Neuron, Vol. 20, 95–102, January, 1998, Copyright ©1998 by Cell Press

RGS9, a GTPase Accelerator for Phototransduction

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Summary

The rod outer segment phototransduction GAP (GTPase-accelerating protein) has been identified as RGS9, a member of the RGS family of Gα GAPs. *RGS9* mRNA expression is specific for photoreceptor cells, and RGS9 protein colocalizes with other phototransduction components to photoreceptor outer segment membranes. The RGS domain of RGS9 accelerates GTP hydrolysis by the visual G protein transducin ($G\alpha_t$), and this acceleration is enhanced by the γ subunit of the phototransduction effector cGMP phosphodiesterase (PDE_γ). These unique properties of RGS9 match those of the rod outer segment GAP and implicate it as a key element in the recovery phase of visual transduction.

Introduction

A challenging problem in phototransduction has been to understand the molecular basis for the temporal resolution of vision. Psychophysical and electrophysiological experiments have revealed that the sequence of light activation and recovery can occur in less than 1 s in mammalian rods and is even faster in cones (Hecht and Verrijp, 1933; Baylor et al., 1984; Kraft, 1988; Schnapf et al., 1990). The molecular properties of the identified mediators of the phototransduction cascade are well-suited to achieving activation on this time scale. Upon light activation, the photon receptor rhodopsin catalyzes rapid release of GDP and uptake of GTP by the G protein transducin (G_t), leading, on a millisecond time scale, to activation of transducin's effector, cGMP phosphodiesterase (PDE). PDE rapidly reduces cytoplasmic [cGMP], causing closure of cation channels cooperatively gated by cGMP. A model capable of remarkably accurate prediction of the activation phase of the light response can be calculated using only parameters derived from careful measurements or reasonable estimates for this limited set of molecules and from the well-supported assumption of a constant rate of cGMP synthesis during this early phase (Lamb and Pugh, 1992; Pugh and Lamb, 1993).

In contrast, the molecular basis for rapid termination of the light response is much less well-understood. Multiple biochemical events appear to be involved, including receptor inactivation and stimulation of cGMP synthesis (both processes are likely to be regulated by changes in internal Ca^{2+}) and GTPase-mediated G protein/effector inactivation. The contribution each makes to inactivation kinetics is unclear, but a "dominant time constant" has been described for recovery from flash-stimulated activation (Pepperberg et al., 1992; Lyubarsky et al., 1996), and it has recently been proposed that this rate-limiting step for the shut off of phototransduction is G protein deactivation via GTP hydrolysis (Sagoo and Lagnado, 1997).

The problem of the molecular mechanism for terminating G protein activation has been particularly perplexing. The intrinsic GTPase activity of $G\alpha_t$ hydrolyzes bound GTP and returns the G protein to the inactive state on a time scale of tens of seconds (Angleson and Wensel, 1993) and is thus too slow to account for observed phototransduction inactivation kinetics. It was proposed a decade ago that the resolution of this discrepancy lies in the action of one or more accessory factors that act on $G\alpha_t$ to accelerate GTP hydrolysis, and, indeed, phototransduction was the first G protein cascade in which the action of a GTPase-accelerating protein (GAP) was demonstrated (Dratz et al., 1987; Arshavsky et al., 1989). However, the identification of the protein or proteins responsible has remained an elusive goal (Arshavsky and Bownds, 1992; Angleson and Wensel, 1993, 1994; Antonny et al., 1993; Yamazaki et al., 1993; Pages et al., 1993; Otto-Bruc et al., 1994; Chen et al., 1996; Faurobert and Hurley, 1997; Nekrasova et al., 1997).

Despite initial reports that $G\alpha_t$ did not require an additional protein for subsecond GTP hydrolysis (Vuong and Chabre, 1990, 1991) or that the PDE_γ subunit was the GAP for $G\alpha_t$ (Arshavsky and Bownds, 1992), there is now considerable evidence that a $G\alpha_t$ GAP distinct from PDE subunits is present in rod outer segment (ROS) membranes (Angleson and Wensel, 1993, 1994; Antonny et al., 1993; Otto-Bruc et al., 1994). Moreover, it is well-established that PDE_γ forms a tight complex with $G\alpha_t$ without accelerating GTP hydrolysis (Angleson and Wensel, 1993, 1994; Antonny et al., 1993; Pages et al., 1993; Yamazaki et al., 1993; Arshavsky et al., 1994; Otto-Bruc et al., 1994; Nekrasova et al., 1997) but can enhance GTP hydrolysis in the presence of an ROS membrane protein (Angleson and Wensel, 1994; Arshavsky et al., 1994; Nekrasova et al., 1997).

Identification of members of the RGS protein family (De Vries et al., 1995; Druey et al., 1996; Koelle and Horvitz, 1996) as GAPs for Gα subunits (Berman et al., 1996; Watson et al., 1996) raised the possibility that the ROS GAP might be an RGS protein. So far, few biochemical studies have been carried out on endogenous RGS proteins in their native environments. However, cloned *RGS* cDNA has been used to express several mammalian RGS proteins or their RGS domains in *Escherichia coli*, allowing them to be assayed for GAP activity toward purified G proteins. Although all the expressed proteins have been soluble, those RGS proteins whose localization in cells has been studied have proven to be membrane-associated (De Vries et al., 1996; Dohlman et al., 1996), as is the ROS GAP (Angleson and Wensel, 1993, 1994). Interestingly, all

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RGS proteins studied to date act as fairly nonspecific accelerators of GTP hydrolysis for all members of the G_i subfamily of $G\alpha$ proteins (Berman et al., 1996; Chen et al., 1996; Hunt et al., 1996; Watson et al., 1996) and also for $G\alpha_q$ (Hepler et al., 1997). $G\alpha_t$, a member of the G_i subfamily, has been shown to serve as a substrate for all RGS proteins tested so far, including RGS1, RGS4, and GAIP (Watson et al., 1996), RGS16 (also referred to as RGS-r; Chen et al., 1996), and RET-RGS1 (Faurobert and Hurley, 1997). This lack of specificity has raised a number of interesting questions about why so many RGS proteins with such similar biochemical properties exist and how specificity for various G protein pathways might be achieved by superficially promiscuous members of the RGS protein family. Another puzzle concerning RGS interactions with $G\alpha_t$ has been the observation that in contrast to PDE_γ enhancement of the activity of endogenous ROS GAP, the $G\alpha_t$-GAP activity of RGS4, GAIP, RGS16, and A28-RGS14 is inhibited by PDE_γ (Natochin et al., 1997; Nekrasova et al., 1997; Wieland et al., 1997), leading to the suggestion that the PDE_γ-dependent ROS GAP may not be an RGS protein (Nekrasova et al., 1997).

We report here the identification of the ROS GAP protein as RGS9. Localization and activity characteristics of RGS9 are consistent with the highly concentrated, specialized, and spatially restricted nature of the phototransduction cascade and its components, as well as with all of the previously established biochemical properties of the ROS GAP, including enhancement by PDE_γ. RGS9 is now a mammalian RGS protein identified with a specific signaling pathway, and its expression pattern and effector subunit recognition suggest general mechanisms for achieving specificity in G protein regulation by RGS proteins.

Results and Discussion

Identification of Retinal RGS cDNAs

We screened cDNA libraries for cDNA encoding RGS domains by degenerate PCR and identified cDNA encoding several different RGS domains: *RGS9*, *RGS7*, *RGS3*, *RGS4*, *RGS6*, *RGS16*, and *RGS11*. These amplification products were then used as probes to isolate cDNA clones for bovine *RGS9*, *RGS4*, *RGS16*, and *RGS7*, as well as for murine *RGS9*, *RGS7*, *RGS6*, and *RGS11*. RGS16 is the name we are tentatively assigning a bovine protein whose sequence is more than 80% identical to murine and human proteins variously termed RGS-r (Chen et al., 1996; Natochin et al., 1997), A28-RGS14 (Buckbinder et al., 1997), and RGS16 (Chen et al., 1997).

Retina-Specific Expression of RGS9

When probes generated from these clones were used to examine mRNA expression by Northern blotting and in situ hybridization, only *RGS9* probes revealed a strong pattern of retinal and photoreceptor specificity (Figure 1). In Northern blots, *RGS9* message was easily detectable with 3 μg of total retinal RNA, while no signal could be detected with 10 μg of RNA from other tissues (Figure 1A). These results indicate that *RGS9* mRNA expression is much higher in retina than in other tissues, including the brain, the one other tissue where *RGS9* RNA has been detected (Koelle and Horvitz, 1996; C. W. C. et al., unpublished data).

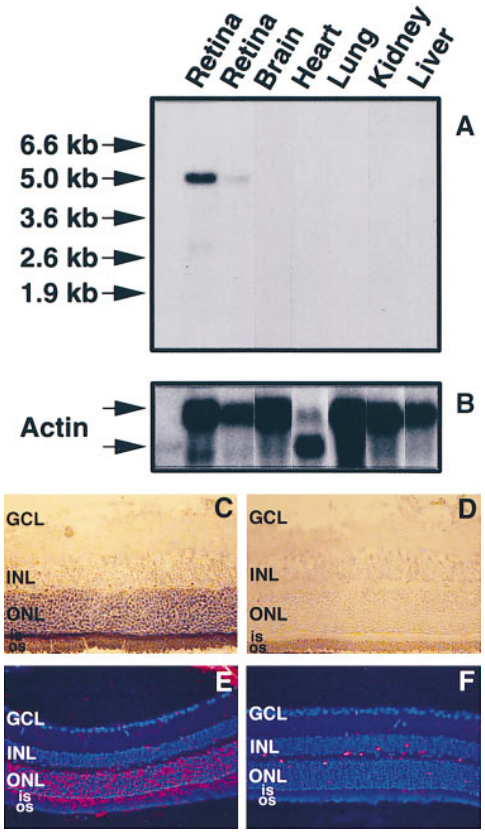

Figure 1. Photoreceptor-Specific Expression of Bovine *RGS9* mRNA

(A) Northern blot analysis of total RNA from (L to R): 10 μg retina, 3 μg retina, 10 μg brain, 6 μg heart, 10 μg lung, 10 μg kidney, 10 μg liver.

(B) Control blot using a human β-*actin* probe.

(C–F) Localization of *RGS9* mRNA to photoreceptor cell layer of the retina. In situ hybridization of murine eye sections using mouse RGS9 antisense (C and E) or sense (D and F) riboprobe, labeled with digoxigenin (C and D) or ^{35}S (E and F). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (contains cell bodies and nuclei of rod and cone photoreceptor cells); is, photoreceptor inner segment layer; os, photoreceptor outer segment layer.

Photoreceptor-Specific Expression of RGS9

Analysis by in situ hybridization localized retinal expression to the outer nuclear layer and inner segment layer, indicating a high degree of specificity for photoreceptor cells (Figures 1C–1F). This same pattern was observed with three different probes, using either digoxigenin (Figure 1C) or ^{35}S labeling (Figure 1E) for probe detection. With the digoxigenin-labeled probes, a highly concentrated signal in the myoid region of the rod inner segments was observed, consistent with results obtained for rhodopsin and $G\alpha_t$. The labeling was sufficiently dense to assure expression in rods but also to make it difficult to determine whether cones were labeled. In

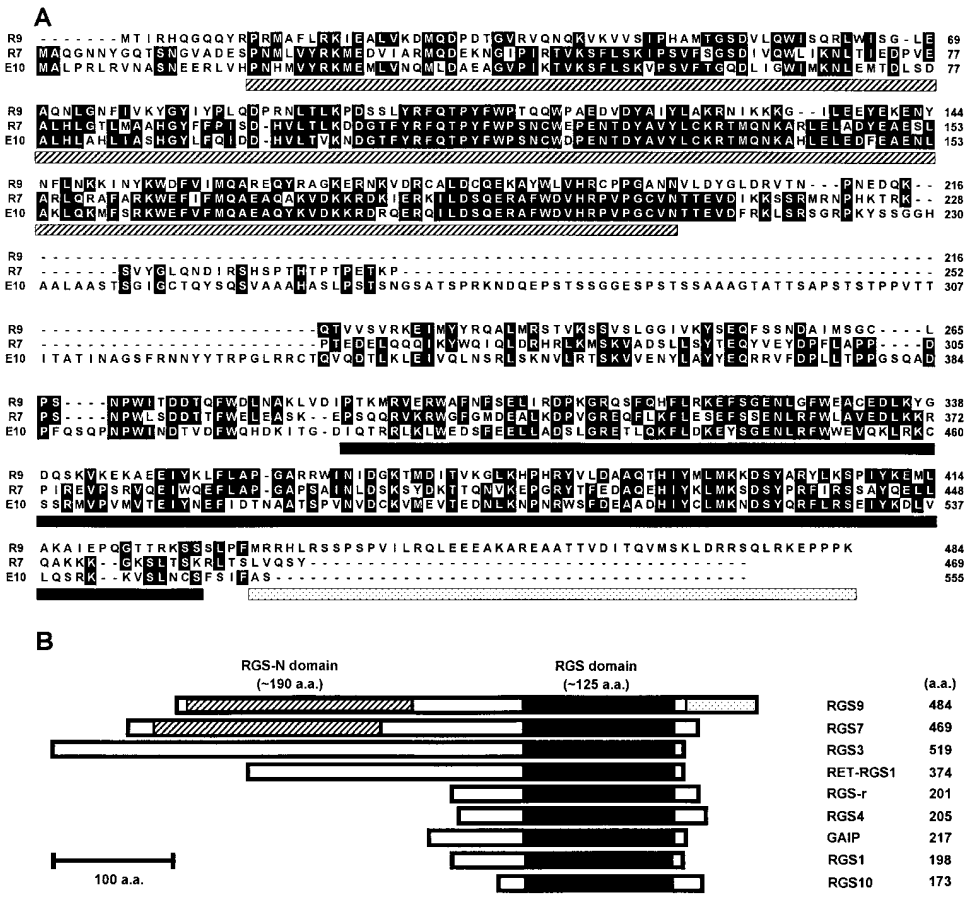

Figure 2. Deduced Primary Structures of RGS Proteins

(A) Predicted amino acid sequence for bovine RGS9 (R9) and alignment with bovine RGS7 (R7) and *C. elegans* EGL-10 (E10). Highlighted sequences are the RGS-N domain (hatched box) and RGS domain (filled box), and the unique RGS9 C-terminal tail (stippled box).

(B) Comparison of domain structures among representative mammalian RGS proteins.

addition to retinal expression, some *RGS9* mRNA is also found in rodent brain (Koelle and Horvitz, 1996), where it displays a highly restricted striatal expression pattern (Gold et al., 1997; C. W. C., unpublished data).

RGS9 Primary Structure Defines an RGS Subfamily

The sequence of the bovine *RGS9* open reading frame predicted a 484 amino acid protein (Figure 2A) of 56.7 kDa calculated molecular mass. The predicted mouse protein is 91.5% identical. Bovine (Figure 2A) and mouse *RGS7* clones predict 469 amino acid proteins, 99% identical to one another and to a partial human sequence (Koelle and Horvitz, 1996). RGS9 and RGS7 share considerable sequence identity outside the RGS domain, especially over a domain of ~190 amino acids near the N terminus, where identity (42%) is nearly as high as in the RGS domain (49%). This conserved RGS-N domain is shared by EGL-10, the *Caenorhabditis elegans* synapse-specific RGS protein (Koelle and Horvitz, 1996), and by partial sequences from RGS6 and RGS11 (W. H., unpublished data). Comparison of RGS domain sequences also suggests that RGS9, RGS7, RGS6, RGS11, and EGL-10 belong to a subfamily of RGS proteins. Within the RGS-N domain, there is an ~80 residue sequence that meets the criteria for a proposed DEP domain found within many signaling pathway proteins (Ponting and Bork, 1996), although the functional significance of this domain is currently unknown. A unique feature of RGS9 among known RGS proteins is a 52 amino acid C-terminal tail.

Localization of RGS9 Protein to Rod Outer Segments

We generated polyclonal antibodies that recognized recombinant RGS9 and used them to determine whether RGS9 protein is present in photoreceptor outer segments. Western blots with anti-RGS9 immune serum but not preimmune serum revealed an immunoreactive band of the same electrophoretic mobility as full-length recombinant His-RGS9 in purified ROS preparations, with no other immunoreactive bands detected (Figure 3A). To verify that the immunoreactive band in ROS was RGS9, we performed partial V8 protease digestion mapping. Digestion patterns for recombinant full-length His-RGS9 and the immunoreactive band in ROS were identical (Figure 3B), verifying the presence of RGS9 in ROS.

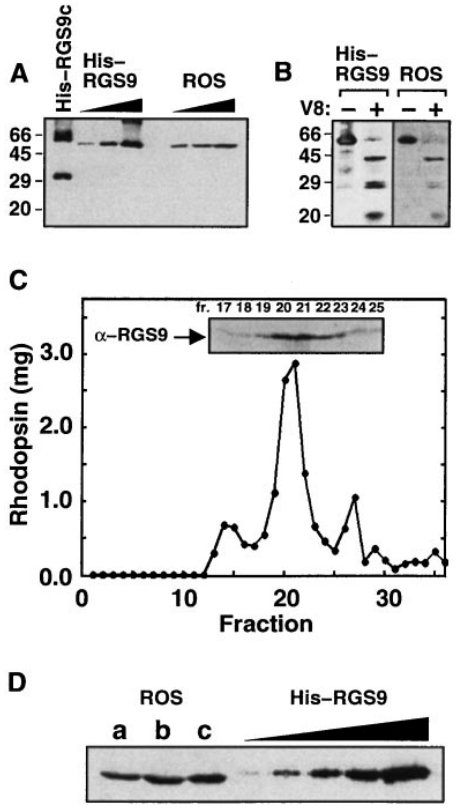

Figure 3. RGS9 Protein in Purified Bovine Rod Outer Segments

(A) Immunoblots of recombinant (lane 1) His-RGS9c used as immunizing antigen for rabbit antisera, (lanes 2–4) full-length His-RGS9, and (lanes 6–8) purified ROS. Amounts of recombinant protein loaded were 3 ng (lane 2), 30 ng (lane 3), or 300 ng (lane 4), and amounts of rhodopsin loaded were 3.4 μg (lane 6), 10 μg (lane 7), or 34 μg (lane 8). Preimmune serum did not detect any of these bands (data not shown).

(B) Immunoblot following treatment with 50 ng V8 protease of His-RGS9 (left panel) or ROS immunoreactive band (right panel).

(C) RGS9 colocalization with rhodopsin in ROS purification from bovine retinas. ROS were purified from bovine retinas by a discontinuous sucrose density gradient (Papermaster and Dreyer, 1974), and fractions were analyzed by change in rhodopsin absorbance at 500 nm after bleach (closed circles). Fractions were also analyzed by immunoblot using anti-RGS9c rabbit polyclonal serum (inset).

(D) Immunoblots of endogenous RGS9 in three different preparations of purified ROS (a, b, c; 30 μg of rhodopsin loaded for each), compared to recombinant His-RGS9 standards (L to R, in ng: 3.3, 10, 33, 100, 333). The average value for the mass ratio of RGS9:rhodopsin, determined by densitometry and interpolation on the standard curve, was 26.5 ± 10 ng RGS9 per 30 μg of rhodopsin (molar ratio 1:1640).

Estimates of the ratio of RGS9 to rhodopsin in three separate ROS preparations were made by densitometric comparison of the immunoblot signals obtained with varying amounts of recombinant RGS9 to those obtained on the same blot from ROS containing an amount of rhodopsin determined by spectrophotometry (Figure 3D). The results gave an estimate of 1 mole of RGS9 per 1640 (± 619) mole of rhodopsin, or about 1 molecule of RGS9 for every 6.6 molecules of holo-PDE ($PDE\alpha\beta\gamma\gamma$) in ROS. Because of the tight membrane association of RGS9 (see below), it seems unlikely that there is much loss during ROS purification.

The similarity in primary structure of RGS9 and other subfamily members (RGS6, RGS7, RGS11) raises the possibility of confusion of these with RGS9 in immunoblots. We tested our RGS9 antibodies for cross-reactivity with recombinant RGS7 and found that more than 100-fold higher levels of RGS7 were needed to obtain signals comparable to those obtained with RGS9. Immunoblots with RGS7-specific antibodies revealed that its levels in ROS are undetectable (i.e., at least 100-fold lower than RGS9 levels), thus ruling out detectable cross-reactivity with RGS7 as a source of confusion in Figure 3 (W. H., unpublished data). As the other subfamily members (*RGS6* and *RGS11*) show even less retinal expression at the mRNA level than *RGS7*, and the other retinal RGS proteins differ greatly in molecular weight from RGS9, it is extremely unlikely that the immunoblotting results shown in Figure 3 could represent cross-reactivity with another retinal RGS protein.

To determine whether RGS9 is an endogenous ROS protein or is simply a contaminant originating from inner segments or other cells in the retina, we used the RGS9 antibodies to assay for RGS9 protein at all stages of a sucrose density purification of ROS from bovine retinas. We found that RGS9 protein copurified with rhodopsin (Figure 3C), the major component of ROS, and was not detectable in any samples enriched in non-ROS components. Thus, RGS9 is predominantly a photoreceptor outer segment protein.

We also used immunoblots to explore the solubility of endogenous RGS9 in ROS. A buffer of moderate ionic strength (buffer A) did not remove a detectable amount of RGS9 from ROS membranes; the membranes used for the immunoblots in Figures 3A and 3B were washed with this buffer. Low salt (buffer B), which removes several other peripheral proteins such as PDE and G_t from dark-adapted membranes, also did not remove a detectable amount of RGS9 from ROS membranes. As observed previously for the ROS GAP (J. K. Angleson and C. W. C., unpublished data), the nonionic detergent octyl glucoside (OG) did solubilize RGS9. Approximately half of the RGS9 was solubilized at 30 mM OG, with maximal solubilization at 40 mM (in buffer C). This is the same concentration at which maximal soluble GAP activity is observed in ROS.

GTPase Accelerating Activity of RGS9

To test for the ability of RGS9 to accelerate transducin GTPase, we used purified recombinant RGS9. All forms of RGS9 expressed so far have been insoluble, apparently sequestered within inclusion bodies in *E. coli*. While full-length RGS9 was completely insoluble after purification and slow removal of denaturant (see Experimental Procedures), two forms (His-RGS9c, His-RGS9d) retained sufficient solubility for their GAP activity to be tested, with His-RGS9d being the more soluble of the two. Both accelerated GTP hydrolysis by $G\alpha_t$. Results with His-RGS9d, a recombinant fragment containing the RGS core domain and an N-terminal His_6 tag, are shown in Figure 4 for a single-turnover GTPase assay (Angleson and Wensel, 1993, 1994) that is sensitive to the hydrolysis of bound GTP rather than the kinetics of nucleotide exchange. In the absence of His-RGS9d, GTP hydrolysis

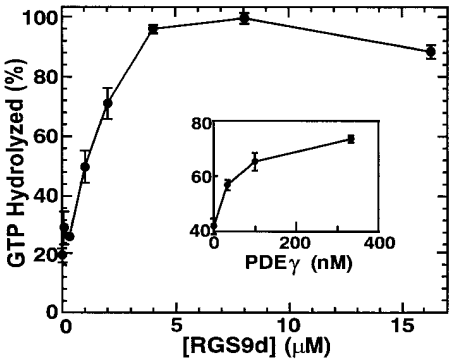

Figure 4. Transducin GTPase Acceleration by the RGS Domain of RGS9 (His-RGS9d)

Data points (closed circles) represent mean percent GTP hydrolysis ± SEM (n = 3), except for 0.33 μM His-RGS9d (n = 1), at 5 s after addition of buffer or His-RGS9d in single-turnover GTPase assays. (Inset) Effect of PDE_γ (recombinant, His_6-tagged) on GTPase acceleration by 1 μM His-RGS9d. Addition of up to 3.3 μM PDE_γ in the absence of His-RGS9d did not significantly affect GTP hydrolysis by transducin.

was slow (k_{inact} = 0.022 s^{-1}), whereas addition of His-RGS9d resulted in dramatic acceleration of GTP hydrolysis by transducin. At 8 μM, His-RGS9d increased the percent of GTP hydrolyzed in 5 s from the basal value of 19.8% ± 2.1%, corresponding to a k_{inact} of 0.022 s^{-1}, to 99.5% ± 1.8%, corresponding to a nominal k_{inact} of 1.04 s^{-1}. Because the value of k_{inact}, calculated as described in Experimental Procedures, becomes much less accurate when more than 90% of the GTP is hydrolyzed, these results allow us to estimate only a lower limit for the maximal GTPase acceleration by His-RGS9d: k_{inact} = 0.71 s^{-1}, corresponding to the lower extreme of the error bar for the 8 μM point in Figure 4. However, it seems likely that maximal GTPase acceleration is even greater than that suggested by the k_{inact} calculated for the average value of GTP hydrolyzed at 8 μM His-RGS9d; i.e., $k_{inact} > 1.04$ s^{-1}, implying acceleration greater than 47-fold. This result does not necessarily represent the maximal GTPase acceleration attainable by RGS9, even at 23°C, as determination of that value will require solubilization and purification in active form of full-length recombinant or endogenous RGS9 as well as the use of assays with greater temporal resolution. The relatively high concentrations of His-RGS9d required for GTPase acceleration raise the interesting possibility that G_t inactivation may be limited by intracellular RGS9 concentrations.

Enhancement of RGS9 GAP Activity by the Effector Subunit PDE_γ

As explained above, k_{inact} values become less accurate using our standard GTPase assay when almost all of the GTP is hydrolyzed before quenching, as occurs at high His-RGS9d concentrations. Therefore, to see if PDE_γ could enhance GAP activity of RGS9, we tested a concentration of His-RGS9d that gave intermediate GTPase acceleration (1 μM; k_{inact} = 0.08 s^{-1}) for the effect of adding PDE_γ. Previous studies (Natochin et al., 1997; Nekrasova et al., 1997; Wieland et al., 1997) have found that PDE_γ inhibits GAP activity of other nonphotoreceptor-specific RGS proteins (RGS4, GAIP, RGS16), while PDE_γ enhances GAP activity of the endogenous ROS GAP (Arshavsky and Bownds, 1992; Angleson and Wensel, 1994). At PDE_γ concentrations in the range of 33–333 nM, significant enhancement of GTPase acceleration was observed (Figure 4, inset), with a maximal effect of about 3-fold (333 nM PDE_γ; k_{inact} = 0.24 s^{-1}). By itself, i.e., without added RGS9, PDE_γ at concentrations up to 3.3 μM did not significantly accelerate transducin GTPase, as reported previously (Angleson and Wensel, 1993, 1994; Antonny et al., 1993). At higher His-RGS9d concentrations (e.g., 2–3 μM) PDE_γ also enhanced GAP activity, but the fold activation was less (1.3- to 1.5-fold), although it is unclear whether the maximum rate constant observed (k_{inact} = 1.04 s^{-1}) reflects an inherent property of the protein complex or limitations of our assay. The GTPase accelerating interactions involving PDE_γ and RGS9 appear to occur rapidly; the acceleration shown in Figure 4 was observed within 5 s after PDE_γ and His-RGS9d were added simultaneously. While PDE_γ enhancement of GTPase acceleration by His-RGS9d is somewhat less than that observed for the endogenous ROS GAP, the recombinant protein is missing more than half of the mass of RGS9, and it is possible that restoration of the missing N- and C-terminal domains and reconstitution of proper membrane association could increase the enhancement.

This observation of enhancement is in sharp contrast to the potent inhibition by PDE_γ of RGS16 (Natochin et al., 1997; Wieland et al., 1997), GAIP, and RGS4 (Nekrasova et al., 1997). Interestingly, only partial inhibition was observed with GAIP and RGS4 at saturating PDE_γ, indicating that an RGS domain and PDE_γ can bind transducin simultaneously, as implied by the enhancement of GAP activity we observe and by direct binding studies with RGS16 (Natochin et al., 1997).

Other Retinal RGS Proteins

In addition to *RGS9*, several other *RGS* cDNAs have been isolated from retinal cDNA libraries. These include *RGS4*, *RGS3*, *RGS7*, *RGS6*, *RGS11* (this work), *RGS16* (this work; Chen et al., 1996; Natochin et al., 1997), and *RET-RGS1* (Faurobert and Hurley, 1997). We explored the expression patterns of *RGS4*, *RGS7*, *RGS6*, *RGS11*, and *RGS16* (data not shown) and found none of them to be expressed in the retina at levels comparable to those of *RGS9*. All were expressed in other tissues at higher levels than in the retina, except for *RGS16*. However, photoreceptor expression of *RGS16* RNA was undetectable using the same in situ hybridization conditions as used for Figures 1C–1F, and when the Northern blot used for Figures 1A and 1B was probed for *RGS16*, its RNA could be readily detected in the liver as well as the retina. In other work, *RGS16* was reported to be specific for the liver and pituitary (Chen et al., 1997), and human *A28-RGS14*, which has been reported to be the human ortholog of *RGS16* (Natochin et al., 1997), shows a widespread pattern of expression in multiple human tissues (Buckbinder et al., 1997). This expression pattern and the inhibitory effects of PDE_γ on RGS16 GAP activity render RGS16/RGS-r an unlikely candidate

for the ROS GAP. Likewise, *RET-RGS1* (Faurobert and Hurley, 1997) does not show the photoreceptor-specific pattern of expression in the retina expected for a major phototransduction component. No evidence has been reported for the presence of any of these proteins other than RGS9 in ROS. It is likely that these multiple RGS proteins expressed at low levels in the retina and elsewhere play important roles in other G protein pathways whose components are less concentrated and specialized than those of phototransduction; alternatively, if they are present in ROS, they may supplement the role of RGS9.

RGS9 as the ROS Transducin GAP

Unlike any other RGS proteins examined so far, RGS9 displays all the hallmarks of an endogenous ROS GAP for transducin: (1) its mRNA expression is the most specific for the retina; (2) within the retina, the expression is restricted to photoreceptors, a pattern it shares with other transduction components, such as transducin α and γ subunits, PDE subunits, rhodopsin, and cone visual pigments, and the cGMP-gated channel subunits; (3) RGS9 protein is predominantly localized in outer segments, as are transducin and the other phototransduction components; (4) RGS9 is tightly membrane-associated; (5) the RGS domain (His-RGS9d) accelerates transducin GTPase >47-fold; (6) like the endogenous transducin GAP, RGS9's GTPase-accelerating activity is enhanced by PDE_γ, while other RGS proteins tested are inhibited by PDE_γ. While the physiological importance of PDE_γ enhancement of the ROS GAP activity remains uncertain, it is clearly a distinguishing feature of the accelerated transducin GTPase activity found in purified ROS (Arshavsky and Bownds, 1992; Angleson and Wensel, 1994). The achievement of highly specific regulation of transducin by RGS9 through a combination of highly restricted mRNA and protein localization and functional discrimination by the effector may represent a general paradigm for assignment of specific functions to superficially promiscuous RGS proteins.

At present, we cannot rule out the possibility that other proteins supplement the role of RGS9 as a $G\alpha_t$ GAP in ROS. While our antibodies do not significantly inhibit GAP activity, it may be possible to prepare antibodies that do inhibit or that can be used for immunodepletion of RGS9 from solubilized ROS. Such experiments would allow an estimate of the fraction of ROS GAP activity due to RGS9.

Another unanswered question concerns the role of RGS9 in setting the dominant time constant of recovery. In amphibians, this value has been established as approximately 2 s over a wide range of light intensities (Nikonov et al., 1998), so even within the limits of our current assays, it seems likely that RGS9-accelerated GTP hydrolysis would be kinetically competent to play this role. Electroretinographic studies in mice suggest it is an order of magnitude faster ($\sim$0.2 s; Lyubarsky and Pugh, 1996), so it will be necessary to determine if at physiological concentrations of RGS9, PDE subunits, and $G\alpha_t$, it is possible to attain GTP hydrolysis on that time scale. It has been reported previously that the GTP hydrolysis rate constant is approximately doubled when temperature is increased from 23°C to 37°C (Arshavsky et al., 1987; Ting and Ho, 1991; Angleson and Wensel, 1993). Therefore, GTP hydrolysis in mammalian retina is likely to be at least twice as fast as the maximum rate constant we have observed with His-RGS9d, corresponding to a time constant of about 0.5 s or less.

RGS9 and GTPase Acceleration in Cones

While the properties of RGS9 match well those of the endogenous GAP of ROS, it or a closely related RGS protein is very likely to be present at high levels in cone outer segments. Preliminary immunocytochemical results with mouse antisera raised against RGS9 reveal that both rod and cone outer segments are stained, but the signal in cones is stronger (K. Palczewski, personal communication). The response kinetics of cones are much faster than those of rods, and GTPase acceleration by RGS9 may be the answer to the long-standing question of how physiological recovery on time scales of tens of milliseconds (Hecht and Verrijp, 1933; Schnapf et al., 1990) can be mediated by G proteins with hydrolysis kinetics on the order of tens of seconds.

Experimental Procedures

Degenerate PCR, Screening of cDNA Libraries, and Northern Blot Analysis

Degenerate primers 5E, 5R, 3T, and 3A (Koelle and Horvitz, 1996) as well as 5′ primers 5W01 (TGGI(A/T)(G/A/C)GCITG(C/T)GA(A/G)GA(T/G/C)T(T/A)CAAG) and 5W02 (TGGI(A/T)(G/A/C)GCITG(C/T)GA(A/G)GA(T/G/C)CT(G/C)A AG) and a 3′ primer 3W01 (AA(A/G)C(T/G)(A/G)(C/G)G(A/G)TAIGA(A/G)TC(C/A/G)(T/ C)T(C/T)T) were used to amplify by PCR cDNA encoding RGS domains from bovine and murine λZAPII retinal cDNA libraries and a Charon BS− murine retina plus pigment epithelium cDNA library. The four 5′ and three 3′ primers were used in all 12 possible combinations. Full-length cDNA clones were isolated by plaque hybridization using the identified RGS domain cDNAs as radiolabeled probes. Northern blots were generated from total RNA of various bovine tissues purified by standard guanidinium isothiocyanate/CsCl precipitation protocol or Qiagen RNeasy Midiprep kit. RNA was separated on a 1% agarose/formaldehyde gel and transferred overnight to supported nitrocellulose membrane. Northern blots were hybridized with a ^{32}P-labeled 275 nucleotide bovine *RGS9* DNA probe. Hybridization was performed at 42°C overnight, and high-stringency washes were performed in 0.1× SSC/0.1% SDS at 65°C. Control hybridizations were performed with a ^{32}P-labeled human β-*actin* probe (Clontech).

In Situ Hybridization

In situ hybridizations were performed on $\sim$7 μm thick wax-embedded sections of adult murine eyes (Novagen) with ^{35}S-UTP-labeled riboprobes generated by in vitro transcription of an $\sim$920 bp *mRGS9* fragment, corresponding to amino acids 150–457 of mouse RGS9 protein (sequence deposited in GenBank) subcloned into pBS KS(−) vector as an EcoRI/XhoI insert, using either T7 (antisense) or T3 (sense) RNA polymerase. Hybridization and wash conditions were performed as described (Albrecht et al., 1997). High-stringency washes were performed at 66°C. Alternatively, hybridization was carried out with digoxygenin-labeled probes as recommended by the reagent manufacturer (Boehringer Mannheim), with visualization by anti-digoxigenin alkaline phosphatase conjugate and NBT/BCIP. The digoxigenin-labeled *RGS9* probes were generated using a 698 bp 3′-untranslated region, beginning at the stop codon, from the *RGS9* cDNA, subcloned into pCR-Script Amp SK(+).

Expression and Purification of Recombinant Proteins

Recombinant His-RGS9c (corresponding to amino acids 226–484), His-RGS9 (corresponding to the full-length protein), and His-RGS9d (corresponding to amino acids 291–418 of the RGS9 core domain)

were expressed in bacteria using pET14b (Novagen), which incorporates an N-terminal His_6 tag. RGS9 recombinant proteins were expressed in BL21(DE3) pLysS cells by addition of 1 mM IPTG at a cell density of $A_{600} = 0.4–0.7$ and growth for 3 hr at 37°C. The recombinant proteins were all solubilized from inclusion bodies and purified with Ni^{2+}-NTA agarose (Qiagen) under denaturing conditions, using the manufacturer's recommended protocol, with final elution in a buffer containing 6 M guanidinium chloride and 0.2 M acetic acid. His-RGS9d was renatured by overnight dialysis at 4°C in buffer D. Insoluble RGS9d was removed by two rounds of centrifugation at 84,000 × g and 4°C for 30 min each. Full-length His-RGS9 was not soluble upon removal of denaturant (urea or guanidinium chloride) under a variety of conditions tried, including stepwise dialysis over several days. His-RGS9c was slightly soluble, although much less so than His-RGS9d.

Recombinant PDE_γ was expressed with an N-terminal His_6 tag using pET14b (Novagen) and purified by Ni^{2+}-NTA agarose (Qiagen) under denaturing conditions and reverse phase HPLC (Angleson and Wensel, 1994). His-PDE_γ gave essentially identical results to those obtained with recombinant PDE_γ (Angleson and Wensel, 1994) for both enhancement of ROS GAP activity and inhibition of trypsin-activated PDE. His-PDE_γ concentration was calculated from a functional protein assay as described (Angleson and Wensel, 1993).

Antibodies and Immunoblot Analysis

Rabbits were injected with purified His-RGS9c at Bethyl Laboratories using a standard immunization protocol. Preimmune and immune sera were tested for immunoreactivity to His-RGS9c by Western blot analysis on nitrocellulose membranes by standard techniques (Harlow and Lane, 1988). Blots were incubated overnight with a 1/1000 dilution of His-RGS9c rabbit antiserum in 5% nonfat dry milk/TBS, followed by incubation for 45 min with 1/10,000 dilution of anti-rabbit peroxidase antibodies (Amersham ECL kit). Western blots were developed using the ECL kit (Amersham) and exposed to Kodak Biomax MR film. Partial V8 protease digestion of proteins excised from gel bands was carried out as described (Harlow and Lane, 1988).

Buffers

All buffers used with proteins and ROS contained solid PMSF and in addition: buffer A: 10 mM MOPS (pH 7.4), 30 mM NaCl, 60 mM KCl, 2 mM $MgCl_2$; buffer B: 5 mM Tris-HCl (pH 7.5), 3 mM EDTA, 1 mM DTT; buffer C (octyl glucoside buffer): 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0, 20, 40, or 60 mM octyl glucoside; buffer D (GAP assay buffer): 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 2 mM $MgCl_2$, 0.02 mM AMP-PNP.

Rod Outer Segment Purification

Rod outer segments were purified from frozen bovine retinas by a standard sucrose gradient technique (Papermaster and Dreyer, 1974). Samples from every stage of the procedure were tested for the presence of RGS9 immunoreactivity by immunoblotting and for rhodopsin by visible absorbance.

Single-Turnover GTPase Assays

Single-turnover GTPase assays were carried out essentially as described (Angleson and Wensel, 1993, 1994). Four molar urea–washed ROS membranes (devoid of endogenous GAP activity) were reconstituted at final assay concentrations of 15 μM freshly illuminated rhodopsin with 1 μM transducin in buffer D. All measurements were performed in a final assay volume of 100 μl at 23°C with continuous mechanical stirring. The GTPase assay was initiated by the addition of 50 nM [γ-^{32}P]GTP (GTP $<<$ $G\alpha_t$), followed by addition of buffer, GTPγS, or accelerating factors (His-RGS9d or His-PDE_γ, alone or together), at 5 s. The reaction was stopped at 10 s by addition of 50 μl of 50% TCA, incubated with 5% activated charcoal, 10% TCA, 5 mM NaH_2PO_4, and free $^{32}P_i$ was determined by scintillation counting. The GTP hydrolysis rate constant was determined according to: $k_{inact} = -$(ln[percent GTP unhydrolyzed at 10 s/percent GTP unhydrolyzed at 5 s])/(5 s).

Acknowledgments

We thank Gregor Eichele and members of his laboratory for assistance with the in situ hybridization experiments, Wolfgang Baehr and coworkers for cDNA libraries, Hong Wang, Brian Perkins, and Chau Le for technical assistance, Krzysztof Palczewski for sharing data prior to publication, and the NIH (EY07981, DK07696) and Robert F. Welch Foundation for funding.

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Received October 6, 1997; revised November 14, 1997.

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GenBank Accession Numbers

The sequences described in this paper have been deposited in GenBank under the accession numbers AF011357 (bRGS9), AF011358 (mRGS9), AF011359 (bRGS7), AF011360 (mRGS7), AF011361 (bRGS16/-r).