C-Terminal peptides of rhodopsin

Determination of the optimum sequence for recognition of retinal transducin**

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In vertebrate retinal rod outer segments, transducin, a guanine-nucleotide-binding protein, mediates signal coupling between rhodopsin and cyclic GMP phosphodiesterase. Whereas the $T\alpha$ subunit (39 kDa) of transducin binds guanine nucleotides and is the activator of the phosphodiesterase, the $T\beta\gamma$ subunits (35 and 10 kDa) may function to physically link $T\alpha$ with photolysed rhodopsin. We have previously reported that a site of binding of transducin is on the C-terminus of bovine rhodopsin. By using competition with synthetic peptides, the recognition region was localized to bovine opsin amino acid residues 317–339. Further studies are detailed which determine the boundaries of this binding site on rhodopsin, as well as some of the critical amino acids needed for transducin binding. These results suggest that the serine and threonine residues in the rhodopsin C-terminal peptides Rhod-1 and Rhod-3 are critical for reconstitution of transducin GTPase activity.

INTRODUCTION

Visual transduction in retinal rods is accomplished through the interaction of photoisomerized rhodopsin with transducin, a GTP-binding protein [1–4]. The resulting activation of cyclic GMP PDE [5–8] causes a decrease both in the concentration of the nucleotide and in membrane hyperpolarization [9, 10]. A cyclic GMP-induced membrane-potential effect has also been noted in retinal cone cells [11, 12].

Photoisomerized rhodopsin does not activate PDE directly, but is coupled 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 molecule for a cytosolic GTP molecule [1]. This GTP- $T\alpha$ complex is thought to be the ultimate activator of the PDE.

Rhodopsin is phosphorylated during this process by an intrinsic opsin kinase; the result is a decrease in the light activation of the PDE [13–16]. The primary site of this phosphorylation is at the C-terminus and one loop region [17–19].

We have previously reported that synthetic peptides which correspond to bovine opsin residues 317–339 inhibited reconstitution of transducin GTPase activity in the presence of rhodopsin [20]. The results suggested that this specific region of the C-terminus of rhodopsin served as a recognition site for transducin.

In order to assess the biochemical requirements of this 'recognition site', we have synthesized analogues of this peptide as well as specific peptides corresponding to the boundaries of the recognition site. The results suggest that the position and presence of key serine and threonine residues are critical for recognition of transducin by rhodopsin.

EXPERIMENTAL

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. The Boc-amino acids and Boc-amino acid resins were from Vega Biotechnologies (Tucson, AZ, U.S.A.). All remaining reagents for peptide synthesis were Sequenal grade from Pierce. Hexyl-agarose was purchased from Sigma.

Preparation of depleted ROS

ROS were purified by discontinuous sucrose-density-gradient centrifugation under dim red light. Isolated ROS membranes were freed of cytoplasmic and membrane-surface-associated proteins by repetitive washings with iso-osmotic buffer containing 120 mM-NaCl, 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 [22]. The ROS were divided into portions and stored at 1.4 mg/ml at –70 °C for 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

Abbreviations used: PDE, phosphodiesterase; $T\alpha$, 39 kDa subunit of transducin; $T\beta\gamma$, 35 kDa and 10 kDa subunits of transducin; PMSF, phenylmethanesulphonyl fluoride; Boc, t-butoxycarbonyl; ROS, rod outer segments; I_{50} , quantity (μ g) of peptide producing 50% inhibition of reconstituted GTPase activity.

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from hexyl-agarose with 300 mM-NaCl [22]. If necessary, this step was repeated until pure transducin was obtained as assessed by SDS/polyacrylamide-gel electrophoresis [23]. Purified transducin was divided into portions and stored at 20 µg/ml at -70 °C.

GTPase assay

GTPase activity was assayed as previously described [24]. The reaction 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 6.4. 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 Whatman GF/A (2.4 cm diameter) glass microfibre filters 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). Radioactive [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.

Peptide synthesis and purification

Peptides were synthesized manually by using the solid-state method of Hodges & Merrifield [25] as modified by Gorman [26] with the exception that cleavage of peptides and protecting groups was performed with HBr and anhydrous trifluoroacetic acid [27]. Peptides were purified by cellulose thin-layer electrophoresis with a buffer of acetic acid/formic acid/water (3:1:16, by vol.). Peptides were revealed with ninhydrin and corresponding unsprayed lanes were scraped from the plates and extracted with 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 6 M-HCl and amino acids were determined by using an h.p.l.c. reverse-phase C₁₈ column (Vydac) and *o*-phthalaldehyde as a detecting agent [28]. All peptides had the expected amino acid compositions as determined after HCl hydrolysis. Quantification was accomplished by peak integration and comparison with known amino acid standards. The regions of bovine rhodopsin which were synthesized are illustrated in Fig. 1.

Other methods

Protein concentration was determined by the method of Bradford [29], with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Table 1 lists the peptides which 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

Fig. 1. Sequence location of rhodopsin peptides and analogues

Residues marked with an asterisk (*) are regions where substitutions have been made.

Table 1. Effects of synthetic peptides on reconstitution of transducin GTPase activity

Peptides were synthesized and purified as described in the Experimental section and by using known amino acid sequence of bovine opsin as a model [17, 18]. Numbering of the sequence starts with the *N*-terminus as residue no. 1 and ends with 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 Experimental section. Peptides were tested for inhibition of reconstituted GTPase activity as described in the text. Results are expressed as the percentage inhibition when compared with a control tube containing 1.5 μg of transducin-depleted ROS, 0.14 μg of purified transducin and 20 μM -[γ - ^{32}P]GTP (1 μCi /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 indicate the total quantity of peptide added to each assay tube (70 μl final assay volume). Results are averages for duplicate samples. Residues marked with an asterisk (*) are regions where substitutions have been made.

Code name [I ₅₀ (μg)]	Amino acid residue nos.	Sequence
Parent peptides		
Rhod-1 (12)	332–339	Glu-Ala-Ser-Thr-Thr-Val-Ser-Lys
Rhod-2 (67)	324–331	Gly-Lys-Asn-Pro-Leu-Gly-Asp-Asp
Rhod-3 (40)	317–321	Met-Val-Thr-Thr-Leu
<i>N</i>-Side boundary peptides		
Rhod-3+2 (42)	315–319	Asn-Cys-Met-Val-Thr
Rhod-9–3 (> 468)	312–316	Gln-Phe-Arg-Asn-Cys
Rhod-3–3 (> 430)	314–318	Arg-Asn-Cys-Met-Val
<i>C</i>-Side boundary peptides		
Rhod-4 (> 600)	341–348	Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala
Rhod-4+1 (> 576)	340–348	Thr-Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala
Rhod-4+2 (> 584)	339–348	Lys-Thr-Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala
Rhod-4+3 (> 600)	338–348	Ser-Lys-Thr-Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala
Analogues of Rhod-1–Rhod-3		
R-1-A (> 450)	—	Glu-Ala-Ser-Ile*-Ile*-Val-Ser-Lys
R-1-B (> 450)	—	Glu-Ala-Val*-Thr-Thr-Val-Val-Val*-Lys
R-2-A (> 140)	—	Gly-Lys-Asn-Ile*-Leu-Gly-Asp-Asp
R-2-B (> 450)	—	Gly-Lys-Asn-Pro-Leu-Gly-Ala*-Ala*
R-3-A (> 450)	—	Met-Val-Ile*-Ile*-Leu

the correct amino acids. Peptides did not alter the pH of the assay. Peptides coded Rhod-1, Rhod-2 and Rhod-3 correspond to the active *C*-terminal region of bovine opsin. Table 1 also lists the I₅₀ for all peptides, which varied from 12 to 67 μg for Rhod-1, Rhod-2 and Rhod-3.

The *N*- and *C*-side peptides include residues which 'span' each side of the Rhod-1–Rhod-3 regions of bovine opsin. On the *N*-end, when residues 320 and 321 are omitted (Rhod-3+2; residues 315–319), the peptide is still active in inhibiting the reconstitution of transducin GTPase activity (I₅₀ = 42 μg). However, when both of the threonine residues are omitted from Rhod-3, as in the synthesis of the analogues Rhod-9–3 and Rhod-3–3, the inhibitory activity is lost (I₅₀ > 468 μg and > 430 μg respectively).

On the *C*-end of the Rhod-1 peptide, synthetic analogues which include residues 338–348 (Rhod-4+3) do not inhibit reconstitution of GTPase activity. This is of interest, since the Rhod-4+3 peptide does include serine and threonine residues. We must conclude that the -Thr-Thr- sequence of Rhod-1 (residues 335 and 336) is needed for activity of this region.

We have further analysed the amino-acid-sequence requirements of the Rhod-1–Rhod-3 region by synthesizing and testing five analogues of these peptides. In synthesizing peptide R-1-A the threonine residues at positions 335 and 336 were replaced by two isoleucine residues, whereas in peptide R-1-B serine residues at positions 334 and 338 were replaced by valine. In both cases the ability to inhibit the reconstitution of GTPase activity was abolished (I₅₀ > 450 μg for each peptide).

Likewise, in peptide analogue R-3-A, the activity is abolished by substitution of threonine residues at positions 319 and 320 with isoleucine.

These results suggest that the presence and position of key serine and threonine residues are important for recognition of transducin by rhodopsin. These data agree with those of previous reports in which it was noted that phosphorylation of rhodopsin or enzymic treatment of the *C*-terminus of rhodopsin alters its ability to activate PDE [30, 15].

Analogues of Rhod-2 were also synthesized in order to determine which amino acids were necessary for its inhibitory activity. In the synthesis of R-2-A, proline-327 was replaced by isoleucine. Although this did not totally abolish activity, the I₅₀ was altered (I₅₀ = 67 μg for Rhod-2 as against 140 μg for R-2-A). By contrast, substitution of the aspartic acid residues (330 and 331) with alanine totally abolished activity (I₅₀ > 450 μg). The two aspartic acid residues may, therefore, be necessary for recognition of transducin by this region of rhodopsin.

It is obvious that a very large number of permutations of these peptides are possible. In choosing a limited number to examine, we were guided by the general rules of protein-structure prediction. Replacement of charged residues by hydrophobic ones, as in R-2-B, or of polar residues by hydrophobic ones, as in R-1-A, would be expected to have a large effect on binding activity. The bulk of the isoleucine molecule is not so much greater than that of threonine (nor that of valine so much greater than that of serine) that this could account for the very striking decrease in affinity for the transducin by R-1-A,

R-3-A and R-1-B. The peptide R-2-A is a particularly interesting case, where putting a slightly more bulky, but flexible, isoleucine residue in place of proline only partially abolishes binding activity. Recognition of small peptides by antibodies entails, in many cases, flexible arrangements of the peptide to fit the antibody [31]. Presumably a similar phenomenon occurs in protein-peptide recognition of the sort studied here.

Residue 319 is clearly important for recognition. The peptide Rhod-3+2 is as effective as Rhod-3 in blocking transducin, whereas Rhod-3-3 is as ineffective as R-3-A. On the C-terminal side the residues 338 and 339 are insufficient to cause blocking, suggesting that the transducin recognition site probably does not extend beyond these. The analogue R-1-B has serine residues at positions 338 and 334 replaced by valine, abolishing blocking activity. The parent peptide Rhod-1 is the most effective blocker of all those tested. If residue 338 is not very important for recognition, then residue 334 must be crucial. Clearly 335 and 336 also play a role, as shown by R-1-A, in which 334 and 338 are intact.

It will be of interest to synthesize longer peptides spanning this entire recognition region. These may be much more potent blockers of transducin activation. In a longer polypeptide the more subtle effects of substitution may be observable as changes in the I_{50} over the full range from strong to weak interaction.

As previously reported by Kühn & Hargrave [32], there is also binding of transducin to a cytoplasmic loop of rhodopsin (residues 230–250). We have also found that a synthetic peptide corresponding to this region inhibits reconstituted GTPase activity (D. J. Takemoto, D. Morrison, L. C. Davis and L. J. Takemoto, unpublished work). Although we have only considered the C-terminal region in the present study, future studies of this cytoplasmic loop and the C-terminus, together, will be of interest. Since there may be at least two recognition regions for transducin, perhaps the addition of peptides corresponding to both of these regions would cause activation of transducin. Clearly, in the above experiments we have only used peptides which mimic a part of the recognition site, though we have previously reported that this is not the GTP-binding site for transducin [20]. Future work with peptides of both regions together may allow specific analysis of rhodopsin-transducin binding and elucidate the mechanism of transducin activation.

In conclusion, we have synthesized peptides that have been used to identify the position and presence of critical serine and threonine residues on Rhod-1 and Rhod-3. Two aspartic acid residues have also been found to be necessary for Rhod-2 function.

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