Regulation of Transducin GTPase Activity by Human Retinal RGS*

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The intrinsic GTPase activity of transducin controls inactivation of the effector enzyme, cGMP phosphodiesterase (PDE), during turnover of the visual signal. The inhibitory γ-subunit of PDE (Pγ), an unidentified membrane factor and a retinal specific member of the RGS family of proteins, has been shown to accelerate GTP hydrolysis by transducin. We have expressed a human homologue of murine retinal specific RGS (hRGSr) in Escherichia coli and investigated its role in the regulation of transducin GTPase activity. As other RGS proteins, hRGSr interacted preferentially with a transiti- 
tional conformation of the transducin α-subunit, Gα_GDPAlFγS, while its binding to Gα_GTPγS or Gα_GDP was weak. hRGSr and Pγ did not compete for the interac-
tion with Gα_GDPAlFγ. Affinity of the Pγ-Gα_GDPAlFγ interaction was modestly enhanced by addition of hRGSr, as measured by a fluorescence assay of Gα_GDPAlFγ binding to Pγ labeled with 3-(bromoacetyl)-7-diethylaminocoumarin (PγBC). Binding of hRGSr to Gα_GDPAlFγ complexed with PγBC resulted in a maximal ~40% reduction of BC fluorescence allowing estimation of the hRGSr affinity for Gα_GDPAlFγ (Kd = 35 nM). In a single turnover assay, hRGSr accelerated GTPase activity of transducin reconstituted with the urea-stripped rod outer segment (ROS) membranes by more than 10-fold to a rate of 0.23 s⁻¹. Addition of Pγ to the reconstituted system reduced the GTPase level accelerated by hRGSr (kcat = 0.085 s⁻¹). The GTPase activity of transducin and the PDE inactivation rates in native ROS membranes in the presence of hRGSr were ele-
vated 3-fold or more regardless of the membrane concentra-
tions. In ROS suspensions containing 30 μM rhodopin these rates exceeded 0.7 s⁻¹. Our data suggest that effects of hRGSr on transducin's GTPase activity are attenuated by Pγ but independent of a putative membrane GTPase activating protein factor. The rate of transducin GTPase activity in the presence of hRGSr is sufficient to correlate it with in vivo turnoff kinetics of the visual cascade.

In vertebrate photoreceptor cells, the signal is transduced from light-activated rhodopsin to the effector enzyme, cGMP phosphodiesterase (PDE), via the heterotrimeric G-protein, transducin (Gαβγ). The GTP-bound α-subunit of transducin (Gα,GTP) relieves the inhibition imposed by two inhibitory PDE γ-subunits (Pγ) on the enzyme catalytic aβ subunits (Pαβ). Activation of PDE leads to a closure of cGMP-gated channels in the photoreceptor plasma membranes (1–3). The inactivation of PDE is a critical component of the turnover mech-
anism in the visual transduction cascade. This inactivation is controlled by the intrinsic GTPase activity of transducin which hydrolyzes GTP to GDP. The GDP-bound Gα (Gα,GDP) has a substantially reduced affinity for Pγ and releases Pγ to re-
inhibit Pαβ (1, 4–6). The rate of GTP hydrolysis by transducin measured in vitro (7, 8) is too slow to account for the fast photoreceptor turnoff in vivo (9, 10). The Pγ subunit (11, 12) and a distinct membrane-associated protein factor (13, 14) have been shown to enhance transducin GTPase activity in the activated membrane-bound transducin-PDE complex to a level comparable with the rate of transducin inactivation in vivo. A recent study has shown that a retinal specific member of the RGS family, RGSr, serves as a GTPase-activating protein (GAP) for transducin, providing an additional dimension to an already complex picture of the regulation of transducin GTPase activity (15). Functional relationships between RGSr, the γ-subunit of PDE, and a putative membrane GAP factor are currently not understood.

Here, we study the interaction between transducin and a human retinal specific RGS (hRGSr), and regulation of trans-
ducin GTPase activity by hRGSr. We examine the effects of Pγ and photoreceptor membrane concentration on modulation of the GTPase activity by hRGSr.

EXPERIMENTAL PROCEDURES

Materials—GTP and GTPγS were products of Boehringer Mannheim. Blue-Sepharose CL-6B was obtained from Pharmacia. 3-(Bromoacetyl)-7-diethylaminocoumarin (BC) was purchased from Molecular Probes, Inc. [γ-32P]GTP (>5000 Ci/mmol) was obtained from Ameri-
sham. [35S]GTPγS (1250 Ci/mmol) was purchased from NEN Life Sciences Products. All other chemicals were from Sigma.

Preparation of ROS Membranes, Gα,GTPγS, Gα,GDP, and PγBC—Bovine ROS membranes were prepared as described previously (16). Urea-washed ROS membranes (uROS) were prepared according to protocol in Ref. 17. Hypotonically washed ROS membranes were prepared as described in Ref. 14. Transducin, Gα,GTPγS, was extracted from ROS membranes using GTP as described in Ref. 18. The Gα,GTPγS was extracted from ROS membranes using GTPγS and pu-
rified by chromatography on Blue-Sepharose CL-6B by the procedure described in Ref. 19. Gα,GDP was prepared and purified according to protocols in Ref. 20. PγBC was obtained and purified as described in Ref. 6. The purified proteins were stored in 40% glycerol at −20 °C or without glycerol at −80 °C.

Cloning and Expression hRGSr—A BLAST search at NCBI (Bethes-
da, MD) to compare the mouse mRGSr cDNA sequence against DNA sequence data bases revealed a human homologue, A28-RGS14p (the GenBank accession number U70426), which is 85% identical to mRGSr. DNA prepared from the amplified human retinal cDNA aqt10 library

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1 The abbreviations used are: PDE, rod outer segment cGMP phospho-
diesterase; Gα, rod GTP-binding protein transducin; Pαβ and Pγ, α, β, and γ subunits of PDE, ROS, rod outer segment(s); uROS, urea-
stripped ROS membranes; dROS, hypotonically washed ROS mem-
branes; PγBC, Pγ labeled with 3-(bromoacetyl)-7-diethylaminocou-
marin (BC); GTPγS, guanosine 5′-O-(3-thiotriphosphate); GST, gluta-
thione S-transferase; h, human; m, mouse.
(kindly provided by J. Nathans, Johns Hopkins University) was used as a template for the polymerase chain reaction amplification with primers that were synthesized based on the A28-RGS14p sequence. The polymerase chain reaction was performed in 30 µl of reaction mixture containing 100 ng of the template DNA and 0.5 µM of the following primers: ATACTCTGACATGCTGCCACCTGTCGG (5') and ATGGCTGAGACTGAGGTTGTTGAGG (3'). The polymerase chain reaction product (620 bp) was digested with XbaI and XhoI (the restriction sites are underlined) and subcloned into the pGEX-KG vector (21) for GST-hRGSr fusion protein expression. The DNA sequence was verified by automated DNA sequencing at the University of Iowa DNA Core Facility using the 3'-GEX sequencing primer (Pharmacia) and the 5'-primer that was synthesized based on the A28-RGS14p at two nucleotide positions of the open reading frame (C130→T and A1700→G), leading to substitutions of amino acid residues Ser42→Phe and Asn54→Asp. Typically, expression host E. coli DH5α cells were grown on 2 × YT medium and induced at OD600 = 0.5 by addition of isopropyl-1-thio-β-D-galactopyranoside (0.4 mM final concentration). After a 4-h induction at 37 °C, cells were harvested and sonicated in 20 mM Tris-HCl containing 10 mM glutathione. GST-hRGSr was then passed through a PD-10 column (Pharmacia) to separate glutathione and digested with thrombin (0.25 NIH units/mg) for 90 min at room temperature. hRGSr was reapplied on a glutathione-agarose column to remove GST.

Binding of Translucin to GST-hRGSr-agarose—Gt6 GDP or Gt2 GDP-S (6 µM final concentration) were mixed with glutathione-agarose retaining ~10 µg of hRGSr in 40 µl of 20 mM HEPES buffer (pH 7.6), 100 mM NaCl, and 2 mM MgCl2 (buffer B). Where indicated, the bound was eluted by adding 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM glutathione. GST-hRGSr was then passed through a PD-10 column (Pharmacia) to separate glutathione and digested with thrombin (0.25 NIH units/ml) for 90 min at room temperature. hRGSr was reapplied on a glutathione-agarose column to remove GST.

Fluorescence Assays—Fluorescence assays were performed on a F-2000 Fluorescence Spectrophotometer (Hitachi) in 1 ml of buffer B essentially as described in Ref. 6. Where indicated, the buffer contained 30 mM AlCl3 and 10 mM sodium fluoride. Typically, hRGSr was added to Py-Fl-βγS solutions of transducer components except when Gt6 GDP and ATPγS were used. In the latter experiments the equilibration buffer with Gt6 GDP activation by ATPγS was used. In the latter experiments the equilibration buffer with Gt6 GDP activation by ATPγS was used.

Analytical Methods—Single turnover GTPase activity measurements were carried out essentially as described in Ref. 22. The reaction was initiated by mixing washed ROS membranes with 200 nM [γ-32P]GTP (~5 × 10⁶ dpm/pmol) in a total volume of 20 µl. The reaction was quenched by addition of 100 µl of 7% perchloric acid. Nucleotides were then precipitated using charcoal, and [32P]P formation was measured by liquid scintillation counting. Concentrations of transducin in different preparations of ROS membranes were determined using the [32S]GTPyS binding assay. ROS membranes were incubated with 2 µM GTPyS (10⁶ dpm/pmol) for 10 min at room temperature in 20 µl of buffer B, and the mixture was mixed onto GF/B filters (Millipore). The filters were washed with 3 ml of 40 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl and 4 mM MgCl2 and counted in a liquid scintillation counter. To determine if hRGSr or Py affects the nucleotide binding to Gt6, 200 nM [32S]GTPyS was added to ROS membranes (5 µM rhodopsin) reconstituted with 0.4 µM Gt6 GDP, and 1 µM hRGSr (and/or 1 µM Py) in 20 µl of buffer B. The mixture was immediately (<2 s) diluted into 3 ml of cold 40 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl, 4 mM MgCl2, and 2 µM GTP-S. The GTPyS binding was then measured as described above. The PDE activity was measured using the protein-evolution assay as described in Ref. 23. Protein concentrations were determined by the method of Lowry (24), using IgG as a standard or using calculated extinction coefficients at 280 nm. SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (25) in 12% acrylamide gels. Rhodopsin concentrations were measured using the difference in absorbance at 500 nm between “dark” and bleached ROS preparations. The Kd and IC50 values were calculated as described in Ref. 26. The results are expressed as the mean ± S.E. of triplicate measurements.

Results

Expression and Purification of hRGSr—The sequence encoding for the human homologue of mouse retinal RGSr protein was polymerase chain reaction amplified from the retinal cDNA library, subcloned into the pGEX-KG vector, and expressed as a GST fusion protein as described under “Experimental Procedures.” Eluate of GST-hRGSr from a column with glutathione-agarose is shown in Fig. 1 (lane 2). GST-hRGSr migrated on SDS gels with the expected mobility of a 52-kDa protein. Appearance of an additional doublet at ~28–30 kDa, which most likely contains GST polypeptide, is typical for the GST fusion expression system (27). hRGSr, purified after cleavage of the fusion protein GST-hRGSr with thrombin, migrated as a 25-kDa protein (Fig. 1, lane 3). The yield of hRGSr was ~6 mg/liter of culture.

Binding of GST-hRGSr to Gt6 GDPAlF4, Gt2 GDPyS, and Gt6 GDP—A number of RGS proteins (RGS1, RGS4, and mouse RGSr) have been shown to interact preferentially with a transitional Gt6 GDPAlF4 or Gt2 GDPyS conformation of Gt6 subunits (15, 28, 29). We examined the ability of GST-hRGSr bound to glutathione-agarose to co-precipitate Gt6 in different conformations. Fig. 2A shows that GST-hRGSr precipitated stoichiometric amounts of Gt6 GDPAlF4, while amounts of Gt2 GDPyS and Gt6 GDP that co-precipitated with GST-hRGSr were significantly lower. In control experiments, Gt6 GDPAlF4, Gt2 GDPyS, and Gt6 GDP did not co-precipitate with glutathione-agarose that contained no bound GST-hRGSr (not shown). The results suggest that the affinity of hRGSr for the Gt6a conformations decreases in the following order: Gt6 GDPAlF4 >> Gt2 GDPyS > Gt6 GDP.

Effects of Py on the Interaction between hRGSr and Gt6 GDPAlF4—To determine if Py can compete with hRGSr for the interaction with Gt6 GDPAlF4, we initially tested effects of Py on Gt6 GDPAlF4 binding to GST-hRGSr. Even at high concentrations (up to 30 µM) Py did not affect binding of Gt6 GDPAlF4 to GST-hRGSr immobilized on glutathione-agarose (Fig. 2B). We next investigated effects of hRGSr on the interaction between Py and Gt6 GDPAlF4 or Gt2 GDPyS using a fluorescence assay. Addition of Gt6 GDPAlF4 to a fluorescently labeled Py, P2BC, produced an approximately 7.5-fold maximal increase in the BC fluorescence (Fig. 3A), while Gt2 GDPyS enhanced the fluorescence of P2BC by more than 6-fold (not shown). The Kd values for the Gt6 GDPAlF4 and Gt2 GDPyS binding to P2BC were 2.8 ± 0.1 and 2.1 ± 0.1 nM, respectively. The dissociation of Gt6 GDPAlF4 binding to P2BC was somewhat higher in the presence of 100 nM hRGSr (Kd = 1.2 ± 0.1 nM), suggesting that hRGSr and Py bind to Gt6 GDPAlF4 noncompetitively (Fig. 3A). Addition of hRGSr had no effect on the fluorescence of P2BC alone (not shown), but resulted in a dose-dependent decrease in the fluorescence enhancement of P2BC caused by the latter binding to Gt6 GDPAlF4 (Fig. 3B). The fluorescence was decreased maximally by ~40% with an

FIG. 1. Expression and purification of hRGSr. SDS-polyacrylamide gel (12%) stained with Coomassie Blue. Lane 1, soluble fraction after sonication of the E. coli cells induced with isopropyl-1-thio-β-D-galactopyranoside as described under “Experimental Procedures.” Lane 2, GST-hRGSr fusion protein. Lane 3, hRGSr purified after cleavage of GST-hRGSr with thrombin.

Table

| kDa | 1 | 2 | 3 |
|-----|---|---|---|
| 46  |   |   |   |
| 48  |   |   |   |
| 50  |   |   |   |

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IC_{50} of ~35 nM. Since hRGSr and Pγ interact with Gt_{GDPAIF₄} noncompetitively, this IC_{50} value may serve as an estimate for the affinity of hRGSr interaction with Gt_{GDPAIF₄}. In control experiments, hRGSr did not affect the fluorescence of the Gt_{GTP}S-PγBC complex (not shown).

**Effects of hRGSr on Transducin’s GTPase Activity**—Single turnover measurements of GTPase activity were carried out as described under “Experimental Procedures.” Under these conditions, Gt_{GTP}S was immobilized on glutathione-agarose was carried out as described under “Experimental Procedures.” Lanes: 1, Gt_{GDP}; 2 and 3, Gt_{GDP} and Gt_{GDPAIF₄} bound to GST-hRGSr, respectively; 4, Gt_{GTP}S; 5, Gt_{GTP}S bound to GST-hRGSr. B, effects of Pγ on binding of Gt_{GDPAIF₄} to GST-hRGSr. Lanes: 1, Gt_{GDP}; 2, Gt_{GDPAIF₄} bound to GST-hRGSr; 3 and 4, Gt_{GDPAIF₄} bound to GST-hRGSr in the presence of 10 and 30 μM Pγ, respectively.

**Fig. 2. Binding of GST-hRGSr to Gt_{GDP-SDS-polycrylamide gel (12%) stained with Coomassie Blue. A, binding of Gt_{GDP}, Gt_{GDPAIF₄}, and Gt_{GTP}S to GST-hRGSr immobilized on glutathione-agarose was carried out as described under “Experimental Procedures.” Lanes:** 1, Gt_{GDP}; 2 and 3, Gt_{GDP} and Gt_{GDPAIF₄} bound to GST-hRGSr, respectively; 4, Gt_{GTP}S; 5, Gt_{GTP}S bound to GST-hRGSr. B, effects of Pγ on binding of Gt_{GDPAIF₄} to GST-hRGSr. Lanes:** 1, Gt_{GDP}; 2, Gt_{GDPAIF₄} bound to GST-hRGSr; 3 and 4, Gt_{GDPAIF₄} bound to GST-hRGSr in the presence of 10 and 30 μM Pγ, respectively.

**Fig. 3. Effects of hRGSr on the interaction between Gt_{GDPAIF₄} and PγBC.** A, the relative increase in fluorescence ([F/F₀]₅₃₀ₕ) of PγBC (5 nM) alone (squares) or in the presence of 50 (triangles) and 100 nM (circles) hRGSr was determined after addition of increasing concentrations of Gt_{GDPAIF₄} and is plotted as a function of the free Gt_{GDPAIF₄} concentration. The assay buffer contained 30 μM AlCl₃ and 10 mM sodium fluoride. The binding curve characteristics are: squares, Kᵦ = 2.8 ± 0.1 nM, maximum Fᵦ/F₀ = 7.5 ± 0.2; triangles, Kᵦ = 1.6 ± 0.1 nM, maximum Fᵦ/F₀ = 5.2 ± 0.2; circles, Kᵦ = 1.2 ± 0.1 nM, maximum Fᵦ/F₀ = 3.9 ± 0.1. B, the relative increase in fluorescence ([F/F₀]₅₃₀ₕ) of PγBC (5 nM) in the presence of increasing concentrations of hRGSr was determined after addition of 10 nM Gt_{GDP}. The assay buffer contained 30 μM AlCl₃ and 10 mM sodium fluoride. The fluorescent change ([F/F₀]₅₃₀ₕ) is plotted as a function of hRGSr concentration. The IC_{50} value of 35 nM is calculated from the curve.

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**Fig. 2.** Binding of GST-hRGSr to Gt_{GDP-SDS-polycrylamide gel (12%) stained with Coomassie Blue. A, binding of Gt_{GDP}, Gt_{GDPAIF₄}, and Gt_{GTP}S to GST-hRGSr immobilized on glutathione-agarose was carried out as described under “Experimental Procedures.” Lanes: 1, Gt_{GDP}; 2 and 3, Gt_{GDP} and Gt_{GDPAIF₄} bound to GST-hRGSr, respectively; 4, Gt_{GTP}S; 5, Gt_{GTP}S bound to GST-hRGSr. B, effects of Pγ on binding of Gt_{GDPAIF₄} to GST-hRGSr. Lanes: 1, Gt_{GDP}; 2, Gt_{GDPAIF₄} bound to GST-hRGSr; 3 and 4, Gt_{GDPAIF₄} bound to GST-hRGSr in the presence of 10 and 30 μM Pγ, respectively.
ROS suspensions containing 30 μM rhodopsin in the presence of 1 μM hRGSr is >0.7 s⁻¹.

**Acceleration of PDE Inactivation by hRGSr**—We measured PDE inactivation in suspensions of bleached native ROS membranes using the proton evolution assay (23). The activation and inactivation of PDE was monitored after addition of GTP under single turnover conditions using a pH microelectrode. The PDE activity was maximal in less than 1 s after addition of GTP. Therefore, we were unable to resolve the activation phase. As shown earlier, the rate of PDE inactivation can be well approximated by fitting the inactivation phase with a single exponential decay function (14). The PDE activity in the assay is proportional to the concentration of active Gα, GTP. The change of pH due to hydrolysis of cGMP under single GTP turnover conditions can be described using an exponential function: \( \Delta pH = \Delta \log H = \Delta[H_\text{max}(1-e^{-k t})] \) or [cGMP]hydrolyzed = \( \Delta[cGMP]_{\text{max}}(1-e^{-k t}) \). The PDE activity represents a derivative of this function, and it decays with the inactivation constant \( k \) from the equation above. In diluted suspensions of native ROS (5 μM rhodopsin), PDE was inactivated with a rate of 0.086 s⁻¹. In the presence of hRGSr, PDE inactivation was enhanced to a rate of 0.31 s⁻¹ (Fig. 5A). The increase in \( k_{\text{inact}} \) (>3-fold) correlated well with the decrease in maximal amounts of cGMP hydrolyzed in the presence of hRGSr (Fig. 5A). Furthermore, the acceleration of PDE inactivation caused by hRGSr was proportional to the elevation of transducin GTPase activity. We next tested effects of ROS membrane concentration on the modulation of PDE inactivation by hRGSr. The PDE inactivation rate (0.26 s⁻¹) was significantly higher in suspensions of ROS membranes containing 30 μM rhodopsin than in diluted ROS suspensions (Fig. 5B). However, this enhanced rate was not accompanied by an equivalent decrease in the maximal amount of hydrolyzed cGMP because the initial PDE activity after addition of GTP was higher. It has been shown previously that PDE activity by transducin is more efficient at higher concentrations of photoreceptor membranes (32–34). Addition of 1 μM hRGSr to ROS membranes containing 30 μM rhodopsin resulted in ~3-fold increase in the PDE inactivation rate. The PDE inactivation rate was as high as 0.75 s⁻¹ and correlated well with the GTPase rate (>0.7 s⁻¹) measured under the same conditions. To determine if hGRSr can serve as an antagonist for PDE and block the enzyme activation, we have measured effects of hRGSr on GTPγS-induced PDE activity in suspensions of ROS membranes containing 5 μM rhodopsin. The rates of the GTPγS-induced cGMP hydrolysis were not significantly affected by hRGSr. In the presence of relatively high concentrations of hRGSr (5 μM) PDE activity was suppressed by only ~15% (not shown).

**DISCUSSION**

Recent findings have established that members of a new family of RGS proteins serve as GAPs for heterotrimeric G-proteins and attenuate G-protein-mediated signal transduction (28, 35, 36). Evidence suggests that RGS proteins accelerate the rate of GTP hydrolysis by Gα subunits but do not affect GDP/GTP exchange induced by activated G-protein-coupled
receptors (35). Precise mechanisms of RGS GAP activity are not yet clear. It has been demonstrated that at least some RGS proteins (RGS1, RGS4, GAP, and mRGSr) interact preferentially with the AlF4−bound conformation of Gα subunits and thus may accelerate GTP hydrolysis through stabilization of the transitional state of G-proteins (15, 28, 29). In several signaling systems the GTPase activity of G-proteins is enhanced by their effector enzymes (11, 37). Regulation of the visual G-protein appears to be even more complicated. The attenuation of GTP hydrolysis were significantly higher in more concentrated suspensions of ROS membranes after addition of GTP were unaffected in the presence of hRGSr. This suggests that hRGSr accelerates the GTPase activity by mouse RGSr have just been reported (45). However, our data indicate that Pγ attenuates effects of RGSr allosterically rather than competitively as suggested by Wieland et al. (45). In agreement with earlier observations, the rates of GTP hydrolysis were significantly higher in more concentrated suspensions of photoreceptor membranes (11–14). hRGSr was equally potent as a GAP for transducin regardless of the membrane concentration, indicating that: (a) a putative membrane GAP factor for transducin represents a distinct non-RGS-like protein, and (b) the membrane factor does not compete with hRGSr for binding to transducin.

The PDE inactivation rates were increased by hRGSr proportions to the acceleration of the GTPase rates. The evidence that transducin’s GTPase activity represents a major mechanism for inactivation of PDE in the turnoff of visual signals (11, 13, 47) has been disputed (48, 49). Our data support the conclusion that transducin’s GTPase activity controls PDE inactivation. The rates of GTP hydrolysis and PDE inactivation (>0.7 s−1) observed at relatively high concentrations of photo-

![Figure 5](image-url)
Functional Characterization of Retinal RGS

receptor membranes in the presence of RGS are adequate to explain fast turnover kinetics in the visual transduction cascade in vivo.

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