several genomic and EST databases indicated that R7BP and R9AP are the only closely related members of this family (Fig. 1C; accession numbers listed in Materials and methods). Both proteins also display weak similarity to SNARE proteins that mediate vesicular trafficking (unpublished data). The predicted R7BP gene contains six exons and is localized to chromosome 5q12.3 in humans, 13D1 in mouse, and 2q13 in rat (unpublished data). R7BP or R9AP homologues were not detected in Caenorhabditis elegans or Drosophila melanogaster, which do possess Gβ5 and R7 homologues. Therefore, R7BP and R9AP evolved after Gβ5 and the R7 family.

We cloned and sequenced a cDNA encoding the R7BP ORF from mouse brain, which confirmed database predictions. A mouse R7BP cDNA sequence assembled from EST databases predicted an mRNA of ~6.4 kb. In the deduced mRNA, the ORF encoding R7BP apparently is preceded by a GC-rich (~66%) stretch of ~720 nucleotides containing four short open reading frames, which could regulate R7BP translation (for review see Meijer and Thomas, 2002).

R7BP and R9AP exhibit similar domain organizations (Fig. 1A). Within their NH2-terminal regions these proteins are 28% identical and 54% similar. Distal to this region is a predicted coiled-coil domain followed by a putative membrane localization domain. The membrane localization domain of R9AP is a transmembrane segment, whereas R7BP contains a COOH-terminal polybasic sequence and a double cysteine motif that is an excellent candidate for lipid modification (Zhang and Casey, 1996; Smotrys and Linder, 2004).

Results of Northern blotting experiments indicated that R7BP is encoded by an ~6.4-kb mRNA expressed highly in brain and at much lower levels in other tissues examined (Fig. 2A), similar to the R7 family and Gβ5 (Gold et al., 1997; Zhang et al., 2000; Larminie et al., 2004). There was no indica-
increased the total levels of G$_7$R family member (Fig. 3 A). Expression of R7BP moderately precipitates indicated that FLAG-R7BP bound G$_7$RGS9-2, or RGS11. Immunoblotting of anti-FLAG immunoprecipitated G from coexpressed FLAG-tagged R7BP (FLAG-R7BP), MYC-coimmunoprecipitation experiments using HEK293 cells that expressed MYC-tagged G$_7$RGS9-2, or RGS11 and each R7 family member at relatively high levels may poorly express R7BP. R7BP mRNA is also detectable in mouse retina, organ of Corti, pituitary, aorta, vein, colon, hematopoetic stem cells, spinal cord, and embryonic heart, as indicated by EST database searches.

R7BP binds R7-G$_7$R5 complexes
To determine whether R7BP binds R7 proteins, we performed coimmunoprecipitation experiments using HEK293 cells that coexpressed FLAG-tagged R7BP (FLAG-R7BP), MYC-tagged G$_7$R5, and HA-tagged RGS6, RGS7, RGS9-1, RGS9-2, or RGS11. Immunoblotting of anti-FLAG immunoprecipitates indicated that FLAG-R7BP bound G$_7$R5 and each R7 family member (Fig. 3 A). Expression of R7BP moderately increased the total levels of G$_7$R5 and R7 proteins, suggesting that R7BP stabilizes G$_7$R5–R7 complexes. In the absence of an R7 protein, G$_7$R5 did not associate with FLAG-R7BP (Fig. 3 B).

When G$_7$R5 was not coexpressed, an R7 family member (RGS9-2) could bind FLAG-R7BP (Fig. 3 B), albeit less efficiently. Therefore, R7BP binds the R7 subunit of the R7–G$_7$R5 complex; G$_7$R5 may promote this interaction by stabilizing or changing the conformation of the R7 subunit. R7BP–R7 interaction was specific because tagged forms of two RGS proteins of a different subfamily (RGS2 and RGS4 of the R4 family) that do not bind G$_7$R5 were undetectable in FLAG-R7BP immunoprecipitates (Fig. 3 C).

R7BP recruits R7 RGS proteins to the plasma membrane
To determine whether R7BP associates with cell membranes and recruits R7 family members, we performed confocal microscopy experiments with cells expressing R7BP tagged at its NH$_2$ terminus with GFP or FLAG. The results showed that GFP- or FLAG-R7BP localized primarily to the plasma membrane as well as the cytoplasm and nucleus (Fig. 4 A). Quantification of confocal images indicated that ~40% of GFP-R7BP (SEM 15%; n = 30 cells) was nuclear, whereas the remainder localized to the plasma membrane or cytoplasm.

Results of similar experiments indicated that R7BP recruits each R7 family member to the plasma membrane. We expressed MYC-tagged G$_7$R5 to stabilize expression of HA-tagged R7 subunits. In the absence of R7BP, R7 proteins failed to associate detectably with the plasma membrane (Fig. 4 B).
and were cytoplasmic (RGS6, 7, 9–1 and 11) or nuclear (RGS9-2), which is in agreement with previous studies. In contrast, coexpression of GFP-R7BP resulted in efficient recruitment of each HA-R7 protein to the plasma membrane coincident with the localization of GFP-R7BP (Fig. 4 B). This effect was specific for the R7 family because FLAG-R7BP failed to recruit GFP-RGS4 to the plasma membrane (Fig. 4 C). Therefore, R7BP is a plasma membrane–anchoring protein specific for the R7 family of RGS proteins.

**Palmitoylation of R7BP**

R7BP possesses a dicysteine motif near its COOH terminus (CCLVSS-COOH) that is a potential lipid modification site (Fig. 5 A). This sequence resembles the COOH terminus of H-Ras (CCAAX-COOH), which is palmitoylated and prenylated (for reviews see Zhang and Casey, 1996; Smotrys and Linder, 2004). However, the COOH-terminal region of R7BP contains four rather than three amino acids after the last cysteine, which should preclude prenylation by displacing this residue from the prenyltransferase active site (Long et al., 2002).

We investigated the roles of COOH-terminal cysteine residues in GFP-tagged R7BP by changing either or both to serine. We then analyzed the localization of wild-type and mutant proteins expressed in HEK293 cells or primary rat hippocampal neurons. Wild-type GFP-R7BP localized to the plasma membrane and nuclei of HEK293 cells and punctate dendritic structures and nuclei of hippocampal neurons (Fig. 5, B and C). In contrast, the C253S single mutant or the C252S/C253S double mutant form of GFP-R7BP did not concentrate on the plasma membrane of HEK293 cells or dendritic foci in neurons but instead was cytoplasmic and/or nuclear localized (Fig. 5, B and C). The C252S mutant exhibited a localization phenotype intermediate between the wild-type protein and other mutants (Fig. 5 B). Quantification of confocal images indicated that whereas 40% of wild-type GFP-R7BP was nuclear in HEK293 cells (SEM 15%; n = 30 cells), 80% of the GFP-R7BP-C252S/C353S double mutant was nuclear (SEM 8%; n = 30 cells). Similar results were obtained using wild type and mutant forms of FLAG-tagged R7BP (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200502007/DC1), indicating that nuclear accumulation was not due to the presence of the GFP tag. Thus, the COOH-terminal cysteine residues profoundly regulate the distribution of R7BP between the plasma membrane, cytoplasm, and nucleus. The R7BP cysteine mutants migrated identically with wild-type R7BP upon SDS-PAGE (unpublished data), suggesting that R7BP is not subject to proteolytic processing characteristic of many prenylated proteins (Zhang and Casey, 1996).

To determine whether R7BP is palmitoylated, we analyzed the localization of GFP-R7BP when cells were treated with agents that block palmitoylation or prenylation. Doses of 2-bromopalmitate (2Br-palmitate) that block the modification of known palmitoylated proteins strikingly decreased plasma membrane localization of GFP-R7BP and increased nuclear localization of the protein (Fig. 5 D). Quantification of confocal images indicated that 35% of GFP-R7BP in vehicle-treated cells was nuclear (SEM 8%; n = 30 cells), whereas 67% of the protein in 2Br-palmitate–treated cells was nuclear (SEM 11%; n = 30). In contrast, neither a selective farnesyltransferase inhibitor (FTI-277) nor a geranylgeranyltransferase inhibitor (GTI-298) resulted in loss of GFP-R7BP from the plasma membrane (Fig. 5 E). However, these inhibitors did disrupt the localization of farnesylated and geranylgeranylated proteins (GFP–H-Ras and -Rap1A, respectively; Fig. 5 F; Fukasawa et al., 2004).

Second, we determined whether FLAG-R7BP expressed in HEK293 cells could be labeled with [3H]palmitate. Fluorography of anti-FLAG immunoprecipitates resolved by SDS-PAGE indicated that 70% of FLAG-R7BP was palmitoylated (data not shown). To confirm this result and to determine if the palmitoylation site was the CCLVSS-COOH motif, we examined the localization of GFP-R7BP when cells were treated with 2Br-palmitate. Treatment of cells with 2Br-palmitate decreased localization of GFP-R7BP to the plasma membrane (Fig. 5 D). Quantification of confocal images indicated that whereas 40% of wild-type GFP-R7BP was nuclear in HEK293 cells (SEM 15%; n = 30 cells), 80% of the GFP-R7BP-2Br-palmitate was nuclear (SEM 8%; n = 30 cells). These data suggest that R7BP is palmitoylated near its COOH terminus.

**Figure 4.** R7BP specifically recruits R7 RGS proteins to the plasma membrane. (A) R7BP is a plasma membrane protein. HEK293 cells expressing GFP-R7BP or FLAG-R7BP were analyzed by laser scanning confocal microscopy. Cells expressing HA-tagged R7 RGS proteins (RGS6, RGS7, RGS9-1, RGS9-2, or RGS11) and MYC-G65 were assayed for localization of the R7 family member by immunofluorescence confocal microscopy in the presence or absence of coexpressed GFP-R7BP. Anti-HA indicates the location of the RGS protein, whereas GFP-fluorescence indicates the localization of R7BP. Colocalization of R7BP and the RGS protein was determined by merging the data from the two channels; colocalization is indicated by yellow. (C) RGS4 is not recruited to the plasma membrane by R7BP. RGS4-GFP was coexpressed with FLAG-R7BP and the localization of these proteins was determined by confocal microscopy.
PAGE revealed that wild-type R7BP but not a control protein (FLAG-ERK2) was labeled with \(^{[3]H}\)palmitate (Fig. 6 A). Palmitate labeling of R7BP was reduced when cells expressed the C252S or the C253S single mutants (Fig. 6 A), suggesting that both cysteines are palmitoylated. Labeling was undetectable when both cysteines were changed to serine (Fig. 6 A). Palmitate labeling of wild-type R7BP was reversed upon treatment with the indicated dose of 2Br-palmitate for 16 h, followed by fixation and analysis by confocal microscopy. (E) Prenylation inhibitors do not block R7BP plasma membrane localization. HEK293 cells expressing GFP-R7BP were treated with the indicated dose of 2Br-palmitate for 16 h, followed by fixation and analysis by confocal microscopy. (F) FTI and GGTI controls. HEK293 cells expressing GFP-H-Ras (farnesylated protein) or GFP-Rap1A (geranylgeranylated protein) were treated with vehicle or the indicated compounds as in E. Cells were fixed and analyzed by confocal microscopy.

Figure 6. R7BP is palmitoylated at its COOH-terminal cysteine residues. (A) Schematic diagram of the R7BP COOH terminus. Cysteine residues that were mutated are indicated with an asterisk. (B) The R7BP double-cysteine motif is necessary for plasma membrane localization. Plasmids encoding GFP-tagged wild type and mutant forms of R7BP were transfected into HEK293 cells and localized by confocal microscopy. The following mutants were used: C252S, C253S, and a C252S/C253S double mutant. (C) R7BP localization in neurons. Neonatal rat hippocampal neurons were transfected with wild type or the C252S/C253S mutant form of GFP-R7BP and analyzed by confocal microscopy. High magnification images of a cell transfected with wild-type GFP-R7BP show localization in the cell soma and dendrites. (D) A palmitoylation inhibitor (2Br-palmitate) disrupts R7BP membrane localization. HEK293 cells expressing wild-type GFP-R7BP were treated with the indicated dose of 2Br-palmitate for 16 h, followed by fixation and analysis by confocal microscopy. (E) Prenylation inhibitors do not block R7BP plasma membrane localization. HEK293 cells expressing GFP-R7BP were treated with 2Br-palmitate for 16 h, followed by fixation and analysis by confocal microscopy. (F) FTI and GGTI controls. HEK293 cells expressing GFP-H-Ras (farnesylated protein) or GFP-Rap1A (geranylgeranylated protein) were treated with vehicle or the indicated compounds as in E. Cells were fixed and analyzed by confocal microscopy.

Because R7 family members in brain localize both to the plasma membrane and nucleus, plasma membrane–nuclear shuttling of R7–Gβ5 complexes may be controlled by the palmitoylation state of R7BP. This hypothesis requires that R7 proteins bind palmitoylated or unpalmitoylated R7BP. Accordingly, we compared the ability of RGS7–Gβ5 and RGS9-2–Gβ5 complexes to bind FLAG-tagged forms of wild-type or nonpalmitoylated (C252S/C253S; “SS” mutant) R7BP in coimmunoprecipitation experiments. The results indicated that RGS7–Gβ5 and RGS9-2–Gβ5 complexes bind wild-type or nonpalmitoylated R7BP (Fig. 7, A and B). Likewise, the nonpalmitoylated R7BP mutant recruited RGS7 or RGS9-1 to the nucleus (Fig. 7 C; RGS9-2 was used rather than RGS9-1 because the latter molecule localizes to the nucleus without R7BP). These results suggested that palmitoylation/depalmitoylation of R7BP provides a mechanism that controls shuttling of R7–Gβ5 complexes between the plasma membrane and nucleus.

To test this hypothesis, we determined whether or not depalmitoylation of wild-type R7BP by endogenous protein-thioesterase activity results in shuttling of R7BP–RGS9-1–
R7BP binds to R7 proteins independently of its palmitoylation state.

(A) RGS7 binds wild type or the C252S/C253S mutant form of R7BP. Wild-type or mutant (SS) FLAG-R7BP was expressed in HEK293 cells with HA-RGS7 and MYC-Gβ5. FLAG immunoprecipitates and total lysates were resolved on SDS–polyacrylamide gels and blotted for the expressed proteins. (B) RGS9-2 binds wild type or the C252S/C253S mutant form of R7BP. Methods were identical to A except that HA-tagged RGS9-2 was expressed. (C) Unpalmitoylated R7BP recruits R7 proteins to the nucleus. Experiments were performed identically to those shown in Fig. 4 B except that the C252S/C253S double mutant form of R7BP was used. (D) Depalmitoylation of R7BP results in translocation of RGS9-1 to the nucleus. HEK293 cells were transfected with plasmids expressing wild-type GFP-R7BP, HA-RGS9-1, and MYC-Gβ5. Cells were treated at t = 0 with cycloheximide (30 μg/ml) and 2Br-palmitate (10 μM) to block, respectively, new protein synthesis and repalmitoylation of R7BP that had been depalmitoylated by endogenous thioesterase activity. Confocal microscopy was used to analyze cells fixed at the indicated times after drug treatment.

Gβ5 complexes to the nucleus. We treated cells coexpressing GFP-R7BP, HA-RGS9-1, and MYC-Gβ5 with cycloheximide to block new protein synthesis. As R7BP was depalmitoylated, it was trapped in the unmodified state by blocking repalmitoylation with 2Br-palmitate. Confocal imaging at various times after drug treatment was used to detect translocation of GFP-R7BP and HA-RGS9-1 from the plasma membrane to the nucleus (Fig. 7 D). Quantification of the images indicated that immediately after drug treatment ~90% of cells showed colocalization of GFP-R7BP and HA-RGS9-1 at the plasma membrane. In striking contrast, within 6 h after drug treatment ~65% of cells exhibited colocalization of R7BP and RGS9-1 in the nucleus. The time course of R7BP translocation to the nucleus was similar to the basal rate with which signaling proteins are depalmitoylated in cells (for review see Smoerys and Linder, 2004). Therefore, these results indicated that R7BP palmitoylation/depalmitoylation regulates plasma membrane–nuclear shuttling of R7BP–R7–Gβ5 complexes.

R7BP augments RGS7-mediated regulation of G protein-regulated inward rectifying potassium (GIRK) channel activity

To determine whether or not R7BP affects R7 protein function, we performed experiments that used G protein–mediated activation of GIRK channels expressed in Xenopus laevis oocytes as an assay (Doupnik et al., 2004). Because R7 proteins are GAPs for Gi/Go subunits (Posner et al., 1999; Rose et al., 2000; Hooks et al., 2003), we examined the effects of R7BP on GIRK channels activated by m2 muscarinic acetylcholine (ACh) receptors selectively coupled to Goq using pertussis toxin (PTX)-insensitive Goxα(C351A) (Zhang et al., 2002). As shown in Fig. 8 A, in this system GFP-R7BP dramatically accelerated the kinetics of ACh-activated GIRK currents; this robust effect depended on coexpression with RGS7–Gβ5 complexes. Coexpression of RGS7–Gβ5 and GFP-R7BP accelerated the kinetics of GIRK channel deactivation, activation, and acute desensitization, which are the hallmarks of GIRK channel modulation by the GAP activity of RGS proteins (Doupnik et al., 1997; Saitoh et al., 1997; Chuang et al., 1998).

To quantify the effects of GFP-R7BP, we varied the expression levels of RGS7–Gβ5 by injecting increasing amounts of cRNA. In the absence of GFP-R7BP, increasing the expression of RGS7–Gβ5 moderately accelerated the rate of GIRK current deactivation (Fig. 8 B), as previously reported (Kovoor et al., 2000). Further increases in RGS7–Gβ5 expression (or RGS7 alone) resulted in marked suppression of GIRK current amplitude (unpublished data), as demonstrated previously (Zhang et al., 2002). At the lowest level of RGS7–Gβ5 expression (0.1 ng
GIRK channel activation kinetics were examined at a fixed RGS7–Gβ5 expression level (1 ng each cRNA/oocyte) by varying the ACh dose (Fig. 8 C). These results indicated that non-palmitoylated GFP-R7BP-SS exhibited significantly reduced activity relative to wild-type GFP-R7BP. The palmitoylation-independent effects of mutant R7BP may occur by stabilizing RGS7–Gβ5 complexes and/or augmenting the GAP activity of RGS7–Gβ5 complexes, similar to what has been shown with R9AP and RGS9-1 (Lishko et al., 2002; Hu et al., 2003). Nevertheless, because wild-type R7BP exhibited significantly greater activity relative to nonpalmitoylated R7BP, we conclude that palmitoylation of R7BP is required for efficient targeting of R7BP–RGS7–Gβ5 complexes to the plasma membrane, thereby facilitating modulation of receptor-coupled GIRK channels.

Discussion

We identify R7BP as a novel palmitoylated protein that regulates plasma membrane–nuclear shuttling of any member of the RGS7 (R7) family of G protein regulators bound to Gβ5. We suggest that R7BP is the principal plasma membrane–anchoring protein for R7–Gβ5 complexes in the nervous system because: (a) R7BP, Gβ5, and R7 proteins are expressed highly only in the nervous system (Gold et al., 1997; Zhang et al., 2000; Larminie et al., 2004), in contrast to the R7 anchor R9AP, which is expressed highly only in retina (Hu and Wensel, 2002); (b) R7BP and R9AP are the only closely related members of this protein family of membrane anchors; and (c) the spatial expression of R7BP, Gβ5, and the R7 family in brain are highly coincident (results presented herein; Gold et al., 1997; Zhang et al., 2000).

In parallel with our studies, Martemyanov et al. (2005) recently identified R7BP as a protein that copurifies nearly stoichiometrically with RGS9–2–Gβ5 complexes isolated from brain extracts. They showed that recombinant R7BP interacts in vitro with each R7 isoform, that endogenously expressed R7BP and each R7 isoform can be coprecipitated from brain membrane extracts, and that R7BP protein is expressed highly in various brain regions and in retina but not in several other tissues. These findings complement our results showing that R7BP is as a palmitoylation-regulated plasma membrane–nuclear shuttling protein that augments R7 protein function by targeting them to the plasma membrane. Thus, there is strong evidence that R7BP is the primary membrane anchoring protein for R7 family members in the nervous system.

Why do vertebrate genomes encode two distinct membrane-anchoring proteins for the R7 family: R9AP in retinal photoreceptor cells and R7BP throughout the nervous system? The nonoverlapping expression patterns and different membrane association mechanisms of R7BP and R9AP indicate that these proteins have distinct physiologic and mechanistic functions. R9AP may function constitutively as a membrane-anchoring protein because it possesses a transmembrane domain. R9AP also may possess unique structural or sequence motifs that target R9AP–RGS9–1–Gβ5L complexes to outer segments of photoreceptor cells, analogous to the outer segment-targeting domain at the COOH terminus of rhodopsin (Tam et al., 2000).
R7BP, in contrast, apparently functions as a regulated plasma membrane–nuclear shuttling protein (Fig. 9). R7BP is palmitoylated, a reversible lipid modification that profoundly affects the localization and function of R7BP–R7–Gβ5 complexes. Palmitoylation may target R7BP–R7–Gβ5 complexes to lipid rafts or other specialized domains of the plasma membrane, similar to other palmitoylated proteins (El-Husseini and Bredt, 2002). Indeed, in hippocampal neurons GFP-R7BP concentrates in dendritic foci, as do the neuronal palmitoylated proteins PSD-95 and GAP-43 (El-Husseini and Bredt, 2002). More strikingly, palmitoylation regulates shuttling of R7BP–R7–Gβ5 complexes between the plasma membrane and nucleus. Accordingly, we hypothesize that reversible palmitoylation of R7BP provides a novel plasticity or “tuning” mechanism that modulates GPCR signaling. Depalmitoylation of R7BP and consequent delocalization of R7BP–R7–Gβ5 complexes from the plasma membrane may sensitize signaling mediated by Gi/o-coupled GPCRs. Palmitoylation of R7BP could have the opposite effect by attenuating or desensitizing signaling via Gi/o-coupled receptors or it could enable R7–Gβ5 complexes transduce signals to plasma membrane effectors.

If R7BP depalmitoylation occurs after GPCR activation, R7BP–R7–Gβ5 complexes would be targeted to the nucleus. Because the R7 protein RGS6 can regulate gene expression (Liu and Fisher, 2004), signal-triggered depalmitoylation of R7BP could provide a novel mechanism for transmitting GPCR signals directly to the plasma membrane to the nucleus. Conversely, signal-regulated palmitoylation of R7BP could recruit quiescent nuclear R7–Gβ5 complexes to the plasma membrane to regulate GPCR signaling. These hypotheses are supported by evidence indicating that endogenously expressed R7 proteins and Gβ5 in neurons localize to membranes and nuclei (Zhang and Simonds, 2000; Bouhamdan et al., 2004; Krumins et al., 2004) and that GPCR activation can regulate the palmitoylation status of signaling proteins (for review see Smotrys and Linder, 2004).

Palmitoylation-regulated translocation of R7BP–R7–Gβ5 complexes to and from the plasma membrane would have profound consequences for GPCR-mediated regulation of ion channels and neuronal excitability. The concentration-dependent kinetic effects of RGS7 toward m2 receptor/Gi/o–coupled GIRK channel activity and the augmented effects caused by R7BP coexpression indicate that palmitoylation-regulated R7BP–R7–Gβ5 translocation controls both the amplitude and kinetics of ion channels regulated by Gi/o-coupled receptors, especially GIRK channels and voltage-gated calcium channels (Wickman and Clapham, 1995). Palmitoylated R7BP also may promote GPCR-selective ion channel regulation by targeting specific GPCR–R7–ion channel complexes to lipid rafts where Go, selected GPCRs, and ion channels colocalize (Cabrera-Vera et al., 2004).

R7BP may prove to be crucial for several functions of the central and peripheral nervous systems. This hypothesis is based on evidence indicating that the R7 substrate Go6 is required in the central nervous system for regulation of motor control, motor behavior, and pain sensation (Jiang et al., 1998), and in retinal “ON” bipolar cells for light response (Dhingra et al., 2002). It also is suggested by evidence indicating that RGS9 is required to regulate antinociception via μ-opioid receptors and locomotor activity via D2 dopamine receptors (Rahman et al., 2003; Zachariou et al., 2003).

In conclusion, many neuronal signaling proteins including β-adrenergic receptors, G protein α subunits, R7BP, several RGS isoforms, PSD-95, nonreceptor tyrosine kinases, and H-ras are palmitoylated (for reviews see El-Husseini and Bredt, 2002; Smotrys and Linder, 2004). The diversity of palmitoylated proteins and the large family enzymes that palmitoylate them (Fukata et al., 2004) raise intriguing questions about how palmitoylation occurs in a specific and regulated manner to modulate nervous and sensory system function. Further analysis of R7BP palmitoylation may reveal novel mechanistic principles applicable to a variety of neuronal signaling proteins.

Materials and methods

Reagents and antibodies

For Western blots, anti-FLAG-M2 HRP conjugate (Sigma-Aldrich) was used at a dilution of 1:5,000, anti-MYC 9E10 HRP conjugate (Roche) at 1:1,000, anti-HA 12CA5 HRP conjugate (Roche) at 1:2,000, and HRP-conjugated goat anti–mouse secondary antibodies (Pierce Chemical Co.) at 1:10,000. For immunofluorescence, the anti-HA-11 antibody (BABco) was used at a dilution of 1:500, anti-FLAG M2 (Sigma-Aldrich) at 1:1,000, and goat anti–mouse Alexa 568 secondary (Molecular Probes) at 1:100. 2β-palmitate was obtained from Sigma-Aldrich. Hydroxylamine and sodium salicylate were purchased from Fisher Scientific. FTI-277 and GGTI-298 were obtained from Calbiochem. Unless noted otherwise, all chemicals were obtained from Sigma-Aldrich.

Cell culture and transfection

HEK293 cells (American Type Culture Collection) were maintained and transfected in DME/F12 with 10% FBS (Atlanta Biologicals) plus penicillin/streptomycin. Cells were grown at 37°C and 5% CO2.

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HEK293 cells was performed using Effectene (QIAGEN) according to the manufacturer’s instructions. Neonatal rat hippocampal neurons were isolated and cultured on glass coverslips coated with laminin and poly-D-L-ornithine as described previously (Wilding and Huettner, 2001). Neurons were transfected with R7BP plasmids via calcium phosphate precipitation.

R7BP cloning

RNA was prepared from adult male mouse brain using TRIZOL (Invitrogen) according to the manufacturer’s instructions. 1 µg of total RNA was used in a reverse transcriptase reaction using oligo-dT primers and Superscript II (Invitrogen) according to the manufacturer’s instructions. cDNA from reverse transcriptase reactions was used as template for PCR to amplify the coding region of R7BP using the following primers that included engineered BamHI and EcoRI sites: 5'-gtgagctctgtcagctgcagctg-3'; 5'-aagaattcagcatagaaacctg-3'. A 904-bp PCR product was generated, which subsequently was cloned into the BamHI–EcoRI sites of pcDNA3.1. The entire insert was sequence and found to be identical to a consensus cDNA sequence for R7BP that was generated by assembling all sequence information available from publicly R7BP homologues in the following species: human (XP_376386), orangutan (CAH92694), chimpanzee (XP_5181291), mouse (BAC32849), rat (XP_215473), African clawed frog (AAH73094), spotted green pufferfish (CAF93443), pig (CO949728), zebrafish (AF286707). This cDNA sequence was used as a template for PCR to amplify the coding region of R7BP. The PCR product was cloned into the BamHI–EcoRI sites of pcDNA3.1 and sequenced with the same primers used for cloning. A PCR fragment containing the complete RGS4-GFP coding region (flanked with BamHI and XhoI sites) from FLAG-GFP plasmid (Clontech) to create a MYC-tagged fusion protein. RGS4-GFP fusion protein was generated by cloning a fragment containing the complete RGS4-GFP coding region from a pFLAG-CMV10 (Sigma-Aldrich). pBlueScript-R7BP was constructed by cloning a BamHI–EcoRI fragment containing the R7BP coding region into pBlueScript II SK (+). Plasmids expressing HA-tagged versions of RGS5, RGS7, RGS9-1, RGS9-2, and RGS11 along with a FLAG-tagged version of G56 were gifts of T.K. Harden (University of North Carolina, Chapel Hill, NC). A plasmid encoding a version of G56 was constructed by amplifying the G56 coding region (flanked with BamHI and XhoI sites) from FLAG-G56. This PCR product was digested and cloned into the BamHI–XhoI sites of pCMV-Tag 3A (Stratagene) to create a MYC-tagged fusion protein. RGS4-GFP fusion protein was generated by cloning a fragment containing the complete RGS4-GFP fusion from pYTV-RGS4-GFP (Srinivas et al., 1998) into pC3X for expression in mammalian cells. GFP-tagged versions of Rap1A and H-Ras were gifts of M. Phillips (New York University School of Medicine, New York, NY). A FLAG-tagged ERK2 plasmid was a gift of M. Weber (University of Virginia Health Sciences Center, Charlottesville, VA). R7BP point mutants were generated using Quikchange (Stratagene) according to the manufacturer’s instructions. All constructs and point mutants were verified by DNA sequencing.

Cell lysis, immunoblotting, and immunoprecipitation

Cells were washed once with ice cold PBS and lysed in lysis buffer (MCLB: 50 mM Tris, pH 8.0, 5 mM EDTA, 0.5% NP-40, 100 mM NaCl, 1 mM sodium orthovanadate, and 1 mM PMSF) supplemented with protease inhibitor tablets (Roche). Cells were incubated at 4°C overnight by end-over-end rotation at 4°C with media (DME/F12 with sodium pyruvate, nonessential amino acids, and 10% dialyzed FBS). Cells were washed with PBS, blocked with 0.1% BSA (in PBS) at 4°C and washed again with PBS. Cells were incubated with primary antibodies (diluted in 5% goat serum) for 20 min at 37°C in a humidified chamber for 10 consecutive washes in PBST. Secondary antibodies (diluted in 5% goat serum) were incubated with cells for 15 min at RT followed by 10 washes in PBST. Slides were mounted for confocal microscopy. Confocal microscopy was performed with a laser scanning confocal microscope (model LSM-510; Carl Zeiss Microimaging, Inc.). GFP-R7BP localization in medial confocal sections of z-axis stacks was quantified by using Adobe Photoshop to determine integrated pixel intensities in the nucleus versus the entire cell and expressing the data as a nuclear/total cell ratio.

Metabolic labeling

HEK293 cells were grown and transfected in 6-well plates for labeling experiments. For [3H]methionine labeling, cells were starved 60 min of methionine (methionine-free DME/F12 with sodium pyruvate and penicillin/streptomycin) followed by labeling for 90 min in labeling media (starvation media plus 10% dialyzed FBS and [3H]methionine (Amersham Biosciences) at 100 µCi per well). After labeling, cells were lysed and processed for immunoprecipitation. For SDS-PAGE, gels were dried and exposed to film at −70°C. For [3H]palmitate labeling, cells were washed with media (DME/F12 with sodium pyruvate, nonessential amino acids, and penicillin/streptomycin) and incubated with labeling media for 90 min in labeling media (starvation media plus 10% dialyzed FBS and [3H]palmitate (Amersham Biosciences) at 10 µCi per well). Cell lysis, immunoprecipitation, and SDS-PAGE were performed as described for [3H]methionine labeling. Gels containing [3H]palmitate were treated with fluor solution (1 M sodium salicylate in 15% methanol) for 30 min before drying and fluorography at −70°C. For hydroxylamine treatment, gels containing either [3H]methionine- or [3H]palmitate-labeled R7BP were treated overnight with 0.5 M NH4OH, pH 7.0, or 0.5 M Tris, pH 7.0, in 50% 2-propanol. Gels were washed several times over 2 d with 50% 2-propanol before fluorography.

Lipid analysis

Cells transfected with either FLAG-R7BP or FLAG-ERK2 were labeled with [3H]palmitic acid. Cells were lysed, immunoprecipitated with anti-FLAG antibodies, and resolved on SDS-polyacrylamide gels. Flag-tagged proteins were excised from the gel and subjected to treatment with NaOH, as described previously (Linder et al., 1995). Lipids were extracted from base hydrolysates with chloroform/methanol (1:2), dissolved in chloroform, spotted on reverse-phase TLC plates (Whatman) along with [3H]-labeled C14:0, C16:0, and C18:0 standards. Plates were developed in acetone/isooctane (9:1) and dried with EN'FLAME (Perkin Elmer), and exposed to film at −70°C.
Electrophysiology
All procedures for the use and handling of X. laevis (Xenopus One) were approved by the University of South Florida Institutional Animal Care and Use Committee in accordance with National Institutes of Health guidelines. Oocytes were enzymatically isolated from ovarian tissue as described previously (Zhang et al., 2002). Stage V–VI oocytes were selected and stored in oocyte culture medium (OCM) at 19°C in 35-mm dishes on an orbital shaker. OCM was changed once to two times daily and was composed of 82.5 mM NaCl, 2.5 mM KCl, 1.0 mM CaCl2, 1.0 mM MgCl2, 1.0 mM NaH2PO4, 2.5 mM Na-pyruvate, and 5.0 mM Hepes, pH 7.5, containing 2% heat-inactivated horse serum.

The effects of R7BP on GIRK channels activated by Gsa-coupled muscarinic m2 receptors were examined with and without RGS7 expression in experimental groups of oocytes injected with different mixtures of cRNAs synthesized in vitro from linearized cDNA vectors (mMessage mMACHINE; Ambion). stock cRNAs for each cRNA were dissolved in DEPC-treated water and concentrations quantified by spectrophotometric absorbance at 260 nm. Absorbance profiles from 220 to 320 nm were performed to assess the quality of the stock cRNAs. Mixtures of different cRNAs were prepared in DEPC-treated H2O so that amounts of each cRNA injected per oocyte reflected that from a 50-nl injection of the cRNA mixture using a positive-displacement nanoliter injector (Nanoinjector; World Precision Instruments). All experimental groups were injected with cRNAs for rat Kir3.1 (0.5 ng/oocyte), mouse Kir3.2a (0.5 ng/oocyte), human m2 muscarinic receptor (0.5 ng/oocyte), PTX-insensitive mouse Gsa (0.5 ng/cRNA/oocyte), and PTX-S1 cRNA (1 ng/oocyte) to inactivate endogenous Gsα/gsubunits (Zhang et al., 2002).

Agonist-activated GIRK currents (IKg) and receptor-independent basal GIRK channel activity (IKbasal) were measured for each of the experimental groups by two-electrode voltage clamp recording (GeneClamp 500; Axon Instruments, Inc.). electrodes were constructed from borosilicate glass capillary tubes (1.5-mm outside diameter, 0.86-mm inside diameter; GC150F-10; Warner Instruments) using a programmable microelectrode puller (P-97; Sutter Instrument Co.). the electrodes had tip resistances of 0.8–1.0 MΩ after filling with 3 M KCl. Membrane currents from voltage clamped oocytes were digitized (Digidata 1200 acquisition system; Axon Instruments, Inc.) and stored on a PC computer running pClamp 10.0 software (Axon Instruments, Inc.).

Oocytes were initially superfused with a minimal salt solution composed of 98 mM NaCl, 1 mM MgCl2, and 5 mM Hepes, pH 7.5 (NaOH). After electrode impalement and clamping the membrane potential to −80 mV, the solution was changed to a high K+ solution composed of 20 mM KCl, 78 mM NaCl, 1 mM MgCl2, and 5 mM Hepes, pH 7.5 (NaOH). high K+ induced an inward current (IKbasal) that is comprised primarily of receptor-independent GIRK channel activity (Dascal et al., 1993). Rapid application and washout of a range of ACh concentrations (Sigma-Aldrich) was performed with a computer controlled superfusion system (SF-77B; Warner Instruments; Doupnik et al., 2004). Voltage ramps from −80 to +20 mV and 1 s in duration were evoked before and during agonist application to monitor inward rectification of the ACh-evoked current. All recordings were performed at RT (21–23°C). GIRK current deactivation kinetics were analyzed using pClAMP software to derive deactivation time constants (τdeact) associated with agonist washout. Statistical comparisons between the various experimental groups were performed by one-way ANOVA where P < 0.05 was considered significant.

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