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MULTIPLE CHROMATOGRAPHIC FORMS OF THE FORMYLPEPTIDE CHEMOATTRACTANT RECEPTOR AND THEIR RELATIONSHIP TO GTP-BINDING PROTEINS

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The radiolabeled formylpeptide chemoattractant receptor, partially purified by wheat germ agglutinin-Sepharose chromatography, eluted in three distinct peaks when chromatographed on DEAE-Fractogel. Incubation of the lectin-Sepharose purified receptor with 100 μM guanosine 5-(3-O-thio) triphosphate markedly altered the distribution of the radiolabeled receptor when chromatographed on the ion exchange resin. Peaks 2 and 3 were reduced by approximately 50% while peak 1 was concomitantly increased. Western blot analysis revealed the presence of a 40 kDa GTP-binding protein α-subunit only in peak 3. Incubation of Western blots with [α-^{32}P]GTP detected low molecular mass GTP-binding proteins (24 and 26 kDa) that coeluted with the receptor in peak 2. Incubation of the peak 1 receptor fractions with fractions containing a mixture of GTP binding proteins resulted in the generation of peaks 2 and 3 when chromatographed on DEAE-Fractogel. These results demonstrate that the chromatographic behavior of the formylpeptide receptor is dependent upon its association with GTP binding proteins and that more than one type of GTP-binding protein may be involved.

Occupancy of formylpeptide chemoattractant receptors on leukocytes results in the activation of various cellular responses including the directed migration of the cell, superoxide production and secretion of lysosomal enzymes (1,2). We have shown that the formylpeptide receptor activates polyphosphoinoside hydrolysis through a pertussis toxin sensitive G protein (3,4) and that partially purified preparations of receptor remain functionally coupled to a G protein with an α-subunit of 40 kDa (5). Recently, GTP-binding activity associated with polypeptides of molecular masses 21-29 kDa has been identified in a variety of tissues (6-13). The specific functions of these proteins in mammalian cells are not well defined, however, in yeast the ras-related SEC4 and YPT1 gene products are known to be involved in the secretory pathway (14,15).

The abbreviations used are: G protein, GTP-binding protein; G_s and G_i, the stimulatory and inhibitory regulatory GTP-binding proteins of adenylate cyclase, respectively; G_o, an abundant pertussis toxin substrate initially isolated from bovine brain; GTPγS, guanosine 5-(3-O-thio) triphosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazine-N'-Z-ethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid.

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It has been suggested that secretion in mammalian cells also involves a low molecular mass GTP-binding protein (16).

The growing evidence for the role of low molecular mass GTP-binding proteins in cellular processes prompted us to examine the formylpeptide receptor for a potential interaction with a low molecular weight G protein. Here we demonstrate that a population of the formylpeptide receptor is regulated by GTP but does not contain a 40 kDa α-subunit. This receptor population coeluted with low molecular mass GTP-binding proteins suggesting a potential role for these molecules in the regulation of the receptor.

MATERIALS AND METHODS

Materials. Fractogel TSK DEAE-650S (DEAE-Fractogel) was purchased from Pierce Chemical Co. Pertussis toxin was from List Biological Laboratories. [α^{32}-P]GTP (3000Ci/mmol) was from Amersham. GTPγS was from Boehringer Mannheim. HA nitrocellulose paper filters (0.45 μM pore size) were from Millipore. All other chemicals were purchased from Sigma.

Labeling, solubilization and chromatography of the formylpeptide chemoattractant receptor. Isolated plasma membranes prepared from differentiated HL60 cells were covalently labeled with formyl-nle-leu-phe-nle-[^{125}I]tyr-lys, solubilized with sodium cholate, and the extract chromatographed on wheat germ agglutinin-Sepharose as described previously (5). The formylpeptide receptor-radioligand complex was eluted from the lectin-Sepharose column with Buffer A (50 mM Hepes, pH 7.2, 1 mM EGTA, 1 mM EDTA, 2 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl flouride, 10 μg/ml each of leupeptin and pepstatin, and 0.1% (w/v) Triton X-100) containing 100 mg/ml of N-acetylglucosamine. The eluate was applied (2 ml/hr) to a 1 ml column of DEAE-Fractogel equilibrated with 10 ml of buffer A. The column was washed with 10 ml of Buffer A and then eluted with a 30 ml gradient of 0-0.2M NaCl in the same buffer. The total radioactivity in each fraction (~0.5 ml each) was determined using a gamma counter. The position of the NaCl gradient on the chromatograms was estimated from conductivity readings performed on selected fractions.

Electroblotting and incubation with antisera and [α-^{32}P]GTP. SDS-PAGE was performed in 12% polyacrylamide slab gels as described (5). Proteins resolved by SDS-gel electrophoresis were transferred to nitrocellulose membranes by electroblotting for 1 hour at 70 V in 25 mM Tris, 192 mM glycine (pH 8.3) containing 20% (v/v) methanol. For detection of GTP-binding proteins nitrocellulose transfers were incubated for 30 minutes in 50 mM Tris-HCl, pH 7.5, containing 2 μM $MgCl_2$, 0.3% (v/v) Tween 20 and 0.3 nM [α-^{32}P]GTP (3000 Ci/mmol) and then washed twice with this same buffer minus the nucleotide. Transfers were air-dried and then exposed to Kodak X-OMAT X-ray film in the presence of a Cronex enhancing screen. Molecular weights of the GTP-binding proteins were estimated by interpolation from a standard curve constructed from the measured relative mobilities and known molecular weights of six low molecular weight standard proteins (Electrophoresis Calibration Kit, Pharmacia Inc.). For detection of α and β subunits of G proteins the electroblots were first blocked with 20 mM Tris, pH 7.5, 0.5 M NaCl (TBS) containing 3.0% (w/v) bovine serum albumin and then incubated overnight in TBS containing a 1/1000 dilution of antiserum A569 (17) and a 1/1000 dilution of antiserum β8 (18). The blots were washed 3 times with TBS containing 0.05% (V/V) Tween 20 and then incubated for 1 hour in this same buffer containing ^{125}I-labeled goat-antirabbit IgG (2.5 x 10^5 counts/min/ml). Following 3 washes in TBS the nitrocellulose blots were exposed to Kodak X-OMAT X-ray film in the presence of a Cronex enhancing screen for 4 hours. Purified pertussis toxin substrates from bovine brain were used as positive controls.

Reconstitution of receptor-GTP-binding protein complexes. Peak 1 fractions obtained from DEAE-Fractogel chromatography were pooled and 1.5 ml aliquots were mixed with 1.5 ml of

either Buffer A containing 0.12 M NaCl or fractions containing GTP-binding proteins that eluted in 0.12 M NaCl on DEAE-Fractogel. Following the addition of 5 mM $MgCl_2$, the samples were incubated on ice for 2 hours and then concentrated to 0.2 ml using an Amicon Centricon CM-10 microconcentrator (Amicon Corp, Danvers, Mass.). Samples were diluted to 2.5 ml with 50 mM Hepes, pH 7.2, containing 5 mM $MgCl_2$, concentrated and diluted once more, and then chromatographed on separate columns of DEAE-Fractogel as described above.

Other methods. Protein assays were performed using a Bio-Rad protein assay kit according to the manufacturer's instructions. Analysis of the formylpeptide receptor-radioligand dissociation was carried out by rapid vacuum filtration as described previously (5).

RESULTS

Formylpeptide chemoattractant receptors on isolated plasma membranes from differentiated HL60 cells were covalently labeled with formyl-nle-leu-phe-nle-[^{125}I]tyr-lys, detergent-solubilized and then chromatographed on wheat germ agglutinin-Sepharose as described previously (5). When the lectin-purified receptor-radioligand complex was chromatographed on DEAE-Fractogel three separate peaks of radioactivity were eluted from the column (Fig. 1). Peak 1 consisted of radioactivity not adsorbed by the resin while peaks 2 and 3 eluted in approximately 30 and 80 mM NaCl, respectively. Selected fractions from the chromatogram were analyzed by SDS-PAGE. The autoradiogram of the SDS-gel shows that the intensity of the 55-75 kDa diffuse band, characteristic of the radiolabeled receptor, correlated with the amount of total radioactivity in each fraction (Fig. 1, inset).

We tested the effect of GTPγS on the three isolated receptor fractions. In these experiments the receptor was not covalently labeled, however, the chromatograms exhibited the same 3 peaks of radioactivity that were observed on chromatography of the cross-linked receptor preparations (not shown). Although no effect was observed for peak 1, receptor-ligand complex taken from both peaks 2 and 3 responded dramatically to the presence of 100 μM GTPγS as evidenced by the increased rate of ligand dissociation (Fig. 2).

We reasoned that the physical association of the formylpeptide receptor with a G protein may contribute to its elution properties on ion exchange chromatography. We tested this by incubating the lectin-Sepharose purified covalently labeled receptor at 20°C for 20 minutes in the absence or presence of 100 μM GTPγS. The two samples were then chromatographed in parallel under identical conditions on DEAE-Fractogel. Pretreatment with GTPγS markedly altered the elution profile obtained with ion exchange chromatography (Fig. 3A) and the covalently labeled receptor itself underwent a similar redistribution in its elution pattern as seen on the autoradiogram obtained following SDS-gel electrophoresis of the chromatography fractions (Fig. 3B).

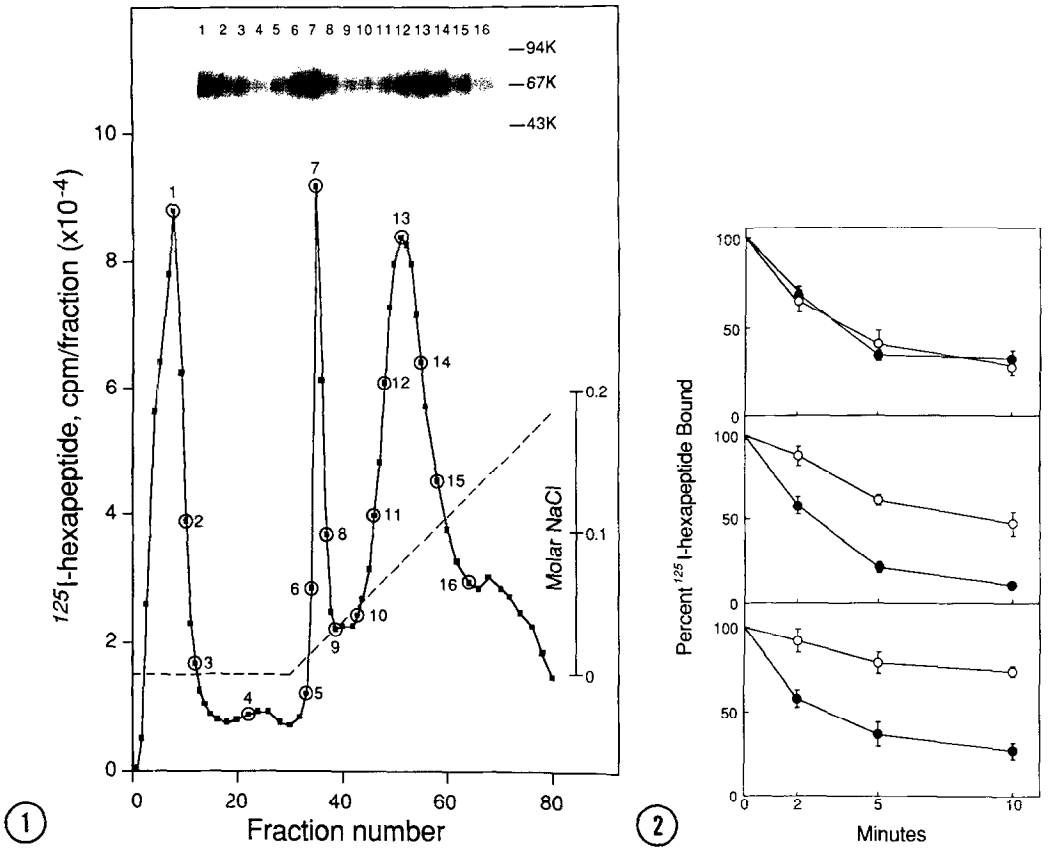

Figure 1. Chromatography of the formylpeptide receptor-radioligand complex on DEAE-Fractogel. Isolated plasma membranes (4 mg total protein) were covalently labeled, solubilized and chromatographed on wheat germ agglutinin-Sepharose and DEAE-Fractogel as described in "Materials and Methods." Total radioactivity was determined for each fraction (■) and aliquots (90 µl each) were taken from the indicated fractions (circled squares) and subjected to SDS-PAGE and autoradiography (inset). Values at right of inset indicate positions and relative molecular weights of applied standard proteins.

Figure 2. Dissociation of the formylpeptide receptor-radioligand complex. Peak fractions were obtained from the chromatographically resolved forms of the noncovalently labeled formylpeptide receptor and the protein-bound radioactivity was determined by filtration following a 2, 5 or 10 min incubation at 30°C in the presence (●) or absence (○) of 100 µM GTPγS. Upper frame, peak 1; middle, peak 2; lower, peak 3. Values are expressed as percentages of protein-bound radioactivity measured at zero-time.

We next attempted to identify the GTP-binding proteins eluted from ion exchange chromatography by performing SDS-gel electrophoresis and immunoblotting on selected fractions. The elution position of the cross-linked receptor was identified by autoradiography of an SDS-gel (Fig. 4A). An electroblot was incubated with a mixture of two antisera; one directed against a sequence common to the α-subunits of G_t, G_i, G_o, and G_s and a second antisera specifically reactive to the β-subunits of G proteins. Although low levels of β-subunit reactivity could be detected in peak 2 no α-subunit reactivity was observed in these fractions (Fig. 4B). Based on the reactivity of

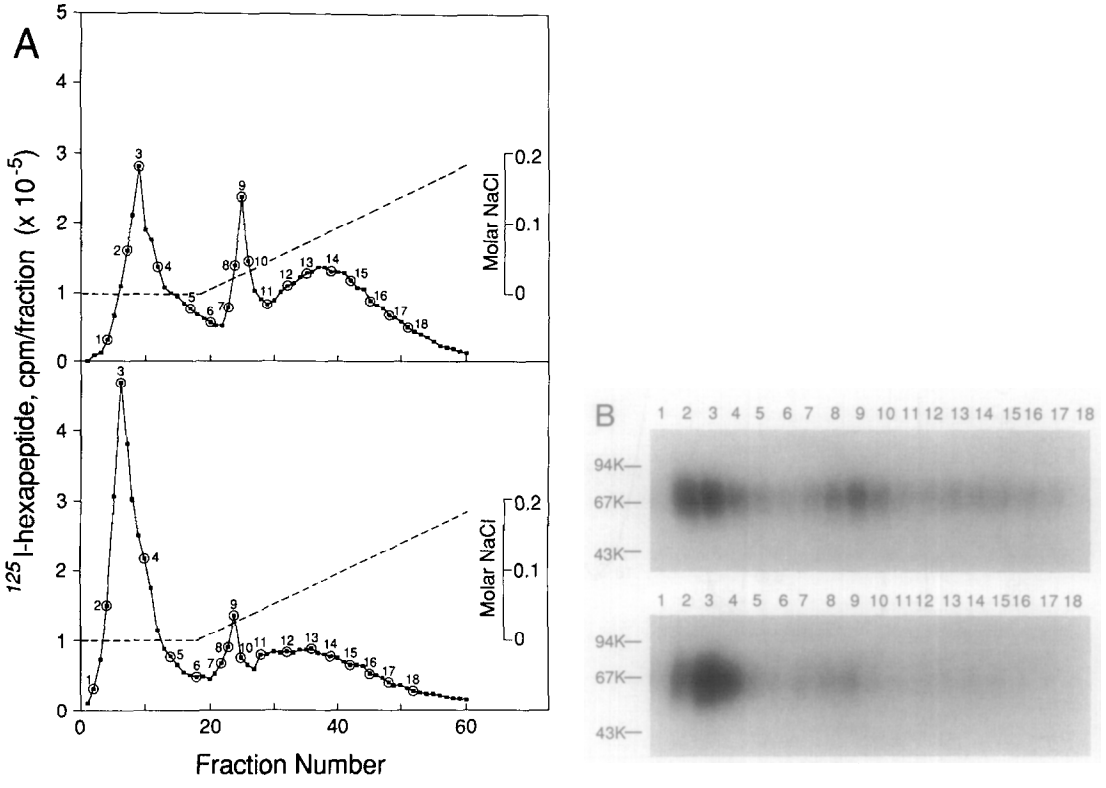

Figure 3. Effect of GTPγS on DEAE-Fractogel chromatography of the formylpeptide receptor-radioligand complex. Isolated plasma membranes (20 mg total protein) were covalently labeled, solubilized and chromatographed on wheat germ agglutinin-Sepharose as described in "Experimental Procedures." The eluate was divided equally and following an incubation in the absence (upper) or presence (lower) of 100 μM GTPγS the two samples were chromatographed on separate columns of DEAE-Fractogel. A, total radioactivity in each fraction was measured; B, 90 μl of each indicated fraction (circled squares) was subjected to SDS-PAGE and autoradiography. Migration of molecular weight markers are indicated at left in fig. B.

known quantities of α-subunit (Giα2) and the specific activity of the receptor radioligand complex, less than 0.1 moles of α-subunit per mole of receptor was estimated for the peak 2 fraction. We also incubated a separate nitrocellulose transfer of these same fractions in the presence of 0.3 nM [α-^{32}P]GTP. GTP-binding activities associated with polypeptides of 21, 24, and 26 kDa were detected with the bulk of the activity eluting after peak 3. In addition, a peak containing the 24 and 26 kDa polypeptides coincided with the elution of the receptor in peak 2 (Fig. 4C).

The results from the experiments presented in Figs. 1-3 suggested that the formylpeptide receptor present in peak 1 may not be coupled to a G protein. We therefore attempted to reconstitute the receptor-G protein complex by combining the peak 1 receptor fractions with fractions that contained substantial amounts of GTP-binding proteins but only low levels of formylpeptide receptor. Almost all of the radioactivity present in the control sample passed unretained through the ion exchange resin (Fig. 5A). However, when the peak 1 fractions were

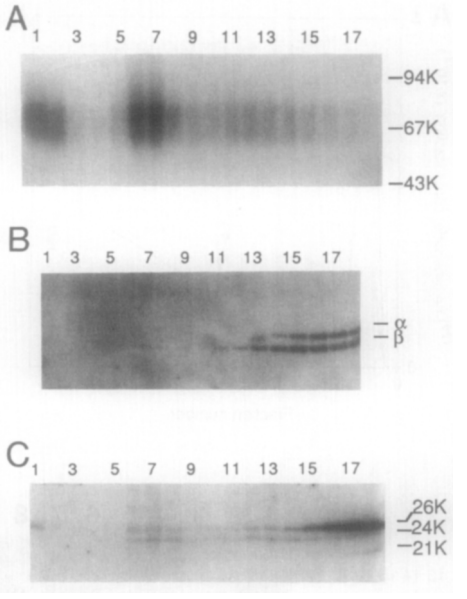

Figure 4. Detection of GTP-binding proteins eluted on DEAE-Fractogel. Isolated plasma membranes (40 mg total protein) were covalently labeled, solubilized and chromatographed on wheat germ agglutinin-Sepharose and DEAE-Fractogel as described in "Experimental Procedures." Aliquots (90 mL) of selected fractions were subjected to SDS-PAGE followed by autoradiography (A) or electroblotting (B and C). Blots were incubated with antisera directed against the α and β subunits of G_t, Gi, Go and Gs (B) or with 0.3 nM [α^{32}P]GTP(D). Relative molecular weights of the [α-^{32}P]GTP-binding proteins (C) are shown at right.

preincubated with fractions containing the GTP-binding proteins the radiolabel was adsorbed and eluted with NaCl at the position of peak 2 and to a lesser extent, peak 3. SDS-gel electrophoresis and autoradiography of selected fractions showed that the eluted radioactivity reflected the elution profile of the labeled receptor (Fig. 5B). We also carried out [α-^{32}P]GTP-binding analysis on electroblots obtained with these same fractions and detected only the 26 kDa G protein in the receptor fractions eluted at the peak 2 position (Fig. 5C). When fractions containing the GTP-binding proteins were applied to the column without prior incubation with peak 1 fractions no [α-^{32}P]GTP-binding proteins were detected in the corresponding peak 2 fractions (not shown).

DISCUSSION

Our data suggests that the chromatographic behavior of the formylpeptide receptor on DEAE-Fractogel is dependent upon its interaction with GTP-binding proteins. This is supported by the observation that the amount of receptor-ligand complex adsorbed on the ion exchange column was reduced substantially following preincubation with GTPγS. The GTPγS-induced reduction in the amount of the receptor eluted in peaks 2 and 3, accompanied by an increase in peak 1, suggests that the receptor which eluted in the flow-through fractions (peak 1) was not associated with a

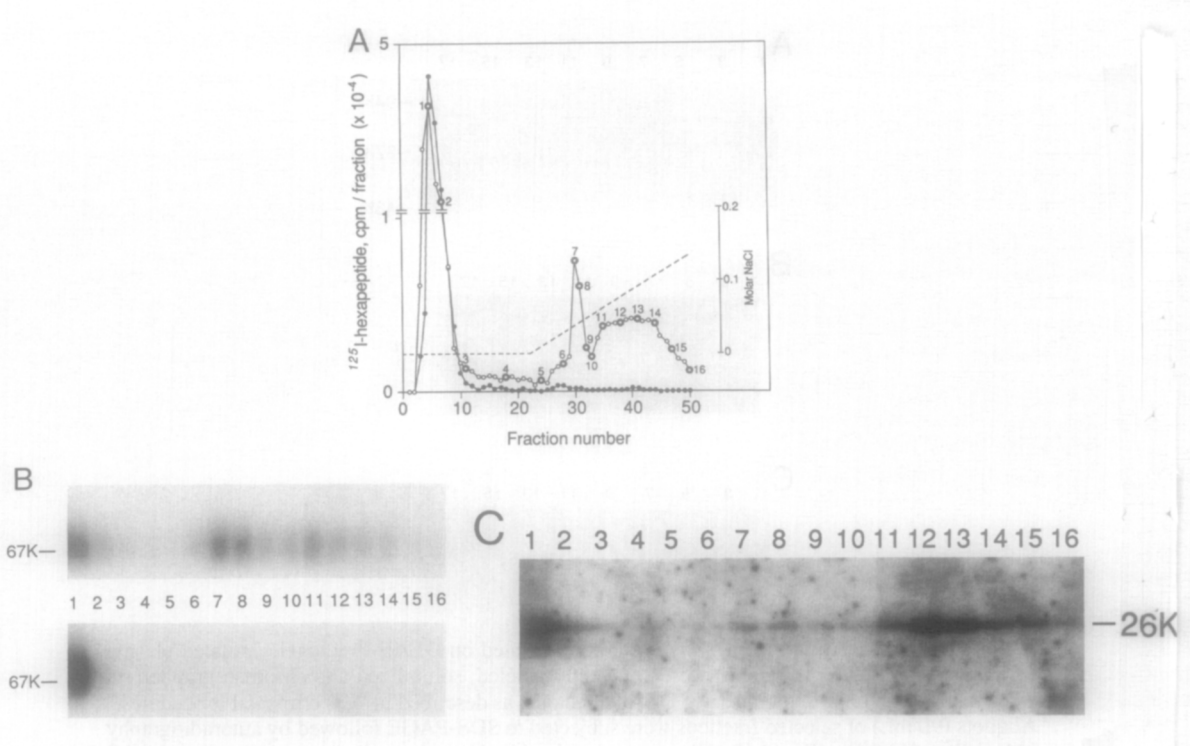

Figure 5. Reconstitution of the formylpeptide receptor-GTP-binding protein complex. Peak 1 fractions (lane 1, Fig. 4) were combined with fractions (lane 18, Fig. 4) containing GTP-binding proteins or with column buffer containing 0.12 M NaCl and treated as described in "Experimental Procedures." A, DEAE-Fractogel chromatography of peak 1 fractions combined with GTP-binding proteins (open circles) or with column buffer (closed circles). B, SDS-PAGE and autoradiography of 90 µl aliquots taken from fractions shown circled in A (upper frame) or from the corresponding set of fractions in the control chromatogram (lower frame). C, [α-^{32}P]GTP-binding to electroblots of SDS-gels following electrophoresis of 90 µl of each fraction shown circled in A.

GTP-binding protein. Indeed, the dissociation of radioligand from the peak 1 receptor was not effected by GTPγS as was the case for peaks 2 and 3. Moreover, when the peak 1 receptor fractions were preincubated with fractions containing GTP-binding proteins the receptor-ligand complex was retained by the ion exchange resin and eluted at the positions of peaks 2 and 3.

The presence of two distinct peaks of receptor which are regulated by GTPγS suggests that the receptor may be complexed with more than one type of GTP-binding protein. Interestingly, the peak 2 receptor fractions did not contain detectable levels of the G_i, G_o, G_t, or G_s α-subunits. We were also unable to detect any substrate for pertussis toxin-catalyzed [^{32}P]ADP-ribosylation in peak 2 (not shown). It is possible that peak 2 contains a G protein α-subunit that is not a pertussis toxin substrate and is unreactive to the common sequence antisera used here. However, the identification of GTP-binding polypeptides of molecular masses 24 and 26 kDa in the peak 2

receptor fractions suggests that the receptor may be complexed with a low molecular mass GTP-binding protein.

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