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AFFINITY PURIFICATION AND STRUCTURAL CHARACTERIZATION OF A SPECIFIC BINDING PROTEIN FOR HUMAN GROWTH HORMONE IN HUMAN SERUM

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Summary: A highly specific binding protein for human growth hormone (hGH) has been isolated from human serum by hGH-affinity chromatography. A purification of ~1500-fold with a 30-40% recovery was obtained with essentially no alteration in binding characteristics. Covalent cross-linking of ^{125}I-hGH to the binding protein, followed by analysis by SDS-polyacrylamide gel electrophoresis and autoradiography, revealed two specifically labeled complexes. Allowing for a 1:1 binding stoichiometry the binding proteins themselves had mean mol wts of 57000 and 69300. These increased slightly to mol wt 60300 and 72000 respectively in the presence of 100 mM dithiothreitol, suggesting the presence of intramolecular but not inter-subunit disulfide linkages. These data confirm the presence of the hGH binding protein(s) in human serum and define their gross structural nature. © 1986 Academic Press, Inc.

A highly specific binding protein for human growth hormone (hGH) has been recently identified and characterized in normal human serum (1,2). This binding protein bound ^{125}I-hGH in a time-, temperature- and serum concentration-dependent manner with an affinity (K_A) of ~0.3 x 10^9 M^{-1}. The binding protein was present in all human sera so far examined (n > 39) (2) and on size exclusion gel chromatography had a molecular weight (mol wt) of 74000-85000 (2) or 60000-65000 (1). The identity of the binding protein, its possible relationship to the hGH tissue receptor, and its physiological role are as yet unknown. To assist in addressing some of these questions, binding protein has now been extracted and partially purified from human serum by affinity chromatography. Its structural nature has also been determined by covalent cross-linking techniques.

ABBREVIATIONS

hGH, human growth hormone; SDS, sodium dodecyl sulfate; DSS, disuccinimidyl suberate; PAGE, polyacrylamide gel electrophoresis.

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MATERIALS AND METHODS

Reagents. hGH (NIAMDD-hGH-I-1) used for iodination was a gift of the National Hormone and Pituitary Program (NIADDK, NIH, Bethesda, MD). hGH used for unlabeled standards and for affinity purification of the binding protein was obtained from the Commonwealth Serum Laboratories, Melbourne, Australia. Affigel-15 chromatography gel and acrylamide were obtained from Biorad Laboratories, Richmond, CA; Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril) and disuccinimidyl suberate (DSS) from Pierce Chemical Co., Rockford, IL; N-N'-methylene bisacrylamide and TEMED from Eastman-Kodak Co., Rochester, NY; SDS and ammonium persulfate from BDH Chemicals Aust., Melbourne, Australia; and dithiothreitol from Calbiochem-Behring Aust., Kingsgrove, Australia. The mol wt protein markers for polyacrylamide gel electrophoresis were obtained as follows: Escherichia coli β-galactosidase (mol wt 116000) from Sigma, St. Louis, MO; rabbit muscle phosphorylase B (mol wt 98000), bovine serum albumin (mol wt 68000), ovalbumin (mol wt 45000) and bovine erythrocyte carbonic anhydrase (mol wt 30000) were from Pharmacia, Uppsala, Sweden.

Iodination and Binding Studies. hGH was iodinated as previously described (2,3), using the Iodogen method (4), to specific activities of 30-50 μCi/μg. Binding studies on whole serum or partially purified fractions were performed as previously described (2). Bound and free hormone were separated by gel filtration on Ultrogel AcA44 mini-columns (0.6 x 22 cm) at 21-23 C as previously described (2,5). All protein estimations were performed using the Coomassie Blue method (6).

Affinity Chromatography of hGH Binding Protein. hGH was covalently attached to Affigel 15 exactly as described previously (3). Normal human serum (9 ml) was applied to a column of hGH-Affigel 15 (0.7 x 19 cm, bed vol. 10 ml) and allowed to interact for 1 h at 21 C. The column was then washed with 50 ml of 25 mM Tris/HCl (pH 7.4) containing 10 mM $CaCl_2$ and 0.02% sodium azide. The bound serum proteins were eluted with 20 ml of 4 M $MgCl_2$ (3,7). Two ml fractions were collected across the whole column. Bound fractions (dialysed against 25 mM Tris/HCl (pH 7.4)) and unbound fractions were then assayed for specific binding of ^{125}I-hGH.

Covalent Cross-Linking of ^{125}I-hGH to Affinity-Purified Binding Protein. ^{125}I-hGH (± unlabeled hGH for determination of specific binding) was incubated at 21 C for 2 h with Affigel-purified binding protein. The incubation mixture was then covalently cross-linked at 21 C using 0.05 mM (final concentration) DSS as described previously (8). The cross-linking reaction was stopped after 15 min by taking 50 μl aliquots and boiling (3 min) in the presence of 2% SDS ± 100 mM dithiothreitol.
SDS-electrophoresis was then performed on vertical slab 10% acrylamide gels (0.5 mm thick) according to the method of Laemmli (9). A 3% acrylamide stacking gel was used. Samples were run in the presence or absence of dithiothreitol and equivalent amounts of radioactivity (65000-100000 cpm) were loaded onto each sample lane. The gels were run, stained with Coomassie Blue to detect the protein markers, dried and then subjected to autoradiography at -70 C for varying times using Kodak AR-5 film and Dupont lightning plus intensifying screens.

RESULTS AND DISCUSSION

When normal human serum was applied to an hGH-Affigel affinity column (Fig. 1), the majority of serum proteins, but not specific hGH binding activity, passed straight through. The column-bound hGH binding proteins were eluted with 4 M $MgCl_2$ - a maneuver known to dissociate GH from solubilized or particulate tissue receptors (3,7). Although the general binding

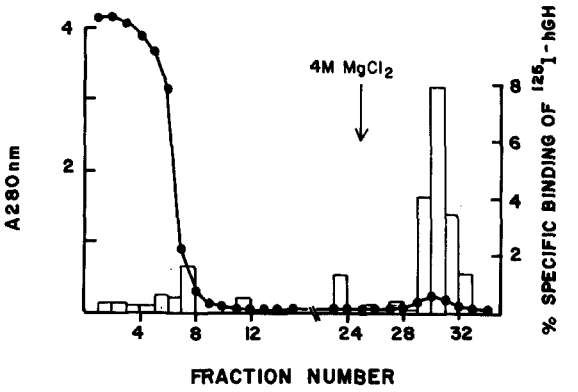

Fig. 1. A representative profile for affinity chromatography of human serum on hGH-Affigel 15. The experiment was performed as described under Materials and Methods. The protein (A_{280}) profile is shown in the solid line; the specific hGH-binding activity is shown by the histogram. The binding of ^{125}I-hGH was determined in each column fraction and is expressed as a percentage of the ^{125}I-hGH cpm added per tube. Identical profiles were obtained in many such experiments.

characteristics of affinity-purified binding protein were unchanged (data not shown) compared to whole serum (1,2) there was a tendency as demonstrated by Scatchard analysis (Fig. 2) for a lower binding affinity (K_A before 0.45-0.49 x 10^9 M^{-1}; K_A after 0.18-0.23 x 10^9 M^{-1}). Scatchard analysis of ^{125}I-hGH binding to whole serum and to affinity-purified binding protein also indicated that, after accounting for the difference in protein content of the samples, a purification of 1000- to 1500-fold was

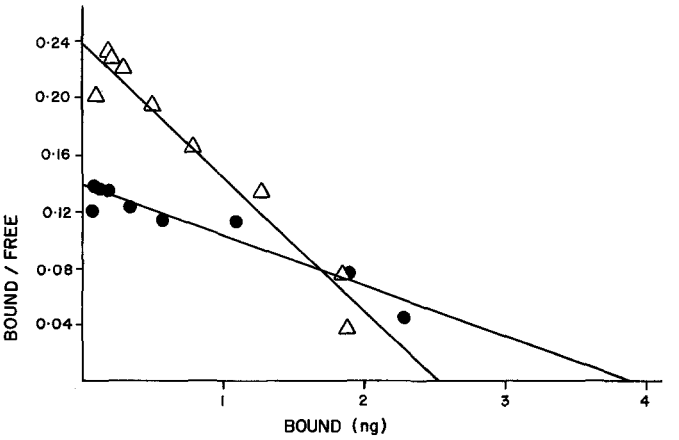

Fig. 2. Scatchard plots of ^{125}I-hGH specific binding to whole human serum (Δ) and to affinity-purified GH binding proteins (●). The protein content of each sample (serum, affinity-purified binding protein) were 7 mg/tube and 11 µg/tube respectively. The lines of best fit were determined by linear regression and values calculated for the binding affinities and binding capacities in two such analyses are given in the text.

achieved (binding capacity before affinity purification being 13-17 fmol/mg protein, and after affinity purification being 17000-20000 fmol/mg protein). Overall recovery was 28-43%, although this is likely to be greater in practice due to an underestimation of hGH binding activity in the purified fraction as a result of the known leakage of hGH off the affinity gel (data not shown).

Affinity-purified hGH binding protein was used to study the structural nature of the binding activity using covalent cross-linking techniques. ^{125}I-hGH, when cross-linked to binding protein and subjected to SDS-PAGE and autoradiography, gave just two specifically labeled bands. On the representative gel shown in Fig. 3, under non reducing conditions these bands were of mol wt 78000 and 93000. Both bands increased slightly in mol wt under reducing conditions (+100 mM dithiothreitol) to mol wt 85000 and 95000 respectively. If one assumes a 1:1 binding stoichiometry between hGH and binding protein, and one subtracts the mol wt of hGH (20000 non-reduced; 22000 reduced; data not shown but determined in separate experiments using 15% polyacrylamide SDS gels), then the calculated mean mol wts (± SEM for 3 gels) for the binding proteins themselves were 57000 ±

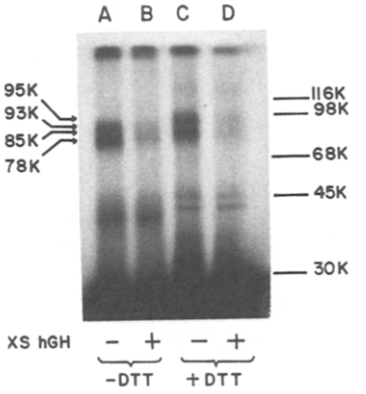

Fig. 3. A representative autoradiograph of SDS-polyacrylamide gel electrophoretic analysis of ^{125}I-hGH covalently cross-linked to affinity-purified binding protein. The cross-linking was performed with 0.05 mM DSS in the absence (lanes A and C) and presence (lanes B and D) of excess unlabeled hGH to determine the specificity of cross-linked complexes. The samples were also run in the non-reduced (lanes A and B) and reduced (+100 mM dithiothreitol, lanes C and D) state. The protein mol wt markers are shown on the right, the mol wt of specifically labeled complexes are indicated by the arrows on the left. Three such gels have given similar results.

1000 and 69300 ± 2300 (non-reduced) and 60300 ± 1800 and 72000 ± 2100 (reduced). The slight increase in mol wt for both bands in the presence of dithiothreitol was consistently observed and, although not significant ($P < 0.1$) by Student's paired t test, suggests the presence of intramolecular disulfide bonds within the binding protein(s). There was no evidence for high mol wt oligomeric disulfide-linked binding subunits. These mol wt compare with those determined (under non-dissociating, non-reducing conditions) by size exclusion chromatography: 60000-65000 (1) and 74000-85000 (2). We do not yet know whether there is a structural relationship between the two binding protein bands - for example, whether they are distinct proteins or different glycosylation states of a single protein.

These data shed some light on the possibility raised previously (1,2) that the serum GH binding protein may represent tissue GH receptor or at least a GH-binding receptor subunit that has been shed or secreted from GH target tissues. GH receptors are known to be easily shed from cultured human IM-9 lymphocytes (10) and to be readily extracted in aqueous buffers from rabbit liver membranes (8,11). In addition, with the exception of the binding affinity (K_A 0.3×10^9 M^{-1}) which is a little lower than for tissue GH receptors ($1\text{-}2 \times 10^9$ M^{-1}), the remaining binding characteristics of the serum binding protein are similar to those for tissue GH receptors (2). In particular, the hormonal specificity of the serum binding protein (2) is essentially identical with that of human liver (12) or IM-9 lymphocytes (13). On the other hand, the structural data suggest a relationship may not exist. Little is known regarding the structural nature of the human tissue GH receptor - the only study carried out to date being on the cultured IM-9 lymphocyte (14). In this tissue, covalent cross-linking indicated that the primary GH binding subunit had a mol wt of 109000 (14) but in a separate study it appeared to exist in a solubilized form as a much higher mol wt (>200000) species (10). These data suggest a distinct structural difference and, therefore, non-identity between the human GH receptor and the serum binding protein.

The data reported in this paper, however, clearly confirm the initial identification of a specific binding protein for hGH in human serum, and demonstrate that it can be readily isolated in an active form. This study provides an important starting point for the purification and complete identification of the binding protein. Its physiological role remains to be determined.

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