Regulation of retinal transducin by C-terminal peptides of rhodopsin*

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Transducin is a multi-subunit guanine-nucleotide-binding protein that mediates signal coupling between rhodopsin and cyclic GMP phosphodiesterase in retinal rod outer segments. Whereas the $T\alpha$ subunit of transducin binds guanine nucleotides and is the activator of the phosphodiesterase, the $T_{\beta\gamma}$ subunit may function to link physically $T\alpha$ with photolysed rhodopsin. In order to determine the binding sites of rhodopsin to transducin, we have synthesized eight peptides (Rhod-1 etc.) that correspond to the C-terminal regions of rhodopsin and to several external and one internal loop region. These peptides were tested for their inhibition of restored GTPase activity of purified transducin reconstituted into depleted rod-outer-segment disc membranes. A marked inhibition of GTPase activity was observed when transducin was pre-incubated with peptides Rhod-1, Rhod-2 and Rhod-3. These peptides correspond to opsin amino acid residues 332–339, 324–331 and 317–321 respectively. Peptides corresponding to the three external loop regions or to the C-terminal residues 341–348 did not inhibit reconstituted GTPase activity. Likewise, Rhod-8, a peptide corresponding to an internal loop region of rhodopsin, did not inhibit GTPase activity. These findings support the concept that these specific regions of the C-terminus of rhodopsin serve as recognition sites for transducin.

INTRODUCTION

Visual transduction in retinal rods involves an internal transmitter that couples the signal of photoisomerization of rhodopsin at the disc membrane to the decrease in Na^+ conductance at the plasma membrane [1,2]. Light-induced activation of cyclic GMP phosphodiesterase (PDE) has been suggested as a key step in this transduction process [3–6]. Activation of this PDE and concomitant hydrolysis of cyclic GMP is mediated by a GTP-binding protein complex referred to as 'transducin' or 'G-protein' [7–11]. Recently, it has been proven that this complex belongs to a family of proteins whose functions are to link external receptors to internal proteins [12–14]. Photoisomerized rhodopsin does not activate the PDE directly, but is coupled to the PDE via the transducin. The resulting interaction of a bleached product of rhodopsin with the $T\alpha$ subunit of transducin results in the exchange of a bound GDP for a cytosol GTP [7]. The GTP- $T\alpha$ complex is the ultimate activator of PDE.

Rhodopsin is known to be phosphorylated during this process by an intrinsic opsin kinase with a half-time of 1–2 min [15]. Although this time-frame is too slow for the phosphorylation to be directly involved in visual transduction, it is thought to be a 'turn-off' signal. Thus phosphorylation of rhodopsin reduces its ability to activate PDE [16–19]. The primary site of this phosphorylation is at the C-terminus [20,21]. C.d. data and other studies indicate that rhodopsin has seven transmembrane helical spans producing three loop regions; these loops, along with the C terminus, are the potential sites for interaction of rhodopsin with either cytosol or peripheral-membrane proteins such as transducin and PDE [22–27].

In order to assess the role of the C-terminus and loop regions in the coupling of rhodopsin to transducin, we have synthesized peptides corresponding to these regions. Three peptides corresponding to regions at the C-terminus of rhodopsin inhibited reconstitution of transducin GTPase activity. These regions contained seven serine and threonine residues that could be potential sites of phosphorylation. These results support the concept that a specific region on the C-terminus of rhodopsin serves as the recognition site for transducin.

EXPERIMENTAL PROCEDURES

Materials

Fresh bovine retinas were obtained from local slaughterhouses. Buffers included Tris and Mops from Sigma. PMSF, GTP, dithiothreitol and other reagents were from Sigma. Guanosine [γ - ^{32}P]triphosphate (14.9 Ci/mmol) was from Amersham International; [$guanylyl$ -8- 3H] 5'-guanylyl imidodiphosphate (8.6 Ci/mmol) was from ICN Radiochemicals. The t-butoxycarbonyl-amino acids and t-butoxycarbonyl-amino acid resins were from Vega Biotechnologies. All remaining reagents for peptide synthesis were Sequenal grade from Pierce.

Preparation of depleted ROS

ROS were purified by discontinuous sucrose-density-gradient centrifugation under dim red light [28]. Isolated ROS membranes were freed of cytoplasmic and membrane-surface-associated proteins by repetitive washings with iso-osmotic buffer containing 120 mM-NaCl,

Abbreviations used: PDE, phosphodiesterase; ROS, rod outer segment; SDS, sodium dodecyl sulphate; $T\alpha$, 39 kDa subunit of transducin; $T_{\beta\gamma}$, 35 and 10 kDa subunits of transducin; Gpp(NH)p, guanosine 5'-[$\beta\gamma$ -imido]triphosphate; PMSF, phenylmethanesulphonyl fluoride.

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2 mM-MgCl₂, 2 mM-dithiothreitol, 0.05 mM-PMSF and 10 mM-Tris, pH 7.5, followed by three to five washes in hypo-osmotic buffer containing 2 mM-dithiothreitol/10 mM-Tris, pH 8.0. ROS were pelleted each time at 27000 *g* for 20 min at 4°C. After the last hypo-osmotic wash, ROS were diluted to twice their volume in buffer A (0.1 mM-GTP/1 mM-dithiothreitol/10 mM-Tris, pH 8.0) and incubated on ice in room light for 10 min. This procedure was repeated until the ROS were completely depleted of GTPase activity [29]. The ROS were divided into portions and stored at concentration of 1.4 mg/ml at -70°C for future use in reconstitution assays.

Purification of transducin

The first supernatant obtained from the ROS washed under light in buffer A was further purified by elution from hexyl-agarose with 300 mM-NaCl [30]. If necessary, this step was repeated until pure transducin was obtained as assessed by SDS/polyacrylamide-gel electrophoresis [31]. Purified transducin was divided into portions and stored at 20 µg/ml at -70°C.

GTPase assay

GTPase activity was assayed as previously described [32]. The reaction mixture typically contained 0.05 µg of dialysed hexyl-agarose-purified transducin and 5 µg of ROS membranes that had been depleted of GTPase activity. This reaction was allowed to proceed for 5 min at 37°C in 70 µl of a buffer consisting of 10 mM-Mops, 2 mM-MgCl₂, 0.1 mM-PMSF and 1 mM-dithiothreitol, pH 7.4. The reaction was initiated by the addition of 20 µM-GTP substrate containing 1 µCi of [γ -³²P]GTP. The reaction was stopped by the addition of 1 ml of solution A (150 mM-HClO₄/0.125 mM-KH₂PO₄) and 1 ml of solution B (10 mM-ammonium molybdate/20 mM-triethylamine hydrochloride). A dense precipitate formed immediately upon addition of solution B. This precipitate was collected on a Whatman GF/A (2.4 cm diameter) glass microfibre filter by using a suction-filtration apparatus. Filters were washed with 50 ml of solution C (10 mM-triethylamine hydrochloride/200 mM-HClO₄/2.5 mM-KH₂PO₄). [³²P]P_i retained on each filter was assessed by liquid-scintillation counting. All procedures were under room light. In some experiments peptides were preincubated with transducin before addition to depleted ROS.

Gpp(NH)p-binding studies

Depleted ROS (5 µg) and purified transducin (0.05 µg) were incubated in GTPase assay buffer (1 ml final vol.) in the dark for 12 h at 4°C. This facilitates binding of transducin to ROS [33]. After incubation, the membranes were pelleted at 45000 *g* for 30 min and the pellet was resuspended in 100 µl of buffer B (60 mM-KCl/30 mM-NaCl/2 mM-MgCl₂/10 mM-Mops/1 mM-dithiothreitol/0.1 mM-PMSF) at pH 7.5. Aliquots of membranes (50 µl) were added to an equal volume of 20 µM-[³H]Gpp(NH)p (containing 600 000 d.p.m.) and immediately illuminated for 30 min at room temperature. The amount of [³H]Gpp(NH)p bound to transducin was assessed by filtration through 0.45 µm-pore-size Millipore HA filters, which were washed three times with 1 ml each of buffer B. In some experiments, synthetic peptides were added several minutes after the time of exposure to room light or when the HA filters were washed. Each filter was counted for radioactivity by liquid-scintillation spectrometry.

Peptide synthesis and purification

Peptides were synthesized manually by using the solid-state method of Hodges & Merrifield [34] as modified by Gormann [35] with the exception that cleavage of peptides and protecting groups was performed by HBr and anhydrous trifluoroacetic acid [36]. Peptides were purified by cellulose thin-layer electrophoresis acetic acid/formic acid/water (3:1:16, by vol.) being used as buffer. Peptides were revealed with ninhydrin and corresponding unsprayed lanes were scraped from the plates and extracted with aq. 0.5% NH₃. These peptides were then freeze-dried and redissolved in distilled water. Peptides were divided into portions and stored at -20°C. To determine amino acid composition and to quantify the peptides, 0.05% of each total sample was hydrolysed in 6M-HCl and amino acids determined by using a reverse-phase C₁₈ h.p.l.c. column (Vydak) and *o*-phthalaldehyde as a detecting agent [37]. All peptides had the expected amino acid compositions when determined after hydrolysis with HCl. Quantification was accomplished by peak integration and comparison with known amino acid standards.

Other methods

Protein concentration was determined by the method of Bradford [38], with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Table 1 lists the peptides that were synthesized and tested for inhibition of reconstituted GTPase activity. All peptides were pure as judged by thin-layer electrophoresis. Amino acid analyses verified that each peptide contained the 'correct' amino acids. The estimated pI values for these peptides ranged from 5.3 to 7.6, and included peptides that were neutral, basic or acidic at the pH of the GTPase assay. The peptides did not alter the pH of the assay mixture. Peptides coded Rhod-1, Rhod-2, Rhod-3, Rhod-9 and Rhod-4 correspond to the C-terminal region of bovine opsin. Peptides coded Rhod-5, Rhod-6 and Rhod-7 correspond to the three hypothesized loop regions that are thought to extend into the outside of the ROS membrane. Rhod-8 corresponds to a hypothesized internal loop region.

Table 2 demonstrates that the Rhod-2 peptide has no effect, alone, on the GTPase assay. Although a marked inhibition of reconstituted GTPase activity was observed (73%), Rhod-2 had no effect on transducin alone, nor when added alone to the substrate. In this assay the depleted ROS did have a low GTPase activity (32% of control as against 13% for the blank), which was also inhibited by Rhod-2. The same results were observed when the other synthetic peptides were used; the GTPase assay (substrate alone) was not altered. These results demonstrate that the peptides alone do not have GTPase activity.

Table 1 further details the I₅₀ (concn. giving 50% inhibition) for all peptides. As illustrated, the I₅₀ varied from 12 to 67 µg for Rhod-1, Rhod-2 and Rhod-3. Rhod-4, the peptide corresponding to the C-terminal end of rhodopsin, did not inhibit reconstituted GTPase activity when added at 650 µg. The peptides corresponding to the three external-loop regions of rhodopsin, Rhod-5, Rhod-6 and Rhod-7, likewise did not inhibit GTPase activity. The I₅₀ values for Rhod-6 and Rhod-7 were all > 650 µg. The peptide corresponding to an

Table 1. Rhodopsin peptides

Peptides were synthesized and purified as described in the text, the known amino acid sequence of bovine opsin being used as a model [20–22, 39]. Numbering of the sequence starts with the *N*-terminus as residue no. 1 and the *C*-terminus as residue no. 348. All peptides were dissolved in distilled water. Concentrations were determined by amino acid analysis as described in the text. Peptides were tested for inhibition of reconstituted GTPase activity at concentration of up to 650 μ g each. GTPase-1, -2 and -3 are peptides that correspond to known sequences of $T\alpha$ of transducin. GTPase-1 is known to be ADP-ribosylated at the Asn residue by islet-activating protein [13]. GTPase-1 corresponds to the ADP-ribosylation site (on the Arg residue) of cholera toxin [40]. GTPase-3 corresponds to a region of $T\alpha$ that has known homology with the homologous adenylate cyclase protein, and with several *ras*-oncogene proteins [41]. G γ -1, -2, -3 and 4 are peptides from the G γ subunit of transducin obtained from the known sequence of this protein [41,42]. Results to determine I_{50} were obtained and expressed as the percentage inhibition when compared with a control tube containing 1.4 μ g of transducin-depleted ROS, 0.14 μ g of purified transducin and 20 μ M-[γ - 32 P]-GTP (1 μ Ci/tube) assayed for 10 min at 37°C. Controls ranged from 10 to 30% of the c.p.m. added to each tube. Each peptide sample was incubated for 10 min on ice with the purified transducin before the addition of depleted ROS and substrate. Concentrations of peptide indicate the total quantity of peptide added to each assay tube (70 μ l final assay vol.). All peptides were in distilled water. Results are the averages for duplicate samples. I_{50} is the quantity (μ g) of peptide producing 50% inhibition of reconstituted GTPase activity.

Code name [concn. (mg/ml); I_{50} (μ g)]	Amino acid residue nos.	Sequence
Rhod-1 (0.56; = 12)	332–339	Glu-Ala-Ser-Thr-Thr-Val-Ser-Lys
Rhod-2 (67.4; = 67)	324–331	Gly-Lys-Asn-Pro-Leu-Gly-Asp-Asp
Rhod-3 (7.9; = 40)	317–321	Met-Val-Thr-Thr-Leu
Rhod-4 (22; > 650)	341–348	Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala
Rhod-5 (52.8; > 650)	245–253	Lys-Ala-Glu-Lys-Glu-Val-Thr-Arg-Met
Rhod-6 (6.1; > 650)	143–150	Met-Ser-Asn-Phe-Arg-Phe-Gly-Glu
Rhod-7 (14; > 650)	66–73	Lys-Lys-Leu-Arg-Thr-Pro-Leu
Rhod-8 (12.5; > 650)	188–194	Gly-Ile-Asp-Tyr-Tyr-Thr-Pro
Rhod-9 (3.6 μ g/ml; > 650)	309–316	Met-Asn-Lys-Gln-Phe-Arg-Asn-Cys
GTPase-1 (–; > 650)	Unknown	Ser-Arg-Val-Lys
GTPase-2 (–; > 650)	Unknown	Glu-Asn-Leu-Lys-Asn-Gly-Leu-Phe
GTPase-3 (–; > 650)	Unknown	Leu-Leu-Gly-Ala-Gly-Glu
G γ -1 (–; > 650)	9–17	Thr-Glu-Lys-Asp-Lys-Leu-Lys-Met-Glu
G γ -2 (–; > 650)	21–28	Leu-Lys-Lys-Glu-Val-Thr-Leu-Glu
G γ -3 (–; > 650)	43–50	Val-Glu-Glu-Arg-Ser-Gly-Glu-Asp
G γ -4 (–; > 650)	58–65	Glu-Asp-Lys-Asn-Pro-Phe-Lys-Glu

Table 2. Effects of Rhod-2 peptide on the GTPase assay

The assay (70 μ l total vol.) contained 1.4 μ g of transducin-depleted ROS and 0.14 μ g of purified transducin. Assay conditions were as described in the text. The control sample contained only the substrate, depleted ROS and transducin incubated with 20 μ M-[γ - 32 P]-GTP (1 μ Ci/tube) for 10 min at 37°C. The control was 7.5% of the c.p.m. added to the tubes. Rhod-2 was added in distilled water at 674 μ g/tube. Transducin was preincubated for 10 min at 4°C with Rhod-2 before the addition of ROS or substrate. Results are the averages for four samples and are expressed as a percentage of the control-tube activity.

Sample	GTPase activity (% of control)
Blank	13
Control + Rhod-2	27
Transducin + Rhod-2	17
ROS + Rhod-2	20
Transducin alone	14
ROS alone	32
Rhod-2 alone	18

internal rhodopsin loop, Rhod-8, had an I_{50} of > 650 μ g; likewise Rhod-9 had no effect. From these data it is evident that sequences corresponding to very specific regions of the *C*-terminus of rhodopsin may serve as recognition sites for transducin.

Examination of pI values of the peptides and their net charges at the pH of the assay revealed that there was no correlation between pI or charge and the ability to inhibit reconstituted GTPase activity.

We have, to date, tested 15 different synthetic peptides for inhibition of reconstituted GTPase activity. Table 1 lists these peptides, their source and sequence. It is evident from these data that very few peptides had any inhibitory activity. Indeed, out of nine rhodopsin peptides, only three were able to inhibit the assay. No effects were seen when peptides of G γ or of $T\alpha$ of transducin were used. These peptides had sequences including known ADP-ribosylation sites. We must, therefore, assume that specific sequences of the *C*-terminus of rhodopsin are important in GTPase reconstitution.

Transducin was preincubated with each peptide before the addition of the depleted and bleached ROS membranes. Therefore the inhibitory peptides may function to block rhodopsin recognition sites on transducin via competition. If transducin-binding sites are blocked, this would prevent the necessary interaction of transducin with bleached rhodopsin, a requirement for reconstituted GTPase activity. Alternatively, the peptides may act at the guanine-nucleotide-binding site on $T\alpha$ of transducin. This would not prevent transducin interaction with rhodopsin, but would inhibit GTPase activity via competition or some alteration of the substrate-binding site. To test this hypothesis, we performed the experiment outlined in Table 3.

Table 3. Effects of rhodopsin peptides on guanine nucleotide binding by transducin

Assays were performed exactly as described in the text. In the sample 'Control + Rhod-2', 667 µg of the peptide were added in 10 µl of distilled water 5 min after exposure of the ROS/transducin/[³H]Gpp(NH)p mixture to room light. The sample 'Rhod-2 alone' contained [³H]Gpp(NH)p plus 667 µg of Rhod-2. Blanks contained only substrate. The 'Control + Rhod-2 wash' consisted of a control sample which was washed with 667 µg of Rhod-2 after filtration of the sample on an HA filter. Results are the average for duplicate samples.

Sample	Radioactivity bound (c.p.m./assay tube)
Control	12640
Blank	380
Control + Rhod-2	15667
Rhod-2 alone	2748
Control + Rhod-2 wash	14606

In the dark, transducin is known to reassociate with dark-adapted and depleted ROS membranes [33]. After exposure to light and Gpp(NH)p, a non-hydrolysable GTP analogue, the transducin T α subunit will bind the guanine nucleotide analogue with a high affinity. In the experiment outlined in Table 3, we allowed [³H]Gpp(NH)p to bind to transducin (control 12640 c.p.m. of [³H]Gpp(NH)p). Once bound, the nucleotide was not removed by the addition of Rhod-2 when added in a further incubation (control + Rhod-2 = 15667 c.p.m.) or when added as a filter wash (control + Rhod-2 wash = 14606 c.p.m.). These results suggest that Rhod-2 does not compete for guanine nucleotide once it is bound to transducin T α . Similar results were observed when Rhod-1 and Rhod-3 were used (results not shown). These results further support the hypothesis that regions on rhodopsin corresponding to the Rhod-1, Rhod-2 and Rhod-3 residues serve as transducin-binding sites. Such a hypothesis is supported by the work of Aton & Litman [19]. In these experiments, enzymic treatment of the C-terminus of rhodopsin altered its ability to activate the ROS PDE.

In conclusion, we have identified a region on rhodopsin corresponding to the C-terminal residues 317–339 which affects reconstitution of GTPase activity. These results suggest very strongly that this region is the recognition site on rhodopsin for transducin.

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