Analysis of hepatic growth hormone binding sites of pregnant rabbit crosslinked to ^{125}I-labelled human growth hormone

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Received 2 July 1982; revision received 19 August 1982

Crosslinking *hGH receptor* *Subunit structure* *Rabbit liver* *Disuccinimidyl suberate* *Immunoprecipitation*

1. INTRODUCTION

We have reported a specific receptor site for human growth hormone (hGH) on liver membrane fractions from pregnant rabbits [1]. The hGH receptor has been solubilized with the non-ionic detergent Triton X-100, and its characteristics have been studied [2–4]. However, little is known about its chemical composition and subunit structure.

In [5] a crosslinking of ^{125}I-insulin to its receptor was described using disuccinimidyl suberate (DSS), a new non-cleavable reagent for crosslinking. Under mild conditions DSS can react rapidly with free amino groups. This affinity crosslinking technique in combination with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) revealed that the insulin receptor on rat adipocytes [6] or IM-9 lymphoblastic cells [7] is composed of insulin binding subunits. Similarly, ^{125}I-human chorionic gonadotropin (hCG) was covalently crosslinked to its receptor on rat testis [8], and the quaternary structure has been analyzed. Thus, the crosslinking of an isotopically labelled hormone to the receptor using the succinimidyl ester proved to be very useful for studying receptor proteins.

Here, we describe the crosslinking of ^{125}I-hGH to the hepatic receptor of pregnant rabbits and present an analysis of the complex. We show that the hGH receptor involves a binding subunit of M_r 59 000.

2. MATERIALS AND METHODS

2.1. *Hormones and reagents*

Human growth hormone (HS 1652 C, 2.0 U/mg) was a gift from the National Pituitary Agency (NIAMDD, NIH). Radio-iodination of hGH was performed as in [3], and ^{125}I-hGH spec. act. was ~ 100 mCi/mg. Na^{125}I was purchased from New England Nuclear (Boston MA). DSS was a product of Pierce Chemicals (Rockford IL). Poly(ethylene glycol) 6000 was purchased from Nakarai Chemicals (Tokyo). Sepharose 6B, protein-A–Sepharose CL-4B, and concanavalin A–Sepharose were obtained from Pharmacia (Uppsala).

2.2. *Preparation of membrane fractions*

Crude membrane fractions were prepared from the livers of pregnant rabbits (New Zealand White, 25–29 days pregnant) as in [1,3]. The fractions were stored at –20°C until use.

2.3. *Crosslinking of ^{125}I-hGH to the membranes*

The crude membranes (1–2 mg protein/ml) were incubated with 5 nM (110 ng/ml) ^{125}I-hGH for 6 h at 20°C in 1.0 ml 50 mM Tris–HCl buffer (pH 7.4) containing 10 mM $MgCl_2$ and 0.1% bovine serum albumin (BSA). At the end of incubation, 3 ml ice-cold Tris–HCl buffer was added and the tubes centrifuged at 3000 × g for 30 min at 4°C to separate bound ^{125}I-hGH from unbound. The ^{125}I-hGH bound membranes were then washed twice with ice-cold Krebs–Ringer

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

phosphate (KRP) buffer and suspended in the buffer at 1 mg protein/ml. Then freshly prepared DSS (0.1 M in dimethyl sulfoxide) was added to make 1 mM final conc. as in [5]. Unless otherwise indicated, crosslinking was performed at 20°C for 30 min, and was terminated by the addition of 5 vol. ice-cold 10 mM Tris, 1 mM EDTA (pH 7.4). The mixture was centrifuged for 30 min at 10 000 × *g* and the pellet washed twice with ice-cold KRP buffer. The dissociation of ^{125}I-hGH from DSS-treated ^{125}I-hGH-bound membranes was determined by the exposure of the membranes to an excess of unlabelled hGH (10 μg/ml), 0.1 N HCl or 5.0 M urea.

2.4. *Solubilization and partial purification of ^{125}I-hGH crosslinked receptor*

^{125}I-hGH crosslinked membranes were solubilized for 60 min at 20°C in 50 mM Tris–HCl buffer (pH 7.4) containing 1% (v/v) Triton X-100, 100 U bacitracin/ml and 500 U aprotinin/ml. Protein concentrations were adjusted to 1–2 mg/ml. The mixture was then centrifuged for 90 min at 100 000 × *g* and the insoluble pellet was discarded. The supernatant was gel-filtered on a Sepharose 6B column (1.5 × 85 cm) at 10 ml/h at 4°C to separate the ^{125}I-hGH:receptor complex from unbound ^{125}I-hGH. The fractions containing ^{125}I-hGH:receptor complex were pooled and subjected to an affinity chromatography on a concanavalin A–Sepharose column (2 × 4 cm) pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.4) containing 0.1% (v/v) Triton X-100 (Tris–Triton buffer). The flow rate was adjusted to 10 ml/h. The column was washed with Tris–Triton buffer at 4°C until no radioactivity was eluted. Elution of the ^{125}I-hGH:receptor complex was achieved with 2 bed vol. of 0.3 M α-methyl-D-glucoside in Tris–Triton buffer at 4°C.

2.5. *Polyacrylamide gel electrophoresis*

^{125}I-hGH crosslinked membranes were solubilized by heating them at 100°C for 5 min in 50 mM Tris–HCl buffer (pH 6.8) containing 10% (v/v) glycerol, 1% sodium dodecyl sulfate (SDS), and 100 mM dithiothreitol. After centrifugation at 10 000 × *g* for 20 min, 50–100 μl supernatant (10 000–20 000 cpm) was applied to a 7.5% polyacrylamide slab gel with a 3% stacking gel, and electrophoresed with the discontinuous buffer system of [9]. After staining the gels with 0.15% Coomassie blue dissolved in 50% trichloroacetic acid and then destaining them with 7% (v/v) acetic acid, they were sliced into 1 mm-thick slices and assayed for radioactivity in a gamma counter. Alternatively, the gels were dried and subjected to autoradiography. The M_r-values of the standards are: filamin (250 000); myosin (200 000); β_1 and β_2 subunits of RNA polymerase (165 000 and 155 000); phosphorylase B (94 000); bovine serum albumin (67 000); and ovalbumin (43 000).

2.6. *Immunoprecipitation of ^{125}I-hGH:receptor complex*

An aliquot of the solubilized and partially purified ^{125}I-hGH:receptor complex was incubated for 18 h at 4°C with several dilutions of rabbit anti-hGH serum in 1 ml of 50 mM Tris–HCl buffer (pH 7.4) containing 0.1% BSA and 0.1% (v/v) Triton X-100. Then 0.1 ml goat anti-rabbit IgG serum or 10 mg protein A–Sepharose CL-4B was added and the tubes incubated a further 2 h at 4°C. The mixture was centrifuged for 20 min at 3000 × *g* and the radioactivity of the pellet was determined.

3. RESULTS AND DISCUSSION

DSS was found to be effective in crosslinking ^{125}I-hGH to the membrane fractions of rabbit livers (table 1). After exposing ^{125}I-hGH bound membranes to 1 mM DSS for 30 min at 20°C, the subsequent dissociation of ^{125}I-hGH was strongly inhibited. Only 30% of the initially bound ^{125}I-hGH was dissociated by 0.1 N HCl from the DSS-treated ^{125}I-hGH-bound membranes, while 90% was removed from the membranes not treated with DSS. The extent of crosslinking increased as a function of [DSS] (fig.1). Exposure to 1 mM DSS for 20–30 min was optimum. Under the conditions, ≥50% of the membrane-bound ^{125}I-hGH was covalently crosslinked to its receptor as judged by the dissociability of ^{125}I-hGH when exposed to HCl or urea. If the membranes were incubated with ^{125}I-hGH in the presence of an excess (10 μg/ml) of unlabelled hGH, no appreciable crosslinking of ^{125}I-hGH was observed.

The ^{125}I-hGH crosslinked membranes were solubilized with 1% (v/v) Triton X-100 as in [3]. About 60% of the total membrane proteins and

Table 1

Dissociation (%) of ^{125}I-hGH from liver membrane fractions

Treatment		Control	DSS-treated
hGH, 10 μg/ml	120 min at 30°C	70 ± 5	18 ± 3
HCl, 0.1 N	10 min at 4°C	88 ± 2	29 ± 4
Urea, 5.0 M	10 min at 4°C	80 ± 5	25 ± 5

Membrane fractions were incubated with ^{125}I-hGH in the presence or absence of unlabelled hGH (10 μg/ml), washed, and then treated with 1 mM DSS for 30 min at 20°C. For control, membranes were incubated with ^{125}I-hGH in the same manner, but treated with dimethyl sulfoxide alone (final conc. 1%). The dissociation of ^{125}I-hGH from the treated membranes was tested by exposure to the agents indicated. ^{125}I-hGH associated membranes were incubated with an excess of unlabelled hGH in 1 ml 50 mM Tris–HCl buffer (pH 7.4) containing 0.1% BSA. Ice-cold Tris–HCl buffer (4 ml) was then added and the tubes centrifuged to separate the dissociated ^{125}I-hGH. Alternatively, membranes containing bound ^{125}I-hGH were exposed to HCl or urea for 10 min. After the addition of 5 vol. ice-cold Tris–HCl buffer, the membranes were separated by centrifugation. Dissociated ^{125}I-hGH was expressed as the percentages of the initially bound ^{125}I-hGH (the mean ± SD of triplicate determinations)

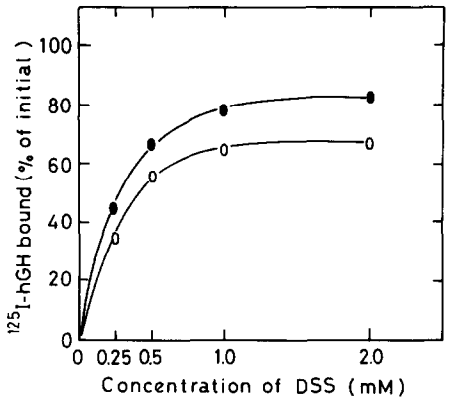

Fig.1. Concentration dependence in crosslinking ^{125}I-hGH to membrane fractions of rabbit livers. ^{125}I-hGH bound membranes were treated with DSS at the indicated concentrations for 30 min at 20°C (•) or 4°C (○). Then the membranes were exposed to 0.1 N HCl for 10 min as described in table 1. Values indicate the percentage of initially bound ^{125}I-hGH not dissociated by HCl (the mean of duplicate determinations).

40% of the membrane-associated radioactivity were solubilized into the 100 000 × g supernatant. When an aliquot of the supernatant was subjected to gel-filtration on a Sepharose 6B column, 3 peaks of radioactivity appeared (fig. 2).The peak appearing in the void volume fractions (peak A) is most likely an aggregate of the ^{125}I-hGH:receptor complex. The second peak (peak B) appeared at the position corresponding to app. M_r 200 000 and represents ^{125}I-hGH crosslinked to the receptor. Peak B ($K_{av.}$ = 0.4) was largely eliminated when the membranes were incubated with ^{125}I-hGH in the presence of an excess amount of unlabelled hGH (10 μg/ml) prior to crosslinking and solubilization. The third peak (peak C) migrated at a position corresponding to that of authentic ^{125}I-hGH.

When peak B fractions of Sepharose 6B column were pooled and applied to a concanavalin A–Sepharose column, ~80% of the radioactivity was adsorbed to the column, and a major part of the adsorbed counts could be dissociated by elution with 0.3 M α-methyl-D-glucoside (not shown). The behavior of ^{125}I-hGH crosslinked receptor in both Sepharose 6B and concanavalin A–Sepharose columns is quite similar to that of its non-crosslinked counterpart [3], indicating that crosslinking using DSS does not cause gross changes in the M_r of the receptor or in its properties as a glycoprotein.

We next tried to determine whether anti-hGH antibodies could bind to ^{125}I-hGH which was covalently crosslinked to the receptor. A solubilized ^{125}I-hGH:receptor complex was incubated with serial dilutions of rabbit anti-hGH serum and then with goat anti-rabbit IgG serum. It can be seen that anti-hGH serum precipitates the ^{125}I-hGH:receptor complex in a dose-dependent manner upon the addition of anti-rabbit IgG (fig. 3). A similar result has been obtained with protein A–Sepharose instead of anti-rabbit IgG (not shown). Immunoprecipitation was strongly inhibited by adding an excess amount of unlabelled hGH prior to the addition of anti-hGH, and normal rabbit serum had no effect in precipitating the

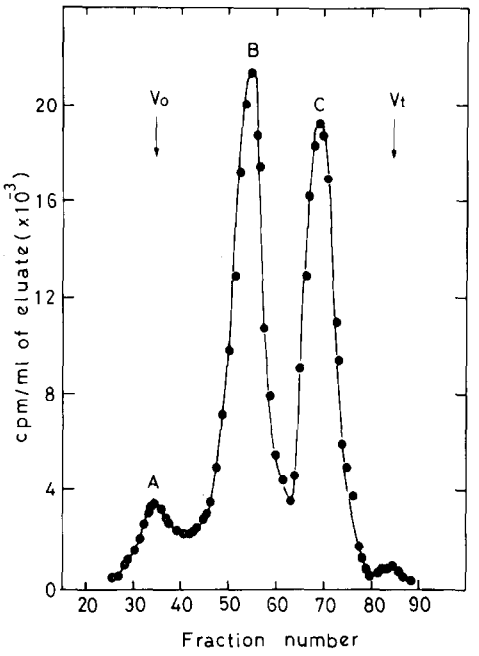

Fig.2. Gel-filtration of solubilized ^{125}I-hGH crosslinked receptor. ^{125}I-hGH crosslinked membranes were solubilized with 1% (v/v) Triton X-100 and centrifuged as described: 3 ml supernatant was gel-filtered on a Sepharose 6B column equilibrated with 50 mM Tris–HCl buffer (pH 7.4) containing 0.1% (v/v) Triton X-100. The 2 ml fractions were collected to determine radioactivity. The column had been calibrated with blue dextran 2000 (for void volume, V_o); bovine thyroglobulin (M_r 670 000), ferritin (440 000); catalase (232 000); BSA (67 000); ovalbumin (43 000); ^{125}I-hGH, and Na^{125}I (for total liquid volume, V_t).

^{125}I-hGH:receptor complex. The binding of anti-hGH to the ^{125}I-hGH:receptor complex indicates that crosslinking does not alter the immunological property of the hGH molecule. Crosslinking and immunoprecipitation may provide a useful method for purifying the GH receptor as has been reported for the insulin receptor [10].

^{125}I-hGH crosslinked membranes were solubilized, reduced and analyzed by SDS–PAGE. Generally, 3 peaks of radioactivity were detected as shown in fig. 4: (A) which barely entered the gel, may represent a high $M_r > 250\,000$) aggregate of the ^{125}I-hGH:receptor complex; (C) representing non-crosslinked ^{125}I-hGH; (B) migrating to the position corresponding to M_r 80 000 ± 3000 (the mean ± SD of 10 expt.) and was largely elimi-

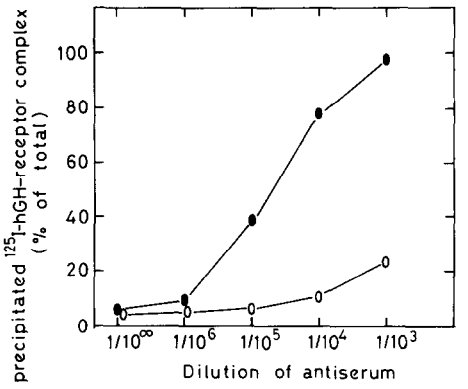

Fig.3. Immunoprecipitation of ^{125}I-hGH:receptor complex; 3000 cpm of ^{125}I-hGH:receptor complex was incubated with rabbit anti-hGH serum at the indicated final dilution in the presence (○) or absence (●) of 10 μg/ml unlabelled hGH. The ^{125}I-hGH:receptor complex had been partially purified by gel-filtration on a Sepharose 6B column and by affinity chromatography on a concanavalin A–Sepharose column as described in the text. Immunoprecipitation of the ^{125}I-hGH:receptor complex was achieved by adding goat anti-rabbit IgG serum (0.1 ml). Radioactivity of the ^{125}I-hGH:receptor complex was completely precipitated with 12.5% poly(ethylene glycol) 6000, indicating the absence of free ^{125}I-hGH. Values are the mean of triplicate determinations.

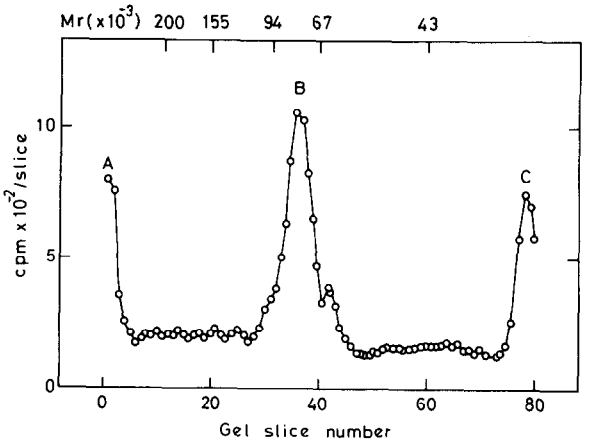

Fig.4. SDS–PAGE of ^{125}I-hGH crosslinked membranes. ^{125}I-hGH bound membranes were treated with 1 mM DSS for 30 min at 20°C and subjected to SDS–PAGE. The samples were electrophoresed at 50 V in the stacking gel and 100 V in the separating gel. After staining and subsequent destaining, the gel was sliced into 1 mm thick slices and assayed for radioactivity.

nated when membranes were incubated with ^{125}I-hGH in the presence of 10 μg/ml unlabelled hGH or bovine GH, indicating specificity in the binding of ^{125}I-hGH to the component. Similar results have been obtained with autoradiography (not shown). Thus, analysis of ^{125}I-hGH crosslinked membranes revealed a complex with M_r 80 000. There was no significant alteration in the electrophoretic behavior of peak B in the absence of the dithiol reductant dithiothreitol (not shown). Therefore, it seems that the hGH binding component is not linked to other components by disulfide bonds. Because hGH M_r is ~21 000, the hGH binding component M_r would be 59 000. An apparent M_r of the Triton-solubilized hepatic GH receptor of pregnant rabbits has been estimated to be 300 000 [2] or 200 000 [3]. The receptor may be composed of M_r 59 000 subunit non-covalently associated with a non-identical component. Alternatively, the receptor may be an oligomer of the M_r 59 000 species forming a complex of app. M_r 200 000.

ACKNOWLEDGEMENTS

This work was supported by grants from the Ministry of Education, and from the Ministry of Health and Welfare.

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