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Rabbit Liver Growth Hormone Receptor and Serum Binding Protein

PURIFICATION, CHARACTERIZATION, AND SEQUENCE*

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Steven A. Spencer, R. Glenn Hammonds, William J. Henzel, Henry Rodriguez, Michael J. Waters‡, and William I. Wood

From the Department of Developmental Biology, Genentech, Inc., South San Francisco, California 94080 and the ‡Department of Physiology and Pharmacology, University of Queensland, St. Lucia, Queensland, 4067 Australia

A putative growth hormone receptor from detergent-solubilized rabbit liver membranes and the growth hormone binding protein from rabbit serum have been purified 59,000- and 400,000-fold, respectively, primarily by affinity chromatography. Both purified proteins exhibit high affinity binding for human growth hormone; K_a = 9–30 × 10^9 M^{-1} for the liver receptor and K_a = 6 × 10^9 M^{-1} for the binding protein. The apparent molecular weight of the liver receptor is 130,000 by reduced sodium dodecyl sulfate gel electrophoresis, while that of the binding protein is 51,000. Both contain *N*-linked carbohydrate. The amino-terminal sequences of the liver growth hormone receptor and the serum binding protein were found to be the same, indicating that the binding protein corresponds to the extracellular domain of the liver receptor. Ubiquitin was found covalently linked to the liver receptor but not to the serum binding protein. The amino acid sequences of several peptides from the liver receptor were also determined after tryptic and V8 protease digestion.

Growth hormone (GH)[1] has been used clinically for many years to treat GH-deficient children, but the mechanism or mechanisms by which it promotes normal growth are not well established. According to the somatomedin hypothesis (1), GH released from the pituitary acts on the liver to induce the synthesis and release of insulin-like growth factor I. This systemic insulin-like growth factor I is then responsible for skeletal growth. However, the discovery of high affinity GH receptors in tissues other than liver (2, 3), as well as the demonstration of direct effects of growth hormone on cartilage *in vivo* (3–5) and recently *in vitro* (6), suggest that the direct action of GH on peripheral tissues may also be important.

One way to address the relative contributions of the systemic and direct actions of GH is to characterize the GH receptor and determine its tissue distribution, to establish whether more than one type of receptor exists, and to determine whether the same receptor exerts different actions in different cell types. To date such studies have not been possible because the receptor has not been isolated and characterized and because the intracellular signaling mechanism for the receptor has not been identified (3). In fact, only a few cell types have been found which produce a biological response *in vitro* to GH binding (2, 3).

The lack of a biological assay for GH receptor function has forced most workers to rely on the specific high-affinity binding of GH to identify the receptor. Using GH affinity-labeling techniques, several groups have partially characterized GH receptors in rat hepatocytes (7), rat adipocytes (8), mouse liver (9), and human IM-9 lymphocytes (10, 11). Others have used this binding assay to partially purify the GH receptor from rabbit liver (12–14), which is a particularly abundant source of GH receptors (15). One complication in the choice of rabbit liver as a source for the GH receptor is the observation that this tissue contains both lactogenic and somatogenic receptors (12, 15). While hGH binds with high affinity to both types of receptor, they can be distinguished because bovine growth hormone binds only to the somatogenic receptor while ovine prolactin shows preferential binding for the lactogenic receptor (15).

In addition to the membrane-bound GH receptor found in liver, a GH binding protein has recently been identified in rabbit (16) and human (17, 18) serum. It is a soluble protein which was not detected earlier because it does not precipitate in the binding assay commonly used to measure the membrane-bound receptor (19). Antibody data show that this binding protein shares several epitopes with the liver receptor (20), indicating the two proteins are related structurally as well as functionally.

In this report we describe the isolation of the putative GH receptor from rabbit liver and the GH binding protein from rabbit serum, as well as the characterization and partial amino acid sequence analysis of the purified proteins. Based on these data, full-length cDNA clones of the rabbit and human GH receptor have been isolated (21).

EXPERIMENTAL PROCEDURES AND RESULTS[2]

DISCUSSION

The putative GH receptor that we have isolated from rabbit liver is a glycoprotein with an apparent size of 130 kDa by reduced sodium dodecyl sulfate gel electrophoresis. This is consistent with the apparent 110-kDa size reported for affinity-labeled GH receptor from rat hepatocytes (7), rat adipocytes (8), mouse liver (9), and human IM-9 lymphocytes (10,

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[1] The abbreviations used are: GH, growth hormone; hGH, human growth hormone; bGH, bovine growth hormone; oPrl, ovine prolactin; KIU, Kallikrein inhibitor units; PMSF, phenylmethylsulfonyl fluoride; Tris-Triton, 50 mM Tris-HCl, pH 7.4, containing 1 ml/liter Triton X-100; SDS, sodium dodecyl sulfate; Mab, monoclonal antibody; HPLC, high performance liquid chromatography.

[2] Portions of this paper (including "Experimental Procedures," "Results," Figs. 1–8, and Tables I–III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

11) but larger than the 50–80-kDa size reported previously for rabbit liver receptor (12–14). The latter size probably reflects the sensitivity of the receptor to proteolysis, which can reduce the 130-kDa receptor to 50–60 kDa without significantly affecting its ability to bind hGH.

The sensitivity of the liver receptor to proteolysis may also explain the mechanism by which the serum binding is produced. We have shown that the amino-terminal 37 residues of the binding protein are identical to the amino terminus of the liver receptor, and preliminary analysis of a mixture of cyanogen bromide fragments from the binding protein (21) indicates that this identity continues through much, if not all, of the binding protein sequence. Thus, a plausible mechanism for generating the binding protein is proteolytic cleavage of the liver receptor near the transmembrane region, thereby releasing the soluble hormone binding domain.

The presence of ubiquitin covalently linked to the GH receptor is unusual but not unique for cell surface receptors, since both the lymphocyte homing receptor (39) and the platelet-derived growth factor receptor (40) are ubiquitinated. While the function for this ubiquitin is unknown, it has been suggested that it may regulate receptor number or produce an intracellular signal upon ligand binding (41). In the case of the GH receptor, ubiquitin could be involved in the rapid receptor turnover observed *in vivo* (42, 43). Alternatively, ubiquitin-mediated cleavage may release the binding protein, either by the direct action of ubiquitin itself (41) or through the ATP-dependent protease pathway (44).

Characterization of the hormone binding properties of the purified liver receptor confirms previous observations of both a somatogenic and a lactogenic binding activity in the rabbit liver (12, 15). The urea fraction from the hGH affinity column contained only somatogenic receptor while the $MgCl_2$ fraction was composed primarily of somatogenic receptor (over 80% of the binding sites) and a small amount of the lactogenic receptor (20%).

The lactogenic receptor is unusual in its ability to bind ^{125}I-hGH so tightly that it is not readily displaceable by a large excess of unlabeled hGH. Its apparent association constant for hGH is over 10^{11} M^{-1} by Scatchard analysis, but since this binding is not at equilibrium, the significance of this value is unclear. This irreversible binding also tends to overemphasize the effects of the lactogenic receptor in the binding assay and makes it difficult to assay mixtures of the two receptors, since receptor concentration, temperature, incubation time, and ligand concentration all affect the relative amount of ^{125}I-hGH bound to the lactogenic receptor. In contrast, the somatogenic receptor behaves normally with respect to reversibility of hGH binding and has an apparent $K_a = 10 \times 10^9$ M^{-1}. The serum binding protein has a slightly lower affinity of $K_a = 6 \times 10^9$ M^{-1}.

The GH receptor described here is the major protein in rabbit liver capable of binding GH, but whether it is the entire receptor or simply the ligand binding subunit has yet to be determined. Proof that the 130-kDa protein is a functional receptor is hampered by the lack of information on the intracellular signaling mechanism of the GH receptor. However, evidence that the GH receptor described here plays an important role in growth comes from recent results showing that Laron-type dwarfs, who have normal hGH levels but no liver GH binding (45), also lack functional serum binding protein (46, 47). These observations, coupled with our demonstration that the serum binding protein is the extracellular GH binding domain of the liver receptor, show that these proteins are intimately involved in the growth process. In addition, antibody binding data indicate that the GH receptor (or a structurally similar protein) is localized to chrondrocytes at the epiphyseal growth plate (48). However, final proof of the role of the GH receptor described here in the transduction of the growth signal must await reconstitution of a functional system containing the cloned GH receptor or genetic analysis of patients with receptor defects.

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Supplementary Material to:

RABBIT LIVER GROWTH HORMONE RECEPTOR AND SERUM BINDING PROTEIN: PURIFICATION, CHARACTERIZATION AND SEQUENCE

Steven A. Spencer, R. Glenn Hammonds, William J. Henzel, Henry Rodriguez, Michael J. Waters, and William I. Wood

Experimental Procedures

Materials–Recombinant human and bovine growth hormones were from Genentech, Inc. Ovine prolactin (31 IU/mg), leupeptin, pepstatin, aprotinin, and glyceryl controlled pore glass (120-200 mesh, 500 Å pore diameter) were from Sigma. Triton X-100 was membrane research grade from Boehringer Mannheim. Centricon 30 centrifugal concentrators were from Amicon. N-glycanase (peptide: N-glycosidase F) was from Genzyme and neuraminidase (*Vibrio cholerae*) from Calbiochem. TPCK-trypsin was from Worthington and *S. aureus* V8 protease from Miles. Frozen rabbit serum was from Pel-Freez. Immunoblotting reagents (Vectastain kit) were from Vector Laboratories. Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) and premixed Bradford protein assay reagent were from Pierce and fluorescamine (Fluram) from Roche. Sephadex and Sephacryl were from Pharmacia.

Iodination–Human growth hormone (hGH) was iodinated by the lactoperoxidase method (22). In brief, 10 μg hGH were combined with 1 mCi Na [^{125}I] and 0.18 μg lactoperoxidase in pH 7.0 phosphate buffer in a total volume of 45 μl, then 25 μl 0.65 mM H_2O_2 added. After 5 min. at room temperature, the reaction was stopped by addition of 0.5 ml phosphate buffered saline containing 0.2 g/l NaN_3 and 1 g/l bovine serum albumin, then fractionated on a 1.5 x 45 cm Sephadex G-75 column run in the same buffer. The peak and trailing 2-3 fractions were pooled and stored at -20°C. Specific activities calculated by the method of Greenwood *et al.* (23) ranged from 80-111 μCi/μg.

Liver GH receptor and serum binding protein samples were iodinated by the iodogen method (24). In brief, 12 x 75 mm polypropylene tubes were coated with 10 μg iodogen in $CHCl_3$ (1 mg/ml), allowed to air dry and rinsed with distilled water just before use. One hundred microliters of a solution containing ~0.5 μg protein dissolved in 50 mM Tris·HCl pH 7.4 containing 0.1% (v/v) Triton X-100 (Tris-Triton) were added followed by 250 μCi Na[^{125}I]. After 20 min. on ice, the reaction mixture was placed in 1 ml ice-cold acetone along with a 100 μl Tris-Triton rinse of the reaction tube. After 30 min. on ice, the tube was centrifuged 5 min. at 16,000 *g* and the supernatant discarded. The pellet was rinsed with 1 ml ice cold acetone and centrifuged again. The pellet was briefly air dried, then dissolved in Tris-Triton and stored at -80°C.

Affinity Column Preparation–Initial hGH affinity columns were only partially successful due to leaching of hGH from the support (Affigel-10); coupling to glyceryl controlled pore glass gave a more stable packing (25). In brief, 1 g glyceryl controlled pore glass was activated for 30 min at room temperature in 10 ml 0.1% sodium metaperiodate then washed quickly with 20 ml cold distilled water. 10 mg hGH in 9.3 ml phosphate-buffered saline pH 7.4 were added to the activated gel along with 5 mg sodium cyanoborohydride. The suspension was mixed gently overnight at 4°C, then free coupling sites were blocked by placing the gel in 10 ml 1 M ethanolamine·HCl pH 8.0 containing 5 mg sodium cyanoborohydride and mixing gently at 4°C for 48 hrs. The affinity gel was washed with three alternating cycles of pH 4.0 sodium acetate and pH 8.5 Tris·HCl, both containing 0.5 M NaCl, then with 5 bed volumes of 6 M urea in Tris-Triton, a Tris-Triton rinse and 5 bed volumes of 5 M $MgCl_2$ in Tris-Triton. The gel was finally rinsed extensively with Tris-Triton and stored at 4°C in this buffer containing 0.2 g/l NaN_3. The affinity gel contained about 3.5 mg hGH/ml.

Binding Assays–The assay method was similar to that described by Herington and Veith (19). Assays were performed in triplicate in 12 x 75 mm polypropylene tubes. Displacing ligands were diluted in assay buffer (50 mM Tris·HCl pH 7.4 containing 10 mM $CaCl_2$, 1 g/l bovine albumin and 0.2 g/l NaN_3) then ^{125}I-hGH was added (30-60,000 cpm/tube, 14-29 pM) followed by the diluted sample to a final volume of 0.5 ml. The tubes were mixed and incubated 24 hr at room temperature unless otherwise indicated. The assays were terminated by adding 0.5 ml 1 g/l bovine γ-globulin followed by 1 ml 300 g/l polyethylene glycol 8000 (both at 4°C in phosphate buffered saline containing 0.2 g/l NaN_3), mixing thoroughly and centrifuging at 4,000 *g* for 20 min. at 4°C. The tubes were inverted to drain, then the pellets were γ-counted for 1 min. For the serum binding protein, monoclonal antibody (Mab) 263 (26) was also included in the incubation mixture at 1/2000 final dilution to make the labeled complex precipitable by polyethylene glycol. Scatchard analysis was performed with the program LIGAND (27).

Protein Assays–Protein concentrations were measured by the dye binding method (28) except for dilute samples (less than 50 μg/ml). For these, the samples were extensively dialyzed at 4°C against 1 mM HCl containing 0.1% (v/v) Triton X-100 to remove all Tris contamination, then assayed either by the standard fluorescamine method (29) or by the more sensitive hydrolysis method (30). Bovine serum albumin was used as the standard throughout.

Liver GH Receptor Purification–All procedures were at 0-4°C except as noted. Fresh livers were removed from 3 or 4 anesthetized young (2 kg) female New Zealand White rabbits and chilled immediately in ice-cold 50 mM Tris·HCl, pH 7.4, containing 0.3 M sucrose and 1 mM EDTA. They were weighed, cut into small pieces, and homogenized until uniform in 5 volumes of the same buffer containing 30,000 Kallikrein inhibitor units (KIU) per liter aprotinin and 2 mM phenylmethylsulfonyl fluoride (PMSF). Benzamidine·HCl was added to 10 mM and the homogenate centrifuged at 14,000 *g* (max) for 20 min., then this supernatant was centrifuged at 142,000 *g* for 60 min. The pellet was uniformly suspended in 1.5 volumes (based on original liver weight) of room temperature, 50 mM Tris·HCl pH 7.4 containing 10 ml/l Triton X-100, 50,000 KIU/l aprotinin, 1 mM PMSF and 7 μg/ml each pepstatin and leupeptin, then benzamidine·HCl was added to 10 mM. The suspension was stirred 20 min. at room temperature then centrifuged at 235,000 *g* for 90 min. at 4°C. The clear extract between the pellet and floating fat layer was carefully removed and brought to 1 mM in PMSF, 12 mM $MgCl_2$ and 150 mM in NaCl, then loaded at 18 ml/hr onto a 1 ml hGH affinity column. The small column size helped to minimize contamination by non-specifically bound proteins without sacrificing recovery. The extract was followed by a 10 ml wash of 50 mM Tris·HCl pH 7.4 containing 10 ml/l Triton X-100, then the flow rate was increased to 150 ml/hr and the column washed with 50 ml of the same buffer containing 0.5 M NaCl followed by 10 ml Tris-Triton. The column was moved to room temperature and eluted with 5 ml room temperature 4.5 M urea in Tris-Triton collected at 0.1 ml/min into 5 ml ice-cold Tris-Triton containing 10 mM benzamidine·HCl. This was followed by 2 ml Tris-Triton, then elution with 5 ml 4.5 M $MgCl_2$ in Tris-Triton. The $MgCl_2$ eluate was also collected into 5 ml ice-cold Tris-Triton containing 10 mM benzamidine·HCl and followed by a 2 ml Tris-Triton wash. The urea and $MgCl_2$ eluates were dialyzed at 4°C against 2 x 1 l Tris-Triton containing 10 mM benzamidine·HCl. For sequence analysis, the dialyzed $MgCl_2$ eluate was concentrated with a Centricon 30 concentrator, then acetone precipitated and run reduced on a preparative sodium dodecyl sulfate (SDS) gel (31) along with about 200,000 cpm of ^{125}I-labeled $MgCl_2$ eluate. The gel was autoradiographed at 4°C and this was used as a guide for excising the 130 kDa protein band. The gel slice was electroeluted at 4°C using the procedure of Hunkapiller *et al.* (32) except the initial buffer containing 2% SDS was omitted. Some samples for amino acid sequencing were reduced and alkylated with iodoacetic acid prior to loading on the preparative gel.

Serum GH Binding Protein Purification–All procedures were at 0-4°C except as noted. 500 ml frozen rabbit serum were thawed at 4°C, during which aprotinin was added to 50,000 KIU/l and benzamidine·HCl to 10 mM. The thawed serum was made to 12 mM in $MgCl_2$ and 2 mM in PMSF, centrifuged at 16,000 *g* (max) for 20 min. and filtered to remove floating fat particles. It was then loaded on a 1 ml hGH affinity column as described for the liver receptor except a 0.5 ml precolumn of blank glyceryl controlled pore glass was used to remove any protein which precipitated during loading. The column was then washed with 15 ml 50 mM Tris·HCl pH 7.4, the flow increased to 150 ml/hr and the column washed with 50 ml Tris-Triton containing 0.5 M NaCl followed by 10 ml 50 mM Tris·HCl pH 7.4. Elution was as described for the liver receptor except the urea and $MgCl_2$ solutions contained no Triton X-100. The eluates were dialyzed at 4°C against Tris-Triton plus benzamidine as before. The $MgCl_2$ fraction was concentrated to about 0.5 ml in a Centricon 30 concentrator and loaded at room temperature onto a 1 x 47 cm Sephacryl S-300 column equilibrated with 50 mM Tris·HCl pH 7.4 containing 0.15 M NaCl and 0.2 g/l NaN_3. The serum binding protein was eluted at 6 ml/hr and 0.5 ml fractions were collected. Based on results of the hGH binding assay and SDS gel analysis, fractions were pooled and concentrated again with a Centricon 30. For sequence analysis the concentrated protein was acetone precipitated and loaded non-reduced onto a preparative SDS gel along with about 200,000 cpm of radiolabeled S-300 pool. The gel was autoradiographed, sliced and electroeluted as described above.

Glycosidase Digestion–Digestion with N-glycanase followed the method supplied with the enzyme except Tris buffer was substituted for phosphate. In brief, an aliquot of ^{125}I-labeled 130 kDa liver receptor (gel eluted) or 51 kDa serum binding protein (S-300 pool) was mixed with about 10 μg aprotinin, acetone precipitated and redissolved in 10 μl of 5 mg/ml SDS containing 0.1 M 2-mercaptoethanol. This was heated 3 min. at 100°C then 10.8 μl 0.55 M Tris·HCl pH 8.6, 3 μl 100 mM 1,10-phenanthroline in methanol and 5 μl of 75 μl/ml NP-40 detergent in water were added and mixed. Finally 0.5 μl N-glycanase (250 U/ml) was added and the sample incubated overnight at 37°C. It was then acetone precipitated and analyzed by SDS polyacrylamide gel electrophoresis followed by autoradiography.

For neuraminidase digestion, the acetone precipitated sample containing aprotinin was dissolved directly in the enzyme solution (1 U/ml in 50 mM sodium acetate pH 5.5 containing 154 mM NaCl and 4 mM $CaCl_2$), incubated overnight at 37°C, acetone precipitated and analyzed as described above.

Amino Acid Sequence Determination–Sequence data were obtained on an automated gas phase sequencer (Applied Biosystems model 470A). Except for one amino-terminal sequence on the $MgCl_2$ eluate of the liver receptor, all sequence analyses were performed on acetone precipitates of SDS gel-eluted 130 kDa liver receptor or 51 kDa serum binding protein. Normally the amount of protein eluted from the gel was not quantitated directly, so the amounts used for sequencing were estimated based on recovery of ^{125}I-labeled protein from the electroelution (normally 50-80%).

To generate internal sequence, the gel eluted 130 kDa liver receptor was digested with TPCK-trypsin or *S. aureus* V8 protease. For the trypsin digest, approximately 200 pmoles of gel-eluted receptor were dissolved in 200 μl Tris-Triton containing 0.3 mM $CaCl_2$ and digested for 22 h at 37°C using 2% (w/w) trypsin added in equal aliquots at zero and 15 h. The digest was then diluted with 220 μl 8 M urea in 50 mM Tris·HCl pH 7.6 containing 25 mM dithiothreitol and fractionated by reverse phase high performance liquid chromatography (HPLC). For the V8 digest, approximately 120 pmoles of gel eluted receptor were dissolved in 250 μl of 0.1 M NH_4HCO_3 pH 8.0 containing 0.1% (v/v) Triton X-100, and digested 18 h at 37°C with 4% (w/w) V8 protease added in equal aliquots at zero and 3 h. The digest was dried under vacuum and redissolved in 200 μl 7 M guanidine·HCl containing 20 mM Tris·HCl pH 8.0 and 20 mM dithiothreitol, then fractionated by reverse phase HPLC.

Results

Liver GH Receptor Purification–The GH receptor was purified from rabbit liver by solubilization in Triton X-100 and affinity chromatography. The results of the purification are summarized in Fig. 1a and Table I. Almost all of the 59,000-fold purification is due to the highly optimized hGH affinity column (see EXPERIMENTAL PROCEDURES). Approximately 20% of the loaded binding activity was recovered in the urea wash while about 50% was eluted with $MgCl_2$ (30% overall recovery). Reduced SDS gel analysis of the purified receptor shows a prominent band at 130 kDa (Fig. 1a). As described previously (21), this 130 kDa band was identified as the receptor by immunoblotting (Fig. 1b, lane 1) with an anti-receptor monoclonal antibody (Mab5) which recognizes the denatured receptor (33). The $MgCl_2$ eluate was estimated to be 40% pure based on a maximum theoretical specific binding of 7,700 pmoles/mg, assuming one hGH binding site per 130 kDa receptor. Final purification for sequence analysis was accomplished by electroeluting the 130 kDa receptor from a reduced SDS gel which would be expected to remove any contaminating lactogenic receptor (see below).

Previous reports of a lower molecular weight (50-80 kDa) for affinity-labeled rabbit GH receptor (12-14) are probably due to its sensitivity to proteolysis. When commercially obtained frozen rabbit liver was used as the starting material, less 130 kDa receptor was found and an immunoblot showed evidence of several lower molecular weight proteins related to the receptor (Fig. 1b, lane 2). In addition, partial trypsin digestion of affinity purified GH receptor led to rapid cleavage of the 130 kDa band and an increase in a broad 50-60 kDa band, with little loss of binding activity. Similar sensitivity to proteolysis has been observed in affinity-labeled GH receptor from rat hepatocytes (34). During the purification, proteolysis was largely prevented by the use of freshly obtained rabbit liver, by the extensive use of protease inhibitors, and by working quickly at low temperatures.

When analyzed by SDS gel electrophoresis under non-reducing conditions, the receptor runs in a large complex at the top of the gel (Fig. 1a, lane 6) and does not resolve into distinct bands even on a low percentage acrylamide gel. Substantial binding activity can be eluted from the top of a non-reduced gel; however, attempts to regain measurable binding activity from reduced, SDS denatured 130 kDa receptor were unsuccessful.

Table I

Liver GH Receptor and Serum Binding Protein Purification Summary[a]

Fraction	Total protein *mg*	High-affinity sites *pmoles*	Specific activity *pmoles/mg*	Purification *fold*	Yield
Liver Receptor					
Liver homogenate	40,000	–	–	–	–
14,000 *g* supernatant	19,000	970	0.051	1	100%
142,000 *g* pellet	4,400	795	0.18	3.5	82%
Affinity load	2,500	595	0.24	4.7	62%
Urea eluate	0.14	110	790	15,500	11%
$MgCl_2$ eluate	0.097	290	3000	59,000	30%
Serum Binding Protein					
Affinity load	24,000	1,200	0.050	1	100%
Urea eluate	0.87	430	494	9,900	36%
$MgCl_2$ eluate	0.15	470	3,130	63,000	39%
S300 pool	0.086	170	20,000	396,000	14%

[a]Values are for 220 g liver or 500 ml serum as the starting material.

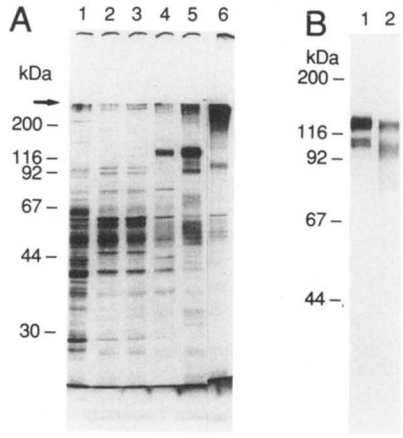

Fig. 1 a. Silver-stained 9.5% polyacrylamide SDS gel of the hGH affinity column fractions for the rabbit liver GH receptor. 1 μg total protein per lane, lanes 1-5 reduced: lane 1, load; lane 2, flowthrough; lane 3, wash; lane 4, urea eluate; lane 5, $MgCl_2$ eluate; lane 6, $MgCl_2$ eluate non-reduced. The arrow indicates the top of the resolving gel.

b. Immunoblot with Mab5 of the affinity column $MgCl_2$ eluate: lane 1, starting with fresh, non-pregnant female rabbit liver; lane 2, starting with frozen (commercially obtained) pregnant rabbit liver. Both samples are reduced.

Binding Protein Purification–The GH binding protein was purified from rabbit serum by the two-step procedure of hGH affinity chromatography followed by gel filtration (Table I and Fig. 2a). The gel filtration step was included to remove a contaminant which comigrated with the receptor on a non-reduced SDS gel (Fig. 3a). Although the broad 51 kDa band was identified as the binding protein (see below), the apparent size by gel filtration is 92 kDa (Fig. 3b), suggesting the binding protein is a non-covalent dimer under these conditions.

In contrast to the liver receptor, the serum binding protein activity was almost equally divided between the urea and $MgCl_2$ eluates from the affinity column, possibly due to the lower affinity of the binding protein for hGH (see below). Recovery from the affinity column was 75% between the two fractions, but since the $MgCl_2$ fraction contained fewer contaminants, it was used exclusively for further purification. The material from the gel filtration column was judged to be at least 70% pure based on SDS gel analysis (Fig. 2a) and based on a maximum theoretical specific binding of 20,000 pmoles/mg assuming one hGH binding site per 51 kDa.

The broad 51 kDa band was identified as the serum binding protein in three ways. First, on gel filtration the binding activity coincided with the intensity of this band on an SDS gel (Fig. 3). Second, crosslinking of ^{125}I-hGH to the binding protein followed by SDS gel analysis (Fig. 2b) showed a single diffuse band at 75 kDa, corresponding to the 51 kDa band plus 22 kDa for growth hormone. Finally, substantial binding activity (over 10% of that loaded) was recovered after electroelution of the 51 kDa band from a non-reduced SDS gel. No other regions containing binding activity were found. The breadth of the band is probably due to partial proteolytic degradation and possibly heterogeneous glycosylation (see below).

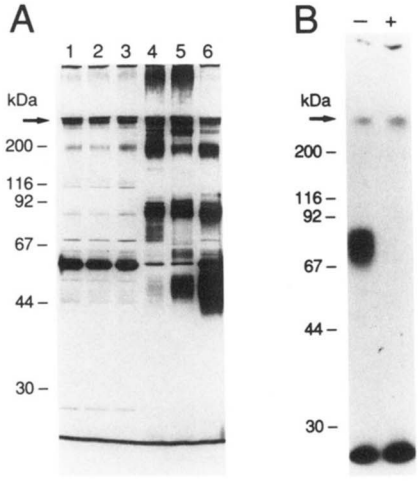

Fig. 2 a. Silver-stained 9.5% polyacrylamide SDS gel of non-reduced fractions from the serum binding protein purification. 1 μg total protein per lane: lane 1, affinity column load; lane 2, flowthrough; lane 3, wash; lane 4, urea eluate; lane 5, $MgCl_2$ eluate; lane 6, S-300 pool. The arrow indicates the top of the resolving gel. The bracketed region indicates the serum binding protein.

b. Autoradiogram for 48 h at -80°C of ^{125}I-hGH (0.35 nM) cross-linked to the serum binding protein $MgCl_2$ fraction in the absence (-) or presence (+) of excess (90 nM) unlabeled hGH. The arrow indicates the top of the resolving gel. Crosslinking conditions have been described previously (21). Approximately 5,000 cpm were loaded per lane.

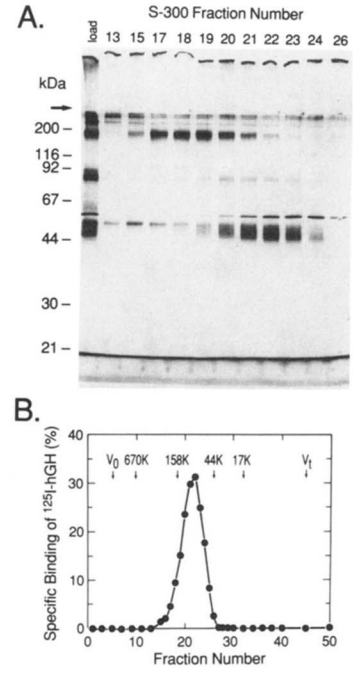

Fig. 3a. Silver-stained 9.5% polyacrylamide SDS gel of non-reduced serum binding protein fractions from the Sephacryl S-300 column. 15 μl loaded per lane except "load" was 1.3 μl to approximate the sample dilution on the column. Fraction numbers correspond to the profile in 3b. The 51 kDa binding protein band peaks in fractions 20-23. The arrow indicates the top of the resolving gel.

b. Binding protein activity profile from the Sephacryl S-300 column (conditions described in the EXPERIMENTAL PROCEDURES section). The molecular weight markers were blue dextran (Vo), thyroglobulin, immunoglobulin G, ovalbumin, myoglobin and vitamin B-12 (Vt) respectively.

Glycosidase Digestion–The ^{125}I-labeled 130 kDa liver receptor (gel eluted material) was digested with either N-glycanase (to remove all N-linked carbohydrate) or neuraminidase (to remove sialic acid). Neuraminidase reduced the 130 kDa band to a sharp band at 116 kDa on reduced SDS gels, while N-glycanase reduced it to a diffuse band centered at 95 kDa (Fig. 4). Sequential digestions with neuraminidase and N-glycanase produced results similar to N-glycanase alone. Similar reductions in molecular weight upon deglycosylation have been reported for affinity-labeled mouse liver (9), rat hepatocyte (34), and IM-9 lymphocyte (11) GH receptors. The affinity purified liver receptor contains a small amount of material around 95-100 kDa which cross reacts with the anti-receptor monoclonal antibody (Fig. 2b), and may correspond to unglycosylated or partially glycosylated receptor. Preliminary results from digesting ^{125}I-labeled serum binding protein with N-glycanase indicate that it also contains N-linked carbohydrate (data not shown).

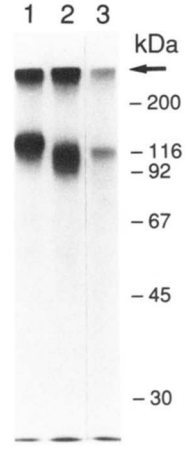

Fig. 4. Autoradiogram for 7 h at -80°C of ^{125}I-labeled 130 kDa liver receptor undigested (47,000 cpm, lane 1), or digested with N-glycanase (46,000 cpm, lane 2), or neuraminidase (32,000 cpm, lane 3) (see EXPERIMENTAL PROCEDURES), then run on a 9.5% polyacrylamide SDS gel. The undigested sample was treated the same as the N-glycanase sample except the enzyme was not added. The arrow indicates the top of the resolving gel.

Characterization of Affinity Column Eluates–Scatchard analysis of competition assays for the purified liver receptor (using ^{125}I-hGH as the tracer and unlabeled hGH as the competing ligand) consistently produced curved plots indicative of more than one class of binding sites (Fig. 5a, b). For both the $MgCl_2$ and urea fractions from the affinity column, a high affinity class of sites ($MgCl_2$, Ka = 30 x 10^9 M^{-1}; urea, Ka = 10 x 10^9 M^{-1}) and a low affinity class of sites (Ka~0.1 x 10^9 M^{-1}) were observed. While statistical analysis indicated the two site fit is significant for both, the effect is barely discernable in the displacement curves (solid versus dashed lines in Fig. 5) and the parameters of the low affinity site are poorly determined. In contrast, the serum binding protein showed a single class of binding sites for the $MgCl_2$ (Fig. 5c) and urea (not shown) fractions with Ka = 6 x 10^9 M^{-1} for both. Repeated assays of the binding protein consistently gave a value for Ka which was slightly lower than that for the purified receptor fractions.

The affinity column fractions were also analyzed in a binding competition assay using ^{125}I-hGH as the tracer and unlabeled hGH, bGH or oPrl as competing ligands. The urea fraction for the liver receptor (Fig. 5e) and both the $MgCl_2$ (Fig. 5f) and urea (not shown) fractions for the serum binding protein behaved as expected for a GH receptor (15), with bGH about a 5-fold and oPrl at least a 200-fold less effective competitor than hGH. The observed competition by oPrl may be due to a slight contamination by oGH (less than 1%).

The most highly purified liver receptor preparation, the $MgCl_2$ fraction, was unusual in that high levels of bGH did not fully compete with ^{125}I-hGH for binding (Fig. 5d) and the extent of this competition decreased with longer incubation times and increasing receptor concentrations. (Under optimal conditions, up to 70% of the ^{125}I-hGH binding could be blocked by saturating levels of bGH.) In addition, the oPrl competition curve was non-parallel, also suggesting the presence of more than one class of binding sites.

Rabbit liver is known to contain both lactogenic and somatogenic receptors to which hGH can bind (12,15). As bGH binds only to the somatogenic receptor (15), the oPrl competition assay was repeated in the presence of a saturating concentration of bGH (46 nM, see Fig. 5d) to assay the contaminating lactogenic receptor. Under these conditions, a normal competition curve (Fig. 6a) was obtained. These data show that, under these assay conditions, 4.4 nM oPrl is sufficient to saturate the lactogenic receptor and that 4.4 nM oPrl plus 46 nM bGH can displace all the specifically bound ^{125}I-hGH. The hGH competition assay was then repeated in the presence of either a saturating concentration of bGH (46 nM) or of oPrl (4.4 nM) to measure the lactogenic and somatogenic receptors respectively. Scatchard analysis of the competition curve in the presence of oPrl (Fig. 6b) showed a considerably improved single site fit to the data compared to the same preparation assayed without oPrl (Fig. 6c). (The residual non-linearity could be due to incomplete blocking of the lactogenic receptor since the receptor was not preincubated with the oPrl before the ^{125}I-hGH was added). The single site fit gave an association constant for the somatogenic receptor of Ka = 9 x 10^9 M^{-1}, close to the value obtained for the urea fraction from the affinity column (Ka = 10 x 10^9 M^{-1}).

Scatchard analysis of the hGH competition curve in the presence of 46 nM bGH (measuring the lactogenic receptor, Fig. 6d) indicated a very large binding constant (Ka ~3 x 10^{11} M^{-1}) but the estimated standard error in this value was large (±85%) due to the nearly vertical slope. Also, this may not be a true equilibrium constant (see below). A poorly defined lower affinity site also appeared to be present (Ka ~0.05 x 10^9 M^{-1}). Based on the relative receptor concentrations obtained from the Scatchard analyses, the somatogenic sites in the $MgCl_2$ fraction constitute over 80% of the hGH binding sites. This estimate was further supported by the observation that an anti-GH receptor monoclonal antibody (Mab7 (33)) could inhibit 70% of the specific ^{125}I-hGH binding in this fraction (M.J. Waters, unpublished).

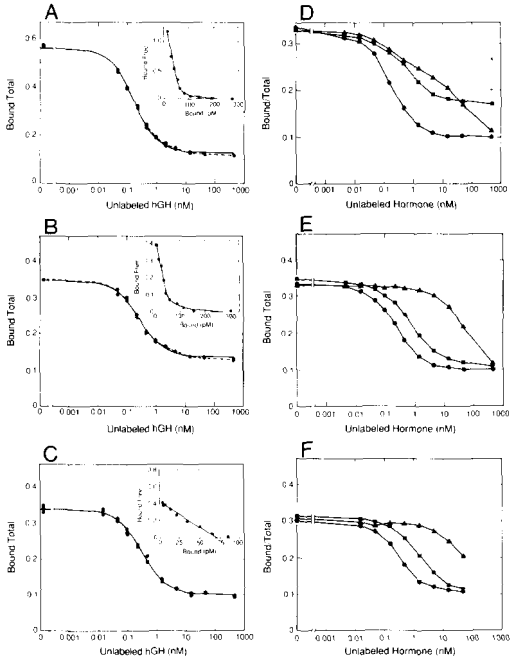

Fig. 5. Human GH competition curves and Scatchard plots (a-c) and hormone competition curves (d-f) for the liver GH receptor $MgCl_2$ (a,d) and urea (b,e) affinity column eluates and the serum binding protein $MgCl_2$ eluate (c,f). The tracer is ^{125}I-hGH in all cases; competing ligands are: recombinant hGH (●), recombinant bGH (■), and pituitary ovine prolactin (▲). Dashed lines (b,e) represent two site fits and solid lines one site fits to the competition curves. Approximate concentrations for the high affinity binding sites were: 57 pM (a), 45 pM (b), 72 pM (c), 36 pM (e) and 58 pM (f). In (d), the isolated symbols show 460 nM bGH endpoints using 57 pM receptor and 24 h incubation (x) or 14 pM receptor and 24 h incubation (+). The full curve is for 14 pM receptor and 4 h incubation (see text).

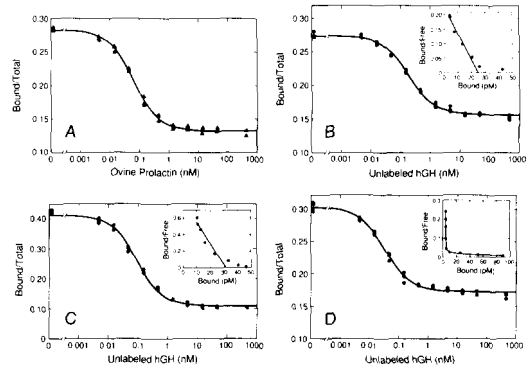

Fig. 6. Binding competition curves for the liver GH receptor $MgCl_2$ eluate from the affinity column. The tracer is ^{125}I-hGH. The competition curves show single site fits only. All measurements were in triplicate. Concentration of the high affinity binding sites was approximately 57 pM throughout.

a. Competition by prolactin in the presence of saturating (46 nM) bGH. Non-specific binding (45 nM hGH) was at bound/total = 0.125.

b. Competition by hGH in the presence of 4.4 nM oPrl (assaying somatogenic sites) and Scatchard plot (single site fit).

c. Competition by hGH in the absence of other hormones. The Scatchard plot shows a single site fit to the data, which is clearly non-linear.

d. Competition by hGH in the presence of saturating (46 nM) bGH (assaying lactogenic sites) and Scatchard plot showing a two site fit to the data.

Indications that a portion of the bound ^{125}I-hGH in the $MgCl_2$ fraction from the liver receptor was not readily displaceable by unlabeled hGH prompted a set of binding experiments in which the liver GH receptor (urea and $MgCl_2$ fractions) or the serum binding protein ($MgCl_2$ fraction) were mixed with ^{125}I-hGH followed by excess unlabeled bGH (4.5 nM) added at subsequent times (Fig. 7). The ^{125}I-hGH which was bound to the serum binding protein or the urea fraction of the liver receptor was almost completely displaced by unlabeled hGH regardless of the interval between the two additions, but the $MgCl_2$ fraction of the liver receptor showed a steady decrease in displaceable binding. This demonstrates that a portion of the binding to the $MgCl_2$ fraction of the liver receptor is not reversible under the assay conditions. When the $MgCl_2$ fraction was reassayed in the presence of 4.4 nM oPrl to measure somatogenic receptor binding (Fig. 7, dashed line), most of the irreversible binding was abolished, indicating this is a property of the lactogenic receptor. Similar irreversible binding has been observed in liver and mammary lactogenic receptors from several species (35-37). This result also explains the decrease in competition by bGH for ^{125}I-hGH binding to this fraction with increasing receptor concentration and longer incubation times (Fig. 5d), since these conditions would favor increased binding of the ^{125}I-hGH to the lactogenic receptor. Under some assay conditions this irreversible binding to the lactogenic receptor can lead to considerable overestimation of the relative amount of lactogenic receptor present (as well as violate the equilibrium conditions assumed for the Scatchard analysis).

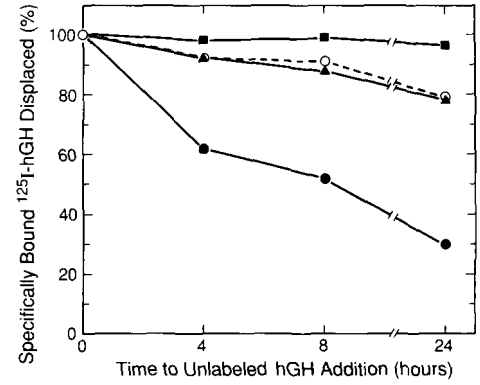

Fig. 7. Binding assay with ^{125}I-hGH (28 pM) added at t=0 h and unlabeled hGH (4.5 nM) added at the times shown. All samples were incubated a total of 48 h at room temperature before termination. Samples were: serum binding protein $MgCl_2$ eluate (■); liver receptor urea eluate (▲); liver receptor $MgCl_2$ eluate (●); liver receptor $MgCl_2$ eluate with 4.4 nM oPrl added at t=0 h (○). Total high affinity binding site concentrations were approximately 50 pM in all samples.

Protein Sequencing Results–Amino-terminal sequence results for the liver GH receptor are shown in Table II. These data are for the 130 kDa receptor eluted from an SDS gel. This procedure should eliminate any contamination by the lactogenic receptor, which has a reported size of 32 kDa (38). As reported earlier (21), sequence analysis of the purified 130 kDa liver receptor also showed the presence of ubiquitin at a level of 20-50% that of the receptor sequence. The variable levels of ubiquitin may indicate either partial loss during the isolation or that only a subset of the receptor is ubiquitinated.

The gel-eluted 51 kDa serum binding protein produced three simultaneous amino-terminal sequences (Table II). The strongest sequence corresponded to the amino terminal sequence of the liver receptor, while the other two corresponded to the sequences following trypsin-like cleavages after arginines 13 and 20. No ubiquitin sequence was observed. Comparison of these sequences with the cDNA sequence for the liver receptor (21) extended the amino-terminal identity between the receptor and the binding protein through residue 37. These data provide direct evidence that the serum binding protein is the extracellular domain of the liver GH receptor.

Internal sequence for the liver GH receptor was obtained by digesting the gel-eluted 130 kDa band with either trypsin or *S. aureus* V8 protease and isolating the peptides by reverse phase HPLC. Six tryptic (Fig. 8a) and five V8 (Fig. 8b) peptides were sequenced, of which five tryptic and two V8 peptides gave useable data, including three mixture sequences. A total of ten internal peptide sequences were obtained (Table III). In addition, peptide V4 was identified as the amino terminus, but the recovery was very low. Peptide T4 was used to successfully clone the liver GH receptor cDNA and all 10 peptides were subsequently found in the translated full-length cDNA sequence (21).

Table II

Amino-terminal Sequences for the Rabbit Liver GH Receptor and Serum Binding Protein

	5	10	15	20	Approximate Initial Yield[c] (pmoles)
GHR-N[a]:	F S G S E A(T)[b] P A T L G R A S E				50
Ub:	M Q I F V K T L T G K X[d](I)				12
		SBP-N2 ↓	SBP-N3 ↓		
SBP-N1:	X X X S E A T P A T L G R A S E S V Q X V H P				110
SBP-N2:	X X E(S)V Q R V H P G L(G)T X S(S)G K P K F T				72
SBP-N3:	X X P X L X(T)X S(S G)K P K F X K				50

[a] GHR-N: liver receptor, amino terminus; Ub, ubiquitin; SBP-N1-3, serum binding protein three simultaneous amino-terminal sequences.
[b] Parentheses indicate uncertain residues. For the serum binding protein, residues are indicated as uncertain if they also appear at the same cycle in one of the stronger sequences. Thr-7 in GHR-N was assigned to ubiquitin because of its low level.
[c] Initial yield is based on HPLC quantitation of phenylthiohydantoin amino acids. Estimated loads were 120 pmoles for the liver GH receptor and 430 pmoles for the serum binding protein. Repetitive yield was 92% for both.
[d] X indicates a residue not identified.

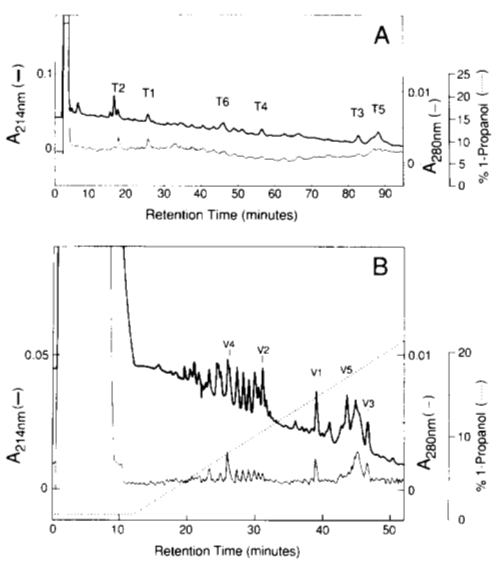

Fig. 8. Reverse phase HPLC of 130 kDa liver receptor trypsin (A) and V8 protease (B) digests. For the tryptic digest, a 0.2 ml sample containing approximately 100 pmoles of receptor was injected onto a Synchrom RP-P column (4.6 x 100 mm, C18). Elution solvents were 0.1% trifluoroacetic acid (solvent 1) and 100% 1-propanol (solvent 2). A linear gradient of 1-30% solvent 2 in 116 min. was used for elution. The column was run at room temperature with a flow rate of 0.5 ml/min. Effluent absorbance was measured at 214 and 280 nm. For the V8 digest, a 0.1 ml sample containing approximately 60 pmoles of receptor was injected onto a Brownlee BU-300 (2.1 x 100 mm, C4) column and eluted with a linear gradient of 1-30% solvent 2 in 58 min. Other conditions were as described above. Most of the peaks eluting between 20-35 min were also in the Triton X-100 blank.

Table III

Tryptic and V8 Peptides from the 130 kDa Liver GH Receptor

		Approximate Initial Yield (pmoles)	cDNA Sequence Position[a]	Repetitive Yield
Trypsin Peptides				
T2.1:	(L)[b][D][K E (Y) (E) V [R] } mixture	65	204-211	76%
T2.2:	(E) V [N][E (T) (Q) [W] K	98	180-187	
T3:	(S) G T A E D A P G S E M P V P D Y	40	561-577	92%
T4:	V E P S F N Q E D I Y I T T E S L T T [T] (A)(E)	60	538-559	92%
T5:	[C] F [S] V E E I V Q P	29	122-131	68%
T6.1:	S P G S V (Q)(L)(F) Y I R } mixture	34	60-70	88%
T6.2:	T [S][C](Y)(E)(P)(D)(I)[L](E) N D F N A [S] D	19	369-385	
V8 Peptides				
V3:	[W] K [E][C] P (D)(Y) V [S](A)(G)(E)(N)[S][C](Y) F	–	80-96	–
V5.1:	(S) T L Q A A P S Q L S N P N S L A N I D F Y } mixture	24	448-469	88%
V5.2:	(F) I E L D I D D	12	327-334	

[a] Reference 21.
[b] Parentheses indicate uncertain residues. Brackets indicate residues not called or incorrectly called; the residue shown is from the cDNA sequence (21).
[c] The absence of Asn may indicate the presence of N-linked carbohydrate.