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SPECIFICITY OF COVALENTLY STABILIZED COMPLEXES OF ^{125}I-LABELED HUMAN SOMATOTROPIN AND COMPONENTS OF THE LACTOGENIC BINDING SITES OF RAT LIVER

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^{125}I-labeled human somatotropin specifically bound to the lactogenic sites of microsomal membranes from pregnant rat liver, originated a radioactive covalent complex of M_r 63,000 upon reaction with dimethyl suberimidate, disuccinimidyl suberate (DSS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The amount of this species was closely parallel with the preexisting amount of the ligand-receptor complex. Photoactivation of a hormone derivative bound to the receptor also gave rise to the 63 K species. A ternary complex of receptor, hormone and Triton X-100 cross-linked with DSS yielded the 63 K species and a new one of 96 K. The data indicate that the 63 K complex involves the radioactive hormone and a constituent of the binding site. The 96 K species could comprise a second component of the receptor.

Lactogenic binding sites have been identified in a variety of tissues (1), and it is well documented the regulatory action of prolactin and other lactogenic hormones, on the synthesis of milk proteins by the mammary gland (2). The action of these hormones on the liver is less clear although it has been reported that they increase RNA synthesis and polyribosome formation (3), somatomedin production (4) and ornithine decarboxilase activity (5,6).

Growing evidence exists (7,8) relating the interaction of the ligand with the receptor and the triggering of the response. Recently, Rosa et al. (9) showed that an antiserum raised against a partially purified prolactin-binding protein is able to elicit an up-regulatory effect when acting on the lactogenic receptors of cultured liver cells.

The abbreviations used are: hGH, human somatotropin; [^{125}I]hGH, ^{125}I-labeled hGH; [^{125}I]AP- hGH, derivatized hGH by succesive reaction with 3,3'-dithiobispropionimidate, dithiothreitol and p-azidophenacyl bromide, and subsequently ^{125}I-labeled; DMS, dimethyl suberimidate.2 HCl; DSS, disuccinimidyl suberate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide.HCl; DFPS, 4,4'-difluoro-3,3'-dinitrophenyl sulfone; Bicine, N,N-bis(2-hydroxyethyl)glicine; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; SDS,sodium dodecyl sulfate; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of 0.1% (w/v) SDS.

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The events subsequent to the ligand interaction and the structure of the receptor itself are not known. An active search for their constituents was conducted in several target tissues (10-13), based on the formation of covalent complexes with the radioactive ligand. Cross-linking, has the potential ability to recognize constitutents of the receptor which do not interact directly with the ligand, hence it becomes important to discriminate whether a covalent complex involves part of the receptor or was formed with a membrane component fo reign to it.

To obtain evidence on the specificity of the covalent complexes we studied the cross-linking of [^{125}I]hGH with the lactogenic receptors present in rat liver using four different cross-linking reagents, and a novel photoactivable derivative of the hormone.

MATERIALS AND METHODS

Human and bovine somatotropins were prepared as indicated elsewhere (14); ovine prolactin (NIH-P-S-3;15 i.u./mg) was obtained from N.I.H., N.I.A.M.D.D. Hormone Distribution program, Bethesda, MD, U.S.A. Bovine serum albumin, suberic acid, N-hydroxysuccinimide and Triton X-100 were obtained from Sigma and p-azidophenacyl bromide, dimethyl suberimidate.2 HCl, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide.HCl and 4,4'-difluoro-3,3'-dinitrophenyl sulfone were purchased from Pierce Chemical Co. Na^{125}I was from New England Nuclear. Molecular weight standards (phosphorilase b, bovine serum albumin, ovalbumin, trypsinogen and β-lactoglobulin) were obtained from Sigma and BioRad Laboratories. Disuccinimidyl suberate was prepared from suberic acid and N-hydroxysuccinimide in the presence of N,N'-dicyclohexylcarbodiimide in dioxane (15). All other chemicals were of A.R. quality.

Preparation of a photoreactive derivative of hGH: Modification of hGH (1 mg) with 3,3'-dithiobispropionimidate was carried out as described elsewhere for the bovine hormone (16). After dialysis against cold distilled water, disulphide bridges were partially reduced (17) by adding 50 µl of 1 M-Bicine/NaOH, 0.05 M-glycine, pH 8.5 and 10 µl of 0.1 M-dithiothreitol, letting the reaction to proceed, under nitrogen, for 1.5 h at room temperature. Then, under yellow safety light, 50 µl of p-azidophenacyl bromide (6 mg/ml in methanol) were incorporated and after 30 min the reaction mixture was dialyzed in the cold against 1 mM-Bicine/NaOH, pH 7.5 and freeze-dried. The ability of this derivative to compete with [^{125}I]hGH for microsomal binding sites was estimated in 40% of that shown by the native hormone.

Binding of ^{125}I-labeled ligands to microsomal fraction or detergent solubilized membranes: Fractionation of pregnant rat (Long-Evans strain) liver membranes, Triton X-100 solubilization of the microsomal fraction and radioiodination of hormonal proteins were effected as previously described (14), except that the amino-containing buffers were replaced by 25 mM-Bicine/NaOH (or 25 mM-Hepes/NaOH), pH 7.5. Aliquots of the microsomal fraction (0.6-1.2 mg of protein) or solubilized membranes (1 mg of protein) were incubated with 1.3-10 ng of either [^{125}I]hGH or [^{125}I]AP-hGH (1.2×10^6dpm) in a total volume of 0.5 ml, with or without unlabeled hormone, and binding of the radioactive ligand was allowed to proceed for 15 h at 25°C. The incubation medium consisted of 25 mM-Bicine/NaOH (or Hepes/NaOH), 10 mM-$CaCl_2$ and 0.1% bovine serum albumin. Four tubes were prepared for each particular condition, one of which was saved for cross-linking. The rest served to estimate the radioactivity bound (14).

Cross-linking procedures: The preincubated membrane preparations were brought to the desired pH by addition of 0.1 M-NaOH or 0.1 M-HCl and diluted with buffer, afterwards varying amounts of stock solution of the cross-linking reagent were incorporated. Total volume was 1 ml. The following specific conditions were employed: 50 mM-Bicine/NaOH, pH 8.5, stock solution 90 mM-DMS in buffer, reaction for 1 h at room temperature; 50 mM-Hepes/NaOH, pH 5.5, stock solution 10 mM-EDC in buffer, reaction at room temperature for 20 min; 20-25 mM Bicine/NaOH, pH 7.5 plus 10% (v/v)-acetone, stock solution 10 mM-DSS in acetone, reaction at 0°C for 30 min; and 50 mM-Bicine/NaOH, pH 7.5 plus 10% (v/v)-acetone, stock solution 3 mM-DFPS in acetone, reaction at room temperature for 12 h. Reactions were terminated by addition of an excess of glycine, followed by either dilution with 4 ml of buffer and centrifugation -in the case of microsomal samples- or freeze-drying -for the solubilized extracts-.

Photolysis of [^{125}I]AP-hGH: The samples were transferred to 3 ml quartz cuvettes and these were placed in a dessicator where dissolved air was eliminated by alternatively applying partial vacuum and restoring atmospheric pressure with nitrogen. After chilling the samples in an ice bath, irradiation took place in a Desaga UVIS apparatus set at 254 nm at 3 cm from each lamp. Then the membrane suspensions were diluted with 4 ml of 25 mM-Bicine/NaOH, pH 7.5 and centrifuged.

Electrophoretic analysis: Polyacrylamide gel electrophoresis in the presence of SDS was performed according to the method of Laemmli (18), by using a linear gradient of polyacrylamide concentration from 7 to 15%. After staining and destaining, the gels were dried in a Pharmacia GSD-4 gel drier and exposed for autoradiography -using Kodak Blue Brand film in combination with Dupont Cronex Hi-Plus intensifying screen (19)- for 2-7 days.

RESULTS

Microsomal preparations of pregnant rat liver membranes with a fraction of their lactogenic binding sites occupied by [^{125}I]hGH were subjected to reaction with different cross-linking agents, and the products analyzed by SDS-polyacrylamide gel electrophoresis. The radioactive species, visualized by autoradiography, are shown on Fig. 1. The homobifunctional reagents DMS and DSS, and the water-soluble carbodiimide EDC, yielded a complex of a molecular weight estimated in 63,000 (63 K). The 63 K band was not formed by omitting the cross-linking reagent or by incorporating an excess (1 µg) of either hGH or ovine prolactin in the incubation media. In the range shown in Fig. 1, the intensity of the 63 K band increased following increments of the reagent concentration. These increments also resulted in the formation of higher molecular weight radioactive species and radioactive aggregated material, that remained on the top of the gel. Omission of the reducing agent during the sample preparation previous to SDS-PAGE did not produce qualitative changes, though more aggregated material was found on the top, concomitant with a fainter radioactivity pattern. Reaction with DFPS did not cause the formation of distinct radioactive bands,

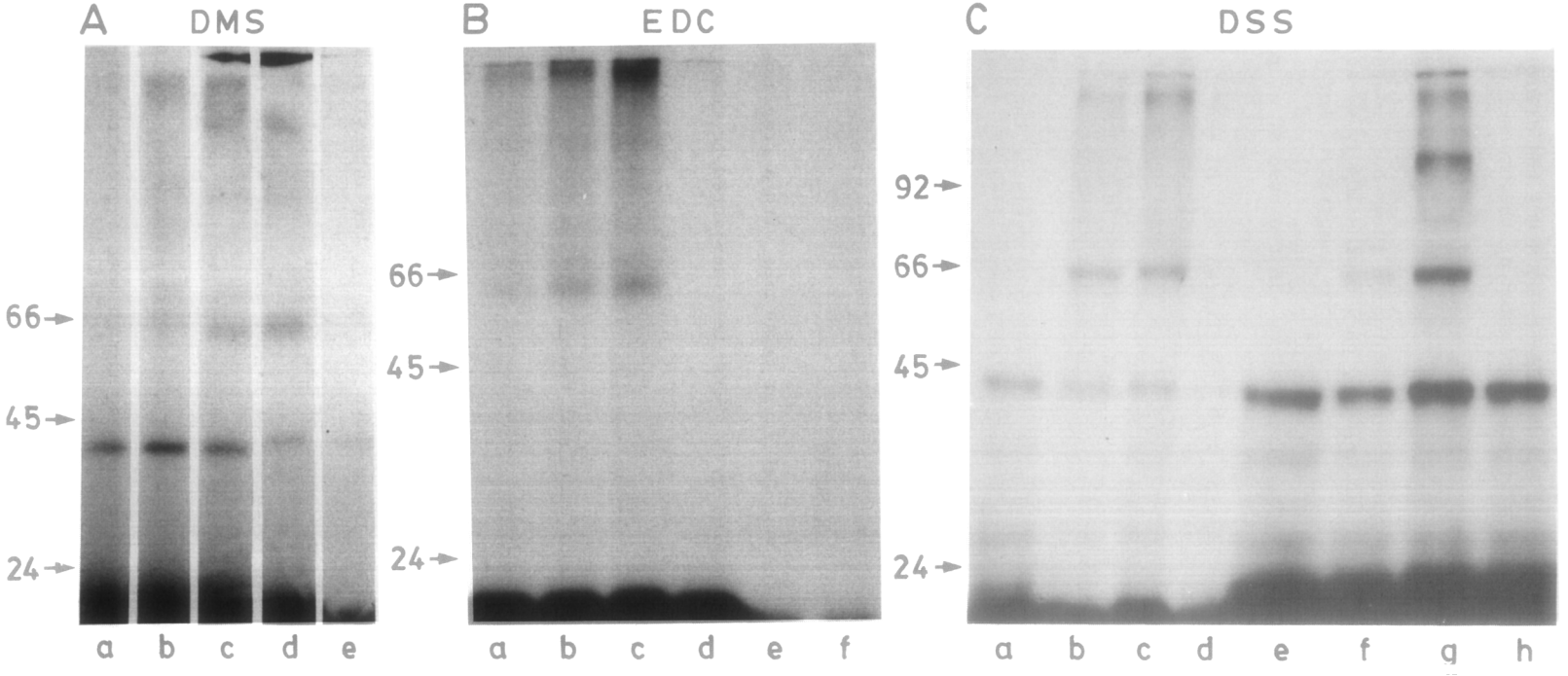

Fig. 1. SDS-PAGE of the cross-linking products of [^{125}I]hGH bound to rat liver membranes or solubilized preparations. Autoradiograms of stained, destained and dried slab gels (7-15% gradient in polyacrylamide). Panel A: microsomal membranes (1.2 mg protein) incubated with [^{125}I]hGH (3.2 ng) in the absence (a through d) or in the presence of unlabeled hGH (1 µg)(e) and cross-linked with DMS at a final concentration of 0 (control) (a), 0.9 (b), 3.6 (c) and 18 mM (d,e). Panel B: microsomal membranes (1.2 mg protein) incubated with [^{125}I]hGH (3.4 ng), in the absence (a through d) or in the presence of either hGH (1 µg)(e) or ovine prolactin (1 µg)(f) and exposed to EDC at the following concentrations: 0 (control)(d), 0.12 (a), 0.25 (b) and 0.5 mM (c,e,f). Panel C: microsomal membranes (1.2 mg protein)(a through d) or Triton X-100 solubilized membranes (1.0 mg protein) (e through h) incubated with [^{125}]hGH (3.2 ng), in the absence (a,b,c,e,f,g) or in the presence of hGH (1 µg)(d,h) and treated with DSS at the concentrations: 0 (control)(a,e), 0.3 (b,f) and 1 mM (c,d,g,h). Samples were reduced by heating at 100°C in the presence of 0.35 M mercaptoethanol and 2% (w/v) SDS, and the aliquots applied to the gels were of 250 µg of protein for solubilized extracts or 300 µg for microsomal preparations. Numbers on the left side represent $M_r \times 10^{-3}$.

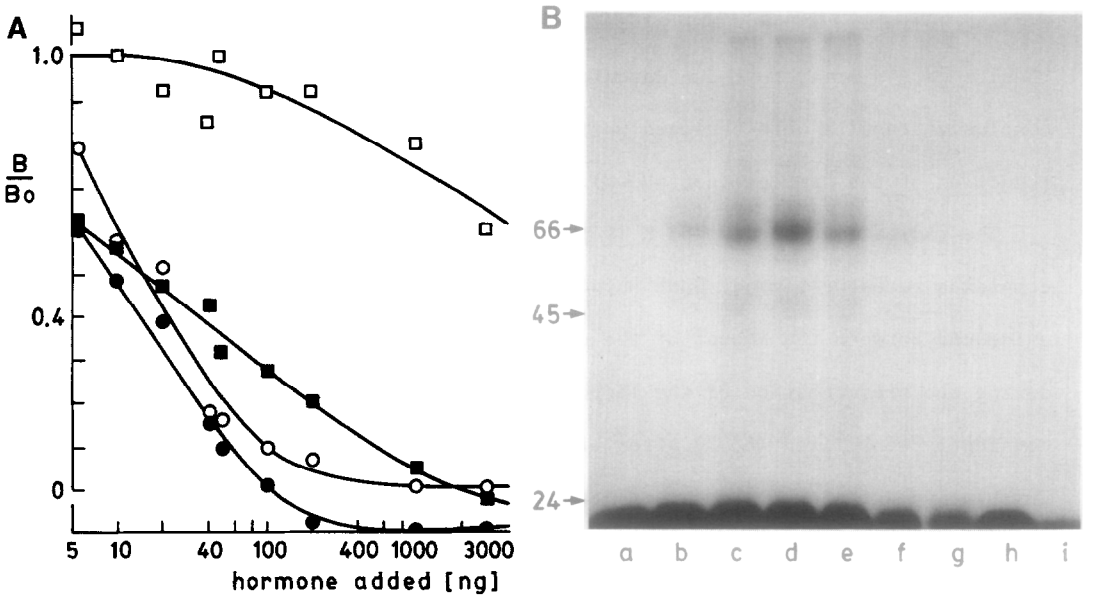

Fig. 2. Specificity of the binding to microsomal sites of the photoactivable derivative of hGH. Panel A: Aliquots of microsomal fraction (0.6 mg protein) were incubated with [^{125}I]AP-hGH (1.5 ng) in the presence of increasing amounts of unlabeled derivative (o), hGH (●), ovine prolactin (■) and bovine somatotropin (□). The ordinate axis represents the ratio of the radioactivity bound to that bound in the absence of unlabeled hormone, both corrected for non-specific binding (radioactivity bound in the presence of an excess (3 µg) of unlabeled derivative). Panel B: Samples (1.2 mg protein, 3.6 ng [^{125}I]AP-hGH) exposed to ultraviolet light (wavelength: 254 nm) and analyzed by SDS-PAGE (7-15% polyacrylamide), followed by autoradiography. Dark incubations were carried out in the absence (a through d) or in the presence of 20 (e), 100 (f) and 3000 ng (g) of unlabeled hGH, and the subsequent iradiation period was extended for 90 (b), 180 (c) or 300 sec (d,e,f,g). Lane (a) shows a control lacking photolysis. Lanes (h) and (i), included for comparison, are equivalent to (d) and (g), but replacing [^{125}I]hGH for the photoreactive derivative. Aliquots containing 300 µg of protein, reduced with mercaptoethanol, were applied. Numbers on the left side of panel B represent $M_r \times 10^{-3}$

although it cross-linked membrane proteins, as inferred by changes in their distribution in stained gels (results not shown).

The (non-covalent) complexes of the photoactivable derivative [^{125}I]AP-hGH with the lactogenic receptors of the microsomal fraction also gave rise to the 63 K species, with a relatively high yield (17%), upon exposition to short wavelength light (Fig.2). Radioactivity in the high molecular weight range or in aggregated material was nearly absent. Lack of irradiation, or incubation in the presence of an excess of native hormone prevented the formation of the 63 K band. Irradiation of [^{125}I]hGH complexes also failed to produce the band.

The products resulting from reaction of the complexes of [^{125}I]hGH and Triton X-100 solubilized receptors with DSS were also examined (Fig.1). A band of

radioactivity was seen at the position corresponding to M_r 96,000, otherwise results were similar to those described for the microsomal preparation. Less conclusive results were obtained when analysis by SDS-PAGE was not preeceded by reduction of the samples (not shown).

The radioactive species 63 K is presumably generated from the non-covalent complexes receptor-ligand. Such supposition is based on the close parallelism evidenced between the amount of the complex [^{125}I]hGH-lactogenic receptor formed during the preincubation of the microsomal fraction and the amount of complexes covalently stabilized (63 K) after cross-linking with DSS as shown in Fig.3. This included the complete disappearance of the 63 K band in the cases in which the preincubation was performed with large amounts of displacing hormone (1 and 3 µg).

DICUSSION

Several experimental results indicate that a membrane component which forms a covalent complex of M_r 63,000 with [^{125}I]hGH on cross-linking, is associated with a high affinity lactogenic site. Cross-linking reagents (including EDC, DMS and DSS) endowed with different chemical specificity formed a covalent species of 63 K. Also, a photoactivable derivative of the hormone yielded a similar species after irradiation of the preformed ligand-receptor complexes. In all these cases preincubation in the presence of an excess of hGH (or prolactin) prevented the generation of the radioactive covalent complex. Addition of increasing amounts of unlabeled hormone to the incubation media disclosed the existence of a close parallelism between the amount of complexes ^{125}I hGH-receptor and the amount of complexes covalently stabilized by reaction with DSS, and thereby that the high affinity sites evidenced by the binding studies (14, 20) are related with the cross-linked product. Possibly this is formed by the labeled hormone and a subunit (or part of it) of the membrane receptor, for which a M_r 77,800 has been proposed (21). Although the possibility that the hormone could establish a covalent link with a molecule foreign to the binding site but close to it cannot be completely ruled out, the following observations do not favor such hypothesis. Reaction of "zero length" cross-linkers, such as

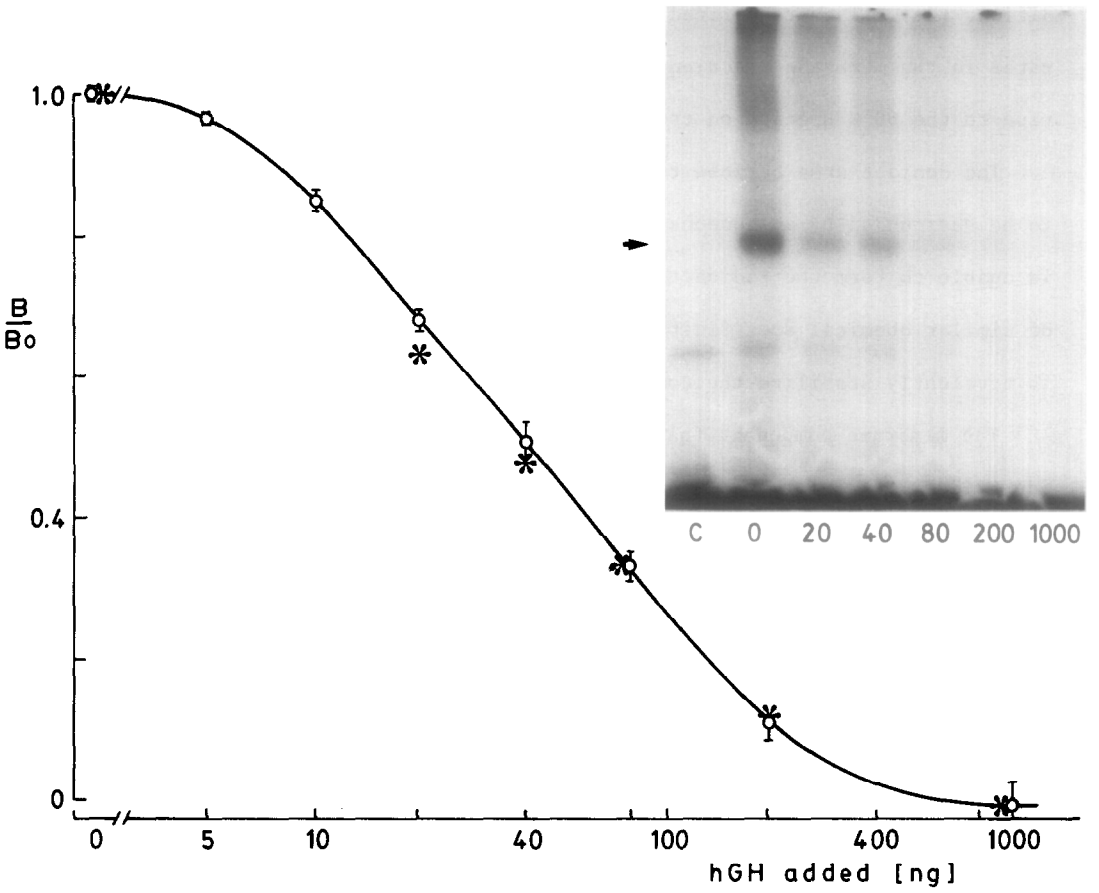

Fig. 3. Comparison of the ability of unlabeled hGH to compete with [^{125}I]hGH for the binding to microsomal sites and to prevent the formation of radioactive, covalently stabilized, complexes of M_r 63,000. Samples of microsomal fraction containing 1.2 mg of protein were incubated with [^{125}I]hGH in the presence of increasing amounts of cold hormone. The fraction of radioligand (non-covalently) bound to microsomal membranes (o) was evaluated as indicated in Fig. 2. Samples simultaneously prepared and cross-linked with 1 mM DSS were analyzed by SDS-PAGE (7-15% polyacrylamide), and after autoradiography (inset), strips containing the radioactive band of M_r 63,000 (indicated by the arrow) were cut from the dried gel. The symbol (*) represents the ratio of the radioactivity found in the 63 K band to the radioactivity present in such position in the lane (0) (inset), corresponding to the sample devoid of unlabeled hormone. Numbers under the inset indicate the amounts of unlabeled hormone, in ng, present during the incubation and (C) is a control without DSS; the ordinate scale represens M_r x 10^{-3}. Aliquots applied to the gel (300 µg protein) were previously reduced with mercaptoethanol.

carbodiimides, are dependent upon a preexisting favorable orientation of the reacting groups (22,23); thus the high efficiency of EDC (4-8 molecules of EDC per molecule of protein of mean M_r 40,000) to generate detectable amounts of the 63 K species could be interpreted as a consequence of the close contact, involving salt bridges, between the hormone and the membrane component. In ad-

dition, the well characterized hormone-receptor-detergent complexes (21) generated in the membrane fraction solubilized with Triton X-100 (14), also gave rise to the 63 K species on cross-linking with DSS.

The contact area between the ligand and the membrane component is believed to be surrounded by an aqueous environment since DFPS, quite insoluble in water, is unable to form the radioactive 63 K species, in contrast with DMS and DSS, of similar chemical specificity and maximal reaction distance, which are able to covalently stabilize the complex.

The membrane component that binds [^{125}I] hGH does not appear to form disulphide bridges, at least not in all cases, with other protein since non-reduced preparations of cross-linked products also evidenced a radioactive band of M_r 63 K. The 96 K band visualized on cross-linking the [^{125}I] hGH-solubilized receptor complex could correspond to a second subunit, made accesible by the action of the detergent, and covalently bound to either the labeled hormone or the 63 K complex; but, although this species fulfills the simplest specificity criteria, further investigation is required. The hGH binding sites probably contain carbohydrates, since solubilized preparations are adsorbed to Con-A Sepharose columns and desorbed by methylglycosides, consequently the M_r estimated for the covalent complexes should be regarded as tentative.

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