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Structure, Function, and Localization of Gβ5–RGS Complexes

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Abstract

Members of the R7 subfamily of regulator of G protein signaling (RGS) proteins (RGS6, 7, 9, and 11) exist as heterodimers with the G protein beta subunit Gβ5. These protein complexes are only found in neurons and are defined by the presence of three domains: DEP/DHEX, Gβ5/GGL, and RGS. This article summarizes published work in the following areas: (1) the functional significance of structural organization of Gβ5–R7 complexes, (2) regional distribution of Gβ5–R7 in the nervous system and regulation of R7 family expression, (3) subcellular localization of Gβ5–R7 complexes, and (4) novel binding partners of Gβ5–R7 proteins. The review points out some contradictions between observations made by different research groups and highlights the importance of using alternative experimental approaches to obtain conclusive information about Gβ5–R7 function *in vivo*.

I. Introduction

A. G Proteins, RGS Proteins, and R7 Family

In the classical paradigm of G protein signaling, the agonist-bound G protein-coupled receptor causes the release of GDP from the G protein α subunit. The subsequent binding of GTP leads to the dissociation of Gα-GTP from the permanently associated Gβγ subunit complex. Both Gα-GTP and Gβγ can modulate the activity of effector enzymes and ion channels, which dynamically control the intracellular concentration of second messengers. The activated state of this pathway is terminated by GTP hydrolysis and reassembly of the inactive Gαβγ heterotrimer. The Gα subunits possess an intrinsic GTPase activity that is too slow to support the rapid termination of most G protein-mediated signaling processes observed *in vivo*. In the 1990s, several groups established that most G protein signaling pathways have a component that accelerates this GTPase activity. In fact, researchers identified a large family of such GTPase activating proteins (GAPs), which are now called regulators of G protein signaling (RGS) proteins. There are more than 30 members of this diverse family, which are defined by the presence of the characteristic domain (RGS box) responsible for the interaction with the Gα subunits and for the GAP activity. RGS proteins have been reviewed quite extensively[1–8] (see other chapters in this volume).

GAP activity toward Gα subunits is the function that defined this class of RGS proteins; however, most of the RGS proteins also contain distinct domains and perform other functions. This chapter will concentrate on a specific subfamily of RGS proteins, the R7 subfamily, which includes RGS6, 7, 9, and 11. This subfamily came into the focus of several laboratories in 1998 when it was discovered that two of its members, RGS7[9] and RGS11,[10] bind to the G protein subunit Gβ5. This article summarizes the most significant results in studies of these protein complexes. I will use the term “Gβ5–R7” when referring to the entire family of these complexes and, for example, “Gβ5–RGS9” when referring to the complex involving a specific member of the R7 family.

B. Gβ5 is a Unique G Protein β Subunit that Interacts with RGS Proteins of the R7 Family

Gβ5 was the last member of the G protein beta subunit family to be cloned.[11] The initial characterization showed that Gβ5 was different in several ways from the four previously identified members of the Gβ subunit family. The amino acid sequence of Gβ5 is only ~50% identical to the rest of the family, whereas Gβ subunits 1 through 4 share ~90% identity to each other. Gβ5 is also longer by about 10 amino acids than the other Gβ subunits. Gβ subunits 1–4 are expressed throughout the body, while endogenous Gβ5 was detected only in nervous tissue or cells of neuroendocrine origin.[5,11,12] There are two products of the Gβ5 gene that result from alternative splicing, Gβ5 ("short," sometimes referred to as Gβ5S) and the longer splice form, Gβ5L, that has a 42 amino acid extension at the N-terminus. Gβ5L is present exclusively in the outer segments of photoreceptor neurons (both rods and cones).[12–15]

One intriguing finding that indicated that Gβ5 could be an unusual member of the Gβ family was its subcellular localization. In native tissues and cells, Gβ subunits types 1–4 associate with the plasma membranes via Gγ subunits, which are prenylated. The only exception is the Gβγ complex of rod photoreceptor transducin (Gβ1γ1), which, due to the unique prenylation of the Gγ1 subunit, can detach from the membranes upon activation and dissociation from Gα-GTP (see Refs. 16–19 for reviews). In all other tissues, Gβγ subunits are tightly associated with the membranes regardless of the G protein activation status.[20] In contrast, upon ultracentrifugation of brain homogenates, Gβ5 distributed almost equally between the soluble (cytosolic) and particulate fractions that presumably represent the membranes. Furthermore, in the retina, Gβ5 is almost entirely soluble. At the same time, the longer splice version of Gβ5, Gβ5L, was entirely membrane-bound and cofractionated with proteins involved in phototransduction such as rhodopsin.[12]

Initial studies showed that, like other Gβ subunits, Gβ5 could interact with Gγ subunits when Gβ5 and Gγ cDNAs were coexpressed in cultured model cell lines *in vitro*. The Gβ5–Gγ2 complex was shown to stimulate PLCβ, a Gβγ effector, and this Gβ5γ2-stimulated PLC activity was reduced upon cotransfection of Gαi.[11,12] These experiments confirmed, that Gβ5 was, in fact, a functional Gβ subunit capable of interaction with Gγ, Gα, and a Gβγ-effector. Further studies demonstrated that Gβ5–Gγ complexes were significantly different from conventional Gβγ complexes, such as Gβ1–Gγ2, in the ability to influence the activity of effectors such as adenylate cyclase or ion channels[21–26] (reviewed in Ref. 27). However, the most significant fact about the complexes of Gβ5 with Gγ subunits is that they have never been detected in native tissues or cells. Instead, Gβ5 was found in a complex with RGS proteins of the R7 family.[9,14,28] Gβ5 associates with the G gamma-like (GGL) domain, that is present in these RGS proteins[10,29,30] (reviewed in Refs. 6, 31, 32). The GGL domains are highly selective for Gβ5 and do not bind to other Gβ subunits. Swapping the GGL domain for a fragment of Gβ1[30] or certain point mutations in the GGL domain[33] can switch the specificity of the RGS subunit from Gβ5 to conventional Gβ. Like Gβ5, the R7 family RGS proteins were only found in the nervous system, where distribute between the membranes in a fashion similar to Gβ5. For example, RGS9-1, which is the GAP for rod photoreceptor G protein transducin, is 100% membrane-bound, and RGS7, which is found in the brain, is distributed between the cytosol and the membranes.

Two strong lines of evidence support the notion that the physiologically relevant binding partners of Gβ5 are GGL domain-containing RGSs rather than Gγ subunits: biochemical purification of native Gβ5–R7 complexes and mutual stabilization of Gβ5 and R7 subunits against proteolytic degradation. Efforts to identify Gβ5–Gγ complexes in native sources by conventional or immunoaffinity chromatography have invariably failed. Gγ subunits were not detected in the final preparations of purified native Gβ5, which were isolated by conventional[14] or immunoaffinity[28] chromatography. Furthermore, the entire pool of native

Gβ5 completely separated from Gβ1 at the initial stages of ion exchange or size-exclusion chromatography.[9,14] What was particularly unusual, was that the native Gβ5 complex bound to anion exchange resins from which it could be eluted by salt. In contrast, the majority of other proteins, including Gβ1, did not bind to the negatively charged matrixes at all. This showed that the physico-chemical properties of the Gβ5 complex are strikingly different from conventional Gβγ, consistent with the idea that instead of Gγ, Gβ5 is associated with a different, larger protein that is positively charged. Indeed, experiments with *in vitro* translated RGS7 and Gβ5 showed that RGS7 was, in fact, responsible for binding of the Gβ5–RGS7 complex to cation exchange resins.[30]

One possible explanation of the separation of the native Gβ5 and Gγ subunits was the reported instability of Gβ5–Gγ complexes in mild detergents such as cholate and CHAPS.[34,35] However, Gβ5 behaved as a complex with RGS in the nonionic detergent Genapol C100 in which recombinant Gβ5–Gγ complexes were stable.[14,34,36] This argues against the idea that native Gβ5–Gγ complexes cannot be detected simply because they are unstable under conditions of cell lysis and protein purification.

Two studies used biochemical and biophysical approaches to compare the affinity of Gβ5 to RGS7 versus Gγ2. Levay et al. showed that RGS7 bound to Gβ5 preferentially when Gβ5, RGS7, and Gγ2 were expressed in reticulocyte lysate, then mixed and analyzed by chromatography.[30] More recently, Yost et al. analyzed the interaction of Gβ5 with several Gγ subunits, RGS7, and R7BP using the bimolecular fluorescence complementation (BiFC) assay in cotransfected HEK cells.[35] In this method, two nonfluorescent “halves” of a fluorescent protein were fused to Gβ and Gγ subunits or RGS7 so that the interaction between Gβ5 and Gγ or Gβ5 and RGS7 reconstitutes the fluorophores. This comprehensive study concluded that Gβ5 had a slight preference for Gγ2 versus RGS7. It was also found that if R7BP was coexpressed together with Gβ5, Gγ2, and RGS7, the affinity of Gβ5 for Gγ2 versus RGS7 appeared to be similar. Noteworthy, the BiFC studies confirmed earlier observations that Gβ5 prefers to dimerize with Gγ2 rather than other Gγ subunits, as shown earlier, for example, in Refs. 11, 12, 26. At the same time, the interaction of Gβ5 with GGL domains of all R7 members was equally robust.[10,13,30,37] Current data show that once Gβ5 binds to a GGL domain the Gβ5–R7 complex can only be dissociated under denaturing conditions.

The second line of evidence that strongly supports the notion that Gβ5 prefers to pair with R7 RGS proteins rather than with G protein γ subunits, is the mutual stabilization of Gβ5 and R7 proteins. If Gβ5 and R7 proteins are expressed together, for example, in transiently transfected COS-7 cells, the expression levels of both proteins are several fold higher compared to when they are expressed separately. As shown by pulse-chase analysis, this occurs due to the more than 10-fold rapid degradation of the monomers compared to the Gβ5–RGS7 dimer.[14] Consistent with this stabilization mechanism, the knockout of RGS9 gene results in the disappearance of the Gβ5 protein (but not the Gβ5 mRNA) from the native cells.[38] Likewise, knockout of Gβ5 leads to disappearance of the entire R7 protein family, while the R7 mRNA levels remain intact.[39] The C. *elegans* Gβ5 and R7 orthologs also mutually stabilize each other.[40] As discussed below, the principle of mutual stabilization is also relevant for the third subunit in the Gβ5–R7 complexes, the membrane anchoring proteins R9AP and R7BP.

It is clear that Gβ5 has the capacity to bind to both Gγ subunits and R7 family RGSs. However, no evidence for the existence of Gβ5–Gγ complexes *in vivo* has been found to date. Therefore, we should consider the Gβ5–RGS complexes to be physiologically relevant entities. At the same time, as pointed out, for example, in reference 27, experiments that utilize recombinant Gβ5–Gγ complexes can identify potential interacting partners of the Gβ–

GGL moiety and shed light on the role of specific structural elements within Gβ5 and its complexes with R7 proteins.

II. Structure of Gβ5–R7 Complexes. The Role of RGS, GGL, and DEP Domains

A. Multidomain Organization of Gβ5–R7 Complexes

The R7 family members are ~55 kDa proteins defined by the presence of three domains: the RGS box localized in the C-terminal portion, the GGL domain localized near the center of the protein, and the N-terminal DEP (first identified in Dishevelled, Egl-10, and plekstrin) domains. These three domains were identified through sequence alignments.[10,29,30] In addition, the recently resolved 1.95-Å crystal structure of the Gβ5–RGS9 complex[41] showed that the relatively long linker between the DEP and GGL domains contains a novel domain termed DEP helical extension (DHEX). Thus, all Gβ5–R7 dimers consist of four distinct structural units: the RGS box, the "Gβγ" represented by Gβ5/GGL moiety, the DEP, and DHEX domains (Fig. 1).

There is a longer splice version of RGS9 that has a 191 amino acid extension at the C-terminus.[42] This ~77 kDa RGS9 gene product is termed RGS9-2 and is expressed in the brain, where it is enriched in striatum.[43–45] The unique C-terminal extension contains the sequence similar to the G protein effector enzyme PDE6, which enhances the GTPase-stimulating activity of the RGS domain.[46] The originally cloned ~55 kDa splice variant of RGS9, RGS9-1, is expressed exclusively in rod and cone photoreceptors.[45,47,48] A recent report showed that RGS9-2 can functionally substitute for RGS9-1 in photoreceptors and, in fact, appears to be a more efficient inhibitor of transducin than RGS9-1.[49]

RGS6 was reported to have as many as 36 splice forms, most of which are shorter than the "normal" ~55 kDa R7 family member and lack portions of the DEP and GGL domains.[50] When these splice forms are expressed in transfected mammalian cell lines, they differentially interact with their binding partners and localize within the cells. However, it is not yet clear if the protein products of these multiple splice forms are expressed in native tissue because immunoblots with antibodies against RGS6 detected either a single[51,52] or 2–3[50,53] distinct bands in the 40–55 kDa range. These bands can represent splice versions of RGS6, but this has not yet been proven.

The crystal structure of the Gβ5–RGS9-1 complex provided the much needed insight into how the domains of the Gβ5–R7 dimers are arranged in space. Since the amino acid homology between Gβ5 and Gβ subunits 1–4 and between the GGL domains and Gγ subunits is substantial, it was predicted that the structure of the Gβ5–GGL complex was similar to that of the conventional Gβγ.[10] The crystal structure confirmed that prediction. Like the originally crystallized Gβγ complex,[54] the Gβ5–GGL moiety consists of seven β-sheet repeats, referred to as "blades" that are arranged into a characteristic fold, the seven-blade propeller. This structure has a distinct void space surrounded by the blades, so it resembles a doughnut. The most striking as well as unexpected insight from the crystal structure of the Gβ5–RGS9-1 complex is that the Gβ5–GGL toroid is sandwiched between the RGS and DEP domains. Both of these domains make distinct contacts with the amino acid residues of the Gβ5–GGL moiety. The RGS domain binds to the broader side (the "bottom") of the Gβ5–GGL doughnut, and the DEP-DHEX domain interacts with the opposing tapered end (Fig. 1). Whereas the discovery of the interaction of the RGS domain with Gβ5–GGL was unexpected, the association between the DEP domain and Gβ5 was hypothesized earlier based upon results of biochemical studies[55] and functional complementation assays.[56] The crystal structure not only confirmed those predictions, but also showed clearly that the binding site for the DEP-DHEX domain overlaps with the

interface that corresponds to the Gα-binding surface of Gβ1.[57] This structural insight can provide one potential explanation of why the Gβ5–GGL moiety has not been found to interact with Gα subunits.

The significance of the elaborate multidomain architecture of the Gβ5–R7 complexes remains to be understood. The overall domain structure of the complex is conserved between round worms,[29] insects,[58] and mammals. Since it was retained during evolution, it stands to reason that the functions acquired in the process of combining a Gβγ complex with DEP/DHEX and RGS domains within one molecule were beneficial. It is unlikely that the increased stability of Gβ5–R7 dimers compared to monomers is the sole advantage offered by this complex formation. The domains must have a role in signal transduction, for example, in regulating protein–protein interactions of the other domains, facilitating assembly of larger signaling complexes, or directing subcellular localization of these molecules. To understand the biological significance of association of Gβ5 and R7 proteins, several laboratories analyzed the role of specific domains. These studies lead to the following insights.

B. RGS Domain and GAP Activity

Early tests of the isolated RGS domain of RGS7 showed that it can accelerate the GTPase activity of purified recombinant Gαo and Gαi.[59,60] It was also shown that the RGS domain of RGS9 has GAP activity toward transducin, a Gi family member that is only expressed in rod photoreceptors.[47] Another member of the R7 family, RGS11, also possessed GAP activity.[10] The fragment of RGS11 containing the RGS and GGL domains and lacking the DEP domain (RGS11ΔD) was expressed and purified as a dimer with Gβ5. The remarkable feature of this Gβ5–RGS11ΔD complex was that its GAP activity was specific toward Gαo. The researchers found that the Gβ5– RGS11ΔD complex did not affect the GTPase activities of Gaq and even Gai. In agreement with this finding, recombinant full-length RGS6 and RGS7 purified from baculovirus-infected insect cells as dimers with Gβ5 also did not affect the GTPase activity of Gαq.[37] These observations were in sharp contrast with control experiments where a different RGS protein, RGS4, robustly stimulated the GTPase reaction of both the Gi and Gq family G proteins. Thus, one notable feature revealed by assays of GAP activity of the R7 family is their apparent selectivity toward the Gi family of G proteins. This feature, however, is not unique for RGS11 or the R7 family as selectivity for specific G proteins was discovered earlier, for example, for RGS2, which was shown to be a selective inhibitor of Gq signaling.[61,62] Another notable feature of the intrinsic GAP activity of the RGS domain of RGS7 is that it was about 10-fold weaker compared to the GAP activity of RGS4 with the same substrate, purified recombinant Gαo, that was used in these analyses.[63]

Consistent with their GAP activity toward Gαi class proteins, RGS7 and RGS9 increased the rate of inactivation of GIRK potassium channel activity in oocytes.[64–66] In these assays, oocytes were injected with cRNA encoding M2 muscarinic receptor, Gβ5, and R7 proteins, and the GIRK subunits. These potassium channels open in response to receptor stimulation because activated Gi generates free Gβγ complexes, which directly bind to the channels and cause them to open.[67,68] Although the mechanism of regulation of GIRK channels by G protein a and bg subunits is rather complex,[69–71] it is reasonable to assume that RGS proteins influence this process by accelerating the G protein GTPase reaction. GTP hydrolysis facilitates the reassociation of Gβγ with Gα-GDP, which forms the inactive Gαβγ heterotrimer. It was found that both the monomeric R7 subunits (RGS7 or RGS9) and their dimers with Gβ5 had the ability to accelerate the kinetics of GIRK channel closure.[64,65]

In transfected cells, the isolated RGS domain of RGS7,[60,72] full-length monomeric RGS7,[14,73] and the RGS7–Gβ5 complex[14,55,74] were reported to inhibit GPCR-mediated

Ca^{2+} mobilization. GPCRs that were used in those studies mobilize calcium from internal stores via PLCβ-mediated generation of IP3, which is a Gq-mediated pathway. It is important to note here also that one of the two R7 RGS orthologs in *C. elegans*, EAT-16, antagonizes the function of Gq (Egl30),[75] providing further evidence that R7 family proteins can regulate Gq-mediated signal transduction. These findings were hard to rationalize because RGS7 did not have GAP activity for Gαq.[37,76] Hooks et al. performed a very thorough characterization of the entire R7 family by reconstituting them together with purified Gαi(1–3), Gαo, Gαq, and Gα11 in liposomes containing muscarinic M1 and M2 receptors.[76] They found that members of the R7 family had different specificities and potencies toward G proteins. For example, all R7 members preferred Gαo as the substrate as compared to the other three Gαi subtypes, and RGS11 was the most potent stimulator of the GTPase reaction among R7 family members. Their results also showed unequivocally that R7 family RGS proteins did not accelerate the GTPase activity of Gαq or Gα11.

If R7 RGS proteins do not possess GAP activity toward the Gq family, what mechanism is responsible for the downregulation of Gq signaling by RGS7? One reasonable idea to explain the discrepancy between the absence of GAP activity of R7 proteins toward Gq versus the negative effects of RGS7 or EAT-16 on Gq signaling in live cells is the requirement of a cofactor that is necessary for the GAP activity. This idea is based on the analogy with regulation of transducin GTPase activity by RGS9-1 in the photoreceptor system. It is known that the full GAP activity of RGS9-1 toward transducin requires the presence of the γ subunit of retinal cGMP phosphodiesterase (PDEγ), the effector enzyme of transducin.[77] PDEγ increases the affinity of RGS9-1 to its substrate, Gαt-GTP.[78,79] A cofactor functionally similar to PDEγ could be missing in the *in vitro* GTPase activity assays utilizing purified R7 proteins and other components. The alternative model explaining the detected influence of R7 proteins with Gq-mediated signaling is that the mechanism underlying these effects does not involve the acceleration of GTPase activity and the RGS domains of R7 proteins.

C. The Role of Gβ5 within the Complex

After the discovery of Gβ5–R7 complexes, researchers thought of them both as the “RGS proteins with attached Gβγ” and “Gβγ fused to the DEP and RGS domains.” In fact, these novel protein complexes have been designated in the literature either as “R7–Gβ5” or “Gβ5–R7.”

The analogy with conventional Gβγ dimers implied that Gβ5–R7 complexes could influence the activity of known effectors of Gβγ and/or interact with some of the multiple binding partners of the Gβγ complexes. To test this concept, in one of the first reports investigating the function of Gβ5–R7 complexes, Posner et al. measured the effect of recombinant Gβ5–RGS6 and Gβ5–RGS7 dimers on the activity of phospholipase C β1 and β2.[37] They found that neither the Gβ5–RGS7 nor the Gβ5–RGS6 dimer could directly influence the activity of these enzymes. However, the researchers noticed that both Gβ5–RGS6 and Gβ5–RGS7 attenuated Gβγ-mediated activation of PLCβ2. This experiment showed that Gβ5–R7 can potentially compete with Gβγ for some of its targets, and therefore must have structurally resembled a Gβγ complex. It seems unlikely that the observed effect mimics the physiological function of Gβ5–R7 because the inhibitory effect was modest, reaching only 30% of Gβγ-stimulated PLC activity. The idea that the Gβ5–R7 complex could compete with Gβγ for the interaction with its effector was in agreement with the finding[24] that Gβ5–Gγ complexes expressed in transfected cells did not activate GIRK channels, but instead inhibited them, evidently by competing with endogenous Gβγ. Whether or not such an antagonism with Gβγ takes place in physiologically relevant systems remains to be seen. So far, none of the performed experiments support the idea that Gβ5–R7 proteins could directly influence the activity of known G protein effectors, bind to Gα subunits, or perform other

functions characteristic of the canonical Gβγ complexes assembled from Gβ1–4 and the Gγ subunits.

Another direction to study the role of Gβ5 within the Gβ5–R7 complex was to determine the effect of Gβ5 or the Gβ5/GGL moiety on the known activity of the R7 subunit, such as the interaction of the RGS domain with G protein α subunits. This approach required comparative measurements of the activity of the monomeric R7 subunit with and without Gβ5. The first such assay tested whether or not Gβ5 interfered with the association of monomeric RGS7 with Gα. RGS7 was expressed in reticulocyte lysate and applied to Ni^{2+}-agarose beads with immobilized recombinant hexahistidine-tagged Gαo. It was shown that the addition of Gβ5 drastically reduced the RGS7-Gαo interaction in this pull-down assay.[30]

Testing the effects of Gβ5 on R7 activity in cellular systems was complicated by the fact that Gβ5 increased the expression level of the RGS7 subunit; therefore the results were hard to interpret.[14,64,65] Measurements of RGS GAP activity in single-turnover GTPase assays required a substantial amount of purified recombinant RGS. While small RGS proteins such as RGS4 or the isolated RGS box of RGS7 could be produced in *E. coli* with relative ease, full-length R7 family proteins were expressed in bacteria in insoluble forms. Furthermore, because of their degradation in the absence of Gβ5, monomeric R7 subunits were expressed at a low level in eukaryotic cells and therefore, difficult to purify in sufficient quantity. These technical difficulties were ultimately overcome by accumulation of the required amounts of monomeric full-length RGS9-1 expressed using the baculovirus/insect cell system. Measurements of RGS9 GAP activity toward transducin confirmed the general notion that Gβ5 can act as a negative regulator of the RGS domain.[80,81] An essential new insight from these studies was that compared to the isolated RGS box of RGS9-1, the Gβ5–RGS9-1 dimer has a better selectivity toward the complex of the GTP-bound Gα subunit with the effector.[78] In other words, one of the roles of Gβ5 within its complex with the R7 protein might be to allow the GTP-bound Gα subunit to interact with its effector before the RGS domain inactivates the G protein, thereby ensuring that the initial signal is not terminated prematurely.

The deciphered crystal structure of Gβ5–RGS9[41] showed that the conformation of the RGS domain within the Gβ5–RGS9-1 dimer is essentially identical to the earlier determined structure of the RGS9 RGS box.[82] *In silico* docking of a Gα–GDP-AlF_4 structure to the RGS domain of the Gβ5–RGS9 dimer showed that there is a clash between Gβ5 and Gα.[41] These results suggest that the inhibition of RGS–Gα interaction by Gβ5 that was observed earlier in the biochemical assays.[30,80,81] may result from steric hindrance between Gβ5 and Gα rather than allosteric regulation of the activity of the RGS domain by the Gβ5/GGL moiety. It was also hypothesized that a small change in the overall conformation of the Gβ5–RGS9 dimer could result in the unmasking of the Gα binding site on the RGS domain. Since Gβ5–R7 dimers have GAP activity, they must transiently interact with Gα subunits. Therefore, it is clear that the proposed conformational change allowing Gβ5 to be displaced by Gα does indeed take place, which indicates that the Gβ5–R7 heterodimer is sufficiently flexible to allow Gβ5/GGL and RGS domains to move relative to each other. It is likely that physiologically relevant molecular events such as interactions with GPCRs, other proteins, or posttranslational modifications can influence the ability of Gβ5/GGL moiety to unmask the RGS domain.

As mentioned earlier, studies showed that Gβ5 can also interact with the DEP domain of the R7 subunit.[41,55,56] Protein–protein interaction assays utilizing FRET and affinity pull-downs indicated that, in contrast to binding to the GGL domain which is irreversible, the interaction of Gβ5 with the DEP domain was dynamic.[55] In a GST pull-down assay the immobilized recombinant DEP domain of RGS7 could bind various forms of Gβ5: Gβ5, Gβ5–Gγ2

complex, or Gβ5 in a complex with RGS7 as well as native Gβ5–RGS7 and Gβ5L–RGS9-1 complexes. Binding of the GST-fused DEP domain was much more robust with Gβ5–RGS7 constructs lacking the DEP domain, indicative of competition between the immobilized recombinant DEP domain and the DEP domain present within the full-length RGS7. This result pointed to the existence of the intra-molecular interaction between the DEP domain of RGS7 and Gβ5, the notion supported by the crystal structure of Gβ5–RGS9-1. The fact that the isolated DEP domain can displace the endogenous DEP domain indicates that the DEP:Gβ5 interaction is sufficiently dynamic to allow the Gβ5–R7 dimer to "open" assuming a conformation where the Gβ5 and DEP domains do not bind to each other. Recent results show that Gβ5 can also inhibit the interaction of the DEP domain with GPCRs;[83] however the effect of Gβ5 on the function of the DEP domain remains to be more fully investigated.

Thus, currently available data suggest that the Gβ5/GGL moiety can influence the activity of the DEP and RGS domains. In this respect, the Gβ5/GGL moiety resembles conventional Gβγ complexes, which have the ability to influence the activity of effectors and Gα subunits.[27] So far, researchers did not find convincing evidence for the physiologically relevant interaction of the Gβ5/GGL moiety with traditional binding partners of Gβγ subunit complexes such as G protein α subunits, effectors, or receptors. The only reported interaction that involved the Gβ5–GGL moiety is the association with the transcription repressor DMAP1 with the N-terminal portion of the GGL domain of RGS6.[84] DMAP1 coimmunoprecipitated with RGS6 from mouse brain lysate, but it has not yet been established if Gβ5 was a part of that native complex and whether this interaction can occur with other R7 family members. At the same time, the evidence for the intramolecular Gβ5 interactions with the DEP/DHEX and RGS domains is quite strong.[41,56,80,81]

It is also worth noting that according to biochemical analyses, the recombinant DEP domain of RGS7 appeared to associate with Gβ5 or Gβ1 equally well.[55] This indicates that the structural elements within the Gβ subunits that are responsible for the contacts with the DEP domain are rather conserved. However, it is not known if the DEP domains can interact with conventional Gβγ complexes under physiological conditions.

D. Function of the DEP Domain

DEP domains were identified in a large number of signaling proteins in a variety of organisms (see Ref. 85 for review). They are generally considered to be protein–protein interaction motifs, and thought to be important for subcellular localization. The role of DEP domains in the R7 family of RGS proteins was poorly understood until the discovery of R9AP and R7BP, novel proteins that bind to the R7 DEP domains and anchor the Gβ5–R7 dimers to the membranes (see Refs. 86, 87 for review).

Hu and Wensel discovered R9AP (RGS9-anchoring protein) by coimmunoprecipitating this ~25 kDa protein together with Gβ5L–RGS9-1 from the membrane extracts of bovine rod photoreceptors.[88] The determined amino acid sequence of R9AP revealed a single C-terminal transmembrane domain and another domain that has structural similarity to the SNARE complex protein syntaxin. It was shown that R9AP binds to the DEP domain of RGS9, tethers the complex to the membranes, and enhances the GAP activity of the Gβ5L–RGS9-1 complex toward transducin by an order of magnitude.[89,90]

Gene knockout studies showed that mice lacking R9AP also do not express Gβ5L and RGS9-1,[91] strongly indicating that R9AP is essential for the stability of the Gβ5L–RGS9-1 complex. To the contrary, the presence of the Gβ5L–RGS9-1 heterodimer is not necessary for the stability and subcellular localization of R9AP. R9AP is expressed in RGS9 knockout mice at the same level as in wild type and is localized in the outer segments.[92]

To study the role of the DEP domain and the importance of the RGS9–R9AP interaction, Arshavsky and colleagues generated a transgenic mouse in which RGS9-1 was substituted in rods by a deletion mutant that lacked the DEP domain.[92] This ΔDEP RGS9-1 construct was expressed at a nearly normal level, but did not localize to the rod outer segments, where the Gβ5L–RGS9-1 complex normally resides. The outer segment of a photoreceptor neuron is a specialized dendritic structure that is densely packed with hundreds of membrane discs. These discs contain the phototransduction machinery including rhodopsin, which is expressed at a uniquely high (3 mM) concentration to provide for efficient photon capture. Instead of localizing to the outer segments, ΔDEP RGS9 distributed throughout photoreceptor inner compartments which contain the nuclei, mitochondria, and other organelles. Photoresponses in these mice had delayed inactivation kinetics, which was similar to those previously registered in the RGS9-1 or Gβ5 knockout animals.[38,39] Thus, the study of the ΔDEP RGS9 identified a new role for the DEP domain: targeting the Gβ5–RGS9 complex to the appropriate location within polarized cells. The role of membrane anchoring proteins in the subcellular localization of R7 complexes will be discussed below in more detail.

Recent studies showed that the interaction of the DEP domain with a membrane-anchoring protein is a common feature of all R7 family proteins. In 2005, two groups independently discovered R7BP (R7 family binding protein), a protein that bore functional and structural resemblance to R9AP. Using an immunoprecipitation approach, Martemyanov and colleagues isolated native R7BP as a component of the brain RGS7 complex.[93] In an independent study, Drennan and colleagues identified the R7BP gene by searching databases for sequences similar to R9AP, then cloned and expressed the cDNA and reconstituted it with the RGS7 complex in transfected cells and in *Xenopus* oocytes.[66] Like R9AP, R7BP facilitated membrane localization of Gβ5–RGS7 dimers and augmented the inhibition of G protein signaling by the Gβ5–RGS7 complex.[94]

So far, researchers have found several differences between R9AP and R7BP. In contrast to R9AP, which is exclusively expressed in photoreceptors, R7BP is widely expressed in the CNS and was shown to bind to all R7 family members.[53] The mechanism of membrane anchoring of R7BP is different from that of R9AP. R9AP has a single C-terminal transmembrane domain, whereas R7BP binds to the membrane via a polybasic region and two palmitoylated cysteine residues. Mutations of these cysteines abolish the membrane binding of R7BP as well as the effects of R7BP on the function of Gβ5–RGS7.[66,94] Another notable difference between R7BP and R9AP is their role in stabilization of the Gβ5–R7 dimer. R7BP is absent in Gβ5 knockout mice[52,95] and therefore appears to be very unstable and requires the presence of Gβ5–R7 complexes for its normal expression. In contrast, R9AP does not require Gβ5–RGS9.[92] Furthermore, knockout of R7BP does not affect the expression level of Gβ5 and RGS7,[96] albeit coexpression of R7BP modestly increases the expression levels of Gβ5–RGS7 in transfected cells.[95] Stability of Gβ5–RGS7 in the absence of R7BP may not be as surprising considering the fact that a large fraction of Gβ5–RGS7 is present in the cytosolic fractions of native cells,[9,14] whereas R7BP is exclusively found in the membranes.[95] Thus, R7BP is stabilized by the Gβ5–R7 dimer, while R9AP does not require this interaction for its stability and its appropriate subcellular localization.

E. Other Binding Partners of the DEP Domain

While the interaction of R7BP and R9AP with the DEP domains of R7 family was established beyond a doubt, there is an increasing body of evidence showing that the DEP domains of R7 proteins have other functions. Recent studies indicate that the DEP domains of RGS proteins can interact directly with G protein-coupled receptors. While investigating the effects of RGS9-2 on signal transduction by the dopamine receptor D2 (D2DR), Kovoor et al. discovered that D2DR facilitates membrane localization of RGS9-2 in transfected cell

lines including CHO and PC12.[97] This effect of D2DR was not observed with other GPCRs such as acetylcholine muscarinic M2 and dopamine D1 receptors. The ability of the D2 receptor to facilitate membrane localization of RGS9-2 was not affected by the activation state of the receptor, as application of dopamine or other agonists of D2DR did not have an effect. Deletion of the DEP domain abolished localization of RGS9-2 to the membrane in the presence of the D2 receptor. Moreover, the GFP-tagged DEP domain alone localized to the membranes in the presence of D2DR. These results showed that the DEP domain was necessary and sufficient to target RGS9-2 to the plasma membranes. This targeting occurred in a selective GPCR-dependent manner, possibly via the direct interaction between the DEP domain of RGS9-2 and the D2 receptor. The effect of Gβ5 on these molecular events was not tested in that study, and so it is not yet clear if the intramolecular interaction of the DEP domain of RGS9 with Gβ5 influences this process. The effects of R7BP were also not tested because the study of Kovoor et al. was conducted before the discovery of R7BP.

The DEP domain is also present in the yeast RGS protein Sst2. Sst2 lacks the GGL domain and is unrelated to the R7 family. This RGS protein antagonizes the signaling pathway from the yeast mating pheromone receptor Ste2, a GPCR coupled to the heterotrimeric G protein Gpa1.[1,98] In addition to a canonical DEP domain, there is also a second DEP-like motif in the N-terminal portion of the protein. Both of these regions are essential for the function of Sst2. Deletion mutants of Sst2 that lack these DEP domains and that contain only the C-terminal fragment with the RGS domain do not oppose activation of Gpa1.[98] A recent study has shown that the DEP domain of Sst2 directly binds to the C-terminal tail of Ste2, the G protein-coupled receptor.[99] The authors conclude that the DEP domains of Sst2 are necessary for positioning of this RGS protein in the proximity of its substrate, GTP-bound Gpa1, so that Sst2 can be effective in promoting GTPase hydrolysis and the subsequent inactivation of the pathway.

Positioning of the RGS domain in close proximity to the site where Gα-GTP originates makes some sense because this would increase the effectiveness of GAP activity. In fact, several groups provided strong evidence that RGS proteins that belong to families other than R7 and do not contain DEP domains, interact with GPCRs.[100–104] For example, it was shown that RGS2 binds selectively to the third intracellular loops of Gq-coupled M1 and M5 acetylcholine receptors, very weakly binds to M3, and does not bind to the Gi-coupled M2 and M4 receptors.[102] Another small RGS protein, RGS8, was also shown to directly bind to the M1 receptor. The interactions of RGS proteins with GPCRs have one common feature, they are highly selective for specific receptor subtypes. The R7 family seems to abide by this rule.

As discussed above, studies showed that the Gβ5–RGS7 complex inhibited Ca^{2+} release elicited by the muscarinic M3 receptor activation.[14,55,74] Our recent report showed that Gβ5–RGS7 can only inhibit the Ca^{2+} signaling mediated by muscarinic M3 receptor, but not by several other receptors including the muscarinic M1 receptor.[83] This selectivity suggested that Gβ5–RGS7 inhibits GPCR-induced Ca^{2+} mobilization upstream of Gq. In the process of investigating this hypothesis, we found that neither RGS nor Gβ5/GGL domains were required for the inhibition of M3-mediated signal transduction. The DEP domain was necessary and sufficient for the robust negative effect on M3 receptor signaling and, in fact, we found that the recombinant DEP domain could directly bind to the third intracellular loop of the receptor. This loop is very long in the M3 receptor and is most divergent from other receptors such as the muscarinic M1 receptor, which is not sensitive to inhibition by Gβ5–RGS7. Evidently, the direct interaction of the DEP domain with this highly divergent loop selectively inhibits the ability of the M3 receptor to activate Gq. Thus, it appears that RGS7 inhibits M3-mediated Ca^{2+} release by preventing Gq activation by the receptor rather than accelerating its inactivation through accelerated GTP hydrolysis. Direct interaction with the

M3 receptor can explain the controversy between the functional effect of Gβ5–RGS7 on Ca^{2+} mobilization and the lack of GAP activity toward Gq, which was discussed earlier in this chapter. It is not clear at the moment whether the Gβ5–RGS7 complex (or the DEP domain) simply prevents Gq from binding to the receptor or somehow inhibits the GDP-GTP exchange. Since studies utilizing FRET have indicated that Gβ5–RGS7 can bind to Gq,[74,105] it is possible that activation of the M3 receptor in the presence of Gβ5βRGS7 can result in an inactive M3βGβ5βRGS7βGq complex. Importantly, R7BP completely prevented the ability of the Gβ5βRGS7 complex to inhibit M3 receptor signaling.[55] This suggests that the interactions of the DEP domain with R7BP and the M3 receptor are mutually exclusive. In other words, the Gβ5βRGS7 dimer can inhibit M3 receptor-mediated signaling, the Gβ5–RGS7–R7BP trimer cannot. One can hypothesize that the interaction with R7BP can control the selectivity of the Gβ5–RGS7 complex by limiting it to Gi-coupled receptors, such as the muscarinic M2 receptor.[66,94]

It is not known if all members of the R7 family can directly interact with GPCRs and whether or not they do so via their DEP domains. So far only the study of the interactions of the dopamine D2 receptor with RGS9[97] and the M3 receptor with RGS7[83] directly support this idea. However, it was also shown that normal localization of RGS11 and RGS7 in retinal bipolar cells requires the presence of the metabotropic glutamate receptor mGluR6,[106] which could be explained by a direct interaction between this receptor with these RGS proteins. The functional role of the interaction of the DEP domains with GPCRs has to be further investigated. On the basis of the analogy with the direct interaction between Sst2 and Ste2[99] it is reasonable to speculate that the interaction of R7 proteins with Gi-coupled GPCRs should make them more effective GAPs toward Gi because of the proximity of the RGS domain to Gα-GTP generated upon receptor activation. In this model, the DEP-mediated association of R7 family RGS proteins with GPCRs could determine the inactivation time ofthe G proteins activated by particular receptors. It is reasonable to speculate that G protein activation by a receptor expressed in neurons would be terminated faster compared to the same receptor in peripheral tissues that do not express Gβ5–R7 proteins. This idea could potentially explain why Gβ5–R7 complexes have only been found in neurons. On the other hand, data also suggest that the interaction of the DEP domains of R7 family RGS protein with GPCRs could represent a distinct mechanism to inhibit GPCRs without involving GAP activity or participate in the assembly and subcellular localization of specific signaling complexes.

The physiologic relevance of the interaction of the R7 family with GPCRs should be considered with caution, because thus far the experiments were performed in reconstituted systems such as transfected model cell lines.[55,74,83,97,105] What can be stated with relative confidence is that inhibition of signal transduction from the M3 receptor by the DEP domain of RGS7 is different from the mechanism employed by Sst2 to inhibit Ste2 signaling. Most importantly, the DEP domain of RGS7 does not require the RGS domain to exert its action, whereas the DEP domain of Sst2 was ineffective in blocking signaling via Ste2 and required the presence of the RGS domain within the same polypeptide.[107] The DEP domain of Sst2 binds to the C-tail of the receptor, whereas the DEP domain of RGS7 binds to the third intracellular loop (although additional binding sites on the receptor might also contribute to the interaction). Interestingly, neither the third intracellular loop nor the C-terminal tail of the dopamine D2 receptor was involved in the interaction with RGS9.[97] So far, these findings indicate that the sites of the interaction with the DEP domains could be present not only in the third loop and C-tail of the receptor, but also in the first and/or second loops. The mechanism of GPCR–DEP interaction could involve other molecules such as G protein subunits and possibly other processes such as GPCR oligomerization. Considering the importance and the emerging variety of signaling pathways stemming from activated

GPCRs,[108,109] the interaction of DEP domains with these receptors certainly warrants further investigation.

In summary, recent investigations identified several novel interactions that involve the domains of Gβ5–R7 proteins (Fig. 1). From these studies, it appears that R7 proteins have two “business ends”: the RGS domain and the DEP domain. RGS domains interact with the Gα subunits of the Gαi family and can serve as a GAP for these G proteins. This activity of the RGS domain is regulated by the Gβ5/GGL moiety, which increases the selectivity toward specific Gαi subtypes and toward the Gα-GTP-effector complexes versus the Gα-GTP. DEP domains of R7 proteins were found to associate with several proteins. Their interaction with the membrane-anchoring proteins R7BP and R9AP was confirmed both by *in vitro* and *in vivo* experimentation. Reported experiments also show that DEP domains bind to snapin and some G protein-coupled receptors. The Gβ5/GGL moiety interacts with both the RGS and DEP domains and serves as a built-in regulator of their activities. As discussed above, Gβ5/GGL increases the selectivity of the RGS domain toward the effector-associated Gα-GTP. Recent results indicate that Gβ5/GGL also negatively regulates the interaction of the DEP domain with the receptor, but this notion requires further examination. There has been no evidence found for the interaction of Gβ5/GGL with traditional binding partners of Gβg subunits, but there has been one report of an interaction of the GGL domain with a novel binding partner, a gene transcription regulator DMAP1. Future experiments will be needed to sort out which of the discussed interactions involve the entire R7 family and which might only occur with specific members. Perhaps the biggest challenge will be to establish which of these protein–protein interactions and regulatory mechanisms occur *in vivo* and what their role is in native cells.

III. Expression and Subcellular Localization of Gβ5–R7 Proteins

A. Regional Expression of R7 Family in the CNS

The initial *in situ* hybridization studies suggested that the specific members of the R7 family are expressed in distinct regions of the rat brain.[44] For example, RGS9 mRNA (the longer RGS9-2 splice form) was almost exclusively found in the striatum.[42,43] RGS6 mRNA was detected in a few striatal cells, olfactory bulb, medial habenula, and in sole reticular thalamic, subthalamic, and pontine nuclei. RGS11 was found in the hippocampus, locus coeruleus, and subfornical organ. The expression of the RGS7 is relatively high compared to other members of the R7 family with respect to both the level and wideness of distribution: high levels of RGS7 were found in the cerebellum, cortex, hypothalamus, and somewhat lower levels were detected in many other regions of the brain and spinal cord.[44,60,110,111] One study compared the total protein expression level of RGS7 in rat brain to that of RGS4 and found that it was 30–40-fold higher.[111] This estimate indicated that RGS7 might be one of the most highly expressed RGS proteins.

As expected from the obligatory subunit of Gβ5–R7 complexes, Gβ5 was found to be broadly expressed throughout the CNS. It is found in all regions expressing the R7 family RGS proteins.[5,51,112,113] This notion also applies to R9AP and R7BP, whose expression patterns generally resemble that of the R7 family (reviewed in Ref. 87). In the mammalian CNS, R7BP is broadly expressed, consistent with its promiscuous interaction with all R7 RGS proteins, whereas R9AP is only found in photoreceptors, in agreement with its preferential binding to RGS9.[53]

Can two or more R7 family RGS proteins be found in the same neuron? After the initial studies by *in situ* hybridization, there was a general feeling that the areas where the individual R7 members localize do not overlap.[44] For instance, in the rat brain, RGS9 gene expression appeared to be restricted to the striatum.[43] However, certain regions of the rat

brain contain mRNA for more than one R7 RGS protein. For example, both RGS7 and RGS11 can be detected in the rat hippocampus.[44,60] Other studies reported an even less region-restricted expression pattern of the R7 family. Lopez-Fando and colleagues studied the expression of RGS9-2 in the mouse brain and found that it was present at appreciable levels not only in the striatum, but also in the cerebral cortex, thalamus, and certain areas of the midbrain.[51] The discrepancies between the findings of the different research groups could be due to the difference in the applied techniques, for example, *in situ* hybridization[44] versus RT-PCR and western blots[51] or the species difference (rat versus mouse). Each of these alternative methods has its shortcomings, that is, *in situ* hybridization is not quantitative, whereas PCR and western blots performed on dissected parts of the brain have limited spatial resolution and may be prone to contamination by neighboring regions. Nevertheless, based on rather extensive investigations by several laboratories, it is clear that one area of the brain can express more than one R7 family member.

B. Expression of R7 Family RGS Proteins in the Retina

Important insights came from studies of the expression pattern of R7 proteins in the retina. This tissue is a very convenient model for protein localization studies because it has a relatively small variety of neurons compared to the brain. In addition, different types of neurons are organized in characteristic layers across the thickness of the retina. Photoreceptors (rods and cones) are organized in a distinct layer, then synapse on the retinal bipolar neurons, which in turn are followed by amacrine and ganglion cells. This simple morphology greatly simplifies histological analyses. Immunological markers are available not only for specific cell types but also for distinct cellular compartments. Initial studies showed that RGS9 was expressed exclusively in photoreceptors and is localized to the outer segments.[45,47] In contrast, RGS7 immunoreactivity was not present in photoreceptors, but localized to other neurons identified morphologically as bipolar and amacrine cells.[14] Like the original mRNA expression analyses in rat brain,[44] these findings suggested that different R7 members are expressed in different neurons. However, more recent and detailed investigations argue that this notion is not exactly correct. In their comprehensive paper, Song et al. showed that RGS11 colocalizes with RGS7 in the bipolar cells.[53] Moreover, RGS11 immunoreactivity was also detected in photoreceptors along with RGS9.

As an alternative approach to immunofluorescence microscopy, Song et al. analyzed the distribution of R7 RGSs and other relevant proteins by tangential sectioning of the retina. In this technique,[114] a small frozen block of flat-mounted retina is sectioned on a microtome perpendicular to the longitudinal axis of the photoreceptor neurons. The 5 μm-thick slices consecutively cut through the photoreceptor outer segments, inner segments, nuclear layer, etc., down to cell layers of the inner retina. These slices are then analyzed by western blot, and so this method is independent of artifacts associated with tissue fixation and antibody penetration. Furthermore, in addition to the strength of the signal, such an experiment provides information about the apparent molecular weight of the antigen, for example, that Gβ5L and Gβ5 isoforms are localized to the outer segments and inner compartments, respectively. According to data obtained using this method, RGS11 was present in slices corresponding to the inner compartments of photoreceptors, but not in the outer segments, which contained RGS9-1. This indicates that R7 RGS proteins can be present in distinct regions of the same cell, presumably playing different roles.

Interestingly, with respect to RGS6, the results of tangential sectioning contradicted the data obtained by immunostaining. An appreciable amount of RGS6 is found in sections corresponding to the outer segments where it was present together with RGS9-1, Gβ5L, and rhodopsin. The bulk of RGS6 was detected in the inner segments and outer nuclear layer (the region containing rod and cone nuclei) where it overlapped with RGS11. However, immunofluorescence did not detect RGS6 in the photoreceptor layer at all and it had a rather

striking localization restricted to the inner plexiform layer where it colocalized with cholinergic amacrine cells. The authors explain this striking contradiction between the serial sectioning and in situ immunostaining for RGS6 by “epitope masking” in the later method. With respect to detection of other R7 proteins, R9AP, transducin, and Gαo and Gβ5, the two methods were remarkably consistent. A point to be made here is that properties of a particular antibody, immunostaining protocols, and other technical issues may lead to different results of experiments that intend to establish the relatively simple fact of the exact localization of one R7 member versus another.

The unexpected finding that RGS11 localized in photoreceptors together with RGS9-1 contradicts the results of Morgans and colleagues who did not detect RGS11 in photoreceptors.[106] Instead, Morgans et al. observed a weak immunostaining of the outer segments with the anti-RGS7 antibody. This discrepancy is likely due to a technical issue related to the properties of the antibodies and potential differences in protocols employed by the two groups to fix, immunostain, and image the tissue. However, there can be little doubt that RGS11 colocalizes with RGS7 in the bipolar cells because this was reported independently by three laboratories,[53,106,115] Furthermore, the presence of RGS7 and RGS11 in bipolar cells was shown not only by immunofluorescence microscopy, but also by tangential sectioning and western blot.[53] These findings show that two members of the R7 family can be expressed in the same neuron.

C. Do Peripheral Tissues Express R7 Family RGS Proteins?

It is now accepted that expression of the R7 family is “predominantly” neuronal. However, it is harder to answer the question whether they are expressed exclusively in neurons. In neurons or neuronal cell lines, R7 members, Gβ5, R7BP, and R9AP were detected by independent and complementary approaches: PCR, Northern blots, *in situ* RNA hybridization, immunoblots, immunoprecipitation, *in situ* immunohistochemistry, immunofluorescence, and immuno-electron microscopy. Compared to this, experimental evidence for expression of these proteins in nonneuronal tissues and/or cell lines is much weaker. Many investigators amplified R7 family mRNA from peripheral tissues by RT-PCR. For example, RGS6, RGS7, and RGS9 mRNAwas detected by PCR in the heart along with RGS proteins that belong to other families.[116–118] However, there has been no reliable evidence by immunoblots or □□□□□□ immunostaining presented that R7 proteins are produced in the heart. Some studies could discern immunoreactive bands corresponding to the molecular weight of Gβ5 on immunoblots of nonneuronal cell lines.[119,120] However, the intensities of these signals were at least 100-fold weaker compared to Gβ5 signals registered from brain or cell lines of neuronal origin. The signals for R7 or R7BP proteins were also very faint or below the detection level.

One obvious problem is that RT-PCR can amplify R7 mRNA originating from neurons that innervate the investigated peripheral tissue. Considering the enhanced sensitivity of some immunodetection methods, contamination can also be a problem for western blots. However, PCR detected R7 mRNA not only in the tissues but also in cultured primary cells contaminated as well as cell lines, which cannot be by neurons. RNA encoding R7 proteins was detected in diverse cells including primary myocytes,[116,117] platelets,[121] ovarian surface epithelial cells,[122] aortal cells,[123] and T lymphocytes.[124] Hooks and colleagues recently reported that RGS6 transcripts were expressed in ovarian cancer cell lines where they were present at dramatically different levels compared to noncancerous immortalized ovarian epithelial cells.[122] Researchers in the Neubig laboratory amplified RGS7 and RGS9, but not RGS6 sequences from total RNA isolated from an A-10 aortal cell line.[123] The absence of RGS6 product indicates that amplification was selective and argues against such trivial artifact as contamination with genomic DNA. In that study, despite the robust detection of RGS7 mRNA, anti-RGS7 antibodies did not detect the protein in the A-10 cells. At the same

time, the same antibodies are known to readily detect RGS7 in the positive control such as brain homogenate. The researchers reported that upon western blot analysis of A-10 cells lysates, anti-RGS9 antibodies detected a band that was close in its molecular weight to the RGS9-2 splice version. It is not clear if that band was identical to RGS9-2 expressed in the striatum and whether it was specific.[123]

The reason for the presence of R7 mRNA in peripheral tissues and cells is unclear. Considering the number of research groups that reported amplification of R7 mRNA from a variety of cells and cell lines, these findings cannot be simply disregarded as an artifact of contamination. One possibility is that this mRNA is never translated or the product is degraded, and therefore the presence of this mRNA is physiologically irrelevant. Many articles reporting the presence of R7 RNA do not test for the presence of Gβ5–encoding RNA or the Gβ5 protein. Yet, we know that in Gβ5 gene knockout animals, R7 proteins are not detected, even in highly expressing tissues such as the brain and retina.[39] Therefore, detection of R7 proteins in peripheral tissues and cells should be performed in conjunction with Gβ5, which acts as the limiting factor for expression of R7 subunits. It is possible, in principle, that the expression level of R7 proteins in nonneuronal tissues is too low to be detected with existing antibodies. Detection at such a low expression level might require enrichment, for example, by immunoprecipitation or partial purification on an ion exchange resin. These approaches work on brain or retina,[9,13,14,28,88,93] but so far have not been reported for nonneuronal tissues. Experiments relying only on detection of R7 or Gβ5 mRNA must be accompanied by negative controls that include established neuronal marker genes, which should help to rule out contamination. Should monomeric R7 RGS proteins be detected in nonneuronal cells, this would contradict the current “dogma” that R7 RGS proteins only exist as dimers with Gβ5 and imply that a special mechanism is engaged to stabilize the R7 subunit against proteolysis. Even more importantly, from a mechanistic point of view, such a finding would be important because, as shown in studies **in vitro**, Gβ5 regulates activity of the R7 subunits.

At the moment, experimental evidence shows that Gβ5–R7 complexes are present only in neurons and neuroendocrine cells. The significance of the strict association of Gβ5–R7 complexes with neuronal signaling remains to be understood.

D. Regulation of R7 Family Expression

The expression of Gβ5, R7 RGS proteins, and R7BP appears very late in embryonic development and is dramatically upregulated in early postnatal development.[5,52,95,125] The timing of expression coincides with the appearance of neuronal markers, particularly proteins localized in synapses such as PSD95. These findings strengthen the notion that Gβ5–R7 proteins are only expressed and/or perform their functions in neurons and lead researchers to hypothesize that the Gβ5–R7–R7BP complex is involved in the process of synapse formation.[87] Molecular pathways driving the expression of R7 proteins in development are unknown. So far, only one research group[126,127] has linked RGS7 upregulation with the activation of the JAK-STAT pathway and suggested that gene expression of RGS7 is mediated by STAT3.

Several laboratories noticed that in addition to dramatic upregulation during neuronal development, expression of the RGS7 gene was upregulated in response to a variety of stimuli. These responses were detected in cultured cells and live animals. For instance, using an *in situ* hybridization approach, Gold and coworkers detected an increase of RGS7 mRNA level following electroconvulsive seizures.[128] This upregulation was restricted to a subdivision of the hypothalamus, indicative of high specificity of this effect. Other researchers found that pharmacological treatments can also cause induction of RGS7 protein and/or mRNA levels. In one of the early investigations of RGS7, Benzing et al. showed that

RGS7 was upregulated in mouse brain after exposure to bacterial endotoxin. This process was mediated by tumor necrosis factor via activation of stress-activated protein kinase p38, which prevented proteasome-dependent degradation of RGS7. Thus, this upregulation of RGS7 occurred at the protein level.[129] Muma and coworkers found that application of olanzapine, an atypical serotonin 2A receptor antagonist, increased RGS7 at the protein level.[126,127] Activation of opioid receptors by morphine altered the expression of RGS9-2 in the nucleus accumbens and other CNS regions. Interestingly, acute and chronic exposure to morphine had opposite effects, with the former increasing and the latter decreasing RGS9-2 protein levels by ~2-fold. Mice lacking RGS9 gene products showed enhanced behavioral responses to both acute and chronic morphine[130](see Chapter 10 in this volume). Injections of morphine also lead to an increase in RGS7 and other members of the R7 family, as well as Gβ5.[51] In their comprehensive analysis these researchers investigated the effects of both acute injections of morphine into cerebral ventricles and subcutaneous application in the form of implanted oily pellets that release morphine slowly. Groups of animals were sacrificed after different time intervals and the expression of the four R7 RGSs and Gβ5 was examined both at the mRNA and protein levels by RT-PCR and western blot analyses. The largest detected increase was the 5-fold upregulation of RGS9-2 in the thalamus, which was observed in the morphine tolerant-dependent mice. Smaller increases in RGS9 were seen in the striatum. Raised levels of RGS7, RGS11, and Gβ5 mRNA were also observed in most neural structures dissected from these mice. In postdependent mice, most of the RGS-R7 and Gβ5 mRNA increases persisted for about 2 weeks after starting the chronic opioid treatment.

Upregulation of RGS7 was also detected in the dentate gyrus of gerbil hippocampus after the animals were subjected to experimentally induced ischemia.[131] According to the estimates of the *in situ* hybridization data, the RNA was upregulated by ~50%. The western blot of isolated hippocampi did not reveal any statistically significant difference. Another recent study also showed that RGS7 was upregulated when animals were stressed by chronic exposure to cold.[132] The detected increase of the RGS7 signal on the immunoblot was only about 20%, but was appreciable particularly because RGS4 and RGS2 levels did not change in the same preparations. Noteworthy, there was no corresponding increase in Gβ5 levels, although the reason for this is unclear. It is unlikely that the additionally expressed RGS7 exists in a monomeric form because RGS7 and other R7 proteins are completely absent in the Gβ5 knockout mice.[39] The simplest explanation is that immunoblots with Gβ5 antibodies did not detect the small difference that occurred upon upregulation of RGS7 because much of the total Gβ5 is dimerized with other R7 proteins present in the dissected tissue. This pool of Gβ5 should not be influenced by RGS7 and therefore the additional amount of Gβ5 that accumulated in the cells with increased RGS7 expression could not be reliably detected.

Thus, many independent investigations support the idea that the level of R7 family proteins can be dynamically regulated in response to external signals. It stands to reason that an increase in the amount of the Gβ5–R7 complex present in cells can serve as an adaptive mechanism to decrease the sensitivity of particular G protein-mediated signaling circuits. In fact, this is not something unusual because many other RGS proteins were shown to be upregulated in response to extracellular stimuli. For example, one of the earliest investigations showed that treatment of PC12 cells caused upregulation of endogenous RGS2, but not RGS7 mRNA.[133] The recent study of Jedema and colleagues indicates that the elevation of RGS7 attenuates signal transduction via the α2-adrenergic receptors in rat locus coeruleus, the area of the brain involved in stress.[132] These presynaptic Gi-coupled receptors provide negative feedback to the process of norepinephrine release. In this model, upregulation of RGS7 under conditions of chronic stress attenuates signaling via the α2-adrenegic receptors by accelerating the GTPase activity of Gi, which would result in a restoration of the system's ability to respond to subsequent stimuli.

A common observation among reports describing influences of external signal on the expression level of endogenous Gβ5–R7 complexes is that the detected changes were quite modest, less than 2-fold.[127,132] Can small changes in the Gβ5–R7 level result in a physiologically meaningful effect on signal transduction? One idea to consider is that a stimulus causing upregulation of R7 could result in the generation of a small excess of the R7 subunit over Gβ5. Without Gβ5–imposed control, the R7 monomer would have a higher GAP activity and/or binding to Gα subunits than the dimeric form and therefore may produce a more substantial inhibition of the G protein signaling. This hypothesis contradicts the presumed 1:1 stoichiometry of the Gβ5 and R7 subunits, the current "dogma" based on protein purification studies and mutual stabilization of the subunits.[9,14,88,93] However, an excess of R7 subunit would likely be transient because the monomer will either degrade or bind to Gβ5. Some investigators report that Gβ5 levels increase coincidentally with R7 subunits,[51] whereas others do not detect the change in Gβ5 levels.[132] Therefore, at the moment, the data are insufficient to conclude whether or not the Gβ5:R7 stoichiometry is altered under conditions that cause upregulation of R7 complexes. It is also possible that the increase in Gβ5–R7 in a certain subset of neurons is high, but does not change in other cells at all, so that the bulk change detected on western blots of brain homogenates appears to be small. Likewise, it is possible that a significant signal-induced increase of Gβ5–R7 concentration occurs locally in specific cellular compartments.

E. Subcellular Localization of Gβ5–R7 Proteins

As mentioned above, the initial characterization of Gβ5 by fractionation of brain and retinal homogenates showed that in contrast to Gβ1, Gβ5 partitioned to both soluble and particulate (membrane) fractions.[12] Likewise, RGS7 can be found in soluble and membrane-associated states[14,95,126,127,134–136] (Fig. 2). In contrast, both splice variants of RGS9, RGS9-1, and RGS9-2, are tightly associated with membranes in photoreceptors and brain.[47,134] In one report, investigators found RGS9-2 in the cytosolic fractions of rat brain.[137] However, it appears that, because the brain lysates were centrifuged for only 10 min at 1500 g, the fractions designated as cytosolic must have also contained plasma and microsomal membranes. The distribution of Gβ5–R7 complexes between membranes and cytosol is closely related to the other aspect of subcellular localization, targeting to specific cellular compartments.

F. Molecular Mechanisms of Gβ5–R7 Membrane Association

Neither Gβ5 nor R7 family RGS proteins have transmembrane domains, and so it was not obvious why they associate with the membranes. In one of the early attempts to understand the mechanism of R7 membrane anchoring, Hepler and colleagues examined biochemical properties of native RGS7 from bovine brain and of recombinant RGS7 produced in Sf9 insect cells.[138] Using detergent extraction and fractionation assays, they demonstrated that RGS7 exists in distinct hydrophilic and hydrophobic forms in brain tissue and when ectopically expressed in Sf9 cells. They found that the membrane-bound form of RGS7 was palmitoylated, while the cytosolic form was not. Mutations of specific cysteine residues resulted in a soluble protein, indicating that they were responsible for acylation by palmitate. Thus, palmitoylation imparted hydrophobicity and membrane association on RGS7, strongly indicating that this posttranslational modification could be responsible for membrane association of the Gβ5–RGS7 complex. The researchers also tested the potential functional effects of palmitoylation. They found that palmitoylation of recombinant RGS7 occurred independently of dimerization with Gβ5 and that it did not affect the GAP activity of RGS7 toward Gαo.[138]

In contrast to other known lipid modifications such as S-prenylation and N-myristoylation, palmitoylation is a reversible process. This reversibility suggested an explanation for the

existence of the soluble and membrane-attached pools of Gβ5–RGS7 and suggested that the membrane attachment of this molecule could be dynamically regulated. Wedegaertner and colleagues studied the effects of Gαo–RGS7 interaction and showed that activated Gαo stimulated palmitoylation of RGS7 in transfected cells.[139] That study also showed that cotransfection of Gαo into COS-7 cells facilitated localization of the Gβ5–RGS7 complex to the plasma membranes. This effect on Gβ5–RGS7 localization was shown by two independent techniques, confocal microscopy and cell fractionation. A Gαo mutant incapable of interacting with RGS proteins failed to recruit the Gβ5–RGS7 complex to the membranes, indicating that this interaction involved the RGS domain of RGS7. At the same time, their studies indicated that the DEP domain of RGS7 was also important for membrane localization of the complex.[139] The crucial palmitoylation site was shown to be located between the DEP and GGL domains. Thus, both palmitoylation of RGS7 and its interaction with Gαo via the RGS domain were implicated in its association with plasma membranes. It should be noted, however, that it has not yet been established if activation of G protein-mediated pathways can influence subcellular distribution of RGS7 or other R7 complexes in native tissues. Treatment of brain homogenates or cultured cells with agonists, GTP, or AMF thus far have not been shown to influence the subcellular distribution of Gβ5–R7 complexes.

G. Nuclear Localization of Gβ5–R7 Complexes

Perhaps the most surprising and intriguing observation made in the studies of subcellular localization Gβ5–R7 complexes was made by Simonds and colleagues who found that Gβ5–RGS7 can localize to the nucleus.[135,136] Importantly, the Simonds group studied the behavior of endogenous Gβ5 and RGS7 proteins in differentiated PC12 cells and in the brain. Using confocal immunofluorescence microscopy, they demonstrated that Gβ5 and RGS7 immunoreactivity localized primarily to the cytosolic space, but a comparable amount of the signal was present within the nuclei. These results were confirmed with two antibodies, one of which was raised against the N- and the other against the C-terminal peptides of Gβ5. It was also reconfirmed by detection of ectopically expressed HA- and GFP-tagged proteins. Furthermore, the results of biochemical fractionation of both PC12 cells and mouse brain homogenates were consistent with the data from microscopy. The cytosolic, membrane and nuclear fractions were subjected to western blot analysis and bands corresponding to Gβ5 and RGS7 were present in all these fractions. In contrast, Gβ1 and Gγ2 were only found in the plasma membrane, as expected. The nuclear marker TBP was present only in the nuclear fraction, which demonstrated that the cell fractionation procedures were effective, ruling out cross-contamination.[135] The subsequent analysis of the mechanism of nuclear localization of the Gβ5–RGS7 complex showed that RGS7 was responsible for the nuclear localization. The researchers also constructed a mutant of Gβ5 that bound to Gγ2 but not to RGS7. While this mutant was indistinguishable from the wild-type Gβ5 in its ability to activate PLCβ2, it failed to localize to the nuclei of either HEK 293 or PC12 cells.[136] These studies convincingly showed that Gβ5–RGS7 complexes could localize to the nuclei.

It is worth noting that nuclear localization is, in fact, a rather common phenomenon among RGS proteins. For example, it was shown that GFP-fused RGS2 can localize to the nucleus.[140] RGS3T, a truncated version of RGS3 (but not the longer form) was also found in the nucleus.[141] RGS10 translocated to the nucleus in a phosphorylation-dependent manner.[142] However, these RGS proteins have a rather small molecular weight and could simply diffuse to the nucleus. Gβ5–R7 complexes are larger than the ~65 kDa cut-off set by the nuclear pore. The potential functional role of Gβ5–R7 complexes as well as other RGS proteins, in the nucleus remains unknown (see Chapter 5 in this volume).

Some insights came from intriguing and promising results obtained in the Fisher laboratory with studies of RGS6. The results of these investigations are discussed in more detail in Chapter 5 in this volume. Briefly, they found that long splice forms of RGS6 (RGS6L) overexpressed in COS-7 cells in the form of a GFP fusion did not localize to the nucleus.[50,143] However, short splice forms of RGS6 (RGS6S), which lack the DEP domain, localized to the nucleus where they concentrated in the nucleoli.[144] Nucleolar localization of all RGS6 splice forms was facilitated by cellular stress to which the COS-7 cells were subjected after transfection. Coexpression of Gβ5 promoted nuclear localization of RGS6L splice forms. These results are particularly interesting in conjunction with the observation that RGS6 was found to interact with DMAP1, a component of a protein complex involved in repression of newly replicated genes. The interaction between RGS6 and DMAP1 was detected in several assays. It was originally identified in a yeast two-hybrid screen, then confirmed by colocalization in transfected cells, coprecipitation of tagged forms of RGS6 and DMAP1, and binding of the endogenous brain DMAP1 to beads with immobilized recombinant RGS6 as well as coimmunoprecipitation of the RGS6–DMAP1 complex from brain lysate.[84] Importantly, coexpression of RGS6 inhibited the transcription repressor activity of DMAP1. Another significant finding about the interaction of RGS6 with DMAP1 is that DMAP1 binds to the GGL domain of RGS6. It does not compete with Gβ5 because DMAP1 binds to the N-terminal part of the GGL domain that is not essential for binding to Gβ5. The GGL domain of RGS6 was also found to interact with SCG10, a neuronal growth-associated protein,[143] but it has not been shown if this interaction occurs between endogenous proteins. Thus, DMAP1 appears to be the only established binding partner of the Gβ5–GGL moiety apart from the DEP and RGS domains.

Another group performed immunocytochemical and immunoblot experiments to study localization of RGS9-2 in rat brain and found that a high proportion of RGS9-2 localized to the nuclei of some striatal or cortical neurons.[137] A large number of cells showed the distribution of RGS9-2 immunoreactivity throughout the neuron cell body. When transfected in COS-7 cells, RGS9-2 also localized to the nucleus. Deletion mutagenesis showed that the nuclear localization of RGS9-2 was determined by the C-terminal domain. RGS9-1, which lacks this region, was completely excluded from the nucleus, in agreement with its localization within photoreceptors.[92] The RGS9 construct consisting of the N-terminus, DEP/DHEX, GGL, and RGS domain, which is common between the two RGS9 slice forms behaved similarly to RGS9-2, indicating that the C-terminus of RGS9-1 was responsible for its exclusion from the nucleus. Finally, the construct consisting of the RGS domain and the unique C-terminus of RGS9-2 showed a particularly striking tendency to localize to the nucleus and was essentially undetectable in the cytoplasm. The importance of the C-terminus of RGS9-2 for nuclear localization was confirmed by biolistic (“gene gun”) transfection of striatal neurons in brain slices. Cotransfection of Gβ5 or Gβ5L (but not Gβ2) enhanced nuclear localization of RGS9-2 in COS-7 cells. The positive effect of Gβ5 on nuclear localization of RGS9-2 is consistent with the reported effect of Gβ5 on nuclear localization of RGS6.[144] At the same time, the idea that Gβ5 is responsible for nuclear localization of Gβ5–RGS9-2 and Gβ5–RGS6 complexes is at odds with the findings the Simonds' group who concluded that the entity responsible for nuclear localization of Gβ5–RGS7 complex is the RGS7 subunit.[136] Thus, it appears that both Gβ5 and R7 subunits can play a role in targeting of the complex to the nucleus, however, the exact mechanism governing nuclear trafficking of Gβ5–R7 complexes has not been elucidated.

Overall, these results suggest that some R7 RGS proteins can play a novel role in the regulation of gene transcription, which might explain the significance of nuclear localization of these complexes. However, the sheer fact of nuclear localization of Gβ5–R7 complexes remains somewhat controversial. Some investigators observe much brighter staining of transfected R7 proteins in the cytoplasm than in the nucleus and interpret this pattern as

cytosolic.[55,74,97,145] Moreover, a carefully controlled fractionation of mouse brain indicated that the nuclear fractions contain very little relative amounts of RGS9-2 compared to its presence in synaptic membranes. In fact, the proportion of RGS9-2 in the nuclear fraction (a pellet obtained at 1000g centrifugation) was similar to that of PSD95, a synaptic protein, which is not known to be localized in the nucleus.[145] The disagreement between the results obtained by different researchers with respect to the nuclear localization of R7 proteins warrants further investigation. It is possible that a careful examination of different experimental protocols will reveal currently unappreciated mechanisms that control subcellular localization of these molecules.

H. R7 Family Membrane Anchoring Proteins, R7BP, and R9AP

The discovery of membrane-anchoring proteins R9AP[88] and R7BP[66,93] brought about a new dimension to our understanding of the subcellular localization of Gβ5–R7 proteins. In native tissues, R9AP and R7BP were found only in the membrane fractions,[95,134,145] and current experimental evidence strongly indicates that these anchoring proteins play a crucial role in plasma membrane association of Gβ5–R7 complexes as well as in their localization to specific regions within neurons.

R9AP localizes exclusively to retinal rods and cones (at least in mammals). Within these cells, it is targeted to the outer segments, the cellular compartment harboring the components of the phototransduction cascade including the Gβ5L–RGS9-1 complex. As discussed earlier, R9AP is required for stability of Gβ5–RGS9 complexes against degradation[91] Since Gβ5–RGS9-1 complexes are simply absent in the R9AP knockout mice, the effect of R9AP on the subcellular localization of the RGS9 complex was investigated by the expression of a DEP-less mutant of RGS9-1. It was found that this ΔDEP-RGS9-1 mutant and Gβ5L localized to the inner compartments of rods and were not present in the outer segments.[92]

The simplest mechanism explaining this mislocalization effect is as follows. R9AP is an integral membrane protein, which partitions to the outer segment discs independently of Gβ5–RGS9 and similarly to the other integral membrane proteins such as rhodopsin or retinal guanylate cyclase.[146] Once it reaches the outer segments, R9AP serves as the membrane “sink” for the otherwise soluble Gβ5–RGS9-1. R9AP is present in the cells at stoichiometric amounts compared to the Gβ5L–RGS9-1 and so R9AP is sufficiently abundant to act as such a “sink” for Gβ5–RGS9. Furthermore, it is likely that any Gβ5–RGS9-1 that is not associated with R9AP and that stays in the cytosol would be eliminated by cellular proteases.[52] If the DEP:RGS9 interaction is abolished by deletion of the DEP domain, the DEP-less Gβ5–RGS9-1 complex diffuses across the cell, similar to other soluble proteins such as phosducin, arrestin, and transducin[147–149] (reviewed in Ref. 150). Martemyanov and colleagues pointed out that such a simple diffusion mechanism would have resulted in an equal distribution of the ΔDEP–RGS9 complex between the outer and inner compartments. They argued that since the ΔDEP–RGS9 construct preferentially localizes to the inner compartments, there should be an additional mechanism that controls its subcellular distribution.[92] While this argument is reasonable, free cytoplasm is not distributed uniformly across the photoreceptor cells. Even neutral cytosolic markers such as green fluorescence protein preferentially localize to regions surrounding the nuclei and are less abundant in the outer segments.[151] The simplest explanation is that the DEP-less Gβ5L–RGS9-1 complex distributes to the areas of photoreceptor cells where more cytoplasmic space is available. Since the outer segments are densely packed with membrane discs that are impenetrable for soluble proteins, it diffuses throughout the inner compartments. This model predicts that the separated DEP domain of RGS9 or any other R9AP-binding construct expressed in photoreceptors would localize to the outer segments.

In contrast to R9AP, R7BP is expressed widely throughout the nervous system and is known to associate with all members of the R7 family.[53,87,134] When expressed in model cell lines such as HEK 293 or CHO K1, R7BP can be found in the plasma membrane, cytoplasm, and nucleus.[66,94,145] Palmitoylation of R7BP is essential for targeting it to the plasma membrane. If palmitoylation is abolished by treatment of the cells with 2-bromo-palmitate or mutations substituting specific Cys residues or the polybasic motif near the C-terminus R7BP, R7BP localizes to the nucleus (reviewed in Ref. 87). It is important to note that in brain homogenates, R7BP partitions entirely to particulate fractions and is not detected in the cytosol. Fluorescence or electron microscopy did not detect endogenous R7BP in the nuclei of neurons in native tissues or cultured neuronal cell lines. The reason for such a discrepancy is not clear, but it could be a technical problem as ectopically expressed proteins often mis-localize in their host cells. One likely explanation is that palmitoylation of R7BP in neurons is more efficient than in transfected cells.

The results of several laboratories showed that in transfected cells, R7BP is necessary for plasma membrane association of Gβ5–R7 complexes.[55,66,120,145] However, a large fraction of Gβ5–R7 is also present in the cytosol. This can be only partially explained by the fact that, in transfected cells, R7BP does not bind to the plasma membrane in its entirety. In brain homogenates, essentially 100% of R7BP is associated with membrane fractions, but a large fraction of Gβ5–RGS7 is present in the cytosol. In this respect, Gβ5–RGS7 is different from either Gβ5–RGS9-1 or Gβ5-RGS9-2 in that both splice forms of RGS9 are only present in the membrane fractions. Evidently, the cytosolic pool of Gβ5–RGS7 can exist in the R7BP-free form. It is not known if the cytosolic form of Gβ5–RGS7 is the dimer or is associated with another protein(s).

As discussed above, the photoreceptor-specific Gβ5L–RGS9-1–R9AP complex localizes to the segment, the cell compartment that represents the dendrites of these highly specialized neurons. Recently performed studies utilizing laser confocal microscopy and immunoelectron microscopy strongly indicate that other Gβ5–R7 complexes and R7BP can localize to distinct regions within other types of neurons. For example, ultra-structural analyses performed on thalamic neurons showed that R7BP was only found in dendrites.[95] However, in striatal neurons, it was also detected both in dendrites and some axons. Another study indicated that R7BP is enriched in postsynaptic densities.[52] Biochemical fractionation of mouse brain showed that R7BP is present in the plasma membrane, microsomes, and synaptosomes, but not in synaptic vesicles. Supporting the notion that R7BP is enriched in synapses, it cofractionated with PSD95, a marker for postsynaptic density. A striking example of concentrated subcellular localization was observed in bipolar cells of the retina where immunofluorescence microscopy studies showed that R7BP, Gβ5, RGS7, and RGS11 localize to the dendrite tips of these neurons.[96,106,115] These results were consistent with the idea that R7BP and R9AP target Gβ5–R7 complexes to specific compartments in cells.[87] The recently performed knockout of the R7BP-encoding gene in mice put this idea to the test, and the results were surprising.[96]

With respect to RGS9, the data supported the notion that R7BP is the crucial factor determining the localization of the complex. As expected, the level of RGS9-2 was dramatically reduced in the brain of R7BP knockout animals,[52,134] consistent with the requirement of R9AP for stability of RGS9 gene products. There was some residual RGS9-2 present in the brain and this pool was largely found in the cytosolic fractions of mouse brain homogenates. These results were in agreement with the previous finding by the same group that deletion of the DEP domain of RGS9-1 resulted in distribution of RGS9-1 to the cytosol.[91,92] Therefore, they strongly supported the notion that an anchoring protein (R9AP or R7BP) is necessary for membrane association of Gβ5–R7 complexes.

However, knockout of R7BP did not affect the expression level of other members of the R7 family, RGS7, 6, and 11. More surprisingly, these Gβ5–R7 complexes still partitioned to the membrane. Furthermore, the Gβ5–RGS7 complex still localized to the dendritic tips of retinal ON-bipolar cells, indicating that R7BP is not required for subcellular targeting of RGS7.[96] This shows that neurons have alternative mechanisms, possibly direct palmitoylation of R7 family members that might be sufficient for its membrane attachment and subcellular localization. Consistent with this idea, the localization of Gβ5–RGS7 and Gβ5–RGS11 was dramatically different in retinal bipolar cells of mice that lack the metabotropic glutamate receptor type 6 (mGluR6).[106] In normal mice, mGluR6 immunoreactivity colocalizes with RGS7 and RGS11 to the dendritic tips of bipolar cells, as detected by analysis of tissue sections and primary cultured bipolar neurons. In contrast, in the mGluR6-deficient mice, immunostaining for RGS7 and RGS11 was diffuse throughout the entire cytosol. Thus, mGluR6, and not R7BP, is essential for the subcellular localization of Gβ5–RGS7 and Gβ5–RGS11. The exact mechanism by which mGluR6 targets the Gβ5–R7 complexes to the dendritic tips has not been delineated. However, it is worth noting that the absence of mGluR6 has the same effect on the subcellular distribution of Gβ5–RGS7 and Gβ5–RGS11 as the absence of R9AP interaction has on Gβ5L–RGS9-1 in photoreceptors.[91] One can speculate that Gβ5–RGS7 and Gβ5–RGS11 can directly bind to the mGluR6 receptor, which acts as the docking site in the dendrites. At the same time, it is also more likely that mGluR6 attracts Gβ5–R7 complexes via activation of Go, which is known to facilitate membrane attachment of Gβ5–RGS7 through enhancement of palmitoylation.

1. Lipid Rafts—Many signal transduction proteins segregate to specific microdomains within the plasma membranes such as caveolae and lipid rafts[152,153] These small areas of the membranes are enriched in cholesterol and sphingolipids; they are thought of as platforms to assemble specific signaling complexes. Heterotrimeric G proteins and other components of G protein signal transduction pathways are known to differentially partition to cholesterol-enriched membrane domains.[154–157] One of the factors playing a role in selective targeting of proteins to distinct membrane microdomains is differential lipidation by fatty acids.[158,159]

The number of experimental approaches to study lipid rafts is limited. The simplest and therefore most popular method is the isolation of detergent-resistant membranes (DRM). In a typical experiment, a membrane preparation or tissue is treated with Triton X 100 or Nonidet P40 (typically, 0.5–1.0%) on ice and the resulting lysate is subjected to ultracentrifugation on a sucrose density gradient.[160,161] According to the lipid raft model of biological membranes, the lipid composition of the raft is such that the raft is more “rigid” because they contain primarily saturated fatty acids tails and are “reinforced” by incorporated cholesterol. The nonraft (fluid) portion is solubilized at a lower detergent concentration, the rafts can still maintain the integrity. Since lipids are less dense than protein, the rafts can float on density gradients, whereas the heavier fractions contain solubilized proteins that presumably reside in the nonraft portion of the membrane. The detergent-insoluble pellets found on the bottom of the ultracentrifuge tubes contain the bulk of microtubules and represent cytoskeleton. Studies utilizing the DRM method are quite sensitive to experimental parameters. An increase in detergent concentration or temperature dissolves lipid rafts. A decrease in detergent concentration or detergent:protein ratio results in “under-solubilization,” so that proteins that are not present in rafts, remain partially associated with lipids, float on the sucrose density gradient, and cofractionate with the DRMs. The behavior of known markers of rafts (such as caveolin) and nonraft proteins serves as the control in these studies. The alternative methods to study lipid rafts involve cholesterol depletion with cyclodextrin, special types of immuno-EM based on imaging of flat-mounted membrane sheets,[162] and biophysical methods that utilize fluorescently-tagged proteins.[163,164]

Studies of photoreceptor lipid rafts showed that the Gβ5L–RGS9-1 complex partitioned to the DRM fraction.[165,166] Other members of the photo-transduction cascade including the G protein transducin were also shown to localize to these rafts, and what is particularly interesting, is that this translocation occurred in a light- and guanine nucleotide-dependent manner.[165,167–169] One study showed that the pool of Gβ5–RGS9-1 present in the DRM increases in light or upon activation of transducin with AMF (Al^{3+}, Mg^{2+}, F^{-}) in the dark-adapted membranes.[165] However, another group found that the same amount of RGS9-1 was present in the rafts isolated from dark- or light- adapted membranes.[166] The reason for this discrepancy is not clear, but it is likely to be a technical one, possibly related to preparation of dark-adapted membranes.

Partitioning of another R7 family member, RGS7, to lipid rafts in brain membranes or transfected cells was also investigated.[95,120,138] One recent study showed that only a very small amount of brain Gβ5–RGS7 and R7BP cofractionated to the lipid rafts fraction with DRM marker proteins including PSD-95, flotillin, caveolin, and Gαi. Consistent with the earlier study by the Hepler group,[138] the bulk of Gβ5–RGS7 and R7BP was found in the heavier fractions, together with the non-DRM marker, the transferrin receptor. The distribution of Gβ5–RGS7–R7BP between the raft and nonraft fractions was not dependent upon treatment of the membranes with AMF, G protein activation, or changing the detergent:membrane protein ratio. These data were interpreted as the absence of the Gβ5–RGS7–R7BP complex in the lipid rafts. At the same time, a different group did detect a portion of the Gβ5–RGS7 complex and R7BP in the DRMs isolated from brain, PC12 cells, or transfected HEK293 cells.[120] Their studies also indicated that palmitoylation of R7BP facilitated partitioning of the R7BP–Gβ5–RGS7 complex to the DRM. Interestingly, these researchers noticed that the DRMs containing Gβ5–RGS7–R7BP were different in buoyancy from those containing PSD95, suggesting that Gβ5–RGS7–R7BP might reside in special microdomains.

Regardless of some disagreement between the published reports, all of them show that a much smaller fraction of brain RGS7 localizes to the DRM as compared to the RGS9-1 complex in photoreceptors. Likewise, the bulk of Gβ5–RGS7–R7BP in transfected cells partitions to detergent-soluble membranes. Noteworthy, RGS9-2 was found in Triton-insoluble fractions of mouse brain homogenates along with R7BP and PSD95.[145] This might indicate that both RGS9-1 and RGS9-2 tend to localize to the more structured portions of the membranes. Although the currently available data are not sufficient to make such conclusions, it is tempting to speculate that distinct members of the R7 family that are expressed in the same cell, can localize within distinct areas in cells, where they could associate with different G proteins and GPCRs.

IV. Other Protein–Protein Interactions and Phosphorylation of R7 Family Proteins

Several research groups used yeast two-hybrid screens to identify new binding partners of R7 family G proteins. For example, using the N-terminal half of RGS7 as the bait, Yong and colleagues identified snapin, a protein associated with the SNARE complex, as a potential binding partner of RGS7.[170] Deletion mutagenesis showed that the interaction is mediated by the N-terminal 64 amino acids, which include the proximal part of the DEP domain. The interaction between RGS7 and snapin was confirmed by pull-down assays in transfected cells, but currently there is still no evidence that it occurs *in situ*. However, because R7BP and R9AP also bear resemblance to the syntaxin family of SNARE proteins, the potential interaction of RGS7 with snapin is interesting. One can speculate that the DEP domains of the R7 family members might participate in differential localization and targeting to

dendrites or other distinct regions of neurons, or in processes associated with membrane fusion and neurotransmitter release.

One of the initial studies of RGS7 investigated polycystin, a chloride channel, and identified RGS7 as its interacting partner.[171] Those early experiments were done in the absence of Gβ5, and the association with polycystin was shown to occur at the same GGL domain in RGS7 that is responsible for the Gβ5 interaction. In our laboratory, a yeast two-hybrid screen identified Gαs as a binding partner of the N-terminal portion of RGS7 (Levay and Slepak, unpublished data). Although *in vitro* analyses suggested that the DEP domain of RGS7 could serve as a guanine nucleotide dissociation inhibitor of Gαs, thus far we were unable to corroborate these results with studies on tissues cells. Another member of the R7 family, RGS6, was found to interact with SCG10, a protein associated with neuronal growth[143] and involved in cytoskeletal functions. A potentially intriguing interaction that involved RGS9 was reported by Yamazaki and colleagues who used a protein overlay assay and found that RGS9 could directly interact with retinal guanylate cyclase.[172,173]

To date, the interactions with snapin, polycystin, Gs, and SCG10 have not been confirmed to occur in native cells. The significance and relevance of all these potential interactions has yet to be determined. It is possible that some of them occur only because these potential binding partners resemble some other molecules. The situation with at least some of these protein–protein interactions could be analogous to the association of Gβ5 with the Gγ subunits, which can produce a functional Gβg dimer simply because they resemble the GGL domains of R7 proteins. Nevertheless, the rich “pipeline” of potential interacting molecules supports the notion that Gβ5–R7 complexes not only serve as GAPs for G proteins but also have additional functions in cells.

Several studies show that R7 family RGS proteins can be phosphorylated and can interact with 14–3–3 proteins. Using an anti-RGS7 antibody, Benzing and colleagues coimmunoprecipitated 14–3–3 from mouse brain lysates. Through mutational analysis of RGS7 in transfected HEK293 cells, they identified a conserved serine that was located in the RGS domain that was essential for binding of 14–3–3. Accordingly, the interaction with 14–3–3 inhibited the GAP activity of the GST-fused RGS domain of RGS7.[174] In a subsequent study, these researchers showed that phosphorylation of RGS7 was dynamically regulated. They reported that treatment of RGS7-transfected HEK 293T with TNF-α decreased the phosphorylation of RGS7. This dephosphorylation coincided with the reduction of the amount of 14–3–3 that could be coprecipitated with RGS7. Consistent with the previous finding that 14–3–3 reduced GAP activity of RGS7, 14–3–3 inhibited the effect of RGS7 on inwardly rectifying K(+) channels (GIRKs) in *Xenopus* oocytes[175]. Previously, this group reported that TNF-α induced phosphorylation of RGS7 in the mouse brain. This phosphorylation was mediated by p38 kinase and resulted in upregulation of RGS7 via reduction of its proteosome-mediated degradation.[129] The researchers proposed that phosphorylation of RGS7 has two effects: increase of expression level and reduction of the GAP activity.

RGS9 was also shown to be phosphorylated. The photoreceptor form of RGS9, RGS9-1 is a substrate for protein kinases A and C.[176–178] Phosphorylation of RGS9-1 occurred preferentially in the dark-adapted cells, required Ca^{2+}, and the phosphorylated form of RGS9-1 localized primarily to lipid rafts. Phosphorylation of RGS9-1 by PKC had little effect on its activity in solution but significantly decreased its affinity for R9AP.[178] These findings led researchers to propose that it was involved in the process of light adaptation.[176,177] The brain form of RGS9, RGS9-2 was also shown to be phosphorylated. Injections of morphine into mouse cerebral ventricles led to phosphorylation of RGS9-2 and increased its coprecipitation with 14–3–3.[179] It should be noted that R9AP is also

phosphorylated by an endogenous kinase, and in photoreceptors, phosphorylated R9AP is localized in the outer segments.[53] Phosphorylation or other posttranslational modifications of Gβ5 has not been reported.

V. Physiological Role of Gβ5–R7 Complexes: A Brief Summary of *In Vivo* Studies

Thus far, most of the information about the physiological role of mammalian R7 proteins come from studies of mouse models with knocked out or overexpressed RGS9.[38,130,180–182] The phenotypes of these animals are described in more detail in other chapters of this volume (see Chapters 7 and 10 in this volume). Briefly, physiological studies of the visual system and behavior of these animals showed enhanced signaling from Gi-coupled GPCRs such as rhodopsin, dopamine, and opioid receptors[38,130,181,182] (for review see Ref. 183). These results were in agreement with the general model that both RGS9-1 and RGS9-2 complexes function as negative regulators of Gi-mediated signal transduction. Immunoprecipitation experiments also indicated that RGS9 associates with Gi proteins *in situ*.[179] This is also consistent with the biochemical studies of recombinant Gβ5-RGS9 complexes that highlighted its GAP activity toward transducin (for review see Ref. 79) and Gi family proteins[76] and with the effect of the Egl-10 mutation in *C. elegans*.[29]

Another relevant mouse model is the knockout of Gβ5, which lacks the entire family of R7 proteins.[39] These animals are viable, but have a number of defects including a very low body weight at birth and difficulties in breeding. Studies on the retina suggested that, in addition to the expected effect on signaling associated with degradation of RGS9 in photoreceptors, the absence of Gb5 causes morphological changes in the synapses of bipolar cells.[115] However, a conclusive interpretation of Gβ5 phenotypes is difficult because these mice lack the entire R7 family and the potential for gross effects in development. Knockouts of other members of the R7 family have not been described.

Mutations of genes encoding R7 family members were identified in humans. Mutations in the RGS9 or R9AP genes resulted in difficulty to adapt to sudden changes in levels of illumination.[184] Studies also suggest that metabolic disorders such as obesity can be associated with RGS7.[185] Immunoblot analyses of autopsies from patients with Parkinson's disease showed that RGS9-2 protein is upregulated in the caudate nucleus and putamen compared to control subjects,[186] consistent with the idea that upregulation of R7 family members is an adaptive mechanism in the nervous system. These findings highlight the significance of the Gβ5–R7 family proteins as interesting molecules for diagnosis and future therapies of neurological disorders.

VI. Conclusions

R7 family RGS proteins exist as obligatory dimers with the G protein subunit Gβ5. They are expressed throughout the CNS, where specific members of the family display regional specificity of expression. Recent studies also show that more than one R7 family member can be present in the same anatomical region and in the same neuron. Gβ5–R7 complexes localize to different cellular compartments such as dendritic tips, plasma membranes, cytosol, and nuclei. The mechanisms governing the subcellular localization of Gβ5–R7 complexes and the physiological significance of localization, particularly, the nucleus, are not yet clear. Physiological experimentation on animal models demonstrated that R7 family members antagonize Gi-mediated pathways underlying sensory transduction and other CNS functions such as addiction, nociception, and control of locomotor behavior. Some experiments suggest that changes of Gβ5–R7 expression levels may serve as a mechanism of neuronal adaptation to stress and other stimuli. At the molecular level, recent research has

led to two main advances: solving the crystal structure of the Gβ5–RGS9 complex and the discovery of membrane anchoring proteins R9AP and R7BP. Progress has also been made in understanding the functional role of the domains constituting the Gβ5–R7 complexes. It is now established that the DEP domain interacts with the membrane anchoring proteins. However, the exact role of R7BP in the regulation of RGS6, 7, and 11 is not well understood. A number of promising binding partners of R7 proteins have been identified in yeast two-hybrid screens and other *in vitro* studies. For instance, according to some studies, DEP domains can interact with some GPCRs. Current data suggest that the Gβ5/GGL moiety can regulate the activity of both the RGS and DEP domains within the Gβ5–R7 complex. The biggest challenge for the future will be to establish which protein–protein interactions and other molecular events have physiologic relevance.

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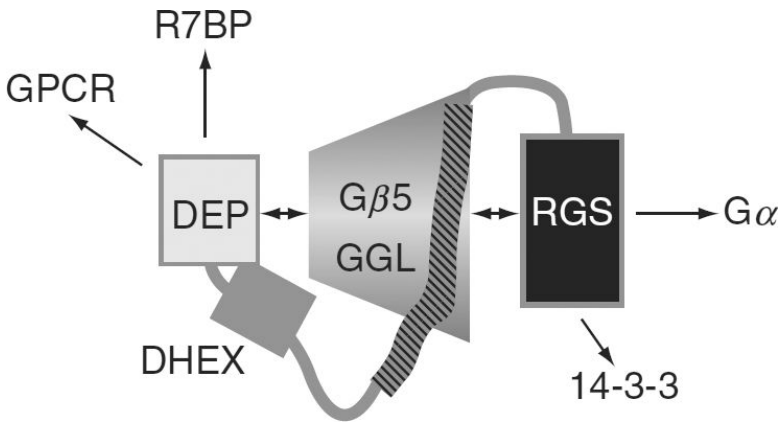

Fig. 1.

Protein–protein interactions of the Gβ5–R7 complex. Schematic drawing showing the composition of a Gβ5–RGS dimer. The RGS polypeptide consists of four domains: DEP, DHEX, GGL, and RGS. The GGL domain is tightly and irreversibly associated with Gβ5. Gβ5 also interacts with the DEP and RGS domains, shown with double-pointed arrows. These interactions are thought to be dynamic. The DEP domain can associate with R7BP (or R9AP), the interaction tethering the complex to the membranes. The interaction with R7BP was proven by a number of approaches, but Gβ5–R7 complexes can also be present in native cells in R7BP-free form. Some DEP domains were also shown to interact with GPCRs and possibly other proteins (see text). The RGS domain of R7 family can bind to G protein alpha subunits of Gi family and serve as a GAP. RGS7 and RGS9 proteins can be phosphorylated and shown to interact with 14–3–3.

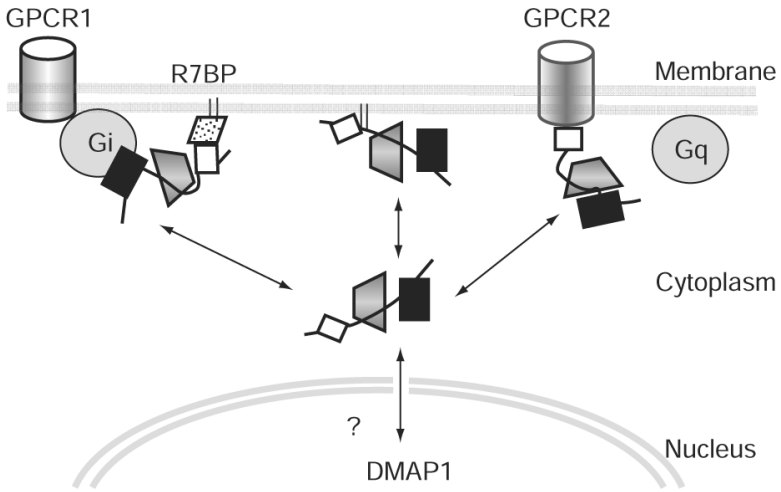

Fig. 2.

Subcellular localization of Gβ5–R7 complexes. This schematic drawing summarized the available information about all the R7 family members and the results of investigations of both native and transfected cells. R7 family membranes were detected at the plasma membrane, in the cytosol and in the nucleus. In the plasma membrane, all R7 members, via their DEP domains, can bind to an anchoring protein, R9AP or R7BP. R7BP attaches to the lipid bilayer via two palmitoyl tails depicted as two parallel lines. R7BP and R9AP were shown to facilitate the GAP activity of Gβ5–R7 complexes toward Gi family G proteins. R7BP was also shown to inhibit the effect of Gβ5–RGS7 on Gq-mediated signaling elicited by M3 muscarinic receptor. RGS9-1 and RGS9-2 require R9AP or R7BP for membrane anchoring, but RGS7 can attach to the membranes via alternative mechanisms that include direct palmitoylation of RGS7 and association with some GPCRs (see text). Evidence from R7BP knockout mice suggests that RGS6 and RGS11 behave similarly to RGS7. The binding partners of Gβ5–R7 in the cytosol are not known, and it is possible that the cytosolic form exists as a dimer. The significance and mechanism of trafficking of the Gβ5–R7 complexes to the nucleus have not been elucidated. It was shown that RGS6 associated with DMAP1, a transcriptional regulator.