Mapping Sites of Interaction between Rhodopsin and Transducin Using Rhodopsin Antipeptide Antibodies*

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Site-directed antipeptide antibodies generated against the predicted cytoplasmic sequences of rhodopsin were used to map the binding domains for transducin, the retinal G-protein, on the photoreceptor. Antibodies against synthetic peptides corresponding to loop 3-4, loop 5-6, and the serine/threonine-rich region of the COOH terminus recognize rhodopsin by immunoblot analysis and also recognize the native protein within the membrane, allowing these probes to be used for functional studies. Rhodopsin reconstituted into phospholipid vesicles binds transducin in the light which significantly reduces the binding of antipeptide antibodies corresponding to loop 3-4 and the COOH terminus of rhodopsin. However, the binding of the antibody raised against a 14-amino-acid peptide corresponding to a sequence within loop 5-6 of rhodopsin was unaffected by the presence of transducin. These results suggest a preferential involvement of regions in or near loop 3-4 and the COOH terminus in the binding of transducin to rhodopsin. In contrast, a significant portion of loop 5-6 does not form a binding domain for the G-protein.

The proposed structure of the photoreceptor, rhodopsin, is an integral membrane protein that spans the lipid bilayer of the rod disc membrane seven times (Ovchinnikov, 1982; Hargrave et al., 1983). Three loops connecting the seven transmembrane α-helices, as well as the COOH terminus of the protein, are exposed on the extradiscal surface and are presumed to be available for interaction with transducin and with other proteins involved in light-mediated signal transduction (Chabre, 1985). The role of these domains in the binding and catalytic activation of transducin has not yet been defined. Recently, several laboratories have reported significant structural homology between rhodopsin, the β-adrenergic receptor and the muscarinic receptor which correspond to their similar modes of signal transduction (Dixon et al., 1986; Koblika et al., 1987; Yarden et al., 1987; Weiss et al., 1987; Kubo et al., 1986; Peralta et al., 1987). In addition, several of these receptors are able to stimulate G-proteins in vitro which do not appear to be normal substrates for activation (Asano et al., 1984; Cerione et al., 1985; Kanaho et al., 1984). This illustrates a high degree of structural conservation that reflects similar mechanisms of activation of G-proteins by their receptors. The relative abundance and ease of purification of the rod cell proteins make the rhodopsin phototransduction system an excellent model for defining the significant structural domains of receptors involved in G-protein-mediated signal transduction.

We have utilized site-directed antipeptide antibodies to begin to define the regions of rhodopsin involved in the binding of transducin and its catalytic activation. A series of antibodies was raised against sequences that correspond to regions of the three extradiscal loops and the COOH terminus. Results from these studies suggest the participation of the COOH terminus and loop 3-4 of rhodopsin, in the binding of the retinal G-protein, but that a significant region of loop 5-6 does not directly interact with transducin.

**MATERIALS AND METHODS**

Generation of Antibodies—Generation of rabbit antiserum against synthetic peptides that correspond to domains of rhodopsin, as well as purification of IgG fractions, are described in Weiss et al. (1987). The peptides used for raising antibodies are as follows: loop 1-2 (amino acid residues 62-73) TVQHKKLRTPLN; loop 3-4 (amino acid residues 141-152) KPMSSFRFGENH; loop 4-5 (amino acid residues 189-200) IDYYPHEETRN; loop 5-6 (amino acid residues 236-249) QQESAINTQKAKE; COOH terminus (amino acid residues 325-343) KNLGDDLEASTTSDKTETS. All sera were tested for immunoreactivity to rhodopsin and transducin by immunoblotting with urea-stripped rod outer segment membranes (ROS)1 and with

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1The abbreviations used are: ROS, rod outer segment; GTPγS, guanosine 5'-O-(thiotriphosphate); KLH, keyhole limpet hemocyanin.
proteins extracted from light-exposed membranes with 40 μM GTP. Gel electrophoresis was performed as described by Laemmli (1970). Protein was transferred to nitrocellulose in a buffer containing 25 mM Tris-HCl (pH 8.3), 192 mM glycine, 20% MeOH, and 0.1% sodium dodecyl sulfate for 1 h at 300 mA.

Purification of Rod Outer Segment Proteins—Rod outer segment membranes were isolated from retina and purified over sucrose gradients according to methods described by Kelleher and Johnson (1986).

Purification of Transducin—Transducin was extracted from ROS membranes purified in the light after washing the membranes with isotonic and hypotonic buffers and then solubilized in 1.5% octyl glucoside. The detergent-extracted proteins were applied to a concanavalin A-Sepharose column, and rhodopsin was eluted with 0.1 M α-methyl mannoside. The peak fractions were reconstituted with egg phosphatidylcholine/phosphatidylethanolamine and dialyzed to remove detergent.

Antibody Binding Assays—ROS membranes or purified rhodopsin reconstituted into phospholipid vesicles were incubated overnight at 4 °C in the dark in the presence of IgG fractions of various antibodies in Tris-buffered saline (pH 7.5) and 5% γ-globulin-free horse serum. The membranes were centrifuged and washed several times with Tris-buffered saline to remove unbound IgG and then reincubated for 1 h at room temperature with 125I-protein A (0.1 μCi/μl). After an additional series of washes and centrifugations, the membranes were counted in a Beckman Gamma 5500 counter.

For some experiments, reconstituted rhodopsin was preincubated for 30 min in the light at 4 °C with heparinase-purified transducin or with the synthetic peptides used to generate the antipeptide antibodies in a buffer containing 10 mM Tris-HCl (pH 7.5), 210 mM NaCl, 2 mM MgCl2, 0.1 mM EDTA, 5 mg/ml bovine serum albumin, and 1 mM dithiothreitol, after which IgG fractions were added for 2 h at 4 °C. Washes and incubation with 125I-protein A were performed as described above.

Transducin Activation—ROS membranes containing approximately 0.3 μM rhodopsin were preincubated in the dark at 4 °C overnight with or without antipeptide antibodies. An aliquot of membranes representing a final concentration within the assay of 10 μM rhodopsin was incubated at room temperature with 0.1-0.5 μM transducin in a buffer containing 10 mM Tris-HCl (pH 7.5), 0.1 mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, and 1 mM dithiothreitol. The reaction was initiated by the addition of 1 μM [35S]GTPγS (20,000 cpm/pmol). Aliquots removed from the reaction mixture were washed on nitrocellulose filters and quantitated by liquid scintillation counting.

RESULTS

Site-directed polyclonal antibodies were generated against sequences corresponding to loops 1-2, 3-4, 5-6 (amino acid residues 63-73, 141-152, and 236-249, respectively) and the serine/threonine-rich region of the COOH terminus (amino acid residues 325-343) by immunization of rabbits with appropriate KLH-conjugated peptides (Weiss et al., 1987). These sequences comprise the predicted cytoplasmic domain of rho- 

rhodopsin recognized by these antibodies, illustrating their monospecificity within the rod outer segment membrane. Neither transducin nor any of the other proteins present in the GTP extract of ROS membranes are recognized by these antibodies. Antibodies generated against KLH do not recognize the ROS membranes or the GTP-extracted material. Antibodies generated against loop 1-2 also recognize rhodopsin (Weiss et al., 1987), but the titre of the serum is very low compared with the other antipeptide antibodies and although useful for Western blotting, has not proven useful for functional studies.

Fig. 2 shows that the antibodies also recognize native, functional rhodopsin. In Fig. 2, stripped ROS membranes were incubated with IgG fractions derived from the anti-L4.5, anti-L4-6, and anti-CT antibodies followed by incubation with 125I-labeled protein A. Compared with a control antibody against KLH that does not recognize any ROS proteins (Fig. 1), the specific antipeptide antibodies showed a 2- to 3-fold greater level of binding to ROS membranes. Evidence supporting the interaction of each of the antipeptide antibodies with rhodopsin is presented in Table I. ROS membranes were preincubated in the dark with IgG fractions derived from each of the rhodopsin antipeptide antibodies or with the anti-KLH antibody, then assayed for the ability to catalyze light-dependent GTPγS binding to transducin. Preincubation of ROS membranes with anti-125I IgG had no effect on transducin activation, whereas the antipeptide antibodies significantly reduced the rate of transducin activation, indicating that the amount of functional rhodopsin available for interaction with the retinal G-protein is reduced. In contrast, antibodies directed against L3-4 (amino acid residues 189-200), a region predicted to lie on the intrasidal side of the membrane, did not bind to ROS membranes and had no effect on transducin activation, presumably because the ROS vesicles are oriented such that this domain is not readily accessible for interaction with antibodies. Antibodies to Loop 4-5 did, however, recognize rhodopsin on Western blots. In similar experiments, incubation with antibodies directed against loops 1-2, 3-4, 5-6, and the COOH terminus also blocked phosphorylation of rhodopsin by rhodopsin kinase (not shown). These results indicate that specific rhodopsin antipeptide antibodies generated against isolated linear sequences of amino acids rec-

FIG. 1. Immunoblot analysis of anti-rhodopsin antibodies against rhodopsin and transducin. Stripped ROS membrane and GTP-extracted transducin, obtained as described under "Materials and Methods," were chromatographed on polyacrylamide gels. The total protein concentration per lane was 3 and 5 μg, respectively. These lanes, which contained 1 μg of rhodopsin (R) and 1 μg of transducin (T) were transferred to nitrocellulose, blocked in Tris-buffered saline containing 10% γ-globulin-free horse serum, and then incubated overnight with a 1:100 dilution of antipeptide antibodies: lane 1, anti-CT; lane 2, anti-L4-6; lane 3, anti-L4-6; and lane 4, anti-KLH. The nitrocellulose was washed, incubated in 125I-protein A, and immunoreactivity detected by autoradiography. R1 and R2 refer to the mobilities of the monomer and dimer forms of rhodopsin. Transducin α and β migrate just above R1.
Sites of Interaction between Rhodopsin and Transducin

C TERMINUS

LOO P 3-4

LO O P 5-6

FIG. 2. Binding of antipeptide antibodies to ROS membranes. Urea-stripped ROS membranes were incubated overnight with IgG fractions purified from sera generated against peptides corresponding to the COOH terminus (upper panel), loop 3-4 (middle panel), and loop 5-6 (lower panel). Procedures for washing and incubation in [125I]-protein A are described under "Materials and Methods." Closed circles, site-directed antipeptide IgG; open circles, anti-KLH IgG.

ncognize cytoplasmically oriented sequences within the native membrane-associated protein, affect rhodopsin's ability to activate transducin, and can be used for studies of receptor-G-protein interaction.

For further experiments, purified rhodopsin was reconstituted into phospholipid vesicles. The advantage of using these vesicles was a higher specific binding of the antipeptide antibodies allowing a more quantitative analysis of the results. The ability of reconstituted rhodopsin to catalyze transducin activation remained virtually identical to that of ROS membranes (Fig. 3). Reconstituted rhodopsin was preincubated with purified transducin under conditions in which rhodopsin and transducin form a stable complex (Kuhn and Hargrave 1981; Wessling-Resnick and Johnson, 1987b) and then incubated with a 1:20 dilution of each of the peptide-specific antibodies in order to examine the domains of rhodopsin involved in the binding of transducin. This amount of IgG, corresponding to 1 mg/ml protein (Fig. 2), is a subsaturating concentration for the binding of each antibody to the amount of rhodopsin present in our assay. Under these conditions,

TABLE I

Inhibition of rhodopsin-catalyzed transducin activation by site-directed antipeptide antibodies

| Experiment | Ab     | Ab dilution | GTPyS bound* |
|------------|--------|-------------|--------------|
|            | None   |             | 1.40         |
| 1          | Anti-CT₁ | 1:500 (0.04) | 1.32         |
| 1          | Anti-CT₂ | 1:20 (0.98)  | 1.27         |
| 1          | Anti-CT₃ | 1:5 (3.92)   | 1.32         |
| 1          | Anti-KLH | 1:500 (0.03) | 1.52         |
| 1          | Anti-KLH | 1:20 (0.82)  | 1.37         |
| 1          | Anti-KLH | 1:5 (3.28)   | 1.42         |
| 2          | None   |             | 3.50         |
| 2          | Anti-L₃₄ | 1:500 (0.04) | 4.50         |
| 2          | Anti-L₃₄ | 1:5 (4.10)   | 1.35         |
| 2          | Anti-KLH | 1:500 (0.03) | 3.30         |
| 2          | Anti-KLH | 1:20 (6.71)  | 3.80         |
| 2          | Anti-KLH | 1:5 (2.84)   | 4.50         |
| 3          | None   |             | 2.75         |
| 3          | Anti-L₅₆ | 1:500 (0.04) | 2.40         |
| 3          | Anti-L₅₆ | 1:20 (0.88)  | 1.45         |
| 3          | Anti-L₅₆ | 1:5 (3.52)   | 0.80         |
| 3          | Anti-KLH | 1:500 (0.03) | 2.32         |
| 3          | Anti-KLH | 1:20 (0.82)  | 2.70         |
| 3          | Anti-KLH | 1:5 (3.28)   | 3.00         |
| 4          | None   |             | 5.05         |
| 4          | Anti-L₄₅ | 1:100 (0.16) | 4.85         |
| 4          | Anti-L₄₅ | 1:20 (0.81)  | 5.65         |
| 4          | Anti-L₄₅ | 1:5 (3.24)   | 5.85         |
| 4          | Anti-KLH | 1:100 (0.11) | 6.25         |
| 4          | Anti-KLH | 1:20 (0.56)  | 5.85         |
| 4          | Anti-KLH | 1:5 (2.22)   | 5.70         |

* Numbers represent the average of duplicate values minus the quantity of GTPyS bound/60 s in the absence of rhodopsin. These values were 0.33, 0.5, 0.25, and 0.15 pmol for experiments 1, 2, 3, and 4, respectively.

FIG. 3. Activation of transducin by ROS and rhodopsin reconstituted into phospholipid vesicles. Preparations of stripped ROS membranes (open circles) or rhodopsin reconstituted in phospholipid vesicles (closed circles and squares) were incubated for 1 min in the presence of 1 μM [35S]-GTPyS (20,000 cpm/pmol) prior to filtration and determination of GTPyS bound to transducin. The rhodopsin concentration was determined by Coomassie staining on 10% polyacrylamide gels.
any site within, or in close proximity to, the transducin binding domain would be inaccessible to the corresponding antipeptide antibody. The results shown in Fig. 4 indicate that preincubation of increased concentrations of transducin with rhodopsin blocked 50% of the binding of both anti-CT, and anti-L3.4 antibodies. However, the binding of anti-L6.6 was unaffected by concentrations of transducin as high as 0.95 μM, 4- to 5-fold higher than the amount required to inhibit anti-CT, and anti-L4.4.

Fig. 5 demonstrates the specificity of interaction of each of the antipeptide antibodies with purified rhodopsin. Saturating concentrations of each synthetic peptide inhibited the corresponding antipeptide antibody 70-95%. This residual binding is consistent with the level of nonspecific binding measured using equivalent concentrations of anti-KLH IgG. In contrast, synthetic peptides which do not correspond to the antipeptide antibody being tested do not significantly affect the binding of these antibodies to rhodopsin. Therefore, the binding of rhodopsin by each of these antipeptide antibodies is dependent on recognition of the domain corresponding to the appropriate synthetic peptide. Transducin at a concentration of 2 μM, twice that of the experiment shown in Fig. 4, blocked specific binding of anti-CT, and anti-L3.4 35 and 50%, respectively. These data suggest that only a fraction of the population of rhodopsin capable of binding antibody is available to interact in a stable manner with transducin (see "Discussion"). Nonetheless, Figs. 4 and 5 demonstrate that transducin partially blocks the specific binding of antipeptide antibodies to the COOH terminus and to loop 3-4, but not to the 14-amino-acid region within loop 5-6. The results indicate that contact sites for transducin are located on rhodopsin in close proximity to the antigenic domains for anti-CT, and anti-L4.4. The region of loop 5-6 involved in binding to anti-L6.6 does not appear to possess a binding domain for transducin.

**FIG. 4. Inhibition of antibody binding to rhodopsin in the presence of transducin.** Purified rhodopsin reconstituted into phospholipid vesicles was preincubated with transducin in the light and then incubated with antipeptide IgG as described under "Materials and Methods." Top panel, anti-CT; middle panel, anti-L3.4; lower panel, anti-L6.6.

**FIG. 5. Inhibition of antibody binding to rhodopsin by transducin.** Reconstituted rhodopsin was preincubated in the light with 2 μM transducin (T) or with the appropriate peptides (CT, L3.4, L6.6) and then incubated with antipeptide IgG fractions as described under "Materials and Methods." The open bars represent the binding of antibody in the absence of transducin or peptide. The concentrations of peptides used in the assay for each antibody were as follows: anti-CT, 100 mM CT, and L3.4 anti-L3.4, 1 mM L3.4 and CT; anti-L6.6, 1 mM L6.6 and CT. Values represent specific binding calculated by subtracting the value for binding of anti-KLH IgG: 21,000 cpm. These values represent 1-15% of the total binding of the peptide-specific antibodies.

**DISCUSSION**

The primary structure of rhodopsin has been defined by several laboratories (Ovchinnikov, 1982; Hargrave et al., 1983). Subsequent predictions of the orientation and conformation of the protein in the membrane are derived from studies using protease sensitivity, chemical modification, and structural predictive methods such as hydropathy analysis (Fung and Hubbel, 1978a, 1978b; Hargrave and Fong, 1977; Ovchinnikov, 1982; Hargrave et al., 1983; Kuhn and Hargrave, 1981; Nathans and Hogness, 1983). In direct analogy with bacteriorhodopsin (Ovchinnikov, 1982), all of these methods predict the presence of 3 loop structures, as well as the COOH terminus at the cytoplasmic surface, where they would be available for interacting with extracellularly (i.e. cytoplasmically) localized ROS proteins involved in the signal transduction process. The recognition of these domains by site-directed antibodies generated against specific linear sequences represents confirmation that these regions are all exposed for interaction with peripheral proteins. Previous studies involving monoclonal antibodies directed against the COOH terminus of rhodopsin (MacKenzie et al., 1984), as well as proteolysis studies (Ovchinnikov, 1982; Kuhn and Hargrave, 1981), have also supported this model.

The ability of antipeptide antibodies to block transducin activation and phosphorylation by rhodopsin kinase indicates that these structures are at least closely associated with the functional domains of the photoreceptor. Molday and MacKenzie (1985) have also reported that monoclonal antibodies directed against the COOH terminus of rhodopsin inhibit the phosphorylation of the photoreceptor. We have utilized site-directed antibodies as tools for probing the interaction of particular rhodopsin domains with its regulatory G-protein, transducin, and have observed that recognition of rhodopsin by antibodies directed against Loop 3-4 and the COOH-terminus, but not Loop 5-6, is inhibited when transducin is bound to rhodopsin. The inability of transducin to block more than 50% of the binding of anti-L3.4 and anti-CT, remains unclear. The possibility that only 50% of the rhodopsin is functional, the rest having been denatured during purification and reconstitution, is unlikely considering that reconstituted rhodopsin and rhodopsin present in ROS mem-
branes possess identical abilities for binding and activating transducin (see Fig. 3 this report; Wessling-Resnick and Johnson, 1987b), and the specific activities of our preparations are similar to those reported by others (Fung, 1983). A second possibility is related to the hypothesis that rhodopsin undergoes a light-dependent oligomerization in order to form high affinity binding sites for transducin. This mode of activation was proposed by Wessling-Resnick and Johnson (1987b) who observed that equilibrium binding between rhodopsin and transducin saturated at 25% of the total rhodopsin present in the assay, although 70% was accessible to V8 protease digestion. Bennett and Dupont (1985) also suggest that oligomerization of rhodopsin may explain its observed binding properties. If oligomerization does occur, this could potentially alter the stoichiometry of transducin binding to rhodopsin, but not necessarily alter the binding of antibodies to specific amino acid sequences within each opsin molecule. Unequivocal interpretation of these results will probably require an understanding of the physical structure of the rhodopsin-transducin complex which is not yet available.

Previous studies (Weiss et al., 1987) suggest that the recognition of rhodopsin by the antibody against the COOH terminus (anti-CT1) involves a Lys-Asn-Pro sequence corresponding to amino acid residues 325–327. Therefore, the results presented in Fig. 3 of this communication, using purified components, indicate a potential interaction of transducin with this region of the photoreceptor located amino-terminal to the serine/threonine-rich region of the COOH terminus. This conclusion is consistent with reports that synthetic peptides corresponding to amino acid residues 324–331 block transducin’s GTPase activity (Takemoto et al., 1985, 1986). In addition, Pellicone et al. (1985) report an increase in sensitivity of Met-317 (located on the NH2-terminal side of the antibody binding site) to CNBr cleavage of photoactivated rhodopsin, suggesting that this region undergoes a light-induced conformational shift consistent with a role in transducin binding. The ability of anti-CT1 to recognize the mouse β-adrenergic receptor (Weiss et al., 1987) suggests that this domain has been conserved in another G-protein-linked hormone receptor and may be important in the signal transduction process.

Our results also suggest that a major binding domain for transducin exists within close proximity to loop 3-4 but does not include a major portion of loop 5-6. Kuhn and Hargrave (1981) showed that long-term thermolysis digestion of rhodopsin, which cleaves between Ser-240 and Ala-241 on loop 5-6, reduced light-dependent binding of transducin and its subsequent GTP-dependent extraction from bleached ROS membranes. Their results suggested that loop 5-6 contains, or is closely associated with, a light-sensitive binding domain for transducin. A reanalysis of these data in view of the present studies suggests that this region of loop 5-6, although not directly responsible for binding transducin, may be important for transferring a conformational shift, induced by the light-dependent isomerization of retinal attached to Lys-296 of the seventh transmembrane helix, to other regions of the receptor. Conformation shifts in this region are supported by reports that Met-253 (helix 6) is more susceptible to CNBr cleavage after photoexcitation of photoreceptor (Pellicone et al., 1985). Comparison of the bovine rhodopsin sequence with that of Drosophila melanogaster (O’Tousa et al., 1985; Zuker et al., 1985), which represents the most evolutionarily distant sequence of the photoreceptors known at present, reveals that Loop 5-6 is the least conserved of the loop structures due to an insertion of 12 amino acids, consistent with a potential role for this domain as a hinge or a region of conformational change rather than as a contact site for transducin.

The data presented in this communication, using purified proteins and site-directed antibodies as probes, begin to define the role of specific rhodopsin domains involved in the interaction of the photoreceptor with transducin. We have begun to investigate further the role of specific rhodopsin sequences in the binding of transducin utilizing site-directed mutagenesis of rhodopsin expressed in mammalian cells. Information from these studies will aid in establishing amino acid sequence requirements for hormone receptors involved in G-protein-mediated signal transduction. The use of site-directed antipeptide antibodies to establish the orientation of specific amino acid sequences within each opsin molecule. Unequivocal interpretation of these results will probably require an understanding of the physical structure of the rhodopsin-transducin complex which is not yet available.

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