The bombesin/gastrin-releasing peptide (GRP) receptor was solubilized from Swiss mouse 3T3 cell membranes in an active form and was purified about 90,000 fold to near homogeneity by a combination of wheat germ agglutinin-agarose and ligand affinity chromatography. The purified receptor displayed a single diffuse band with a $M_\text{r}$ of 75,000–100,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After treatment of the receptor with $N$-glycanase, removing $N$-linked oligosaccharide moieties, the receptor with a $M_\text{r}$ of 38,000 band. These results agree with the $M_\text{r}$, value estimated for the GRP receptor that was labeled on Swiss 3T3 cells by cross-linking to $\text{^{125}I-GRP}_{1-27}$. GRP$_{1-27}$ bound to the purified receptor with a $K_\text{d}$ of 0.038 ± 0.019 nM. By comparison, the soluble receptor in unfractionated extracts and intact membranes displayed a $K_\text{d}$ for GRP$_{1-27}$ of 0.036 ± 0.003 nM and 0.13 ± 0.04 nM, respectively. The relative potencies of a series of GRP analogs for the soluble receptor and intact membranes indicated that the extraction procedure did not significantly alter the receptor’s ligand binding specificity. However, coupling of the receptor to its guanyl nucleotide regulatory protein was not maintained in the soluble extract, and a G-protein did not co-purify with the receptor. Physiological concentrations of NaCl greatly inhibited the binding of some GRP analogs to the receptor, while the binding of other analogs was not affected. A domain on the GRP molecule involving Lys-13 or Arg-17 was identified which promoted binding to the GRP receptor under conditions of low ionic strength. These findings aided the development of an effective ligand affinity resin for the purification of the GRP receptor.

Gastrin-releasing peptide (GRP) is a member of a family of peptides found in mammals, including neuromedin B, that is homologous to the amphibian peptide bombesin. Although the GRP molecule contains 27 amino acids, the biologically active region of the molecule has been localized to the C-terminal 8 amino acid residues. GRP has been found to elicit a wide range of biological responses such as the stimulation of gastrointestinal hormone release, induction of smooth muscle contraction, and, when administered to the central nervous system, alteration of homeostasis and thermoregulation. Furthermore, GRP has been shown to be a potent mitogen for human small cell lung cancer cells (SCLC) (11, 12), and bronchial epithelial cells (13). Potentiation of the growth of some SCLC cell lines by GRP has been found to occur in an autocrine manner (12). The possibility of controlling the growth of SCLC by GRP has stimulated the development of potent antagonists of the cell surface GRP receptor (14, 15).

Radiolabeled GRP binds to a number of target tissues such as a clonal line of pituitary cells (16), pancreatic acinar cells (17), central nervous system cells (18), and Swiss 3T3 cells (19). In addition, neuromedin B receptors in rat esophageal muscle have been characterized which also bind GRP and may be highly homologous to the GRP receptor (19). The GRP receptor and its signal transduction mechanism have been studied most extensively in Swiss 3T3 fibroblasts. GRP binding to Swiss 3T3 cells promotes the breakdown of inositol phospholipids, resulting in the release of Ca$^{2+}$ from intracellular stores, the activation of protein kinase C, and the induction of cellular oncogenes fos and c-myc (20-25). The synergistic effect of forskolin and bombesin on cAMP levels, and the stimulation of cell division by GRP, suggest that several signal transduction pathways may be involved in eliciting these complex cellular responses (25, 26). Experiments cross-linking $\text{^{125}I-GRP}_{1-27}$ to high affinity binding sites on Swiss 3T3 cells indicate that the GRP receptor is a glycoprotein ($M_\text{r}$ = 75,000-85,000) that has a protein core with a $M_\text{r}$ of 45,000 (27) or 43,000 (28, 29).

Fischer and Schonbrunn have shown that the ligand affinity of the GRP receptor is reduced by guanyl nucleotides in membranes from GH$_3$, pituitary cells and, to a lesser extent, from HIT islet cells and Swiss 3T3 fibroblast cells (30). Such regulation is considered a hallmark of the coupling of a receptor to guanyl nucleotide-binding regulatory proteins (G-proteins) (31, 32). In addition, the GDP analog GDP$\beta$S was shown to block the stimulation of protein kinase C activity by bombesin in permeabilized Swiss 3T3 cells (33). These results suggest that enzymes which hydrolyze inositol phospholipids can be regulated by G-proteins coupled to the GRP receptor in a manner that is analogous to the coupling of adenylate cyclase to receptors via G-proteins, which has been characterized in detail (31, 32).

To identify the components of the GRP receptor system and study their structure and function, it is necessary to purify the receptor. In this paper, we report the solubilization
of the GRP receptor from Swiss 3T3 cell membranes in a form that retains high affinity GRP binding activity and its purification to homogeneity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Gastrin-releasing peptides GRP1-27, GRP14-27, GRP18-27, N-acetyl-GRP20-27, and GRP1-16, physalaemin, substance P, and [d-Arg-d-Pro-d-Typ-Leu] substance P agonist were purchased from Peninsula Laboratories (Belmont, CA), GRP19-27, [Nle'4,27]GRP13-27-OH, [Nle'4,27]GRP13-27, and GRP1-27 (0.02 nM) and was terminated after 60 min at 37°C by the addition of 10 μl of ice-cold 50 nM HEPES, pH 7.5, 7 mM MgCl₂, 2 mM EDTA, 10 mg/ml bovine serum albumin, and 30 μg/ml bacitracin in a final volume of 250 μl. The binding reaction was initiated by adding [125I]-GRP1-27 (20 pmol) and was terminated after 60 min at 37°C by the addition of 10 μl of ice-cold 50 nM HEPES, pH 7.5, 7 mM MgCl₂, 2 mM EDTA, 10 mg/ml bovine serum albumin, and 30 μg/ml bacitracin. The membranes were then pelleted at 39,000 g for 10 min at 4°C. The supernatants were aspirated, and the radioactivity in the pellets was determined. For the other experiments presented in this paper, the binding reactions were conducted as described above, with the exception that the ice container used was not available. The final volume was 500 μl. The reactions were terminated by cooling the reaction mixtures on ice and filtering them through P Entreated Whatman GF/F glass fiber filters. The filters were rapidly washed four times with 4 ml of ice-cold 50 mM Tris-HCl, pH 7.5, and counted for radioactivity. Using either assay method described above, nonspecific binding was determined by addition of 100 nM unlabeled GRP1-27 and was about 1-2% of the total [125I]-GRP1-27 added.

**125I-GRP1-27 Binding to Soluble Membrane Extracts—Specific [125I]-GRP1-27 binding to detergent-solubilized receptors was assayed in 50 mM HEPES, pH 7.5, 2 mM EDTA, 10 mg/ml bovine serum albumin, 30 μg/ml bacitracin, 20 μM [125I]-GRP1-27, and a concentration of CHAPS and CHS of 0.075% and 0.0075%, respectively, unless indicated otherwise in the figure legends. After incubation at 15°C for 30 min, samples were cooled to 0°C, and bound ligand was recovered by rapid filtration through P Entreated Whatman GF/F filters, followed by four washes with 4 ml of ice-cold 50 mM Tris-HCl, pH 7.5. Finally, radioactivity on the filters was determined. Nonspecific binding was determined by inclusion of 100 nM unlabeled GRP1-27 and was typically 1.5-2% of the total [125I]-GRP1-27 added.**

**Cross-linking of [125I]-GRP1-27 to GRP Receptors on Swiss 3T3 Cells and in Soluble Extracts**—To affinity label the soluble GRP receptor, soluble membrane protein (40 μg) was incubated for 30 min at 15°C in 50 mM HEPES, pH 7.5, 2 mM EDTA, 0.075% CHAPS, 0.015% CHS, 30 μg/ml bacitracin, and 0.2 nM [125I]-GRP in a volume of 500 μl. The binding reaction was then cooled to 0°C, and the bifunctional cross-linking reagent Bis(sulfosuccinimidyl)suberate was added to yield a final concentration of 3 mM. Cross-linking was quenched after 15 min by addition of 0.10 ml of 1.0 M Tris-HCl, pH 7.5. After another 10 min, 0.1 ml of trichloroacetic acid (100%) was added, and the solution was further incubated at 0°C for 30 min. Precipitated material was collected by centrifugation, washed with 95% ethanol, dried, and heated at 95°C for 5 min in SDA PAGE sample buffer (60 mM Tris/HCl, pH 6.8, 10% w/v glycerol, 2% w/v SDS, 0.7 M β-mercaptoethanol).

The [125I]-GRP was cross-linked to its receptor on intact Swiss 3T3 cells essentially as described by Kris et al. (27), except that the [125I]-GRP was bound to the cells at 15°C, and the resin container used was not available. The final volume was 500 μl. The reactions were terminated by cooling the reaction mixtures on ice and filtering them through P E Entreated Whatman GF/F glass fiber filters. The filters were rapidly washed four times with 4 ml of ice-cold 50 mM Tris-HCl, pH 7.5, and counted for radioactivity. Using either assay method described above, nonspecific binding was determined by addition of 100 nM unlabeled GRP1-27 and was about 1-2% of the total [125I]-GRP1-27 added.**

**Preparation of [125I]-GRP1-27—Agarose Resin**—Ten ml of Actigel Superflow (Sterogen, San Gabriel, CA), a beaded agarose matrix (30-100 μm) containing aldehyde moieties at the end of five atom hydrophilic spacers, was washed with 5 volumes of 100 mM NaPO₄, pH 7.0, and then incubated with 10 ml of 100 mM NaCNBH₃, pH 7.0, containing 2 mg/ml [Ni(EDTA)GRP1-27 for 2 h. The resin was then washed three times with 10 ml of 50 mM Tris-HCl, pH 7.0, followed by alternating washes with a low pH buffer (100 mM NaAc, pH 4.0, 0.5 mM NaCl) and a high pH buffer (100 mM Tris, pH 8.0, 0.5 mM NaCl). Over 95% of the peptide was coupled to the resin. The resin was stored in 100 mM NaPO₄, pH 7.0, and 0.04% sodium azide at 4°C.

**Desalting of the GRP Receptor**—Crude membranes were prepared from 2 x 10⁶ cells cultured in 200 roller bottles (1300 ml) as described above. Before extracting the GRP receptor, the membranes were washed twice in high salt buffer (50 mM HEPES, pH 7.5, 2 mM EDTA, 1.0 M NaCl, 50 μg/ml leupeptin, 2.5 μg/ml pepstatin, 10 μg/ml aprotinin, and 0.5 mM PMSF), washed once in the same buffer,
but without NaCl, and finally suspended at 7 mg/ml in 50 mM HEPES, pH 7.5, 2 mM EDTA, 1 mM EGTA, 100 mM NaCl, 30 μg/ml bacitracin, 25 μg/ml leupeptin, 10 μg/ml aprotinin, 2.5 mM β-mercaptoethanol, and 0.5 mM PMSE. This procedure reduced the protein content of the membrane preparation by about 50% with loss of GRP receptor binding activity. The membranes were solubilized by slowly adding a mixture of CHAPS and CHS, in a 10:1 ratio, to the membranes until the final detergent concentration was 0.75% CHAPS and 0.075% CHS. After incubating the mixture for 30 min at 91 °C, insoluble material was removed by centrifugation at 100,000 x g for 60 min.

The soluble extract was then fractionated by polyacrylamide gel electrophoresis (PAGE, 8% gel in 7 M urea). It was loaded onto the column at 0.03 ml/min, and the column was washed with about 10 column volumes of buffer and eluted with column buffer + 5 mM EDTA, 0.25% CHAPS, and 0.025% CHS. Therefore, the results reflected the number of detergent concentrations that extracted 40% of the total membrane protein. Solubilization of membranes with higher

The GRP receptor was further purified by ligand affinity chromatography. A column (1.5 x 9 cm) containing wheat germ agglutinin (WGA)-agarose resin (3-5% of lectin/ml of wet gel) (E-Y Laboratories, San Mateo, CA) was equilibrated with 50 mM HEPES, pH 7.5, 2 mM EDTA, 0.075% CHAPS, 0.0075% CHS, 5 μg/ml leupeptin, and 10 μg/ml bacitracin at 4 °C. The soluble extract was diluted with 1 volume of column buffer, and the final detergent concentration was adjusted to 0.25% CHAPS and 0.0075% CHS. The sample was applied to the lectin column at a flow rate of 1.5 ml/min. The column was washed with about 10 column volumes of buffer and eluted with column buffer + 5 mM N,N',N'-triacetylchitotriose. Fractions containing GRP receptor binding activity were pooled and diluted with 2.3 volumes of 25 mM HEPES, 25 mM Tris, pH 7.5, 2 mM EDTA, 5 μg/ml leupeptin, and 10 μg/ml bacitracin, reducing the detergent concentration to 0.075% CHAPS and 0.0075% CHS.

The GRP receptor was further purified by ligand affinity chromatography. [Nle4,27]GRP13-27-agarose, prepared as described above, was poured into a column (1.5 x 5 cm) and equilibrated with 25 mM Tris, 25 mM HEPES, pH 7.5, 2 mM EDTA, 0.075% CHAPS, 0.0075% CHS, 5 μg/ml leupeptin, and 10 μg/ml bacitracin at 4 °C. The WGA-agarose-purified extract was loaded onto the GRP affinity column at a 0.1 ml/min, and the column was washed with about 20 volumes of the equilibration buffer. The bound receptor was eluted from the column with equilibration buffer + 0.5 mM NaCl at a flow rate of 0.2 ml/min. Fractions containing [125I]-GRP binding activity were pooled (10-15 ml) and concentrated to about 1 ml by ultrafiltration using a Centricon-10 device (Amicon, Danvers, MA). To desalt the sample, it was diluted with 15 volumes of affinity column equilibration buffer and concentrated again to a volume of 1 ml. This step was repeated, and the resulting 1-ml sample was diluted to 5 ml with affinity column equilibration buffer.

The protein was then solubilized by ligand affinity chromatography, the receptor was chromatographed on a second [Nle4,27]GRP13-27-agarose column (1.0 x 3 cm), prepared as described above. The sample was loaded onto the column at 0.05 ml/min, and the column was washed with 20 column volumes of equilibration buffer. Bound receptor was eluted from the column with equilibration buffer + 0.5 mM NaCl at a flow rate of 0.1 ml/min. Fractions containing GRP receptor binding activity were pooled (about 6 ml) and concentrated to about 1 ml using a Centricon-10 ultrafiltration device (Amicon). The sample was then diluted to 3 ml using a Centricon-10.

Following ligand affinity chromatography, the GRP receptor preparation was chromatographed on a Superose-6 HR 10/30 column (Pharmacia LKB Biotechnology Inc.). The column was equilibrated with 20 mM HEPES, pH 7.5, 2 mM EDTA, 0.075% CHAPS, 0.0075% CHS, and 100 mM NaCl. The receptor was chromatographed at 0.4 ml/min, and fractions containing the receptor were determined by assays of [125I]-GRP binding activity.

Fractionation of the Purified GRP Receptor—IODO-GEN (Pierce) was dissolved in dichloroethane at a concentration of 1 mg/ml. The solution was then concentrated to about 6 ml using a Centrisep-10 device (Amicon), followed by concentration to 0.3 ml using a Centricon-10. Following ligand affinity chromatography, the GRP receptor preparation was chromatographed on a Superose-6 HR 10/30 column (Pharmacia LKB Biotechnology Inc.). The column was equilibrated with 20 mM HEPES, pH 7.5, 2 mM EDTA, 1 mM EGTA, 100 mM NaCl, 30 μg/ml bacitracin, 25 μg/ml leupeptin, 10 μg/ml aprotinin, 2.5 mM β-mercaptoethanol, and 0.5 mM PMSE. This procedure reduced the protein content of the membrane preparation by about 50% with loss of GRP receptor binding activity. The membranes were solubilized by slowly adding a mixture of CHAPS and CHS, in a 10:1 ratio, to the membranes until the final detergent concentration was 0.75% CHAPS and 0.075% CHS. After incubating the mixture for 30 min at 91 °C, insoluble material was removed by centrifugation at 100,000 x g for 60 min.

The soluble extract was then fractionated by polyacrylamide gel electrophoresis (PAGE, 8% gel in 7 M urea). It was loaded onto the column at 0.03 ml/min, and the column was washed with about 10 column volumes of buffer and eluted with column buffer + 5 mM EDTA, 0.25% CHAPS, and 0.025% CHS. Therefore, the results reflected the number of detergent concentrations that extracted 40% of the total membrane protein. Solubilization of membranes with higher detergent concentrations was normalized to 0.1% CHAPS and 0.02% CHS, and the final detergent concentration was kept at one-fifth level of CHAPS. The protein content of the solubilized receptor was achieved at 0.75% CHAPS, a concentration of detergent that extracted 40% of the total membrane protein. Solubilization of membranes with higher

RESULTS

Solubilization of the GRP Receptor—A mixture of the zwitertilic detergent CHAPS and the cholesterol ester, cholesteryl hemisuccinate, was found to solubilize [125I]-GRP1-27 binding activity from Swiss 3T3 membranes (Fig. 1). Extracts were prepared by incubating membranes with various concentrations of detergent followed by centrifugation at 100,000 x g for 60 min to remove insoluble material. Before extracts were assayed for [125I]-GRP1-27 binding activity, the concentration of detergent was normalized to 0.1% CHAPS and 0.02% CHS. Therefore, the results reflected the number of receptor molecules extracted and not the effect of detergent on the ligand binding properties of the receptor. The best yield of solubilized receptor was achieved at 0.75% CHAPS, a concentration of detergent that extracted 40% of the total membrane protein. Solubilization of membranes with higher detergent concentrations was normalized to 0.1% CHAPS and 0.02% CHS, and the final detergent concentration was kept at one-fifth level of CHAPS. The protein content of the solubilized receptor was achieved at 0.75% CHAPS, a concentration that extracted 40% of the total membrane protein. Solubilization of membranes with higher detergent concentrations was normalized to 0.1% CHAPS and 0.02% CHS, and the final detergent concentration was kept at one-fifth level of CHAPS.
concentrations of detergent yielded extracts that exhibited less ligand binding activity, possibly due to the irreversible inactivation of the receptor.

No detectable specific $^{125}\text{I-GRP1-27}$ binding activity was observed in the complete absence of CHS. However, receptors solubilized without CHS regained $^{125}\text{I-GRP1-27}$ binding activity when assayed in a medium that contained the cholesterol ester. The amount of binding activity seen in this case was about 30% of the level observed in control experiments where CHS was present during the solubilization step (data not shown). These results indicate that CHS can promote a change in the receptor structure which enables it to bind GRP with high affinity. It may also play a limited role in protecting the receptor from irreversible denaturation by CHAPS. Other detergents, including Triton X-100, Nonidet P-40, and digitonin were not able to solubilize $^{125}\text{I-GRP1-27}$ binding activity from Swiss 3T3 membranes. However, the possibility that active GRP receptors could be extracted from membranes by a combination of CHS with these detergents was not addressed.

The concentration dependence of CHAPS on $^{125}\text{I-GRP1-27}$ binding to the GRP receptor is shown in Fig. 2. CHS was present in the binding medium at a level equivalent to one-fifth of the concentration of CHAPS. The specific $^{125}\text{I-GRP1-27}$ binding activity of the soluble GRP receptor was maximal at 0.075 to 0.15% CHAPS and fell to negligible levels at concentrations of CHAPS above 0.4%. As also evident from Fig. 2, the level of nonspecifically bound $^{125}\text{I-GRP1-27}$ increased significantly at concentrations of CHAPS greater than about 0.2%, possibly due to the formation of detergent aggregates that trapped $^{125}\text{I-GRP1-27}$ on PEI-treated filters used to separate the receptor-ligand complexes from unbound ligand. The inhibition of $^{125}\text{I-GRP1-27}$ binding caused by CHAPS was completely reversed by reducing the concentration of detergent by either dilution or dialysis. The effect of various concentrations of CHS on the binding activity of the soluble receptor at a constant CHAPS concentration (0.075%) was also investigated. Maximal binding activity was observed at a CHAPS to CHS ratio of 10 to 1 (data not shown).

**Ligand Specificity of the GRP Receptor in Soluble Extracts and Intact Membranes**—The ligand specificity of the soluble GRP receptor was investigated by analyzing the ability of various peptides and GRP analogs to compete with $^{125}\text{I-GRP}$ binding (Table I). GRP1-16, substance P antagonist, and physalaemin did not compete with $^{125}\text{I-GRP1-27}$ binding to the soluble receptor at concentrations less than 1 nM. [Nle$^{14,27}$]GRP13-27, an analog of GRP13-27 that is more stable to oxidation, inhibited the binding of $^{125}\text{I-GRP1-27}$ to the soluble receptor with an $IC_{50}$ of 0.10 nM. The affinity of this analog for the soluble receptor was very similar to that of GRP1-27 ($IC_{50} = 0.13$ nM). In contrast, [Nle$^{14,27}$,Leu$^{15}$]GRP14-27, which does not contain the lysine 13, and has leucine substituted for arginine 17, displayed an $IC_{50}$ of 10 nM, which was about 2 orders of magnitude higher than that exhibited by [Nle$^{14,27}$]GRP13-27. The results indicate that lysine 13 and/or arginine 17 provides determinants on the GRP molecule that are important for high affinity receptor binding under the conditions examined. Consistent with this finding, N-acetyl-GRP20-27 ($IC_{50} = 20$ nM) also bound to the receptor with a much lower affinity than [Nle$^{14,27}$]GRP13-27. Bombesin and [Lys$^{3}$]bombesin exhibited intermediate $IC_{50}$ values of 1.3 nM and 3.8 nM, respectively, while GRP18-27 (neuromedin C) displayed an $IC_{50}$ of 10 nM.

The ligand specificity of the GRP receptor in isolated membranes was also examined using conditions that were comparable to those employed above (Table I). The relative affinities of each of the peptides tested for the soluble and the membrane-bound forms of the GRP receptor were similar, indicating that the binding specificity of the receptor was not significantly altered by the solubilization procedure.

**Inhibition of GRP Receptor Binding by NaCl**—Binding of $^{125}\text{I-GRP1-27}$ to the soluble GRP receptor and intact Swiss 3T3 membranes was inhibited by NaCl with an $IC_{50}$ of 150 mM and 30 mM, respectively (Fig. 3). Other salts such as KCl and NaAc also inhibited the binding of $^{125}\text{I-GRP1-27}$ to membranes (data not shown). 150 mM NaCl increased the $K_d$ of membranes for GRP1-27 by a factor of about 40 (Table II) without altering the total number of binding sites present (data not shown). NaCl produced analogous effects on $^{125}\text{I-GRP1-27}$ binding to the receptor in intact Swiss 3T3 cells. However, the binding of N-acetyl-GRP20-27 to the receptor was unaffected by salt (Table II). Accordingly, GRP1-27 exhibited a 50-fold higher affinity than N-acetyl-GRP20-27 under low salt conditions, but had about the same affinity in the presence of 150 mM NaCl. The experiments discussed...
Inhibition constants of various peptides in the presence and absence of 150 mM NaCl

| Peptide     | Membranes | Soluble extract |
|-------------|-----------|-----------------|
|             | High salt | Low salt        | High salt | Low salt |
| GRP1-27     | 4.5 ± 1   | 0.12 ± 0.02     | 3.1 ± 2   | 0.26 ± 0.2 |
| N-Ac-GRP20-27| 5.7 ± 1   | 0.6 ± 1        | ND        | ND       |

Kᵢ (equilibrium inhibition constant) values were determined from competitive displacement of 125I-GRP binding to membranes or intact cells as described under "Experimental Procedures." Under high salt conditions, 150 mM NaCl was present in the assay medium.

Purification of the GRP Receptor—Out of a total of 10 independent receptor purifications performed, the data from a representative preparation are summarized in Table III. Crude membranes were washed with high salt buffer to remove extrinsic membrane proteins from the preparation and were solubilized with CHAPS and CHS. The extract was further fractionated by precipitation with PEG, giving a 2-fold purification of the receptor with negligible loss of GRP receptor binding activity. PEG precipitation and suspension of the pellet in a smaller volume of buffer were also convenient methods of concentrating the extract. In addition, the detergent concentration could be reduced 10-fold, which significantly enhanced the stability of 125I-GRP binding activity. After precipitation by PEG, the soluble extract was chromatographed on a WGA-agarose column. The column bound greater than 95% of the 125I-GRP binding activity in the extract, and elution of the column with N,N',N"-triacetylchitotriose (5 mM) produced a large peak of glycoproteins which generally included over 60% of the total 125I-GRP binding activity applied to the column. In contrast, all attempts to elute the column with N-acetyl-d-glucosamine resulted in unacceptably poor recovery of 125I-GRP1-27 binding activity from the column. The eluate from the WGA-agarose column was then chromatographed on a GRP affinity column. The ligand used for the column was [Nle4,27]GRP13-27, which had similar affinity for the soluble GRP receptor as GRP1-27 (Table I). [Nle4,27]GRP13-27 was coupled to an activated aldehyde-agarose resin (Actigel Superflow) via a reduced Schiff base linkage to either the ε-amino group of lysine 13, or the N-terminus of the peptide.

Application of WGA-agarose purified receptor to the ligand affinity column resulted in the binding of about 80% of the 125I-GRP binding activity in the extract to the column (Fig. 5). After extensive washing, the column was eluted with column buffer + 0.5 M NaCl. Yields of 125I-GRP binding activity recovered from the column ranged from 35 to 50% of the total activity loaded. The ability to elute the GRP receptor from the column by salt is consistent with the finding that
Pooled fractions containing "GRF binding activity showed that numerous contaminants remained in the preparation. Absorbance at 280 nm detected in the elution peak came from a absorbance monitored during the run (Fig. 5), which indicated purification achieved since a significant portion of the ab-

by salt along with the receptor. SDS-PAGE analysis of the binding activity was estimated from the trace of 280 nm eluted from the column in fractions containing ""GRF binding activity. This figure should be taken as a lower limit of the

by a salt wash of the column without also eluting the GRF bound nonspecifically to the affinity resin could not be eluted affinity column was difficult to improve since the protein that

in an attempt to reduce the level of protein that bound

GRP19-27, GRP18-27 (neuromedin C), [Lys3]bombesin, and

GRP1-27 binding to the receptor is reversibly inhibited by NaCl (EC50 = 150 mM) (Fig. 3). The total amount of protein eluted from the column in fractions containing 125I-GRF binding activity was estimated from the trace of 280 nm absorbance monitored during the run (Fig. 5), which indicated that the specific activity of the receptor was increased about 300-fold. This figure should be taken as a lower limit of the

by ultrafiltration. The recoveries of 125I-GRF binding activity in this step varied from 50-100%. The desalted sample was applied to a smaller version of the first [Nle4~27]GRP13-27 agrose column and was again eluted with column buffer + 0.5 M salt. The recovery of GRP receptor binding activity from the second affinity column was generally better than that obtained with the first ligand affinity column step, ranging from 50-80%.

The fractions containing 125I-GRF binding activity were pooled, concentrated by ultrafiltration, and analyzed by SDS-PAGE. The gels showed presence of a single diffuse band of silver-stained material, characteristic of a glycoprotein, with a M, of 75,000-100,000 (Fig. 6, lanes D and E). The protein appeared to be free of significant contaminants. Cross linking of 125I-GRF to the GRF receptor in unfractionated soluble extracts (Fig. 6, lane F) or intact Swiss 3T3 cells (Fig. 8 and Refs. 27-29), labeled a glycoprotein with a SDS-PAGE mobility corresponding to that of the purified receptor protein. In both cases, the production of cross-linked receptor was competed by unlabeled GRP1-27 (Fig. 6, lane G; data not shown for intact cells), indicating that the 125I-GRF1-27 was cross-linked to high affinity GRF receptor binding sites.

The eluate from the [Nle4~27]GRP13-27 affinity column was chromatographed on a Superose 6 gel filtration column (Fig. 7), which gave essentially quantitative recovery of 125I-GRF1-27 binding activity. This step desalted the sample and removed some of the detergent accumulated when the ligand affinity column eluate was concentrated by ultrafiltration. The apparent molecular weight of the soluble receptor was estimated from the column to be 250,000, which was several times larger than the size of the receptor determined by SDS-PAGE. Elution of the purified receptor from the Superose 6...
Purification. Lane A, soluble extract after PEG fractionation; lane B, the crude soluble extract was affinity-labeled by cross-linking to lz51-eluate; lane D, second [Nle'4,s7]GRP13-27-agarose column eluate; lane E, receptor after Superose 6 chromatography. The GRP receptor in receptor purification and products of lz51-GRP cross-linking to the soluble GRP receptor.

6 chromatography and the assay of '251-GRP1-27 binding were performed as described under "Experimental Procedures." Each fraction contained 0.5 ml.

The gel results also showed that the peak of detergent aggregates did not contain detectable amounts of protein.

The total yield of GRP receptor isolated was estimated by quantitating its 280 nm absorbance from the Superose 6 chromatogram and assuming that the receptor displayed the same extinction coefficient as bovine serum albumin and chicken ovalbumin standards. By this method of analysis, the preparation summarized in Table III yielded 0.73 ng of purified receptor protein. This value is the same as the amount of receptor determined from 125I-GRP1-27 binding data (Table III), using 38,000 g/mol as the molecular weight of the deglycosylated receptor, which was determined from the analysis described below. The data indicate that a large fraction of the purified receptor remained in an active conformation.

N-Glycanase Treatment—The presence of N-linked carbohydrate on the purified GRP receptor was investigated by treating the protein with N-glycanase and analyzing shifts in mobility of the protein on SDS-PAGE. N-Glycanase hydrolyzes the glycosylamine linkages between the oligosaccharide chains and asparagine residues on a protein without any apparent specificity toward the structure of the carbohydrate moiety present. To enhance the detection of receptor bands and avoid interference from the N-glycanase protein present in the sample (Mₚ = 35,000), the experiment was performed with purified receptor protein that was 125I-labeled. As shown in Fig. 8 (right panel), treatment of 125I-GRP receptor with N-glycanase changed its mobility on the gel from Mₚ = 75,000-100,000 to Mₚ = 38,000. The same result was obtained when unlabeled purified receptor was used in the experiment, and deglycosylated products were visualized by silver staining of the gel (data not shown). In addition, affinity-labeled GRP receptor, produced by cross-linking 125I-GRP to the GRP receptor in intact Swiss 3T3 cells, was also subjected to treatment with N-glycanase (Fig. 8, left panel). The deglycosylated form of 125I-GRP affinity-labeled receptor also exhibited a Mₚ of 38,000, strongly indicating that the GRP receptor labeled by cross-linking and the purified GRP receptor are the same protein.

Scatchard Analysis of the Purified GRP Receptor and the Crude Soluble Extract—Data for the binding of 125I-GRP to purified GRP receptor in the presence of various amounts of unlabeled GRP1-27 are shown as a Scatchard plot in Fig. 9A. The equilibrium dissociation constant of the purified receptor for 125I-GRP, Kₐ, was found to be 0.038 ± 0.019 nM. Under similar conditions, GRP1-27 bound to the GRP receptor in isolated membranes with a Kₐ of 0.120 ± 0.02 nM and to intact Swiss 3T3 cells with a Kₐ of 0.26 ± 0.2 nM (Table II, low salt).

The binding of 125I-GR to soluble receptors in an unfractiated extract was also analyzed by the method of Scatchard (Fig. 9B). The crude soluble receptor exhibited a Kₐ for GRP1-27 of 0.036 ± 0.003 nM indicating that the ligand binding properties of the soluble receptor were not altered by purifying the protein to homogeneity. The number of receptor sites (Bmax) present in the crude extract was extrapolated to be 0.60 pmol/mg of membrane protein.
The GRP receptor was solubilized from Swiss 3T3 fibroblast membranes in an active form with CHAPS and the cholesterol ester, CHS. The receptor was then purified about 90,000-fold homogeneity by a combination of WGA-agarose affinity chromatography. The soluble receptor exhibited the same specificity for a series of GRP analogs as displayed by the membrane-bound form of the receptor. After purification, the receptor still bound GRP \( K_d = 0.038 \pm 0.019 \text{nM} \) with the same affinity as the receptor in the unfractionated soluble extract \( K_d = 0.036 \pm 0.003 \text{nM} \). In addition, the purified receptor exhibited an affinity for GRP similar to that displayed by Swiss 3T3 membranes \( K_d = 0.12 \pm 0.02 \text{nM} \) and intact cells \( K_d = 0.26 \pm 0.2 \text{nM} \), assayed under similar conditions.

In order for the GRP receptor to bind \(^{125}\text{I}-\text{GRP}1-27\) after solubilization with CHAPS, it was necessary to include CHS in the medium. CHS was found to promote formation of the active conformation of the receptor, possibly by directly interacting with hydrophobic domains on the protein. CHS was also found to be crucial for the solubilization of the neurotensin receptor from rat brain in an active form (42) and promoted the stability of the dithiothreitol-reduced form of the \( \beta \)-adrenergic receptor after reconstitution into phospholipid vesicles (43).

On SDS-PAGE gels, the purified receptor runs as a single diffuse band with a \( M_r = 75,000 \) to 100,000. These results agree with estimates of GRP receptor size made by cross-linking \(^{125}\text{I}-\text{GRP}\) to the receptor in intact Swiss 3T3 cells (Fig. 8) (27-29), or soluble membrane extracts (Fig. 6), and analyzing the labeled species by SDS-PAGE and fluorography. The deglycosylated form of the purified GRP receptor, generated by treatment of the protein with N-glycanase, exhibited a \( M_r = 38,000 \). Receptor labeled on intact Swiss 3T3 cells by cross-linking to \(^{125}\text{I}-\text{GRP}\) was also converted by N-glycanase treatment to a species with a \( M_r = 38,000 \), providing additional evidence that the \( M_r = 75,000-100,000 \) protein that was purified is the GRP receptor. In similar cross-linking experiments, others have found slightly higher \( M_r \) values for deglycosylated receptor of 43,000 (29) or 45,000 (27), possibly due to the use of different cross-linking reagents or SDS-PAGE conditions. It has also been observed that N-glycanase treatment of the cross-linked receptor generates a single deglycosylation intermediate, suggesting that at least two \( N \) linked oligosaccharide chains are present on the receptor molecule (29). The purification of the GRP receptor should make it possible to perform a more comprehensive carbohydrate analysis of the protein.

The apparent molecular weight of the native receptor determined by gel filtration was 250,000, or 2 to 3 times higher than that determined by the SDS-PAGE analysis discussed above. The same value for the size of the GRP receptor was estimated from chromatographic runs of unpurified receptor (data not shown). However, with crude extracts, it was necessary to maintain a relatively high CHAPS concentration \( (0.25\%) \) during the chromatography to prevent aggregation of the receptor with other proteins in the preparation. The apparent size of the receptor estimated from gel filtration data may be larger than that determined from SDS-PAGE because enough detergent binds to the receptor to greatly increase its hydrodynamic radius. Alternatively, the native receptor may exist as a dimer in membranes or in solution. However, the fact that \( M_r = 250,000 \) dimers are not produced in ligand-receptor cross-linking experiments argues against this possibility, since the homobifunctional cross-linking reagent used would be expected to also cross-link receptor subunits. In addition, the mobility of the purified receptor on SDS-PAGE was the same, whether or not the protein was run in a reduced form (data not shown). Therefore, the receptor is not a disulfide-linked homodimer and did not form such a species by oxidation in the course of purification.

The amount of protein obtained in purified preparations of the GRP receptor was estimated from \(^{125}\text{I}-\text{GRP}\) binding data, assuming that each receptor molecule has a single ligand binding site and a molecular weight of 38,000 g/mol. The yield of receptor derived in this manner was approximately the same as that estimated from the absorbance of the receptor at 280 nm. Both these methods of receptor quantitation are subject to relatively large errors, but the close agreement of the values obtained, coupled with data from the cross-linking, SDS-PAGE, and carbohydrate analysis described above, strongly indicate that the receptor preparation is free of major contaminants. The data also indicate that a large fraction of the purified receptor maintains its native conformation.

As observed previously (30), treatment of Swiss 3T3 membranes with guanyl nucleotides converts the GRP receptor from a high affinity to a low affinity form, demonstrating that the receptor is coupled to a guanyl nucleotide regulatory protein. This G-protein is likely involved in coupling the receptor to the breakdown of inositol phospholipids (33). Kinetic analysis of ligand dissociation from the Swiss 3T3 membranes in the presence and absence of Gpp(NH)p showed that disruption of G-protein coupling decreased the affinity of the receptor for \(^{125}\text{I}-\text{GRP}1-27\) about 10-fold (30). However, the affinity of the soluble receptor was not affected by guanyl nucleotides and thus the receptor was no longer coupled to its G-protein. It was not surprising, therefore, that the CRP receptor did not co-purify with an associated G-protein. The fact that the purified receptor displays a marginally higher affinity for ligand than the G-protein-coupled membrane form of the receptor is consistent with the notion that the receptor adopts its high affinity conformation when extracted from the

\(^2\text{J. Wu and R. Feldman, unpublished observations.}\)
membrane bilayer. However, it is more likely that other factors acting on the receptor in solution alter its affinity and
more than compensate for the loss of affinity due to the disruption of G-protein coupling.

A number of different G-proteins appear to be able to regulate inositol phospholipid metabolism, since the stimula-
tion of protein kinase C in some, but not all, systems studied is inhibited by pertussis toxin (44-47). Despite reports that
pertussis toxin inhibits the stimulation of DNA synthesis in
Swiss 3T3 cells (48), other work indicates that it does not
significantly alter bombesin's ability to stimulate phospho-
oositide turnover (49) and does not affect the ligand affinity of
the GRP receptor (50). It has also been reported that the
p21 gene product of the N-ras proto-oncogene, when overex-
pressed in NIH 3T3 cells, can couple the GRP receptor to the
regulation of protein kinase C (50); however, the role of p21
in GRP receptor function in Go.

vesicles. Such methods may make it possible to identify
specific members of the G-protein family that are important in
GRP receptor function in vivo.

Sodium chloride, at a concentration of 150 mM, strongly
inhibited the binding of GRP1-27 to the GRP receptor. In
contrast, the binding of truncated analogs, such as N-acytyl-
GRP20-27, was not affected by salt. N-Acetyl-GRP20-27 was
shown previously to contain all of the determinants for recep-
tor binding present on its parent molecule, GRP1-27, under
physiological conditions (2). However, the data described in
this paper indicate that additional determinants are used to
bind GRP1-27 to the receptor in media containing low ionic
strength. This enhanced binding affinity, likely involving
lysine 13 or arginine 17 of GRP1-27, was eliminated in the
presence of 150 mM NaCl. An understanding of the binding
properties of different GRP analogs in relationship to salt
concentration was useful in developing an effective ligand
affinity chromatography method to purify the GRP receptor.
The complex effects of salt noted here underscore the impor-
tance of maintaining standard assay conditions that are func-
tionally equivalent to physiological conditions before making
conclusions regarding the relative potency of different recep-
tor ligands in vivo.

The purification of the GRP receptor in an active form,
described in this paper, is a significant step toward the reso-
lution of other components involved in the signal transduction
pathway of the receptor. This work will also facilitate obtain-
ing an amino acid sequence of the receptor that could be used
to clone the GRP receptor gene.

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