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Identification of the Glucagon Receptor by Covalent Labeling with a Radiolabeled Photoreactive Glucagon Analogue†

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ABSTRACT: The photoreactive ^{125}I-labeled glucagon-NAPS [^{125}I-labeled 2-[(2-nitro-4-azidophenyl)sulfenyl]-Trp^{25}-glucagon] was used to label the glucagon receptor sites in rat liver plasma membranes. The proteins labeled were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis with or without reduction with dithiothreitol. The photoaffinity peptide specifically labeled a number of bands with apparent molecular weights >200 000 and probably at least two protein bands in the molecular weight range 52 000–70 000. The relative amounts of radioactivity associated with these bands and their relative mobilities differed in samples from reduced and unreduced membranes. Their relative mobilities also differed with percent acrylamide cross-linking, suggesting a glycoprotein nature and the presence of intramolecular disulfide bonds. A nonspecifically labeled band with an apparent molecular weight of 27 000–28 000 also displayed a similar behavior. Photolabeling in the presence of 0.1 mM guanosine 5′-triphosphate (GTP) decreased the amount of radiolabeling of these bands, suggesting their involvement in the glucagon stimulation of adenylate cyclase. The photolabeled receptor in the membranes, solubilized with Lubrol-PX and fractionated on an Ultrogel AcA22 column, eluted with an apparent molecular weight of 200 000–250 000. Addition of GTP to the solubilized glucagon receptor of nonirradiated membranes caused complete dissociation of the complex. Gel electrophoresis of the partially purified radiolabeled receptor identified the same protein components observed in photolabeled membranes. These results indicate that the glucagon receptor is an oligomer probably composed of at least two different subunits that are linked together or greatly stabilized by disulfide bonds. They also show that ^{125}I-labeled glucagon-NAPS can be used effectively to covalently label the putative glucagon receptor and thus aid in its further characterization.

Several studies have shown that the first step in the action of many hormones is binding to specific sites on target cell membranes containing adenylate cyclase systems responding to these hormones [see, for example, Rodbell (1980)]. The binding properties of glucagon receptor(s) in rat liver plasma membranes (Rodbell et al., 1971, 1974; Lin et al., 1977; Sperling et al., 1980), crude liver microsomal membranes (Desbuquois et al., 1974), intact hepatocytes (Sonne et al., 1978), and adipocytes (Birnbaumer & Pohl, 1973) have been extensively studied, but very little is known about the identity and character of the glucagon receptor itself. Initial attempts to characterize the glucagon receptor in Lubrol-PX-solubilized liver plasma membranes by gel fractionation have identified a macromolecule with a molecular weight of 190 000 (Blecher et al., 1974). Similar studies with the myocardial adenylate cyclase–glucagon complex have identified two components, one having a catalytic activity and a molecular weight greater than 100 000 and the other, a glucagon-binding fraction with a molecular weight of 24 000–28 000 (Levey, 1975). Active glucagon–agarose preparations have also been studied for their possible use in affinity chromatography (Krug et al., 1971; Johnson et al., 1972). Labeling of rat liver plasma membranes with radioactively labeled iodoacetamide or iodoacetic acid in the presence and absence of glucagon has identified a 240 000 molecular weight protein by sodium dodecyl sulfate ($NaDodSO_4$)[1]–polyacrylamide gel electrophoresis as being an integral component of the adenylate cyclase–glucagon receptor complex (Storm & Chase, 1975). Photoaffinity labeling of the glucagon receptor with the inactive derivative ^{125}I-labeled N^{ϵ}-(4-azido-2-nitrophenyl)-Lys^{12}-glucagon, which was able to bind to liver plasma membranes, has identified two components with a molecular weight range of 23 000–25 000 as the receptor sites for glucagon (Bregman & Levy, 1977). Recently, using photoaffinity cross-linking with hydroxysuccinimidyl *p*-azidobenzoate, Johnson et al. (1981) have identified an ^{125}I-labeled glucagon-binding membrane protein having a molecular weight of 53 000.

In this study, we have used a photoreactive radiolabeled glucagon derivative, ^{125}I-labeled 2-[(2-nitro-4-azidophenyl)-sulfenyl]-Trp^{25}-glucagon (^{125}I-labeled glucagon-NAPS)(Demoliou & Epand, 1980), to covalently label the glucagon receptor(s). The criteria and advantages of using the pho-

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[1] Abbreviations: ^{125}I-labeled glucagon-NAPS, ^{125}I-labeled 2-[(2-nitro-4-azidophenyl)sulfenyl]-Trp^{25}-glucagon; BSA, bovine serum albumin; App(NH)p, adenosine 5′-(β,γ-imidotriphosphate); cAMP, adenosine cyclic 3′,5′-phosphate; GTP, guanosine 5′-triphosphate; Gpp(NH)p, guanosine 5′-(β,γ-imidotriphosphate); Tes, 2-[[tris(hydroxymethyl)-methyl]amino]ethanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; DNP-glycine, *N*-(2,4-dinitrophenyl)glycine; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3CCOOH, trichloroacetic acid; KIU, kallikrein inactivator units; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

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toaffinity labeling technique for the in situ labeling of receptors have been previously discussed (Knowles, 1972; Bayley & Knowles, 1977). The glucagon-NAPS derivative fulfills the criteria of a glucagon analogue for it can specifically bind and saturate the glucagon receptor sites and it can activate the adenylate cyclase system in rat liver plasma membranes having both a slightly higher affinity and potency than the native hormone (Demoliou-Mason & Epand, 1982). The photoaffinity-labeled bands in plasma membranes were compared by $NaDodSO_4$–polyacrylamide gel electrophoresis with the bands of the partially purified detergent-solubilized glucagon receptor, which is GTP sensitive (Welton et al., 1977).

Materials and Methods

The synthesis, purification, and iodination of glucagon-NAPS as well as the iodination of glucagon have been previously reported (Demoliou & Epand, 1980). Bovine serum albumin (fraction V), bacitracin, trypsin (type III), soybean trypsin inhibitor, guanosine 5′-triphosphate, and aminophylline were purchased from Sigma; Trasylol (10000 KIU/mL) was from Boehringer; Ultrogel AcA22 (1.0×10^5–1.2×10^6 range) was from LKB; *N*,*N*′-methylenebis(acrylamide) and *N*,*N*,*N*′,*N*′-tetramethylenediamine were from Bio-Rad; ultrapure urea was from Schwarz/Mann; protein molecular weight standards were from BRL; Lubrol-PX was kindly provided by ICI America; bovine–porcine glucagon was purchased from the Elanco Corp. and its purity tested as previously reported (Demoliou & Epand, 1980). N^{α}-(2,4,6-Trinitrophenyl)-glucagon (Cote & Epand, 1979) and $Asp^{9,15,21}$-triglycinamide–glucagonylglycinamide (Epand & Epand, 1972) were synthesized as previously reported.

Rat Liver Plasma Membrane Preparation. Partially purified rat liver plasma membranes were prepared from Wistar rats (150–200 g) according to the procedure of Neville (1968) as previously described (Demoliou & Epand, 1980).

Photoaffinity Cross-Linking of ^{125}I-Labeled Glucagon-NAPS to Rat Liver Plasma Membranes. Plasma membranes (0.3–0.6 mg/mL) were preincubated with ^{125}I-labeled glucagon (1.5×10^{-9} M, specific activity 2.0×10^6 cpm/pmol) or with ^{125}I-labeled glucagon-NAPS [7.2×10^{-10}–1.0×10^{-8} M, specific activity (3.0–3.5) $\times 10^6$ cpm/pmol] at 30 °C, in the dark, for 10 min. The incubation medium was 30 mM Tris-HCl, pH 7.0, buffer containing either 750 KIU/mL Trasylol and 0.25 mg of BSA/mL or the reagents present in the adenylate cyclase assay including 30 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM mercaptoethanol, 0.2 mM cAMP, 0.56 mM ATP, 5 mM creatine phosphate, 0.64 mg/mL creatine phosphokinase, 1 mM aminophylline, 7 mM bacitracin, and 4 mg/mL BSA either with or without 0.1 mM GTP (Demoliou-Mason & Epand, 1982). The total incubation volume was 0.2–0.3 mL. After incubation, the membrane samples were cooled in ice for 5 min, saturated with N_2 gas (water saturated), and irradiated for 2 min under the conditions previously described (Demoliou-Mason & Epand, 1982) or kept in the dark. The membranes were subsequently diluted with 10 mL of 30 mM Tris-HCl, pH 7.0, containing 0.1 mM GTP and incubated at 30 °C for 15 min, in the dark to dissociate noncovalently bound ligand. The membranes were centrifuged at 12000*g* at 4 °C for 30 min, and the pellets were washed once with 30 mM Tris-HCl, pH 7.0, and resuspended in 6 M urea containing 10% (w/v) $NaDodSO_4$ for gel electrophoresis. Control samples included membranes preincubated with 1000 × excess unlabeled glucagon, glucagon-NAPS, N^{α}-(2,4,6-trinitrophenyl)glucagon, and $Asp^{9,15,21}$-triglycinamide–glucagonylglycinamide, membranes preincubated with prephotolyzed ^{125}I-labeled glucagon-NAPS, and trypsinized membranes. When dithiothreitol was used as a scavenger during irradiation, it was added to the incubation sample immediately before irradiation to a final concentration of 50 mM.

Membrane Trypsinization. Liver plasma membranes (0.3 mg/mL) were incubated in 30 mM Tris-HCl, pH 7.0, with 0.10 mg/mL trypsin (10000 units/mg) at 30 °C for 30 min. Trypsinization was inhibited by the addition of 0.12 mg/mL soybean trypsin inhibitor. The trypsinized membranes were subsequently used for photoaffinity labeling as described above.

Solubilization of the Glucagon Receptor. The glucagon receptor was solubilized from liver plasma membranes according to Welton et al. (1977). Membranes (5–10 mg/mL) were incubated with ^{125}I-labeled glucagon (1.7×10^{-9} M, specific activity 3.7×10^6 cpm/pmol) or with ^{125}I-labeled glucagon-NAPS (1.23×10^{-9} M, specific activity 3.3×10^6 cpm/pmol) in 0.6 mL of 30 mM Tris-HCl buffer, pH 7.0, containing 0.04% BSA, 0.1 mM App(NH)p, 10 μM cAMP, 30 μM Gpp(NH)p, and 5 mM $MgCl_2$. Incubation was at 30 °C for 20 min, in the dark. After incubation, the membrane samples were cooled in ice for 5 min, saturated with N_2 gas, and irradiated or kept in the dark as described above. The membranes were then pelleted by centrifugation (50000*g*) at 4 °C for 20 min, the supernatant was discarded, and the membranes were resuspended in 1.0 mL of 10 mM Tes buffer, pH 7.5, containing 1 mM DTT and 25% (w/w) sucrose. When GTP was included during solubilization, the buffer contained 0.1 mM GTP. Lubrol-PX (10% w/v) in 10 mM Tes buffer, pH 7.5, was added to the membrane to give a final concentration of 1.0% (w/v). The membrane–detergent mixture was blended on a Vortex mixer for 1 min and sonicated for another minute in a Bransonic-12 bath-type sonicator. Unsolubilized membranes were pelleted by centrifugation (100000*g*) at 4 °C for 30 min. The supernatant fluid containing the solubilized glucagon receptor (40–50% of radiolabeled ligand) was then fractionated on an Ultrogel AcA22 column as described below. Control samples included membranes which had been incubated in the presence of 1000 × excess unlabeled glucagon.

Fractionation of Detergent-Solubilized Plasma Membranes. The solubilized membranes prepared as described above were chromatographed on an Ultrogel AcA22 column (1.5 × 90 cm) at 4 °C according to the procedure of Welton et al. (1977). Fractions (1.0 mL) were collected at a rate of 10–15 mL/h. The elution buffer was 10 mM Tes, pH 7.5, containing 1 mM EDTA, 1 mM DTT, 25% (w/w) sucrose, and 0.01% (v/v) Lubrol-PX. When GTP was present during solubilization, the elution buffer also contained 10 μM GTP. The elution profile was monitored by ^{125}I radioactivity and protein content in 150-μL samples of the collected fractions. The column was calibrated with dextran blue, apoferritin, human γ-globulin, BSA, and DNP-glycine in the absence of detergent and DTT. From the ^{125}I elution profile obtained, the fractions of each radiolabeled peak were pooled into 30-mL Corex tubes, and the protein was precipitated with saturated ammonium sulfate solution. The precipitated protein fractions were centrifuged at 12000*g* at 4 °C for 1 h and redissolved in 6 M urea/10% (w/v) $NaDodSO_4$ for gel electrophoresis.

Gel Electrophoresis. Radiolabeled monomeric and oligomeric species of ^{125}I-labeled glucagon and ^{125}I-labeled glucagon-NAPS were separated by gel electrophoresis in 12.5% acrylamide cross-linked cylindrical gels (14 × 0.6 cm) containing 6 M urea and 0.1% (w/v) $NaDodSO_4$. The gels were prepared according to the procedure of Swank & Munkres (1971). Various concentrations of glucagon (10^{-9}–10^{-6} M)

in 0.2 mL of 30 mM Tris-HCl, pH 7.0, containing 750 KIU/mL Trasylol and radiolabeled ^{125}I-labeled glucagon or ^{125}I-labeled glucagon-NAPS [(3.0–7.0) × 10^{-11} M, specific activity (2.8–3.0) × 10^6 cpm/pmol] were incubated at 30 °C for 10 min, cooled in ice for 5 min, and subsequently saturated with N_2 gas and irradiated or kept in the dark as previously described. The irradiated or nonirradiated samples were dried with N_2 gas, in the dark, redissolved in 0.1 mL of 8 M urea and 10% (w/v) $NaDodSO_4$, heated for 30 min at 60 °C, and left at room temperature overnight. Before electrophoresis, to each sample were added 10 μL of 0.01 M phosphate buffer, pH 6.8, containing 1.0% (v/v) mercaptoethanol and 5 μL of 0.1% (w/v) bromophenol blue. The samples were electrophoresed for 18–20 h at 5–10 mA/gel. After electrophoresis, the gels were fixed with 10% Cl_3CCOOH for 3–4 h, frozen, cut into 2-mm slices with a Bio-Rad gel slicer (Model 190), and counted in a Searle γ counter. Protein bands were detected by staining with 0.25% (w/v) Coomassie blue R-250 dye/10% acetic acid/50% methanol and destained with 8% acetic acid/25% methanol.

Photoaffinity-labeled BSA, membranes, detergent-solubilized membranes, and soluble receptor fractions were electrophoresed on cylindrical gels (14 × 0.6 cm) or slab gels (≃14 cm) by using a modification of the Laemmli (1970) method. Separating gels contained 7.5%, 10%, and 12.5% (w/v) acrylamide, 0.27% (w/v) *N,N'*-methylenebis(acrylamide), 0.1% (w/v) $NaDodSO_4$, 0.03% (v/v) *N,N,N',N'*-tetramethylenediamine, 0.01% (w/v) ammonium persulfate, 0.1% (w/v) glycerol, 0.5 M urea, and 0.38 M Tris-HCl, pH 8.8. Stacking gels (2 cm) contained 5% (w/v) acrylamide, 0.15% (w/v) *N,N'*-methylenebis(acrylamide), 0.01% (w/v) $NaDodSO_4$, 0.05% (v/v) *N,N,N',N'*-tetramethylenediamine, 0.06% (w/v) ammonium persulfate, 0.05% (v/v) glycerol, 0.5 M urea, and 0.12 M Tris-HCl, pH 6.8. The electrode buffer was 25 mM Tris, 0.192 mM glycine, and 0.1% (w/v) $NaDodSO_4$. Protein samples in 6 M urea and 10% (w/v) $NaDodSO_4$ were heated for 30 min at 60 °C and left at room temperature overnight. Before electrophoresis, unless otherwise stated, the protein samples were either reduced with 10 mM DTT for 30 min under N_2 gas or reduced and alkylated with 18 mM iodoacetamide for 1 h. Cylindrical gels were run 12–16 h at 3.0 mA/gel; slab gels were run at 60 V for 18–20 h. Gels were either fixed with 10% Cl_3CCOOH for 3–4 h, frozen, cut into 1- or 2-mm slices, and counted or stained with 0.25% (w/v) Coomassie blue R-250/50% ethanol/10% acetic acid and destained with 25% ethanol/10% acetic acid. Cylindrical gels were scanned at 550 nm with a Gilford spectrophotometer with linear transport before cutting and counting. Results were corrected for any discrepancies between the recorded lengths of gels and the number of slices obtained. After slab gels were stained and destained, they were dried on a Bio-Rad gel drier and then exposed to Kodak XR-2 film for 10–15 days. Molecular weight standards were myosin (H chain) (200 000), phosphorylase *b* (92 500), bovine serum albumin (68 000), ovalbumin (43 000), α-chymotrypsinogen (25 700), β-lactoglobulin (18 400), lysozyme (14 300), cytochrome *c* (12 300), bovine basic pancreatic trypsin inhibitor (6200), and insulin (3000).

Results

Characterization of Molecular Weight Species of Irradiated ^{125}I-Labeled Glucagon-NAPS by Gel Electrophoresis. Polyacrylamide gel electrophoresis in 8 M urea/12.5% acrylamide of ^{125}I-labeled glucagon-NAPS identified three major radioactively labeled components (Figure 1), a 20 000–26 000-dalton and a 6000-dalton component, each representing about 3% of the total counts applied on the gel, and a third major component where monomeric glucagon runs having a molecular weight ≤3000 and representing 55–60% of total radioactivity. Monomeric glucagon in 8 M urea gels runs anomalously with a molecular weight of ≃1800 (Swank & Munkres, 1971). When ^{125}I-labeled glucagon-NAPS was irradiated in the presence of unlabeled glucagon (10^{-10}–10^{-6} M), there was an increase in radioactivity associated with the 6000-dalton component, up to 200% for the highest peptide concentration. No appreciable differences were observed for the 20 000–26 000-dalton component. These results suggested that the 6000 molecular weight component could arise, at least in part, from the covalent cross-linking of glucagon. The bands observed in unphotolyzed samples (Figure 1) are most likely cross-linked by radical formation during storage since their amounts varied with days of storage even after repurification of radiolabeled peptide (Demoliou & Epand, 1980). Additional protein bands stained with Coomassie blue dye >200 000, 40 000–50 000, and ~11 000 daltons were observed in highly overloaded gels with glucagon, which were attributed to aggregates spontaneously formed in concentrated glucagon solutions. When BSA was included in the incubation mixture, this protein was labeled presumably by covalent cross-linking to ^{125}I-labeled glucagon-NAPS (Figure 2). In addition, there was also a small amount of radioactivity incorporated into higher molecular weight bands. This was observed even with samples which were not photolyzed (Figure 4, lane L) and may arise from contaminants or from $NaDodSO_4$–protein–peptide aggregates which do not rapidly dissociate.

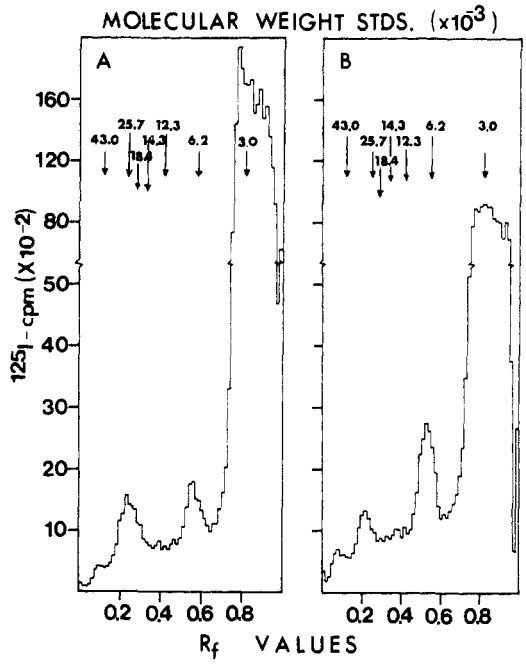

FIGURE 1: Urea/12% acrylamide gel electrophoresis of irradiated ^{125}I-labeled glucagon-NAPS. ^{125}I-Labeled glucagon-NAPS (7.2 × 10^{-11} M, specific activity 2.8 × 10^6 cpm/pmol) was incubated with or without native glucagon (1 × 10^{-5}–5 × 10^{-2} mg/mL) in 30 mM Tris-HCl, pH 7.0, containing Trasylol (750 KIU/mL) at 30 °C for 10 min, in the dark. Samples were subsequently irradiated for 2 min or kept in the dark, dried under N_2 gas, and treated for gel electrophoresis as described under Materials and Methods. For brevity, only the radioactivity profiles of 2-mm sliced gels of representative samples are shown. (A) ^{125}I-Labeled glucagon-NAPS, nonirradiated; (B) ^{125}I-labeled glucagon-NAPS irradiated in the presence of 0.05 mg/mL native glucagon.

Molecular Weight Characterization of ^{125}I-Labeled Glucagon-NAPS Covalently Labeled Membrane Components. Figure 3 shows the Coomassie blue staining pattern and ra-

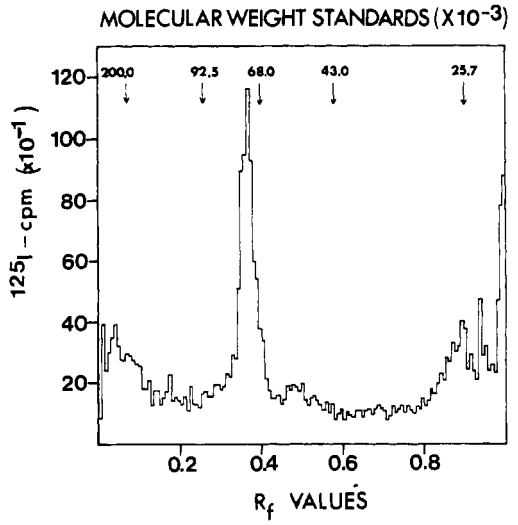

FIGURE 2: Gel electrophoresis of covalently labeled bovine serum albumin with ^{125}I-labeled glucagon-NAPS in 10% acrylamide/NaDodSO$_4$ cylindrical gels. ^{125}I-Labeled glucagon-NAPS (1.7×10^{-8} M, specific activity 3.0×10^6 cpm/pmol) was preincubated with 0.25 mg of BSA/mL in 30 mM Tris-HCl, pH 7.0, containing Trasylol (750 KIU/mL) in the presence and absence of native glucagon (10^{-6} M), at 30 °C for 10 min in the dark. Samples were subsequently irradiated for 2 min, dried under N_2 gas, and treated for gel electrophoresis as described under Materials and Methods. The radioactivity profile was determined from 1-mm slices of gels. The radioactivity profile in the presence of native glucagon was similar to that in its absence and is not shown for brevity.

dioactivity profile in 10% acrylamide/NaDodSO$_4$ gels of irradiated rat liver plasma membrane proteins after binding of ^{125}I-labeled glucagon-NAPS in the absence and presence of 10^{-6} M unlabeled glucagon. The effect of irradiation on the Coomassie blue staining pattern of the membrane proteins can be observed by comparing lanes G–K with A′ in Figure 4. There was a considerable decrease in the intensity of low molecular weight bands and the formation of some high molecular weight aggregates in irradiated membranes. Whether this is a result of membrane irradiation or membrane pretreatment before gel electrophoresis is under investigation. A similar phenomenon has been observed by Johnson et al. (1981) in the irradiation of membranes in the presence of the photoaffinity-cross-linked hydroxysuccinimidyl *p*-azidobenzoate. The formation of high molecular weight aggregates during irradiation may explain the 15% loss of adenylate cyclase activity observed in irradiated membranes (Demoliou-Mason & Epand, 1982).

The radioactivity profile of sliced gels (Figure 3) as well as the autoradiogram (Figure 4) shows that irradiation of membranes in the presence of ^{125}I-labeled glucagon-NAPS resulted in the labeling of a major broad band of 52000–70000 daltons. Other bands labeled less intensely had apparent molecular weights of 27000–28000, 46000, and greater than 200000, and another band was found in the interphase between the stacking and the separating gels (Figure 4). High molecular weight aggregates were observed on top of stacking

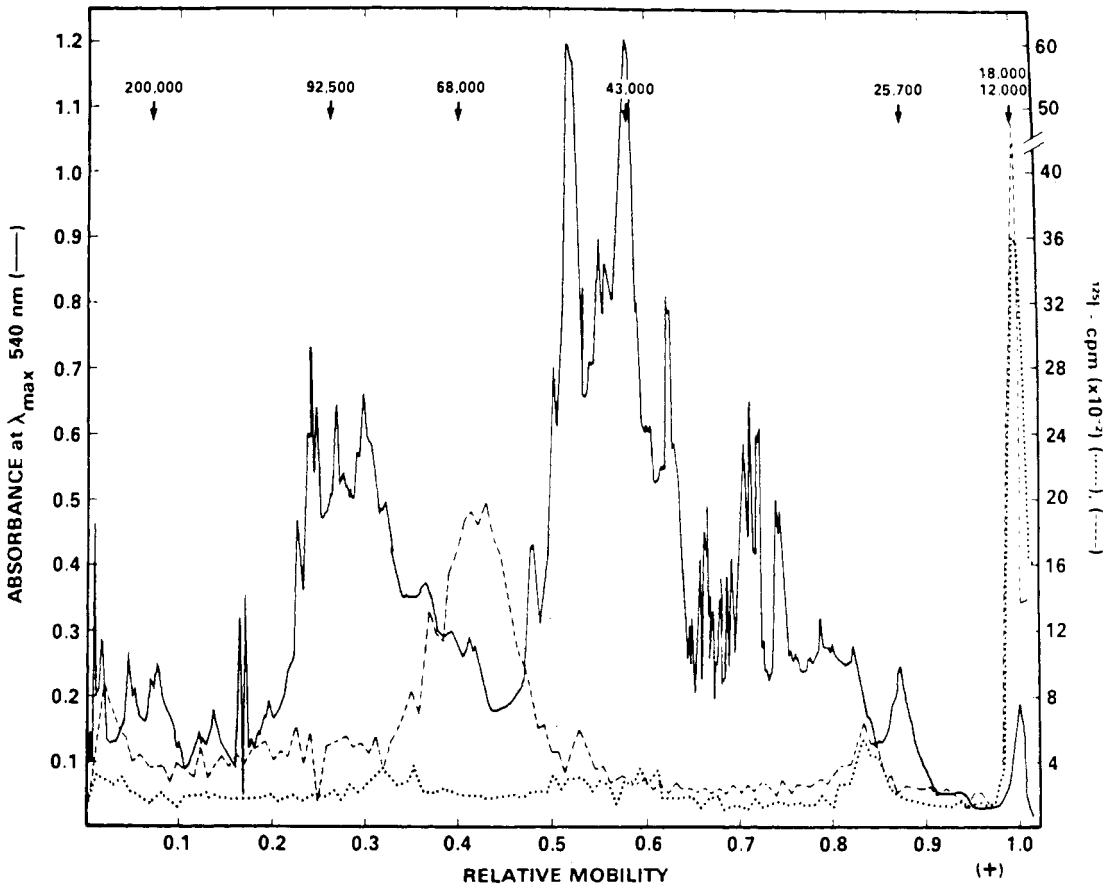

FIGURE 3: Gel electrophoresis profile of covalently labeled plasma membranes with ^{125}I-labeled glucagon-NAPS in 10% acrylamide/NaDodSO$_4$ cylindrical gels. Liver plasma membranes (0.4 mg/mL) were preincubated with ^{125}I-labeled glucagon-NAPS (7.2×10^{-10} M, specific activity 2.9×10^6 cpm/pmol) in the presence and absence of native glucagon (10^{-6} M) in 30 mM Tris-HCl, pH 7.0, containing 0.25 mg of BSA/mL and Trasylol (750 KIU/mL), at 30 °C for 10 min in the dark. After irradiation for 2 min, the membranes were subjected to the GTP dissociation step (see Materials and Methods), and after being pelleted by centrifugation, they were solubilized in 6 M urea/10% NaDodSO$_4$, heated at 30 °C for 60 min, and left at room temperature overnight. Solubilized membranes were reduced with 10 mM DTT before electrophoresis. (—) Densitometric scan at λ 540 nm of nonirradiated membranes stained with Coomassie blue; (- - -) ^{125}I counts per minute profile of 1-mm sliced gels of membranes preincubated and irradiated in the absence of glucagon; (···) ^{125}I counts per minute profile in the presence of glucagon.

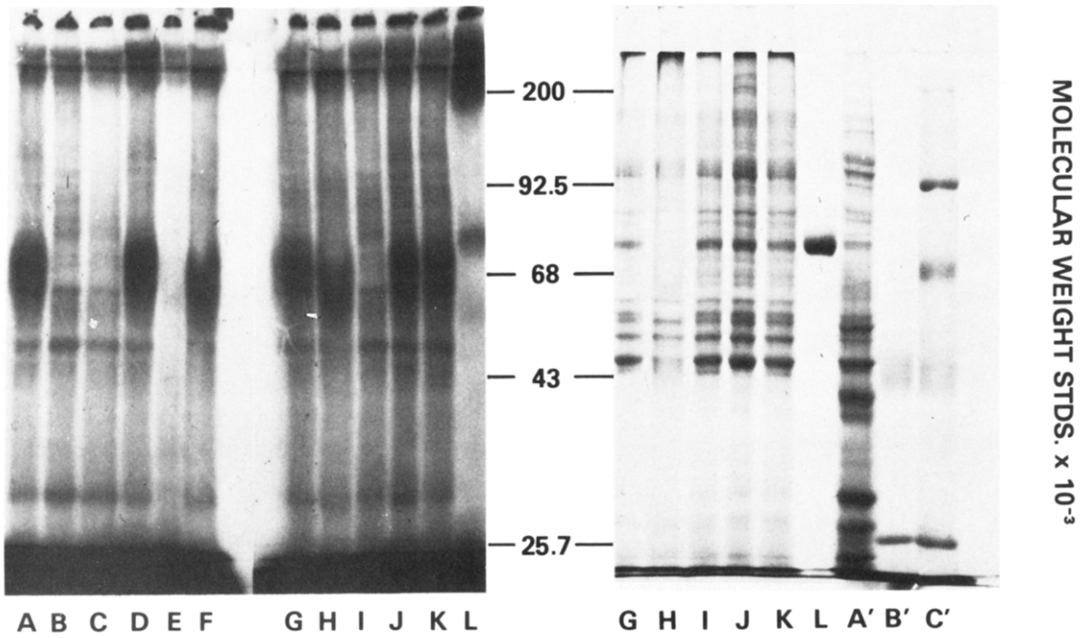

FIGURE 4: Autoradiogram (left) and Coomassie blue stained protein profile (right) of photolabeled membranes, electrophoresed in 10% acrylamide/$NaDodSO_4$ slab gels. Liver plasma membranes (0.4 mg/mL) were preincubated with ^{125}I-labeled glucagon-NAPS (1.6×10^{-9} M, specific activity 2.5×10^6 cpm/pmol) for 10 min at 30 °C, in the dark, in the adenylate cyclase assay medium (see Materials and Methods) ± 0.1 mM GTP. Irradiated membranes were treated for gel electrophoresis as described in the legend to Figure 3. (A and G) Membranes preincubated with ^{125}I-labeled glucagon-NAPS; (B and I) membranes preincubated with ^{125}I-labeled glucagon-NAPS and 10^{-6} M native glucagon; (C) membranes preincubated with ^{125}I-labeled glucagon-NAPS and 10^{-6} M N^{α}-(trinitrophenyl)glucagon; (D) membranes preincubated with ^{125}I-labeled glucagon-NAPS and 10^{-6} M $Asp^{9,15,21}$-triglycinamide–glucagonylglycinamide; (E) trypsinized membranes preincubated with ^{125}I-labeled glucagon-NAPS; (F and H) membranes preincubated with ^{125}I-labeled glucagon-NAPS, electrophoresed without reduction; (G–I) preincubated in the presence of 0.1 mM GTP; (J) membranes preincubated with ^{125}I-labeled glucagon-NAPS in assay medium containing 30 mM Gpp(NH)p and no GTP; (K) membranes preincubated as in (J) in the presence of GTP; (L) ^{125}I-labeled glucagon-NAPS in 30 mM Tris-HCl, pH 7.0, containing 1 mg of BSA/mL and Trasylol (750 KIU/mL), nonirradiated; (A′) nonirradiated membranes; (B′ and C′) molecular weight standards.

gels, and a number of other protein bands appeared to be labeled upon long exposure of gels (Figure 4). The 52 000–70 000 molecular weight band and that with a molecular weight greater than 200 000 were specifically labeled. The specificity of labeling was ascertained from the fact that the radioactivity associated with these bands was significantly reduced when the membranes were preincubated and irradiated in the presence of excess unlabeled glucagon (10^{-5}–10^{-6} M), in the presence of the glucagon derivative N^{α}-(2,4,6-trinitrophenyl)glucagon, which behaves as a glucagon antagonist possessing 0.1% of glucagon adenylate cyclase activity and 7% of glucagon binding (Epand et al., 1981) (Figure 4, lane C), and in the presence of unlabeled glucagon-NAPS (Figure 6). Furthermore, the derivative $Asp^{9,15,21}$-triglycinamide–glucagonylglycinamide with 0.004% and 0.05% glucagon adenylate cyclase and binding activity, respectively (Epand et al., 1981), was unable to displace bound ^{125}I-labeled glucagon-NAPS (Figure 4, lane D). Although not readily apparent in the autoradiogram (Figure 4), the ^{125}I counts per minute profile of sliced gels (Figures 3 and 5) suggests that the major band (52 000–70 000 daltons) may be composed of more than one component. Because of the breadth of the bands, accurate molecular weights could not be determined. Since a high concentration of BSA as well as other proteins was used in the assay medium, it is possible that the receptor-labeled bands are contaminated with labeled soluble protein, thus contributing to the broadness of the observed bands. This possibility is unlikely for a number of reasons. First, the membranes are centrifuged and washed with a GTP solution, thus removing the soluble proteins, before the electrophoresis. In addition, a BSA–glucagon adduct would have a molecular weight of 71 500 and would migrate slightly less in the gel (see Figure 4, lane L) than the receptor-labeled bands. Finally, we have also photolyzed ^{125}I-labeled glucagon-NAPS in the presence of membranes but in the absence of BSA and have observed similar broadness of the labeled bands (data not shown). The breadth of the labeled bands observed is also not due to the gels themselves since the Coomassie blue staining patterns (Figures 3 and 4) suggest good resolution for the high-abundance proteins. It is possible that the glucagon receptor is composed of a number of subunits with similar molecular weights or that some heterogeneity results from the proteolysis of a single protein component. Alternatively, they may be glycoproteins since, besides their breadth, they also displayed anomalous electrophoretic mobilities with varying percent of acrylamide cross-linking (Figures 3, 5, and 6). Although the apparent molecular weights did not vary much with gels of different percent of acrylamide, the Ferguson plot (Ferguson, 1964) showed that their mobilities at zero acrylamide concentrations were different from the mobilities of the marker proteins, suggesting anomalous bindings to $NaDodSO_4$. The 27 000–28 000 molecular weight band also displayed anomalous electrophoretic mobility with percent acrylamide cross-linking. The relative mobility of this band as well as of the 50 000–70 000-dalton band(s) and of that found in the stacking gel was changed when membranes were not reduced before gel electrophoresis (Figure 4, lanes F and H, and Figure 5), suggesting the presence of intramolecular disulfide links. It has been shown in studies with model proteins that the extent of reduction may influence the mobility of proteins containing cystine bonds (Griffith, 1972).

High molecular weight bands (>200 000) were also observed, including those in the electrophoretic profile of nonirradiated ^{125}I-labeled glucagon-NAPS (Figure 4, lane L), and one may suggest that they are artifactual due to aggregated peptide. However, there are a number of reasons to suggest that they are membrane components related to the glucagon receptor: ^{125}I-labeled glucagon-NAPS (70–75% of

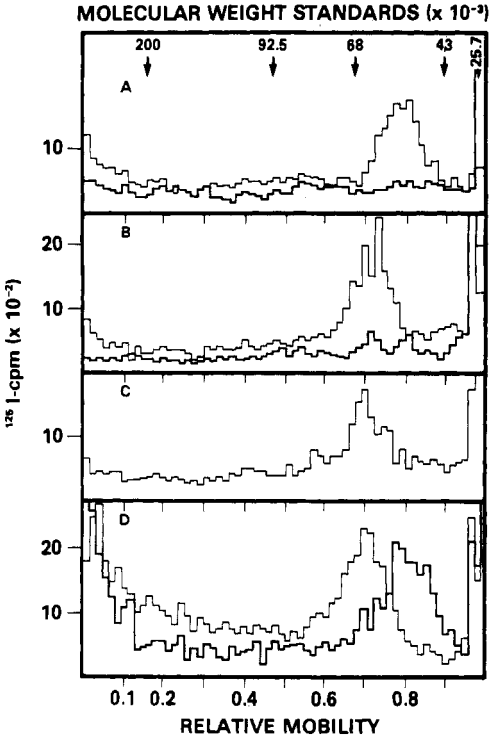

FIGURE 5: Gel electrophoresis of photolabeled membranes and soluble receptor in 7.5% acrylamide/NaDodSO$_4$ cylindrical gels. Liver plasma membranes (0.4 mg/mL) were preincubated with ^{125}I-labeled glucagon-NAPS (1.0×10^{-8} M, specific activity 3.0×10^6 cpm/pmol) in the presence (heavy line, panels A and B) and absence of 10^{-6} M native glucagon and irradiated as described in the legend to Figure 3. The ^{125}I counts per minute profile was obtained from 2-mm sliced gels. (A) Membranes electrophoresed without reduction; (B) membranes electrophoresed after reduction and alkylation; (C) membranes irradiated in the presence of 0.1 mM GTP, reduced; (D) receptor fraction of Ultrogel-chromatographed irradiated plasma membranes solubilized with 1% (w/v) Lubrol-PX, after precipitation with ammonium sulfate, electrophoresed with (light line) or without (heavy line) reduction.

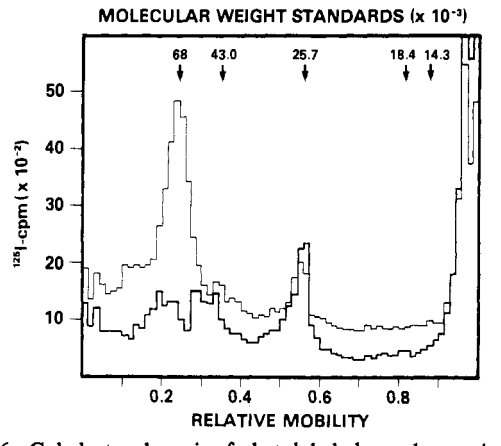

FIGURE 6: Gel electrophoresis of photolabeled membranes in 12.5% acrylamide/NaDodSO$_4$ cylindrical gels. Liver plasma membranes (0.6 mg/mL) were preincubated with ^{125}I-labeled glucagon-NAPS (1.7×10^{-8} M, specific activity 3.0×10^6 cpm/pmol) in the absence (light line) and presence (heavy line) of 10^{-6} M unlabeled glucagon-NAPS and subsequently irradiated and electrophoresed as described in the legend to Figure 3. The ^{125}I counts per minute profile was obtained from 1-mm sliced gels.

total) is removed by membrane centrifugation before electrophoresis; less radioactivity is associated with these bands in reduced samples and in the presence of excess native glucagon (Figures 3, 5, and 6); their relative mobility changes when membranes are electrophoresed without reduction (Figure 4, lanes F and H).

The minor bands of 27 000–28 000 and 46 000 molecular weight were not displaced in the presence of excess unlabeled peptide. The 27 000–28 000-radiolabeled band had a different mobility (Figure 3) than the band which appeared in this region when ^{125}I-labeled glucagon-NAPS was photolyzed in the absence of plasma membrane (Figure 2).

Photolabeling of plasma membranes under the adenylate cyclase assay condition or in 30 mM Tris-HCl buffer, pH 7.0, resulted in the radiolabeling of the same membrane components (Figure 3 vs. Figure 4). When 0.1 mM GTP, however, was included in either of the media, there was a reduction of label incorporation especially in the 50 000–55 000 molecular weight band (Figure 4, lanes G and H, and Figure 5). Gpp(NH)p did not seem to have any effect. This finding requires further investigation.

When trypsinized membranes were preincubated with ^{125}I-labeled glucagon-NAPS and then irradiated, although 18% of radiolabeled peptide remained bound to the membranes after the GTP dissociation step, no label was incorporated into any of the specifically or nonspecifically labeled membrane proteins seen in nontrypsinized membranes (Figure 4, lane E).

Reduction of nitrenes with 50 mM DTT prior to irradiation inhibited covalent incorporation into specifically labeled bands by more than 85%, into the 46 000-dalton bands by about 50%, and into the 27 000–28 000-dalton bands by about 15% (data not shown).

Covalent labeling in the absence of GTP and at 7.0×10^{-10} M ^{125}I-labeled glucagon-NAPS of the 52 000–70 000 molecular weight band(s) was approximately 10–15% as calculated from sliced cylindrical gels, corresponding to 0.15 ± 0.02 pmol of bound ligand per mg of membrane protein applied on the gels. This amount, however, may be underestimated since both autoradiography and protein staining showed aggregates on top of the gels. In nonirradiated membranes and in irradiated membranes which were incubated with prephotolyzed ^{125}I-labeled glucagon-NAPS, less than 0.10 ± 0.05% of total bound peptide (32–35% of total ligand was bound to membrane at 1.7×10^{-8} M ^{125}I-labeled glucagon-NAPS) was associated with the specifically labeled bands. Similarly, irradiation in the presence of ^{125}I-labeled glucagon (10^{-8} M) resulted in about 0.6–1.0% incorporation of total bound ligand most likely as the result of ^{125}I migration during irradiation. The pattern of radiolabeled bands did not change when BSA was omitted from the incubation medium of irradiated membranes or when membranes were irradiated for a longer time, up to 10 min. In the latter case, however, there was a progressive reduction of label incorporation with time of irradiation as the result of photolytic decomposition of ^{125}I-labeled peptides (Demoliou & Epand, 1980) (data not shown).

Receptor Solubilization. The elution profile of covalently labeled membranes with ^{125}I-labeled glucagon-NAPS solubilized with 1% (w/v) Lubrol (Figure 7) indicated that the glucagon receptor eluted from the gel in the included volume. The receptor was identified on the basis that radioactivity was completely dissociated when nonirradiated membranes were solubilized in the presence of 0.1 mM GTP and eluted in the presence of 0.01 mM GTP or in the presence of excess (10^{-6} M) unlabeled glucagon or glucagon-NAPS. From the elution profile of molecular weight markers used, the receptor was estimated to be 200 000–250 000 daltons. Gel electrophoresis of the covalently labeled fraction identified as the glucagon receptor (Figure 5D) showed that it was composed of the same subunits observed in covalently labeled membranes (Figure 5A,B). Both specifically and nonspecifically labeled bands were observed as well as the same effect of reduction on

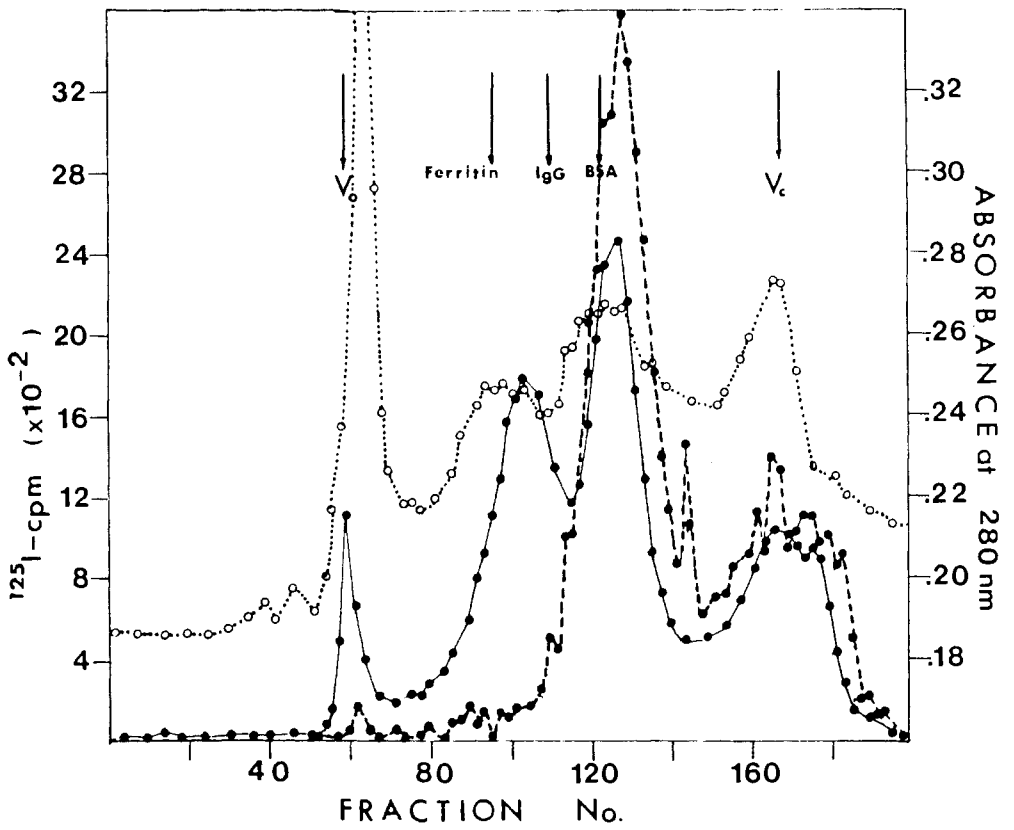

FIGURE 7: Elution profile of Ultrogel AcA22 of detergent-soluble plasma membranes photolabeled with ^{125}I-labeled glucagon-NAPS. Liver plasma membranes (6.0 mg/mL) were preincubated with ^{125}I-labeled glucagon-NAPS (1.2×10^{-8} M, specific activity 3.3×10^6 cpm/pmol), irradiated, and subsequently solubilized with 1% (w/v) Lubrol-PX as described under Materials and Methods. The (100000g) nonpelleted soluble membrane fraction was chromatographed on an Ultrogel AcA22 (1.5 × 90 cm) column. Fractions of 1.0 mL were collected, of which 0.5 mL was counted. (●—●) ^{125}I counts per minute profile of photolyzed membranes solubilized in the presence of 0.1 mM GTP, eluted in the presence of 0.01 mM GTP; (●---●) ^{125}I counts per minute profile of nonirradiated membranes solubilized and eluted in the presence of GTP as above; (○) absorbance at λ 280 nm of elution profile (1.0-mL fraction).

electrophoretic mobility and the same anomalous variation of electrophoretic mobility with percent acrylamide cross-linking of gels.

After preincubation and irradiation and before solubilization, approximately 90–95% of radiolabeled ligand (10^{-9} M) remained bound to the membranes. Lubrol (1% w/v) solubilized 15–20% of the membrane protein; 35–40% of the radioactivity was associated with the soluble fraction. Approximately 30% of that was associated with the peak of the peptide–receptor complex in the elution profile of the Ultrogel (Figure 7).

The elution profile of solubilized membranes preincubated with ^{125}I-labeled glucagon-NAPS which was not irradiated, as well as those of irradiated and nonirradiated membranes preincubated with 10^{-9} M ^{125}I-labeled glucagon was the same in the absence of GTP as those from samples of membranes photolyzed with ^{125}I-labeled glucagon-NAPS. In the presence of the nucleotide, bound peptide was displaced from the peak identified as the receptor from nonphotolyzed samples (as well as from the photolyzed ^{125}I-labeled glucagon sample), in agreement with the results of similar studies of Welton et al. (1977).

Discussion

The major 52 000–70 000 molecular weight components of the glucagon receptor which were covalently photolabeled in membranes (Figure 5A,B) have an identical mobility on the same gel system as the partially purified receptor solubilized by nonionic detergent (Figure 5D). Photoaffinity labeling of these components (Figures 3, 4A, 5A, and 6) is inhibited by native glucagon (Figures 3 and 4B), the glucagon-NAPS agonist (Figure 6), and the partial antagonist N^{α}-(2,4,6-trinitrophenyl)glucagon (Figure 4C) but not by the inactive Asp^{9,15,21}-triglycinamide–glucagonylglycinamide derivative (Figure 4D). GTP specifically inhibits covalent labeling of these proteins in membranes (Figures 4G and 5C) and in the soluble receptor (Figure 7), in agreement with the decrease in glucagon affinity for the receptor in the presence of GTP (Rodbell et al., 1971; Welton et al., 1977). Membrane trypsinization also inhibits covalent labeling of these components, thus demonstrating their protein nature (Figure 4E). All of the above provides convincing evidence that the labeled proteins are components of the glucagon receptor.

The presumably intact receptor complex can be identified as a 200 000–250 000 molecular weight species after Ultrogel chromatography of a photolabeled Luberol-solubilized membrane (Figure 7). This value of the molecular weight may be inaccurate because of the presence of bound detergent and phospholipids (Welton et al., 1978). A similar molecular weight has also been found by other procedures (Blecher et al., 1974; Storm & Chase, 1975). The gel electrophoresis pattern of radiolabeled $NaDodSO_4$-solubilized membranes also identified a labeled molecular weight component >200 000 which was displaced in the presence of native glucagon (Figure 4). It is possible that this component may represent an oligomeric glucagon receptor. Johnson et al. (1981), using a photoaffinity bifunctional reagent, have identified a 53 000 molecular weight component as the glucagon receptor which was GTP sensitive, in agreement with our observation of labeled protein in the 52 000–55 000 molecular weight range. Higher molecular weight components, however, were not reported. We believe that the difference in the higher molecular

weight components may be the result of the extent of reduction of membranes since our samples were treated differently before gel electrophoresis. Similar cases have been reported for the insulin receptor (Jacobs et al., 1979) and the acetylcholine receptor from *Torpedo californica* (Chang & Bock, 1977; Hamilton et al., 177; Witzemann & Raftery, 1978). It is possible that the glucagon oligomeric receptor, like the insulin receptor, is composed of subunits which are linked or greatly stabilized by disulfide bonds. This would explain the altered electrophoretic mobility of the labeled subunits in unreduced preparations. If the stability of the disulfide bonds is essential for binding of glucagon to the receptor, it would also explain part of the inhibition (85%) of radiolabeling of the 52 000–70 000 molecular weight components in the presence of 50 mM DTT which was added to act as a scavenger immediately before irradiation (Staros et al., 1978). We thus believe that the DTT is altering the receptor to prevent photolabeling, rather than acting as a scavenger for the azide, since the largely hydrophobic-modified COOH-terminal region of the bound peptide is not likely to be exposed to the solvent. Storm & Chase (1975) have reported the inhibition of glucagon-stimulated adenylate cyclase in membranes pretreated with sulfhydryl reagents, thus indirectly supporting the fact that these labeled species are part of the glucagon receptor that mediates activation of adenylate cyclase.

Recently, it has been reported that the GTP regulatory protein in rabbit liver is composed of three polypeptides of 35 000, 45 000, and 52 000 molecular weight, all of which are required for guanine nucleotide, fluoride, and hormone-stimulated adenylate cyclase activity in reconstitution studies (Gilman et al., 1981). Since in detergent-solubilized preparations a GTP functionally linked site with adenylate cyclase and the glucagon receptor elute together in gel filtration (Welton et al., 1977; this work), we would like to suggest that the 52 000–55 000 labeled component (Figure 4) may be the subunit of the GTP regulatory protein which may couple glucagon receptor binding to adenylate cyclase stimulation. In this case, the question arises whether this binding site may contribute to the low-affinity–high-capacity sites defined in the glucagon adenylate cyclase and binding studies and in the evidence for distinct GTP sites in the regulation of the glucagon binding (Lad et al., 1977; Welton et al., 1977; Demoliou-Mason & Epand, 1982). This question is under investigation.

Levey (1975) has reported an ^{125}I-labeled glucagon binding component of 24 000–28 000 molecular weight. Bregman & Levy (1977) have also reported the photoaffinity labeling of the glucagon receptor with a molecular weight range of 23 000–25 000, and a similar size component was observed in some membrane preparations in the photoaffinity studies of Johnson et al. (1981). We have also observed the radiolabeling of a similar protein in all irradiated membrane preparations as well as in the detergent-soluble membrane fractions and in the partially purified soluble receptor. In agreement with Johnson et al. (1981), we were not able to displace labeling of this component in the presence of excess native glucagon, glucagon-NAPS, or any of the other derivatives tested. However, the extent of labeling of this component differed in reduced vs. unreduced preparations, and the band displayed anomalous electrophoretic mobility with percent acrylamide cross-linking. Although this component may have nothing to do with the glucagon receptor, it is also possible that it is closely associated with the receptor and is therefore labeled and copurified with it. Alternatively, it may be cross-linked by disulfide bonds to the 52 000–55 000 component, thus resulting in the 60 000–70 000 molecular weight band which we observed to display the same anomalous electrophoretic mobility with percent acrylamide cross-linking and reduction. It is also interesting that labeling of this protein is not greatly inhibited in membranes reduced before irradiation. At present, we cannot explain the lack of displacement of radioactivity from this component in the presence of excess native hormone. One possibility to be considered is that this component may be part of the glucagon receptor displaying high affinity and having the slow dissociation rate constant (half-life, 75 min) reported by Sonne et al. (1978), and therefore, a longer incubation time may be required for hormone binding to reach equilibrium. At present, we are investigating the possible relevance of this component to the glucagon receptor.

We have shown in these studies that ^{125}I-labeled glucagon-NAPS can be used effectively as a photoreactive glucagon agonist to label the glucagon receptor sites, and therefore, it can aid in its further characterization and purification and in the assessment of the biological relevance of the labeled membrane components to hormone binding and adenylate cyclase stimulation.

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Phosphorylation of the Receptor for Immunoglobulin E†

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ABSTRACT: Specific immune precipitation of immunoglobulin E (IgE)–receptor complexes from detergent extracts of ^{32}P-labeled rat basophilic leukemia cells yielded a phosphoprotein of M_r ~35 000 on gel electrophoresis in sodium dodecyl sulfate. This phosphoprotein was shown by several criteria to be the β chain of the receptor for IgE. Phosphorylation occurs at a serine residue (or residues) in a region (β_2) of the β chain that is thought to be exposed on the cytoplasmic face of the plasma membrane. Our results suggest that phosphorylation probably takes place after the insertion of the β chain into the membrane. The IgE-binding α chain of the receptor and the IgE associated with it are not phosphorylated. We have so far been unable to detect any changes in the state of phosphorylation of either chain of the receptor or of IgE itself after IgE-mediated triggering of the cells.

Mast cells and basophils have on their surfaces a receptor which binds immunoglobulin E (IgE)[1] with high affinity. This receptor is made up of two polypeptide chains denoted α and β (Metzger et al., 1982). The α chain of the receptor binds IgE and is a glycoprotein with a molecular weight of about 50 000. The recently identified β chain of the receptor has a molecular weight in the region of 35 000 and does not appear to be exposed on the outside of the plasma membrane.

When receptors for IgE are aggregated either indirectly by using specific antigen or anti-IgE which cross-link via IgE (Ishizaka & Ishizaka, 1971) or directly by using antibodies to the receptor itself (Ishizaka & Ishizaka, 1978; Isersky et al., 1978), the cells are stimulated to secrete histamine, serotonin, and other mediators of immediate hypersensitivity by a noncytoxic, calcium-dependent mechanism (Mongar & Schild, 1958). The sequence of biochemical events occurring between receptor activation and the final secretory response still remains to be elucidated, although a number of potential intermediate steps have been described. These include activation of a serine esterase (Becker & Austen, 1966), phospholipid methylation (Ishizaka et al., 1980), adenylate cyclase activation, changes in cyclic nucleotide levels and activation of phosphoprotein kinases (Holgate et al., 1980a,b), phosphatidylinositol turnover (Cockcroft & Gomperts, 1979), and an increase in membrane permeability to calcium (Foreman et al., 1973). In addition changes in protein phosphorylation have been observed during secretion from mast cells (Sieghart et al., 1978).

The role played by the receptor itself in any of these processes is entirely unknown. Furthermore, designing appropriate experiments to determine the way in which this receptor might function is not straightforward. One obvious approach is to search for structural changes in the receptor molecule which occur on stimulation, and the present study was one of several initiated for that purpose. The demonstration that the nicotinic acetylcholine receptor can be phosphorylated (Gordon et al., 1977a,b; Teichberg et al., 1977) and the important role of protein phosphorylation in a variety of regulatory mechanisms (Krebs & Beavo, 1979) encouraged us to examine the state of phosphorylation of the receptor for IgE in rat basophilic leukemia (RBL) cells. IgE-mediated secretion from these tumor cells is similar in all important respects to that from normal mast cells and basophils (Fewtrell & Metzger, 1981), and the availability of large numbers of pure cells from tissue culture makes this a particularly attractive system for such studies.

A preliminary account of some of these results has already been given (Fewtrell et al., 1981).

Materials and Methods

IgE, IgE Derivatives, and Anti-IgE Antibodies. Rat monoclonal IgE (IR162) and human monoclonal IgE (PS) were purified as described previously (Kulczycki & Metzger, 1974). A mouse hybridoma anti-dinitrophenyl (DNP) IgE selected and characterized by Liu et al. (1980) was isolated and purified as described previously (Holowka & Metzger, 1982). Iodinated and amidinated IgE's were prepared as described by Kulczycki & Metzger (1974) and Holowka et al. (1980), respectively. Iodination of mouse IgE by the Chloramine T

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[1] Abbreviations: RBL, rat basophilic leukemia; IgE, immunoglobulin E; $NaDodSO_4$, sodium dodecyl sulfate; DTBP, dimethyl 3,3′-dithiobis(propionimidate); DNP, dinitrophenyl; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.