The Hepatic Glucagon Receptor

SOLUBILIZATION, CHARACTERIZATION, AND DEVELOPMENT OF AN AFFINITY ADSORPTION ASSAY FOR THE SOLUBLE RECEPTOR

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The hepatic glucagon receptor was covalently labeled with [125I-Tyr10]monoiodoglucagon ([125I]MIG) by use of the heterobifunctional cross-linker hydroxysuccinimidyl p-azidobenzoate. Labeling of the $M_r = 63,000$ peptide was sensitive to glucagon and GTP at concentrations at which they affect [125I]MIG binding to the receptor. The labeled receptor was solubilized with Lubrol-PX, and the hydrodynamic characteristics of the receptor were determined. The molecular parameters of the solubilized receptor are: $s_{20w} = 4.3 \pm 0.1$, Stokes radius $= 6.3 \pm 0.1$ nm, frictional coefficient $f/f_o = 1.8$, and a calculated $M_r = 119,000$. Incubation of liver membranes at 32°C for 15 min prior to the addition of [125I]MIG permitted us to identify the high molecular weight form ($M_r \approx 113,000$) of the receptor by direct sodium dodecyl sulfate-gel electrophoretic analysis.

The $M_r = 63,000$ peptide can be adsorbed to wheat germ lecin-Sepharose. The glycoprotein nature of the receptor has been utilized to develop an assay for the detergent-solubilized receptor that uses wheat germ lecin-Sepharose as a solid matrix to adsorb the [125I]MIG-receptor complex. The free hormone remains in the liquid phase and is removed in the supernatant after low speed centrifugation. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) solubilizes receptors with retention of [125I]MIG binding activity. [125I]MIG binding to the CHAPS-solubilized receptor is specifically affected by unlabelled glucagon. Interaction of [125I]MIG with the soluble receptor is insensitive to the presence of GTP. $I_{50}$ for glucagon using the soluble receptor was 33–70 nM, irrespective of the presence or absence of GTP, while when the membrane-bound receptor was used, the $I_{50}$ in the absence of GTP was 2–4 nM and in the presence of GTP was 35–80 nM.

These data allow us to conclude that the hepatic glucagon receptor in the membrane and in the non-denaturing detergent solution is a dimer of the $M_r = 63,000$ hormone-binding subunit and a glycoprotein. The soluble receptor does not display any functional interaction with the stimulatory regulator.

Glucagon is the major regulator of carbohydrate metabolism in the liver. Glucagon initiates its action by binding to receptors on the plasma membranes. The glucagon-bound receptor activates the stimulatory regulator of adenyl cyclase (1) which, in turn, associates with and activates the catalyst, resulting in enhanced cAMP synthesis rates (2). A detailed understanding of the primary events in glucagon action requires the isolation, characterization, and reconstitution of the purified components of the system. While the stimulatory regulator has been purified and extensively characterized (3–5), relatively little has been known about the glucagon receptor.

Recently, we have used the procedures of Johnson et al. (6) to covalently attach [125I-Tyr10]monoiodoglucagon to its receptor using the heterobifunctional cross-linker HSAB (7). This resulted in identification of a $M_r = 63,000$ peptide as the hormone-binding subunit of the glucagon receptor. In the previous studies, we had shown that only a portion of the $M_r = 63,000$ protein was required for hormone binding and interaction with the stimulatory regulator; however, since all analysis of the receptor was carried out by SDS-gel electrophoresis, it was not possible to study the subunit structure of the receptor. In this article, we have analyzed the glucagon receptor in non-denaturing detergent solutions and found the native receptor in the membrane and in detergent solution to be a dimer of the $M_r = 63,000$ peptide.

EXPERIMENTAL PROCEDURES

Materials

HSAB and CHAPS were obtained from Pierce Chemical Co. Dimethyl sulfoxide was obtained from Mallinkrodt Chemical Works. Calibrating proteins for hydrodynamic measurements, D2O, Lubrol-PX, digitonin, octyl glucoside, cholic acid, deoxycholate, Triton X-100, Sepharose 6B-CL, Hepes, GTP, insulin, ACTH, WGL, GlcNAc, marker enzyme substrates, and diisopropyl fluorophosphate were obtained from Sigma. [8-Aarginine]vasopressin and Nonidet P-40 were purchased from Calbiochem. Markers for SDS-gel electrophoresis were obtained from Pharmacia. Chemicals for SDS-gel electrophoresis were purchased from Bio-Rad. Sources of other materials have been previously reported (8,9). [α-32P]ATP was synthesized according to the procedure of Walshet and Johnson (10). [125I]MIG was synthe-

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The abbreviations used are: HSAB, hydroxysuccinimidyl p-azidobenzoate; BSA, bovine serum albumin; GMP-P(NH)P, guanyo-5'-yl imidodiphosphate; [125I]MIG, [125I-Tyr10]monoiodoglucagon; SDS, sodium dodecyl sulfate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; WGL, wheat germ lectin; GlcNAc, N-acetylglucosamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ACTH, corticotropin; GTP, guanosine 5'-O-(thio-triphosphate); PAGE, polyacrylamide gel electrophoresis.
sized and purified by reverse chromatography over a C18 μBondapak column as described in detail elsewhere (11). WGL-Sepharose was synthesized according to the procedure of Adair and Kornfield (12) with some minor modifications. One hundred mg of WGL were coupled to 40 ml of packed CNBr-activated Sepharose 6B-CL in the presence of 10 mM NaHCO3, 100 mM NaCl, and 200 mM GlcNAc overnight at 4 °C. After the coupling, the gel was washed with 2 liters of ice-cold 10 mM NaHCO3, 100 mM NaCl, and then reacted with 1.5 g of glycine in 30 mM NaHCO3, 100 mM NaCl overnight. After the reaction, the gel was washed with 2 liters of 10 mM NaHCO3 and 100 mM NaCl and stored at 4 °C as a 1:2 suspension in the same solution. Sepharose was activated with 100 mg of CNBr per ml packed gel in the presence of 2 mM NaCO3, according to the procedure of Parikh et al. (13). The WGL-Sepharose retained its binding characteristics for 4–6 weeks and showed no significant leaching of the WGL.

Preparation of Membranes

Rat liver plasma membranes were prepared according to the procedure of Neville (14) as described by Pohl et al. (15). Prior to use, the membranes were suspended in 20 mM phosphate buffer, pH 7.5 (10 mg of protein/ml) and mixed with 100 mM diisopropyl fluorophosphate dissolved in propylene glycol, such that the final concentration of diisopropyl fluorophosphate was 10 mM. The mixture was held on ice for 10 min. Subsequently, the membranes were washed twice with 10 volumes of 20 mM phosphate buffer, pH 7.5, and diluted and the pellet was resuspended in 1 ml of 20 mM phosphate buffer, pH 7.5, at a concentration of 5–10 mg of protein/ml. This procedure resulted in total solubilization of the M, = 63,000 protein per ml and stirred for 30 min at 0–4 °C. The detergent suspension was centrifuged at 100,000 × g for 60 min. The clear supernatant was removed carefully and made 16% (w/v) with respect to sucrose. Aliquots were quick frozen in a dry ice-acetone bath and stored at −70 °C until use. Typically, under these conditions, about 50% of the membrane protein is solubilized and 25–30% of specific glucagon binding sites are recovered in the supernatant.

Solubilization of Membranes Containing Covalently Labeled Receptor—Liver membranes (5–10 mg of protein/ml) that had been cross-linked to [3H]MIG were solubilized in the presence of 25 mM NaHepes, pH 8.0, 1% Lubrol-PX. The samples were held on ice for 30 min with periodic agitation. Subsequently, the mixture was centrifuged at 100,000 × g for 60 min. This procedure resulted in total solubilization of the M, = 63,000 peptide, since no labeled peptide was observable in the 100,000 × g pellet. The supernatant was removed and used as the source of solubilized glucagon receptor.

Preparation of CHAPS Extract—Liver membranes were suspended in 20 mM CHAPS, 0.5 mM NaCl, 0.5 mM EDTA, 1.0 mM MgCl2, and 25 mM NaHepes, pH 8.0, at a final concentration of 6–8 mg of protein per ml and stirred for 30 min at 0–4 °C. The detergent suspension was centrifuged at 100,000 × g for 60 min. The clear supernatant was removed carefully and made 16% (w/v) with respect to sucrose. Aliquots were quick frozen in a dry ice-acetone bath and stored at −70 °C until use. Typically, under these conditions, about 50% of the membrane protein is solubilized and 25–30% of specific glucagon binding sites are recovered in the supernatant.

Binding Assay for the Solubilized Receptor—[3H]MIG was used in the absence of CHAPS extract protein, 0.08 to 0.3% (600–2500 cpm out of 720,000 cpm) of the total counts adsorbed to the gel in a manner unaffected by the absence or presence of 0.1 mM GlcNAc.

Sucrose Density Gradient Ultracentrifugation—The solubilized receptor preparation (150 μl) was mixed with 10 μl of malate dehydrogenase (10 mg/ml; 443 units/μg), 5 μl of catalase (10 mg/ml, 11,000 units/μl), 10 μl of fumarase, 10 μl of catalase, 10 μM of [125I]MIG, 2 mM MgCl2, 1 mM EDTA, 25 mM NaHepes, pH 8.0, and 12% (w/v) sucrose. [3H]MIG was added in 30 μl of 0.1% BSA. Incubations were carried out in plastic centrifuge tubes (16 × 100 mm; Sarstedt 25.488) for 90–180 min on ice. At the end of the incubation, 300 μl of a 1:4 (v/v) suspension of WGL-Sepharose in 25 mM NaHepes, 8% (w/v) sucrose, 0.5% Lubrol-PX, 50 mM KCl, 1 mM EDTA, and 0.5% Me2SO (wash buffer) were added to each tube. Prior to use, the WGL-Sepharose was washed with 10 volumes of the wash buffer. The samples were then shaken for 2 h in the cold at a moderate speed so as to impede the gel from settling. At the end of the 2-h period, the samples were diluted to 5 ml with ice-cold wash buffer and centrifuged at 1500 × g for 15 min in a refrigerated IEC centrifuge. The supernatant was aspirated, and the gel was resuspended again in 5 ml of wash buffer. After a second centrifugation, the supernatant was removed and the gel was counted in a Tracor y counter. When [3H]MIG was used in the absence of CHAPS extract protein, 0.08 to 0.3% (600–2500 cpm out of 720,000 cpm) of the total counts adsorbed to the gel in a manner unaffected by the absence or presence of 0.1 mM GlcNAc.

Cross-linking of Bound [3H]MIG to Liver Membranes

HSAB was weighed in the dark and dissolved in dimethyl sulfoxide in a brown bottle to give a concentration of 20 mM. The membranes in 13-mL ultracentrifuge tubes were mixed with the HSAB solution such that the final concentration was 200 μM. The samples were kept in the dark for 3 min. Subsequently, they were irradiated in a final volume of 100 μl under a 150-W Osram low-pressure mercury arc lamp (Arthur H. Thomas 6281-H10) at a distance of 16 cm. The samples were kept on ice during the UV irradiation. After the irradiation, the samples were diluted to 10 ml with 25 mM Tris- HCl, pH 7.5, 100 μM GTP, and 1 μM glucagon. This mixture was incubated for 15 min at 32 °C. The membranes were then sedimented by centrifugation at 100,000 × g for 20 min. This procedure resulted in 1–3% of the bound hormone being covalently attached to the M, = 63,000 peptide.

Detergent Solubilization
cuvette at room temperature. Catalase—5-10 µl of sample were added to 1 ml of 50 mM Tris-HCl, pH 7.5, and 1.2-7 mM H2O2. After a 5-min incubation at room temperature (22-24 °C), the decrease in absorbance at 240 nm was recorded.

Fumarase—5-10 µl of sample were added to 1 ml of 50 mM Tris-HCl, pH 7.5, and 40 mM Tris malate. After incubation for 3.5-5 min at room temperature, the reaction was stopped by the addition of 50 µl of 1 N HCl. The increase in absorbance at 250 nm was measured.

β-Galactosidase—20-50 µl of sample were added to 1 ml of 50 mM Tris-HCl, pH 7.5, and 2 mM o-nitrophenyl-β-D-galactopyranoside. The samples were incubated at 32 °C until a visibly clear color change occurred (5-10 min). The reaction was stopped by the addition of 0.5 ml of 1 M Na₂CO₃. Increase in absorbance was measured at 405 nm. Details of the marker enzyme assays were kindly provided by Dr. Eva Neer (Department of Cardiology, Harvard Medical School, Boston, MA 02115).

**SDS-Polyacrylamide Gel Electrophoresis**

SDS-PAGE was run according to the procedure of Laemmli (18). Routinely, samples were analyzed on a 10% gel with a 3% stacking gel. When membranes were loaded onto the gel, 25-50 µg of protein per sample in a volume of 50 µl were used. When sucrose density gradient fractions were used, 35 µl of sample were mixed with 35 µl of buffer containing 2% SDS and 5% β-mercaptoethanol. 200-µl aliquots of the gel filtration column fractions were mixed with 50 µl of 1 mg/ml of BSA and 2 ml of cold (−10 °C) acetone. The precipitated proteins were collected by centrifugation at 1500 × g for 10 min at −10 °C. The precipitates were dissolved in a buffer containing 1% SDS and 2.5% β-mercaptoethanol. Prior to application onto the gel, all samples were incubated for 1 h at 32 °C in the presence of 1% SDS and 2.5% β-mercaptoethanol. Heating at higher temperatures was avoided since this led to aggregation and subsequent retention of the samples in the stacking gel during electrophoresis.

After electrophoresis, the gels were stained with Coomassie Blue, destained, and dried. The gels were then exposed to Kodak XAR-5 film, in cassettes equipped with intensifying screens (Cronex Xtra-Lite). Generally, exposure was for 0.2 h exposure allowed for scanning within the linear ranges of the instrument.

**Assay for the Stimulatory Regulator of Adenylcyclase**

The regulatory component was assayed by its capacity to reconstitute the cya S49 cell membrane adenyl cyclase in the presence of 10 µM GTPγS and 20 mM MgCl₂, as described in detail elsewhere (2). [³²P]cAMP formed was quantified according to the method of Salomon et al. (19) as modified by Bockaert et al. (20).

**Protein Measurement**

Proteins were measured by the procedure of Lowry et al. (21). When CHAPS was present during estimation, its concentration was 0.2 mM. This concentration did not interfere with the protein estimation.

**Replication of Results**

All experiments were performed at least three times. Experiments with the CHAPS extract were performed 4 to 6 times using three different batches of CHAPS extract protein from different membrane preparations and using two different batches of WGL-Sephase. While there was variability in the extent of binding obtained as well as the level of nonspecific binding (20-50% of total binding), all the basic characteristics of the binding activity were observed throughout. Representative experiments are shown.

**RESULTS**

**Covalent Labeling of the Glucagon Receptor**

When liver membranes were exposed to [¹²⁵I]MIG and binding was measured by removal of unbound label by filtration or centrifugation, similar levels of specific receptor sites per mg of protein were observed irrespective of the method used to separate bound label from free label (Table I). Since it is relatively easy to recover the membranes after centrifugation, we routinely separated the membrane-bound [¹²⁵I]MIG from free [¹²⁵I]MIG by centrifugation. When the membranes that had [¹²⁵I]MIG specifically bound were treated with HSAB and then analyzed by SDS-PAGE on 10% gels, the label was associated with one macromolecular species which migrated with an apparent Mr = 63,000. When excess unlabeled glucagon was included during the binding reaction, the labeling of the Mr = 63,000 peptide was abolished. However, insulin, ACTH, and vasopressin had no effect on the labeling of the Mr = 63,000 peptide (Fig. 1). This indicated that the Mr = 63,000 peptide specifically recognized glucagon or its radiolabeled analog. The inclusion of unlabeled glucagon during the binding reaction not only abolishes the labeling of the Mr = 63,000 peptide but also significantly decreases the amount of label associated with the 63,000 peptide.

**TABLE I**

| Additions to assay | Method of separation | [¹²⁵I]MIG bound (fmol/mg protein) |
|-------------------|----------------------|----------------------------------|
| None              | Filtration           | 227 ± 6                          |
| 100 µM GTP        | Centrifugation        | 236 ± 7                          |
| 1 µM Glucagon     |                      | 55 ± 3                           |
|                   |                      | 51 ± 5                           |

*a The assay was carried out in a final volume of 1 ml at 0.6 mg of protein/ml.

*b 100-µl aliquots were withdrawn at the end of the incubation and filtered as described under "Experimental Procedures."

*c 200-µl sample was diluted to 2.0 ml and centrifuged as described in detail under "Experimental Procedures."

**Fig. 1. Effect of various peptide hormones on the binding of [¹²⁵I]MIG to live membranes and the HSAB-mediated covalent attachment of [¹²⁵I]MIG to a liver membrane protein.** Liver membranes (0.32 mg/ml) were incubated with 1 nM [¹²⁵I]MIG without and with the other specified peptide hormones. The hormones used were: insulin (1.8 µM), ACTH (2.2 µM), [8-arginine]vasopressin (8-AVP) (8.6 µM), and glucagon (3 µM). After the incubation, the membranes were washed free of unbound label and incubated with HSAB in the dark and under UV illumination. The membranes were then washed, and protein and bound counts in the final pellets were estimated. It was found that incubation in the absence of any unlabeled hormone or in the presence of insulin, ACTH, or [8-arginine]vasopressin resulted in 29,000–32,000 cpm bound per 40 µg of membrane protein. When glucagon was present, 1200 cpm were bound to 40 µg of membrane protein. Forty µg of protein for each sample were loaded on the gel. The samples were then subjected to electrophoresis followed by autoradiography. An autoradiogram (48-h exposure) is shown.
amount of label that travels with the dye front. This indicates that the label associated with the dye front probably represents the fraction of the receptor-bound \(^{125}\text{I}\)MIG that is not cross-linked by HSAB, rather than nonspecifically adsorbed \(^{125}\text{I}\)MIG.

We then studied the effect of inclusion of various concentrations of unlabeled glucagon during the binding reaction on the extent of the labeling of the \(M_r = 63,000\) peptide by 1 nM \(^{125}\text{I}\)MIG. We found that the concentration range in which glucagon affected labeling of the \(M_r = 63,000\) peptide (Fig. 2) agreed with the range in which it interacts with the glucagon receptor and stimulates adenylyl cyclase (22).

To establish that the \(M_r = 63,000\) peptide labeled is a part of the guanine nucleotide-sensitive glucagon receptor, we compared the effect of various concentrations of GTP on the amount of \(^{125}\text{I}\)MIG bound to receptors in the membrane and on the amount of labeling obtained after cross-linking. One such experiment is shown in Fig. 3. In this experiment, \(^{125}\text{I}\)MIG binding was performed in the presence of varying concentrations of GTP. Aliquots were withdrawn, and the amount of \(^{125}\text{I}\)MIG bound per mg of protein was determined. Another aliquot of membranes was incubated with HSAB in the dark first and under UV light afterwards to covalently attach the bound \(^{125}\text{I}\)MIG. The peptide(s) labeled by this treatment were then analyzed by SDS-PAGE and autoradiography. As illustrated, increasing concentrations of GTP lead to a progressive decrease in binding and in the labeling of the \(M_r = 63,000\) peptide. The concentration range in which GTP affected the labeling of the \(M_r = 63,000\) peptide (top) was very similar to the range in which GTP affected \(^{125}\text{I}\)MIG binding to the receptor (bottom).

Subsequent analyses of the receptor by sucrose density gradient centrifugation and by gel filtration columns were carried out by monitoring for the presence of the \(M_r = 63,000\) band with \(^{125}\text{I}\)MIG cross-linked to it. Receptors that had \(^{125}\text{I}\)MIG covalently attached were solubilized using 1% Lubrol-PX. This detergent was chosen because the partial specific volume of Lubrol-PX is known (0.956 ml/g), and it has been reported that the glucagon receptor maintains guanine nucleotide sensitivity after solubilization with Lubrol-PX (23).

Hydrodynamic Measurements

**FIG. 2.** Effect of varying concentrations of glucagon on the extent of HSAB-mediated \(^{125}\text{I}\)MIG cross-linked to the \(M_r = 63,000\) peptide. Liver membranes were incubated with 1 nM \(^{125}\text{I}\)MIG and indicated concentrations of unlabeled glucagon. After the binding reaction, the membranes were washed free of unbound label. The bound \(^{125}\text{I}\)MIG was cross-linked by incubation with HSAB. The membranes were washed free of cross-linker and the proteins estimated in the final pellets. Equivalent amounts of proteins (35 \(\mu\)g) were applied to the gel for electrophoresis. After electrophoresis, the gels were dried and subjected to autoradiography (48-h exposure). The autoradiogram was scanned using a Kontes fiberoptics scanner equipped with a Hewlett-Packard integrator. The areas under individual peaks for the \(M_r = 63,000\) peptide are shown as a function of the glucagon concentration used. The region of autoradiogram used for the scanning is shown as the inset.

**FIG. 3.** Effect of varying concentrations of GTP on the extent of \(^{125}\text{I}\)MIG covalently attached to the \(M_r = 63,000\) peptide (top) and binding of \(^{125}\text{I}\)MIG to liver membranes (bottom). Liver membranes (0.25 ml/mg) were incubated with 1 nM \(^{125}\text{I}\)MIG, 1 mM ATP, a nucleoside triphosphate-regenerating system consisting of 20 mM creatine phosphate, 0.2 mg/ml of creatine phosphokinase, and 0.02 mg/ml of myokinase and indicated concentrations of GTP in a final volume of 3 ml. After 15 min at 32 °C three 100-\(\mu\)l aliquots were withdrawn and filtered to estimate hormone binding to membranes (bottom). The remaining membranes were washed free of unbound \(^{125}\text{I}\)MIG and incubated with HSAB in the dark and under UV illumination. The membranes were then washed to remove the cross-linker, and proteins were estimated in the final pellet. Thirty-one \(\mu\)g of protein for each sample were applied to the gel. The gel was dried and then exposed to x-ray film. The autoradiogram (48-h exposure) was scanned using a Kontes fiberoptics scanner equipped with a Hewlett-Packard integrator. The areas under individual peaks for the \(M_r = 63,000\) peptide are shown as a function of the GTP concentration (top). The region of autoradiogram used for the scanning is shown in the inset.
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Fig. 4. Gel filtration profile of the glucagon receptor. [125I]MIG was covalently attached to the receptor, and the labeled receptor was solubilized with 1% Lubrol-PX as described under “Experimental Procedures.” 500 μl of the detergent extract and marker proteins were applied to a AcA34 column (48 × 1 cm). One hundred fractions of 450 μl each were collected. The samples were analyzed for the positions of the marker proteins. Aliquots (200 μl) of individual fractions were then precipitated with cold acetone (−10 °C) in the presence of 5 μg of BSA. The acetone precipitate was dissolved in a buffer containing 1% SDS and 2.5% β-mercaptoethanol, incubated for 1 h at 32 °C, and then subjected to electrophoresis. Subsequently, the gel was dried and exposed to the x-ray film for 30 h. The autoradiogram was scanned using a Kontes fiberoptics scanner. The scan of the M, = 63,000 peptide as a function of fraction number is shown. The autoradiogram of the M, = 63,000 peptide is also shown (inset). Positions of the marker proteins are indicated by arrows. The abbreviations used are: β-GAL, β-galactosidase; CAT, catalase; MDH, malate dehydrogenase; and Cyt, cytochrome c. For other details, see “Experimental Procedures.”

Fig. 5. Standard curve for sucrose density gradient ultracentrifugation. Migration distances of marker proteins (fraction numbers) are plotted as a function of s,wp value in H2O and D2O. The s,wp values for M, = 63,000 peptide are shown. For other details, see “Experimental Procedures.” Cat, catalase; Fum, fumarase; MDH, malate dehydrogenase; Cyt C, cytochrome c.

Subunits of the Glucagon Receptor

In order to test if the subunits of the receptor were linked via disulfide bridges, we analyzed the receptor by SDS-PAGE before and after treatment with varying concentrations of β-mercaptopethanol (Fig. 6). It was found that addition of varying concentrations of β-mercaptopethanol during solubilization of the sample did not result in any decrease in the size of the band in the M, = 63,000 region. In fact, with increasing concentrations of β-mercaptopethanol, there was a small increase in the apparent molecular weight (~3000). The calculated M, of the labeled peptide in the absence of β-mercaptoethanol is 60,000, while at 10, 25, and 50 mM, β-mercaptopethanol is 63,000. Such increases in apparent molecular weights are attributed to the breakage of intrachain disulfide bridges (27). We then searched for the M, = 120,000 form in the membranes to determine if the high molecular weight form was an artifact of the solubilization procedure. For this pur-

AcA 34 gel. The elution pattern of the glucagon receptor as determined by the appearance of the labeled M, = 63,000 band is shown in Fig. 4. Standard curves of −log Ks (Ks = distribution coefficient) versus Stokes radius of the marker proteins were constructed and found to be linear. By interpolation, the Stokes radius of the glucagon receptor was found to be 6.3 nm.

Sucrose Density Gradient Ultracentrifugation—The [125I]MIG-glucagon receptor complex was cross-linked with HSAB, solubilized with Lubrol-PX, and centrifuged through 5–15% sucrose gradients in H2O and D2O in the presence of 0.5% Lubrol-PX. The gradients were fractionated and analyzed by SDS-PAGE followed by autoradiography for the appearance of the M, = 63,000 peptide. The receptor displayed very similar sedimentation coefficients in H2O and D2O (4.3 ± 0.15 (n = 5) in H2O and 4.2 ± 0.2 S (n = 3) in D2O), the difference between these values being statistically not significant (Fig. 5). The stimulatory regulator of adenylyl cyclase migrated in sucrose gradients (using H2O as the solvent) with a sedimentation coefficient of 5.2 ± 0.2 S (n = 3), a value previously observed by us (24). Treatment of liver membranes cross-linked to [125I]MIG with 100 μM GMP-P(NH)P and 25 mM MgCl2 for 20 min at 32 °C prior to extraction with Lubrol-PX followed by centrifugation through sucrose gradients containing 100 μM GMP-P(NH)P and 5 mM MgCl2 resulted in a decrease in the sedimentation coefficient of the regulatory component from 5.2 ± 0.2 to 4.4 ± 0.3 S (n = 3). No change in the apparent S value of the receptor was observed (data not shown).

Calculation of Molecular Parameters—The sedimentation coefficient of the receptor in D2O was not significantly different from that in H2O. This observation indicates that the M, = 63,000 peptide in Lubrol-PX solution has a partial specific volume similar to that of standards used (25, 26). Hence, the average partial specific volume of the calibrating proteins (0.738) was used for calculating the molecular weight of the receptor (25). The s,wp value used for these calculations was the experimentally determined value using H2O as solvent (Fig. 5). From the Stokes radius and sedimentation coefficient, an M, = 119,000 was calculated (Table II). These data indicate that the M, = 63,000 peptide is part of a larger protein of M, = 119,000.
The Coomassie Blue stained gel (top) and autoradiogram (48-h exposure) of the glucagon receptor in rat liver membranes as described under "Experimental Procedures." The membranes samples (23–25 μg of protein) were incubated with 1% SDS and the indicated concentration of β-mercaptoethanol (β-ME) for 1 h at 32 °C. The samples were then subjected to electrophoresis and autoradiography. A photograph of the Coomassie Blue stained gel (top) and autoradiogram (48-h exposure) (bottom) is shown.

Fig. 6. Effect of varying concentrations of β-mercaptoethanol during SDS solubilization. [125I]MIG was cross-linked to glucagon receptor in rat liver membranes as described under "Experimental Procedures." The membrane samples (23–25 μg of protein) were incubated with 1% SDS and the indicated concentration of β-mercaptoethanol (β-ME) for 1 h at 32 °C. The samples were then subjected to electrophoresis and autoradiography. A photograph of the Coomassie Blue stained gel (top) and autoradiogram (48-h exposure) (bottom) is shown.

Fig. 7. Identification of a high molecular weight form of the glucagon receptor by SDS-gel electrophoresis. Liver membranes (0.6 mg/ml) were incubated at 32 °C for 20 min in 20 mM phosphate buffer, pH 7.5, 1 mM EDTA, and 0.1% BSA. After this incubation, the membranes were then exposed to 1 nM [125I]MIG under standard binding conditions without any other additives (−) or in the presence of 100 μM GTP, 3 μM glucagon, or 1.8 μM insulin. The membranes were then washed free of unbound [125I]MIG and then treated with HSAB in the dark and under UV illumination. After cross-linking, the membranes were washed and subjected to SDS-gel electrophoresis on 5% gels. The gels were stained, destained, dried, and then subjected to autoradiography. A 72-h exposure is shown.

Fig. 8. Sugar specific adsorption of the M, = 63,000 receptor to wheat germ lectin-Sepharose. Liver membranes that had [125I]MIG covalently attached were extracted with 1% Lubrol-PX. The Lubrol-PX extracts were incubated with WGL-Sepharose in the absence and presence of various sugars. Equivalent amounts of the gel supernatants were analyzed by SDS-gel electrophoresis for the presence of the M, = 63,000 peptide. It was found that the M, = 63,000 receptor was not present in solution after exposure to WGL-Sepharose. However, addition of GlcNAc but not glucose or galactose allowed for the hormone-binding subunit to remain in solution (Fig. 8). This experiment indicates that the receptor is a glycoprotein that can specifically interact with WGL. The specific sugar sensitivity of the labeling of this M, = 113,000 band was tested by the inclusion of GTP, glucagon, or insulin during the binding reaction. Labeling of the M, = 113,000 band was decreased by the inclusion of GTP, abolished by the inclusion of glucagon, and unaffected by insulin. This indicates that the M, = 113,000 protein also recognized glucagon specifically in a GTP-dependent manner as is observed with the M, = 63,000 peptide (Fig. 7).

Since many hormone receptors are glycoproteins, we tested if the glucagon receptor was also a glycoprotein. We extracted membranes that contained receptors that had [125I]MIG covalently attached with 1% Lubrol-PX. The Lubrol-PX extracts were incubated with WGL-Sepharose in the absence and presence of various sugars. Equivalent amounts of the gel supernatants were analyzed by SDS-gel electrophoresis for the presence of the M, = 63,000 peptide. It was found that the M, = 63,000 receptor was not present in solution after exposure to WGL-Sepharose. However, addition of GlcNAc but not glucose or galactose allowed for the hormone-binding subunit to remain in solution (Fig. 8). This experiment indicates that the receptor is a glycoprotein that can specifically interact with WGL. The specific sugar sensitivity of the

### Table II

| Molecular parameters for glucagon receptor |
|-------------------------------------------|
| Values are average ± S.D. of number of determinations indicated within parentheses. |
| Stokes radius (nm) | 6.3 ± 0.1 (3) |
| Sedimentation coefficient, s_{20,0} |
| M, calculated | 119,000 |
| Frictional ratio, f/f₀ | 1.80 |
| M, SDS-PAGE measurement | 63,000 |

*Stokes radius was obtained from a standard curve of S = log K_D (K_D = distribution coefficient) versus Stokes radius of the marker protein.

Since there was no significant differences in the experimental S values in H2O and D2O, the H2O value was used as s_{20,0}.

Calculated from the following equation,

\[
M_t = \frac{6 \pi N x_{20,0}}{1 - \frac{\rho}{\rho_0}} \frac{a}{a - a_{w20,0}}
\]

where \( a \) (partial specific volume (ml/g)) was assumed to be 0.738, the average for all the marker proteins.

Calculated according to the equation,

\[
f/f₀ = \frac{4 \pi N}{3 M_t (1 + \delta)}
\]

where \( \delta \), the solvation factor, was assumed to be 0.2 g of solvent/g of protein.
binding reaction is in agreement with the known specificity of WGL (12) and indicates that the glucagon receptor contains GlcNAc and/or sialic acid residues.

Assay for the Solubilized Receptor

Since the receptor is a glycoprotein, we reasoned that if we could solubilize the receptor in a state that still retained hormone-binding capability, we should be able to separate the receptor-bound [125I]MIG from free [125I]MIG by adsorption onto WGL-Sepharose. This method for the separation of bound label from free label would then be based on an affinity chromatography of the receptor and would lend specificity to the separation procedure. Such specificity could be particularly valuable since standard methods based on size such as gel filtration yield unacceptably high backgrounds with labeled glucagon due to aggregation of glucagon. Precipitation with polyethylene glycol also yields high backgrounds. Consequently, no specific binding of the labeled glucagon to solubilized receptor can be detected in a routine manner using these assays.

We made extracts of liver membranes using a variety of detergents (Fig. 9). The extracts were incubated with 0.2 nM [125I]MIG in the absence and presence of 3 μM unlabeled glucagon for 3 h on ice. After the binding reaction, the mixture was exposed to WGL-Sepharose for 2 h to adsorb the hormone-receptor complex. The gel was then washed and counted. Using this procedure, we found significant differences between the [125I]MIG bound to the gel in the absence versus that bound in the presence of excess unlabeled glucagon. The difference varied with the detergent extract used and was the greatest when CHAPS extracts were used. Even though the difference in [125I]MIG bound to the gel was due to the addition of unlabeled glucagon, this observation alone is not sufficient to conclude that the difference in [125I]MIG bound in the absence and presence of unlabeled glucagon is due to the receptor. In order to establish that the binding observed in the CHAPS extract does indeed represent [125I]MIG interaction with the solubilized glucagon receptor, we tested 1) if the binding could be inhibited by excess unlabeled glucagon but not by other peptide hormones when added during the hormone-binding reaction and 2) if the binding could be abolished by the inclusion of 0.1 M GlcNAc along with WGL-Sepharose. In the experiment shown in Fig. 10, we exposed the CHAPS extract to [125I]MIG without any other hormones and in the presence of glucagon, insulin, ACTH, and vasopressin. After incubation for hormone binding, the reaction mixtures were exposed to WGL-Sepharose in the presence and absence of 0.1 M GlcNAc. Of all the hormones tested, only glucagon was able to abolish [125I]MIG adsorption to WGL-Sepharose in the absence of 0.1 M GlcNAc. Inclusion of GlcNAc during exposure to WGL-Sepharose reduced binding in all cases to the level observed in the presence of unlabeled glucagon. Thus, the nonspecific component of the total binding to the gel was the same, irrespective of whether we blocked [125I]MIG interaction with the receptor or adsorption of the [125I]MIG-receptor complex to the gel. The sensitivity of the [125I]MIG binding to WGL-

![Fig. 9. Use of various detergent extracts of rat liver membranes for the measurement of the [125I]MIG-binding activity, using the WGL-Sepharose adsorption assay.](image)

![Fig. 10. Effect of various peptide hormones on [125I]MIG binding to the solubilized receptor and the effect of GlcNAc during the adsorption to WGL-Sepharose.](image)
TABLE III

Lack of effect of GTP on \(^{125}\)I-MIG binding to CHAPS extract protein

| Additions during binding reaction | \(^{125}\)MIG adsorbed to WGL-Sepharose |
|----------------------------------|--------------------------------------|
| 100 \(\mu\)M GTP                | 6068 ± 225                           |
| 3 \(\mu\)M Glucagon              | 5856 ± 572                           |
| 100 \(\mu\)M GTP + 3 \(\mu\)M glucagen | 1601 ± 63                           |

Fig. 11. Effect of various concentrations of unlabeled glucagon on \(^{125}\)I-MIG binding to liver membranes (top) and CHAPS extract (bottom) in the absence (A, O) and presence (\(\Delta, \bullet\)) of 100 \(\mu\)M GTP. \(^{125}\)I-MIG binding to membranes and CHAPS extract protein was carried out in the presence of 25 mM NaHepes, 1 mM EDTA, 2 mM Mg\(_2\)Ac, a nucleoside triphosphate-regenerating system consisting of 20 mM creatine phosphate, 0.2 mg/ml of creatine phosphokinase, and 0.02 mg/ml of myokinase was present. 180 \(\mu\)g of CHAPS extract protein were used per assay tube. Values are mean ± S.D. of triplicate determinations.

TABLE IV

CHAPS extract was prepared as described under “Experimental Procedures” and heated at room temperature (22–24 °C) for 15 min to inactivate intrinsic adenylyl cyclase activity. The CHAPS extract was then diluted 3-fold with 55 mM NaHepes, pH 8.0. 200 \(\mu\)l of the diluted extract were mixed with 200 \(\mu\)l of cyc- membranes in 25 mM NaHepes, 1 mM EDTA. The final concentration of cyc- membrane protein was 2.2 mg/ml and that of CHAPS extract protein 0.67 mg/ml. The reconstituted mixture was held on ice for 20 min. Subsequently, 20 \(\mu\)l of the mixture were added to 30 \(\mu\)l of solution containing the other reagents required for the measurement of adenylyl cyclase and incubated for 30 min at 30 °C. Final concentration of Mg\(_2\)C\(_2\) was 10 mM, GTP and GTP\(_\gamma\)S, 10 \(\mu\)M, and that of isoproterenol (ISO), 50 \(\mu\)M. Values are mean ± S.D. of triplicate determination.

Binding Characteristics of the Solubilized Receptor

We characterized further the \(^{125}\)I-MIG-binding activity with respect to its guanine nucleotide sensitivity, since in the intact membrane, the glucagon receptor binds \(^{125}\)I-MIG in a GTP-sensitive manner (28). We found that the specific binding of \(^{125}\)I-MIG in solution did not display GTP sensitivity (Table III). We then compared the effect of varying concentrations of unlabeled glucagon on the extent of binding in the absence and presence of GTP. Using the CHAPS extract, it was found that glucagon competed with an IC\(_{50}\) of 56 nM in the presence and absence of GTP (Fig. 11, bottom). Using liver membranes, the IC\(_{50}\) of glucagon was 3.3 nM in the absence of GTP and 35 nM in the presence of GTP displaying the characteristic differential affinity induced by guanine nucleotides (Fig. 11, top). In three other experiments, the IC\(_{50}\) with which glucagon inhibited \(^{125}\)I-MIG binding to the solubilized receptor was 33–70 nM while the IC\(_{50}\) with which it inhibited \(^{125}\)I-MIG binding in the presence of GTP to the membrane-bound receptor was 35–80 nM. Thus, the solubilized receptor displays the low affinity GTP-insensitive binding of agonist.

We tested whether the lack of guanine nucleotide effect on the solubilized receptor was due to the lack of co-extraction of the stimulatory regulator of adenylyl cyclase. To this end, we tested for the presence of the stimulatory regulator in the CHAPS extract by assaying for reconstitution of cyclase S49 cell membrane adenylyl cyclase. As shown in Table IV, the glucagon receptor containing CHAPS extract also contains the stimulatory regulator of adenylyl cyclase.

DISCUSSION

Size and Subunit Structure of the Glucagon Receptor—Receptors that stimulate adenylyl cyclase bind hormone in a guanine nucleotide-sensitive manner. Current evidence indicates that this guanine nucleotide sensitivity to hormone binding is conferred by the stimulatory regulator of adenylyl cyclase (29). Interaction between the hormone-occupied re-
ceptor and the regulatory component results in facilitation of the Mg and guanine nucleotide-mediated activation (1). Thus, guanine nucleotide sensitivity of the binding reaction is related to the capacity of the hormone-occupied receptor to activate the stimulatory regulator of adenyl cyclase. Cross-linking of $^{[125]}$MIG to the $M_r = 63,000$ peptide was shown above to have the same guanine nucleotide sensitivity as $^{[125]}$MIG binding to receptors in membranes. This observation indicates that the labeled $M_r = 63,000$ peptide represents the structural correlate of the interaction of the $^{[125]}$MIG with the membrane-bound receptor. The inhibition of the labeling of the $M_r = 63,000$ peptide by low concentrations of glucagon (1-100 nM) and the lack of effect of other peptide hormones on this labeling indicate that the $M_r = 63,000$ peptide specifically recognizes glucagon. Taken together, all of these observations strongly suggest that the $M_r = 63,000$ peptide is the hormone-binding subunit of the glucagon receptor that interacts with the stimulatory regulator.

During hydrodynamic measurements, the receptor was monitored by the presence of the $M_r = 63,000$ peptide along the density gradient or gel filtration column fractions. It appears that in the presence of non-denaturing detergents, the $M_r = 63,000$ peptide behaves as part of a larger protein. Since the hydrodynamic measurements were performed in the presence of 0.5% Lubrol-PX, it does not seem likely that the larger size is due to nonspecific aggregation of hydrophobic protein. It also does not appear that the larger size is due to a receptor-detergent complex since no significant differences in the $S$ values were measured using $H_2O$ and $D_2O$ as solvents during centrifugation. The lack of difference in $S$ values indicates that the partial specific volume of the glucagon receptor is the same as that of the globular proteins used as standards and suggests that there is no significant binding of detergent to the protein; if the protein bound significant amounts of Lubrol-PX, the partial specific volume of the protein-detergent complex would be greater than 0.74, since the partial specific volume of Lubrol is 0.956 ml/g (27). This would have resulted in different $S$ values in $H_2O$ and $D_2O$. The lack of difference in measured $S$ values using $H_2O$ and $D_2O$ as solvents was rather unexpected. It is also possible that since the glucagon receptor is a glycoprotein, the partial specific volume of the receptor is lower than that of globular protein and the observed lack of shift in $H_2O$ versus $D_2O$ is because the receptor-detergent complex coincidentally has a partial specific volume of 0.74. This appears unlikely since we can also observe the high molecular weight form in the membrane by direct SDS-gel electrophoretic analysis. There appears to be no $a$ priori way of predicting whether a certain membrane protein, after solubilization, will bind significant amounts of detergent. Glycoprotein IIb and III of platelets which are known to be integral transmembrane proteins show no differences in $S$ values between $H_2O$ and $D_2O$ in the presence of Triton X-100 and their calculated molecular weights from hydrodynamic parameters agree with the observed molecular weights on SDS gels (30).

The data indicate that the $M_r = 63,000$ peptide is a part of a larger protein ($M_r = 110,000-120,000$) both in detergent solution and in the membranes. The identification of a high molecular weight form in the membranes indicates that the observed size of the receptor in detergent solution is not an artifact of solubilization. Furthermore, the size of the glucagon receptor determined by hydrodynamic measurements is similar to the molecular weight obtained for the functional receptor by target size analysis (31).

To observe the presence of the high molecular weight species of the receptor in the membranes after solubilization with SDS, it was essential that membranes be incubated at 32 °C prior to the addition of $^{[125]}$MIG. This would suggest that specific aggregation of the receptor occurs in the vacant state and that addition of $^{[125]}$MIG allows dissociation of the aggregates as suggested by Rodbell (32). Alternatively, incubation in the absence of $^{[125]}$MIG may permit the receptor to orient itself such that it makes available reactive groups to which HSAB can attach to cross-link the subunits of the receptor. Our data do not allow us to distinguish between these two alternate explanations.

The identity of the second subunit of the receptor protein merits comment. Since we are measuring the size of the agonist-occupied receptor, it may be suggested that a portion of the stimulatory regulator could account for the higher molecular weight. The experimental procedures we use preclude this possibility. After covalent attachment of $^{[125]}$MIG to receptor, we treat the membranes with GTP and excess unlabeled glucagon. Treatment with guanine nucleotide dissociates the receptor-regulator complex. Further, when guanine nucleotides are not included in the gradient, the stimulatory regulator displays a $s_{20,w}$ of 5.2, while the receptor has a $s_{20,w}$ of 4.3. The position of the receptor is unaffected by inclusion of guanine nucleotides during centrifugation. If the second subunit is not part of the stimulatory regulator, then the possibility exists that it is a subunit dissimilar from the hormone-binding subunit. While this possibility cannot be conclusively precluded for the glucagon receptor, it appears unlikely. Recent studies on the pure &beta-adrenergic receptors have shown that the hormone-binding subunit is sufficient to confer hormone responsiveness to the system, indicating that there are no additional components that intervene between the receptor and the stimulatory regulator (35, 34). At the present time, there is no reason why this situation should not be applicable to the glucagon receptor as well. Another possibility is that the native protein is a dimer of the hormone-binding subunit. In view of the size of the low ($M_r = 63,000$) and high ($M_r = 110,000-120,000$) molecular weight form, this appears to be a reasonable explanation. Definitive proof to support this contention must await purification of the glucagon receptor.

**Solubilization and Assay for Functional Glucagon Receptor in Solution**—The results show that specific $^{[125]}$MIG-binding activity has been solubilized by exposure of rat liver membranes to CHAPS. As with other systems, the choice of detergent appears to be crucial for the successful solubilization of the active receptor since significant binding activity could only be obtained when CHAPS was used. Repeated attempts to use Lubrol-PX or cholate during solubilization have not yielded solubilized receptors that retain hormone-binding activity. The binding activity does not sediment when subjected to centrifugation at 220,000 x g for 2 h and freely passes through a 0.22-μ filter, indicating that it is truly soluble. The binding activity in solution displays characteristics of the glucagon receptor. These are: 1) specificity of competition by peptide hormones; 2) similar rates of glucagon binding; and 3) similar affinities for glucagon as observed in the membrane in the presence of GTP.

The method described in this article for the measurement of $^{[125]}$MIG binding to the receptor represents a new and novel approach. It is based on the observation that the receptor is a glycoprotein, as demonstrated by the GlcNAc-sensitive adsorption of the receptor to WGL-Sepharose. We have utilized this structural feature of the receptor to develop an assay for receptor function (i.e. hormone binding), demonstrating the utility of structural studies on covalently labeled trace proteins. In the assay described here, we use the WGL-
Sepharose as an insoluble matrix onto which the hormone-receptor complex is adsorbed under conditions where the adsorption of the free hormone is minimal. This allows for the removal of the free hormone in the supernatant after a low speed centrifugation. A different effect of lectin has been observed in another receptor system. It has been reported that concanavalin A stabilizes the conformation of epidermal growth factor receptor in detergent solution such that hormone binding is facilitated (35). No such effect of WGL was observed on the glucagon receptor. Addition of WGL during the binding reaction did not allow us to observe specific binding of $[^{125}]$MIG to the receptor when polyethylene glycol precipitation technique was used to separate the free ligand from the bound. Thus, WGL does not appear to promote $[^{125}]$MIG interaction with the CHAPS-solubilized receptor.

While this study has specifically focused on optimizing conditions for the measurement of the glucagon receptor, the general principle used for the measurement of hormone binding to the solubilized receptor should be useful for other receptors and glycoproteins as well. The procedure will work if the appropriate lectin-Sepharose is used to absorb the glycoprotein of choice. The simplicity of this method compares favorably with that of the polyethylene glycol precipitation technique which indeed is not always usable. In the case of the glucagon receptor, the availability of a simple and reproducible binding assay after solubilization represents a major step toward the purification of the receptor.

**Binding Characteristics of the Solubilized Glucagon Receptor**—It is noteworthy that the glucagon receptor in solution displays low affinity guanine nucleotide-insensitive binding of $[^{125}]$MIG. Studies on the $\beta$-adrenergic system had shown that solubilization of the unoccupied receptor results in a form that binds agonists with low affinity unaffected by guanine nucleotides (36). Genetic manipulations on the S49 lymphoma cell system have shown that such low affinity binding represents binding to receptor unaffected by a functional stimulatory regulator of adenyl cyclase (15, 37). This was substantiated by Shorr et al. who showed that the purified frog erythrocyte $\beta$-adrenergic receptor binds agonists with low affinity in a guanine nucleotide-insensitive manner (38). Thus, by a number of criteria, low affinity nucleotide-insensitive agonist binding is a characteristic of the free receptor. The studies presented in this article demonstrate that the solubilized glucagon receptor also binds hormone with low affinity in a guanine nucleotide-insensitive manner, indicating that it is a free receptor. If follows, therefore, that when agonist probes such as $[^{125}]$MIG are used to study receptor behavior in intact membranes in the absence of guanine nucleotides, the data obtained represent the behavior of the receptor-stimulatory regulator complex and not that of the receptor itself. The functional characterization of the soluble receptor as "free" agrees with the structural studies which also indicate that the $[^{125}]$MIG receptor in detergent solution is not the receptor-stimulatory regulator complex.

The lack of guanine nucleotide effect in solution is not due to the functional absence of the stimulatory regulator in the CHAPS solution, since it can reconstitute guanine nucleotide, and, more importantly, hormonal sensitivity to cAMP S49 cell membrane adenyl cyclase. It appears that reconstitution into a lipid environment may be necessary for such receptor-stimulatory regulator interactions to occur, as has been found to be the case for the $\beta$-adrenergic receptor.

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**REFERENCES**

1. Iyengar, R., and Birnbaumer, L. (1980) Proc. Natl. Acad. Sci. U.S.A. 79, 5179-5183
2. Iyengar, R. (1981) J. Biol. Chem. 256, 11042-11050
3. Northup, J. K., Sternweis, P. C., Smigel, M. D., Schleifer, L. S., Ross, E. M., and Gilman, A. C. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 6516-6520
4. Sternweis, P. C., Northup, J. K., Smigel, M. D., and Gilman, A. G. (1981) J. Biol. Chem. 256, 11517-11526
5. Northup, J. K., Smigel, M. D., Sternweis, P. C., and Gilman, A. G. (1983) J. Biol. Chem. 258, 11418-11423
6. Johnson, G. L., MacAndrew, V. L., and Pick, P. F. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 8705-8710
7. Iyengar, R. (1984) J. Biol. Chem. 259, 5222-5229
8. Brandt, D., Minza, P. D., Swartz, T. L., and Birnbaumer, L. (1980) J. Biol. Chem. 255, 11875-11882
9. Iyengar, R., Abramowitz, J., Bordelon-Riser, M., Blume, A. J., and Birnbaumer, L. (1980) J. Biol. Chem. 255, 10312-10321
10. Walbath, T. F., and Johnson, R. A. (1979) Bioclini. Biochim. Acta 562, 11-31
11. Rojas, F. J., Swartz, T. L., Iyengar, R., Garber, A. J., and Birnbaumer, L. (1983) Endocrinology 113, 711-719
12. Adair, L. L., and Kornfeld, S. (1974) J. Biol. Chem. 249, 4696-4701
13. Parikh, I., March, S., and Cuatrecasas, P. (1974) Methods Enzymol. 44B, 77-102
14. Neville, D. M., Jr. (1969) Biochim. Biophys. Acta 154, 540-552
15. Pohl, S. L., Birnbaumer, L., and Rodbell, M. (1971) J. Biol. Chem. 246, 1849-1856
16. Ross, E. M., Maguire, M. E., Sturgill, T. W., Biltonen, R. L., and Gilman, A. G. (1977) J. Biol. Chem. 252, 5761-5775
17. Iyengar, R., Bhat, M. R., Raser, M. E., and Birnbaumer, L. (1981) J. Biol. Chem. 256, 4839-4845
18. Laemmli, U. K. (1970) Nature ( Lond.) 227, 680-685
19. Salomon, Y., London, C., and Rodbell, M. (1974) Anal. Biochem. 58, 541-548
20. Bockaert, J., Huntecker-Dunn, M., and Birnbaumer, L. (1976) J. Biol. Chem. 251, 2923-2926
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
22. Iyengar, R., and Birnbaumer, L. (1980) J. Biol. Chem. 255, 3558-3564
23. Welton, A. L., Lad, P. M., Newby, A. G., Yamanaka, H., Niclos, S., and Rodbell, M. (1977) J. Biol. Chem. 252, 5947-5950
24. Bhat, M. K., Iyengar, R., Abramowitz, J., Bordelon-Riser, M. E., and Birnbaumer, L. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 3836-3840
25. Clarke, S. (1975) J. Biol. Chem. 250, 5459-5469
26. Neer, E. J. (1974) J. Biol. Chem. 249, 6257-6261
27. Phillips, D. R., and Agin, P. P. (1977) J. Biol. Chem. 252, 2121-2126
28. Rodbell, M., Krans, H. M. J., Pohl, S. L., and Birnbaumer, L. (1971) J. Biol. Chem. 246, 1871-1876
29. Ross, E. M., Howlett, A. C., Ferguson, K. M., and Gilman, A. G. (1978) J. Biol. Chem. 253, 6401-6412
30. Jennings, L. K., and Phillips, D. R. (1982) J. Biol. Chem. 257, 10458-10460
31. Schlegel, W., Kempen, E. S., and Rodbell, M. (1979) J. Biol. Chem. 254, 5168-5176
32. Rodbell, M. (1980) Nature (Lond) 284, 17-21
33. Brandt, D. R., Asano, T., Pedersen, S. E., and Ross, E. M. (1983) Biochem. Int. 22, 457-467
34. Cerione, R. A., Strulovicz, B., Benovic, J. L., Lifkowitz, R. J., and Caron, M. G. (1983) Nature (Lond) 304, 562-565
35. Kempner, E. S., Conolly, D. T., and Rodbell, M. (1982) J. Biol. Chem. 257, 11469-11466
36. Caron, M. G., and Lifkowitz, R. J. (1976) J. Biol. Chem. 251, 2374-2384
37. Haga, T., Ross, E. M., Anderson, H. J., and Gilman, A. G. (1977) J. Biol. Chem. 252, 5761-5775
38. Shore, R. G., Lifkowitz, R. J., and Caron, M. G. (1981) J. Biol. Chem. 256, 5820-5826