Identification and partial characterization of a growth hormone-binding protein in rat serum

Morgan Emtner and Paul Roos

Department of Biochemistry, University of Uppsala, Biomedical Centre, Uppsala, Sweden

Abstract A binding protein for growth hormone in serum from female rats has been identified and partially characterized. Serum was incubated with 125I-labelled human GH and fractionated on an agarose HPLC column. Complexes between the binding protein and 125I-hGH were detected as a peak eluted at a volume corresponding to a relative molecular weight of 159 000 ± 11 000 (N=8). The peak was not seen when the incubation was carried out in the presence of excess unlabelled hGH. The 125I-hGH bound with high affinity (Kd=0.87 ± 0.3 l/nmol; N=3) and the binding was time- and dose-dependent. Bound 125I-hGH was displaced by rat GH and bovine GH, but not by rat prolactin. The protein was not detected in radioreceptor assay by the commonly used polyethylene glycol precipitation technique and was not recognized by a monoclonal antibody raised against lactogenic receptors from female rat liver. Covalent cross-linking of 125I-hGH to serum revealed in SDS electrophoresis two labelled complexes with molecular weights of 62 300 ± 3900 and 77 600 ± 4100, respectively (N=10).

Prolactin and growth hormone are two related polypeptide hormones produced in the pituitary. After secretion into the blood they are transported to their target tissues, where they bind to specific receptors situated in the plasma membranes. The subsequent cellular transduction mechanisms are not known.

The plasma membrane receptors have been found in many tissues and species (1) and receptors from some sources as rabbit (2) and rat (3) liver have been well characterized.

Some years ago a specific GH-binding protein was identified in rabbit serum (4). It was partially purified and characterized, and it turned out to be identical with the extracellular part of the liver receptor (2). Corresponding protein has also been identified in serum from man (5) and mouse (6).

Although a substantial part (40-60%) of circulating GH in man is bound to carrier proteins (7), the physiological significance of such GH complexes has yet not been clarified.

Here we report on GH-binding activity in rat serum. Such an activity, which is apparently an intrinsic property of specific carrier protein molecules, has not been identified and characterized previously in rat serum.

Materials and Methods

Materials
Human GH (hGH; 8), rat PRL (rPRL; 9) and rat GH (rGH; 10) were prepared as described previously. Monoclonal antibody (Mc-ab) was prepared and purified according to Emtner et al. (11) and rabbit anti-hGH was supplied by Dr J Brandt (Kabi, Stockholm, Sweden). 125I-labelled recombinant hGH (Somatomorm®) was donated by Dr C.-Å. Isacson (Kabi), recombinant bovine GH (bGH, American Cyanamid, USA) was a gift from Dr U. Schröder, University of Lund, Sweden and the detergent heptaoxyethylene lauryl ether (G3707) (Atlas Chemie, Everberg, Belgium) was provided by Dr D. de Coster. All other materials were obtained from commercial suppliers.

Treatment of serum
Serum was collected from female Sprague-Dawley rats 2-3 months of age. To increase the life-span of the HPLC
columns and to strip endogenous rGH from its binding protein, MgCl₂ was dissolved (final concentration 4 mol/l) in the serum (2 ml) which was thereafter applied to a Sephadex G-75 (Pharmacia LKB Biotechnology, Uppsala, Sweden) column (1.9 × 14 cm) equilibrated with 25 mmol/l TRIS-HCl buffer, pH 7.4, containing 10 mmol/l MgCl₂ (TRIS/MgCl₂ buffer). The elution was carried out at a flow rate of 12 ml/h and the void volume peak, localized by spectrophotometry at 280 nm, was collected (stripped serum). Serum used in cross-linking experiments was treated in the same way, but the TRIS/MgCl₂ buffer was substituted by phosphate buffered saline (PBS; 137 mmol/l NaCl, 3 mmol/l KCl, 8.4 mmol/l Na₂HPO₄, 1.6 mmol/l KH₂PO₄, pH 7.4).

Binding assay by HPLC on agarose gel beads (HPLC assay)
Serum (100 µl) was incubated overnight at 24°C with 75 µl TRIS/MgCl₂ buffer containing 0.1% (w/v) bovine serum albumin (BSA) and 125I-l-hGH (=0.4 ng). After the incubation free and bound 125I-l-hGH were separated on an agarose (9%, cross-linked) HPLC column (12) equilibrated in TRIS/MgCl₂ buffer containing 100 mmol/l NaCl. The column (0.6 × 50 cm) was eluted with the same buffer at a flow rate of 0.2 ml/min. Fractions of 0.2 ml were collected and the radioactivity in each fraction was measured. Specific binding was calculated as the difference between the amount of radioactivity in fractions containing the binding protein (peak I, Fig. 1) and the amount obtained in corresponding fractions in runs where the incubation had been carried out in the presence of excess (5 µg) unlabelled hGH (the non-specific binding). The specific binding was expressed as percentage of the sum of binding (peak I) and free (peak II) 125I-l-hGH. The column was calibrated with thyroglobulin, catalase, aldolase, BSA, and ovalbumin.

Binding assay by precipitation with polyethylene glycol
The assay was performed as described previously (15). In short, samples were incubated overnight with 125I-l-hGH (30 000-60 000 cpm) in a volume adjusted to 0.5 ml with the TRIS/MgCl₂ buffer containing 0.016% gamma-globulin (w/v) and 0.1% BSA (w/v). The complexes formed were precipitated by addition of 1.0 ml of an aqueous solution of polyethylene glycol (PEG) 6000 (18.75%; w/v). Specific binding was calculated as the difference between the total and the non-specific binding measured in the presence of an excess of unlabelled hGH (10 µg). The specific binding was expressed as a percentage of the total cpm added.

Radioimmunoassay of GH-binding protein
Each sample to be assayed, was incubated overnight at 24°C together with 125I-l-hGH (30 000-60 000 cpm) and Mc-ab (0.02 µg) in a total volume of 150 µl TRIS/MgCl₂ buffer containing 0.1% (w/v) BSA. The hormone-receptor-Mc-ab complexes were precipitated by adding 50 µl donkey anti-mouse antibodies immobilized on cellulose particles (Sac-Cel, Welcome Research Laboratories, Beckenham, Kent, UK). After 30 min, 2 ml PBS was added and the tubes were centrifuged at 2 000 × g for 2 min. Tubes containing non-specific antibodies at the same dilution were incubated as controls and treated in the same way. The radioactivity of the pellets was measured and the specific binding was given as the difference between the sample and the control.

Radioimmunoassay of 125I-hGH
Of each fraction (containing radioactivity) 100 µl was incubated overnight at 24°C together with 50 µl of rabbit anti-hGH serum (diluted 1:1000). The procedure was identical with the one described above except for the immobilized antibody, which was a donkey anti-rabbit antibody.

Calculation of affinity constant
Affinity constants and binding capacities were calculated using a computer programme (14). The analyses were based on HPLC assays (see above) wherein 125I-labelled hGH was displaced by different amounts of unlabelled hGH.

Radioactive labelling
Labelling of hGH with 125I was performed using the iodoagen method which yielded moniodinated hormone (15). The specific activity was 60-80 µCi/µg.

Covalent cross-linking of GH-binding protein to 125I-hGH
Cross-linking experiments were performed using disuccinimidyl suberate (DSS; ICN Biomedicals Inc. Planview, NY). Stripped serum (100 µl) was incubated overnight with 125I-labelled hGH (300 000 cpm) at room temperature in a total volume of 250 µl PBS containing MgCl₂ (10 mmol/l). On the next day, 50 µl of DSS (2.5 mmol/l freshly dissolved in dimethyl sulfoxide) was added and the tubes were incubated for 15 min at 0°C. The reaction was terminated by the addition of 15 µl 2 mol/l TRIS-HCl buffer, pH 7.5, containing 1 mmol/l EDTA. Controls where the incubation was carried out in the presence of an excess (15 µg) of unlabelled hGH were run in parallel.

SDS electrophoresis
Following cross-linking, electrophoresis was performed by the procedure of Laemmli (16) in a polyacrylamide gel (T = 10% (w/v), C = 2.7% (w/v); 17). In short, the samples (315 µl) were mixed with "sample cocktail" (75 µl of 0.3 mol/l TRIS-HCl buffer, pH 8.8, containing 14% (w/v) SDS, 20% (w/v) mercaptoethanol, and 36% (w/v) sucrose) and boiled for 5 min. After alkylation with iodoacetamide (60 mmol/l) for 15 min at 24°C the electrophoresis (aliquot of 40 µl) was run at 200 V for 45 min using a Mini-Protean II apparatus (Bio-Rad Laboratories, Richmond, CA). Prior to autoradiography (Kodak X-AR 5.
Results

HPLC of complexes between $^{125}$I-hGH and rat serum

Female rat serum, pre-incubated overnight with $^{125}$I-hGH and fractionated on the agarose HPLC column, gave three major peaks containing radioactivity (Fig. 1). Peaks II and III eluted in the same positions as the markers $^{125}$I-hGH and Na$^{125}$I, respectively. Peak I was shown to represent a specific hGH-binding protein complex since it was not seen when the pre-incubation was carried out in the presence of excess (5 μg) unlabelled hGH. The $K_d$-value for the complex between the binding protein and $^{125}$I-hGH was 0.33 ± 0.02 (N=8) which corresponds to a molecular weight of 159 kD ± 11 if compared with $K_d$-values for the calibration proteins.

When stripped serum was incubated with $^{125}$I-hGH and subsequently chromatographed on the agarose HPLC column, the same three peaks were observed, but stripping of the serum approxi-

Fig. 1.
Female rat serum (100 μl) was incubated overnight with $^{125}$I-hGH (30 000 cpm) and subsequently fractionated on an agarose HPLC column at a flow rate of 0.2 ml/min. Fractions of 0.2 ml were collected and assayed for radioactivity ( ). A parallel incubation was made in the presence of excess unlabelled hGH ( ). For the denotations I-III see the text.

mately doubled the amount of specific binding in peak I. Furthermore, one peak, comparable in size to peak I, eluted in the void volume. Since only about half of the radioactivity in this unretarded peak was specifically bound and was, moreover, assumed to be associated with aggregated molecules, the major interest was focused on the activity in peak I. That the labelled molecule in the complex in peak I (stripped serum) was intact GH was demonstrated in two different ways. Firstly, the labelled complex in peak I was, in RIA, recognized by polyclonal antibodies towards hGH (not shown). This was the case also for free hGH (peak II) but not for free $^{125}$I (peak III). Secondly, when material from peak I was subjected to SDS electrophoresis and the gel subsequently autoradiographed, the labelled molecules had a molecular weight of 22 kD, in agreement with the value for intact hGH (Fig. 2).

Chromatography on the HPLC column of the serum alone (no addition of $^{125}$I-hGH) and assays of each fraction (the HPLC assay) gave hGH-binding activity in the molecular weight range 90-115 kD (not shown).

Precipitation with PEG

Examination of fresh untreated serum for hGH-binding, using the PEG precipitation assay, gave no detectable activity. However, assays of stripped
serum (50 µl), performed in the same way gave binding activity at a significant level (7.8 ± 1.4%; N=8). By applying stripped serum to the agarose HPLC column and examining the eluates, the PEG precipitable hGH-binding activity was located in the void volume peak. In contrast, the activity in peak I was not precipitated by PEG under the conditions utilized (not shown).

Interaction with a Mc-ab raised against lactogenic liver receptors
RIA of untreated or stripped serum using the purified Mc-ab (11) revealed no binding, although control tubes with microsomal receptors from female rat liver gave a positive response. Moreover, the Mc-ab in combination with Sac-Cel did not precipitate any cross-linked 125I-hGH protein complex prior to SDS-electrophoresis, which is further evidence for the binding protein being without the epitope recognized by the Mc-ab.

Binding characteristics
The characterization of binding between the serum protein (peak I, stripped serum) and the hormones was carried out by using the HPLC assay. The binding between hGH and the protein had an affinity constant of 0.87 ± 0.3 l/nmol (N=3) calculated from experiments where labelled hGH was displaced by the unlabelled hormone. The data were consistent with one class of binding sites and the binding capacity was 60 ± 30 pmol/l (N=3). The binding was dependent on the amount of serum used in the assay (Fig. 3) and on the incubation time (Fig. 4) with the maximum binding reached within 5 h. The incubation could be carried out for another 25 h without any noticeable decrease in the specific binding.

The concentration of Ca2+ or Mg2+ ions had no major influence on the binding (not shown).

The binding protein showed specificity towards somatogenic hormones since labelled hGH was displaced by an excess (5 µg) of rGH or bGH (91.2 ± 6.2% and 86.7 ± 9.4% as efficiently as by hGH, respectively; N=3). The corresponding value for rPRL was 12.8 ± 5.5%, whereas BSA or myoglobin had no effect.

SDS-electrophoresis of cross-linked binding protein to 125I-hGH
Analysis by SDS-electrophoresis of stripped serum incubated with 125I-hGH and subsequently cross-linked revealed two protein complexes with molecular weights of 62.3 ± 3.9 (major constituent) and 77.6 ± 4.1 kD (N=10; Fig. 5, lane a). The labelling was specific, since addition of excess unlabelled

![Fig. 3.](image)

Different amounts of stripped serum from female rats were incubated overnight with 125I-hGH (30 000 cpm) and subsequently fractionated on an agarose HPLC column. Specific binding was calculated as described in the text. The values are means of 3 experiments and the bars indicate the sd.

![Fig. 4.](image)

Stripped serum (100 µl) was incubated for different periods of time with 125I-hGH (30 000 cpm) and subsequently fractionated on an agarose HPLC column. Specific binding was calculated as described in the text. Values are means of 3 experiments and the bars indicate the sd.
hGH during incubations with \(^{125}\text{I}\)-hGH eliminated the two bands from the autoradiograms (lane c).

Cross-linking of \(^{125}\text{I}\)-hGH to fractions obtained by the HPLC of stripped serum and subsequent electrophoresis showed the same two bands in lanes where samples from peak I were applied (Fig. 5, lane e), but not in lanes which contained samples from other fractions, including samples from the void volume peak. Cross-linked complexes originating from this peak were not able to enter the separation gel (Fig. 5, lane d).

Discussion

We have shown, for the first time, that serum from female rats contains a GH-binding protein. The concentration of the protein in untreated serum was estimated at 0.4 nmol/l, based on the binding capacity calculated for stripped serum. Since the mean plasma level in female rats (age 90 days) is 4 nmol/l (18), it follows that less than 10% of circulating rGH can be bound. Labelled hormone incubated in a serum-containing medium may be degraded. Therefore, it was important to show that the labelled complex in peak I contained intact GH. The transference of the labelled component to anti-hGH is supporting evidence that the hormone was not degraded and also suggests that the specific binding calculated was not due to incubation damage described in connection with RIA (19,20). Furthermore, our SDS electrophoresis showed that the labelled component had the same size as intact hGH. Taken together, these experiments established that peak I contains bound intact hGH. However, these findings do not exclude some degradation of labelled hGH during the incubation, which, for instance, was visualized as a small increase in peak III with time (not shown).

To get reproducible results in the assay of the binding activity we had to use the HPLC technique described. This method was very time-consuming and laborious, and therefore we looked for another, more efficient detection procedure. Since we have in our hands a Mc-ab raised against purified lactogenic receptors from rat liver (11) and since the sequences of the lactogenic receptor from rat liver (3) and the binding protein from rabbit serum (2) are partly homologous, we tried to use the Mc-ab in an RIA. However, the Mc-ab did not recognize the GH-binding protein and could therefore not be used for this purpose. Efforts to simplify the detection in other ways, including molecular-sieve chromatography on mini-columns or binding of the protein to hydroxyapatite, were not successful.

The value obtained in this study for the affinity constant (0.87 l/nmol) for the binding between hGH and rat serum is in the same range as those between the hormone and the binding protein from man (0.4 l/nmol; 21) and rabbit (1.6 l/nmol; 5).

Similarly to the binding proteins from rabbit (5) and mouse serum (6), the protein from rat serum showed specificity towards somatogenic hormones. The small amount of labelled hGH displaced by rPRL can most probably be attributed to trace amounts of rGH in the PRL preparation used and consequently does not reflect a specificity of the binding proteins for lactogenic hormones. In the rabbit, the binding protein has been shown to be identical to the extracellular part of the liver receptor (2). In the rat, the difference in specificity between the binding protein in serum and that of
the predominating liver receptor, which is lactogenic (11), indicates that the binding protein in this species is not identical to the extracellular part of the lactogenic receptor. This is supported also by the lack of interaction between the binding protein and the Mc-ab with specificity for this receptor. More probably, the binding protein is related to the somatogenic receptor which is present in rat liver in small amounts. The molecular weight of the somatogenic receptor has been proposed to be 86 kD (22). If the mol wt values of the putative intracellular and membrane-spanning domains are subtracted (≈43 kD; calculated from the sequence reported by Mathews et al.; 23), the extracellular part would have a mol wt of 43 kD. This rough estimate agrees reasonably well with the mol wt of the major component (40.3 kD after subtraction of the mol wt of hGH) seen after the cross-linking experiments with rat serum (Fig. 5, lane a).

These experiments also indicate the existence in rat serum of two binding units of different size, neither of which is identical to any of the binding units from the microsomal female rat liver fraction (Fig. 5, lane b) characterized previously (11). Also in human (24) and mouse (6) serum the cross-linking technique has identified complexes of more than one size. The relationship between the units is not known. The bands may originate from unrelated molecules or from one molecular species at different stages of glycosylation or proteolysis.

Evidently the binding units in the rat are associated with each other and/or non-binding unit(s) under the conditions used in HPLC (mol wt ≈ 100 kD). Whether this is the case also in vivo we do not know. For the time being it is not meaningful to speculate in the stoichiometry of the native protein, since the estimates of the molecular weights obtained by the HPLC procedure are not very precise. The mol wt span of the fraction containing peak 1, for instance, was about 20 kD.

The next steps in the investigation of the binding protein from rat serum will be its purification and further characterization. However, before this goal can be achieved a more efficient method for the detection of the protein has to be developed.

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Dr Paul Roos,
Department of Biochemistry,
University of Uppsala,
Biomedical Centre,
P.O. Box 576,
S-751 23 Uppsala,
Sweden.