Identification of a rabbit liver cytosolic binding protein for human growth hormone

Susie I. YMER, Janet L. STEVENSON and Adrian C. HERINGTON
Medical Research Centre, Prince Henry's Hospital, Melbourne, Victoria, Australia 3004

(Received 16 January 1984/Accepted 5 April 1984)

A specific growth hormone (GH) binding protein of M_r approx. 100 000 has been demonstrated in the cytosolic fraction (200 000g supernatant) of pregnant-rabbit liver by gel filtration techniques. This binding species was detectable by a standard charcoal separation procedure but not by the widely used poly(ethylene glycol) precipitation method. The GH binding protein had similar binding characteristics to those of classical membrane-bound GH receptors. The kinetics of association and dissociation, binding affinity (2.56×10^9 l/mol) and hormonal specificity have been established. There appears to be equal or greater amounts of GH binding protein in the cytosol than in the membrane fraction. The presence of the GH binding protein in rabbit liver cytosol was substantiated by its selective purification on a GH-Affigel 15 affinity column. This technique has resulted in a 200–300-fold purification with no substantial change in binding affinity. The ability of a concanavalin A-Sepharose affinity column to also bind the cytosolic binding protein indicates that, like the membrane-bound GH receptor, it is a glycoprotein. This is the first report of a cytosolic binding protein for GH and raises important questions regarding its potential physiological role in the mechanism of action of GH.

Using gel chromatographic techniques we have recently identified and partially characterized a previously unreported specific GH binding protein of M_r 100 000–124 000 in both aqueous and detergent-solubilized membrane preparations of pregnant female rabbit liver (Ymer & Herington, 1984). Furthermore, we have shown that poly(ethylene glycol) precipitation, which is used widely as a method for detection of soluble hormone–receptor complexes (Cuatrecasas, 1972; Shiu & Friesen, 1974; Herington & Veith, 1977), is unable to detect this particular binding protein. The possibility, therefore, that a similar soluble GH-binding protein might be present in the intracellular cytosolic fraction (post-200 000g cell supernatant) of rabbit liver was re-examined: previous studies using the poly(ethylene glycol) precipitation method had failed to detect any such soluble GH-binding protein (Tsushima, 1978). The studies reported here describe for the first time the presence in rabbit liver cytosol of a high-affinity

GH-binding protein with characteristics similar to those of classical membrane-bound or -associated GH receptors. The data therefore raise important questions regarding the possible mechanism(s) of action of GH, in particular the initiation of intracellular biological actions.

Experimental

Hormones and reagents

hGH (NIH-GH-H52160E) used for iodination, and oPRL (NIH-P-S-15), bGH (NIH-GH-B-18) and rGH (NIH-rGH-B6) used for unlabelled preparations were gifts of the National Hormone and Pituitary Program (NIADDKD, NIH, Bethesda, MD, U.S.A.). hGH (M_r 21 000) for unlabelled standards was a gift of the Commonwealth Serum Laboratories, Melbourne, Australia. Iodination of hGH was by the Iodogen method (Salacinski *et al.*, 1981) using an equimolar ratio of hGH to Na^{125}I . The reaction mixture was separated on a 10 ml Sephadex G-50 column equilibrated in 25 mM-Tris/HCl pH 7.5. The protein peak was then further purified on Ultrogel ACA54. The specific activity of the iodinated hGH monomer peak ranged between 50 and 100 Ci/g. Other

Abbreviations used: GH, growth hormone; PRL, prolactin, (the prefixes b, h, o and r refer to the bovine, human, ovine and rat hormones, respectively); PMSF, phenylmethanesulphonyl fluoride; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

reagents were obtained as follows: Ultrogel ACA34 and ACA54, LKB Produkter AB, Bromma, Sweden; concanavalin A-Sepharose, Sephadex G-50, dextran T-70 and poly(ethylene glycol), Pharmacia, Uppsala, Sweden; Affigel 15, Biorad Laboratories, Richmond, CA, U.S.A.; activated charcoal, Merck, Darmstadt, Germany; Trasylol, Bayer AG, Leverkusen, Germany; PMSF, thyroglobulin (porcine, M_r 660000), ferritin (horse spleen, M_r 450000), catalase (bovine liver, M_r 210000), aldolase (rabbit muscle, M_r 158000), ovalbumin (M_r 45000), Sigma, St. Louis, MO, U.S.A.; bovine serum albumin (M_r 68000), Miles Laboratories, Elkhart, IN, U.S.A.; methyl α -D-mannopyranoside, Calbiochem-Behring Corp., La Jolla, CA, U.S.A.

Preparation of rabbit liver cytosol

Microsomal membranes (100000g) were prepared from the livers of 25–28 day pregnant female New Zealand White rabbits as described previously (Tsushima & Friesen, 1973) in the presence of 1 mM-PMSF and 1000 k.i.u. of Trasylol/ml. The 100000g cytosol obtained was recentrifuged at 200000g and the resulting supernatant filtered through a 0.22 μ m Millipore filter. Protein estimations were carried out by the Coomassie Blue (Bradford, 1976) or Lowry (Lowry *et al.*, 1951) methods.

Measurement of 125 I-hGH binding

Rabbit liver cytosol (100 μ l; 2 mg of protein/ml) was incubated with 125 I-hGH (1000–20000 c.p.m.; 0.2–0.4 ng) in 25 mM-Tris/HCl, pH 7.5, containing 10 mM-CaCl₂, 0.1% bovine serum albumin and 0.02% NaN₃ in a final volume of 500 μ l at 23°C for 2 h. Separation of bound and free hormone was achieved by the addition of an equal volume of 1% (w/v) charcoal coated with 0.1% (w/v) T-70 dextran. After 5 min at 4°C the mixture was centrifuged (10000g, 2 min) in a Beckman Microfuge and the free 125 I-hGH absorbed to the charcoal pellet was determined. Previous studies (results not shown) had determined these conditions to be optimal. Specific binding of hGH was determined by the difference between binding in the absence (total binding) and presence (non-specific binding) of a large excess (1 μ g) of unlabelled hGH. 125 I-hGH binding to rabbit liver particulate fractions (e.g. the 100000g pellet) was determined as described previously (Tsushima & Friesen, 1973).

Detection of hGH binding activity by gel chromatography

Gel filtration of 125 I-hGH-binding protein complex. Cytosol (50 μ l, 2 mg of protein/ml) was incubated with 125 I-hGH in the presence and

absence of excess unlabelled hGH (1 μ g) as described above but in a final volume of 250 μ l and with the CaCl₂ replaced by MgCl₂. After a 2 h incubation, the whole mixture was chromatographed at 4°C on an Ultrogel ACA34 column (1 cm $\times$ 90 cm) equilibrated in 50 mM-Tris/HCl buffer (pH 7.5). Fractions (1 ml) were collected and the radioactive elution profile was determined. Routinely, 80% of applied radioactivity was recovered. The ACA34 column was calibrated with Dextran Blue (V_0), riboflavin (V_r) and the M_r markers listed above.

Gel filtration of liver cytosol. In some experiments liver cytosol alone (1 ml; 10–20 mg of protein/ml) was gel chromatographed on the same ACA34 column described above. Fractions (1 ml) were collected and 100 μ l aliquots were analysed for 125 I-hGH binding activity by using the charcoal-precipitation method described above.

Affinity chromatography

Concanavalin A-Sepharose. Cytosol (2–4 ml, 10–20 mg of protein/ml) was passed through a column of concanavalin A-Sepharose (0.7 cm $\times$ 22 cm) equilibrated in 25 mM-Tris/HCl 10 mM-Ca²⁺, pH 7.4. Fractions (15 $\times$ 2 ml) were collected and then elution (15 $\times$ 2 ml fractions) was carried out in the presence of 0.1 M-methyl α -mannopyranoside and absence of calcium. An aliquot (100 μ l) of each fraction (both 'bound' and 'unbound' peaks) was assayed as above for 125 I-hGH binding.

hGH-Affigel 15. hGH (70 mg) was covalently linked to 17 g of Affigel 15 at pH 7.5 (0.1 M-Hepes buffer) with gentle agitation for 1 h at room temperature. Any free, active esters remaining on the gel were blocked with 1.2 ml of 7 M-ethanolamine/HCl (pH 8). Approx. 91% of the hGH remained linked following thorough sequential washing with 25 mM-Tris/HCl, pH 7.4, and 4 M-MgCl₂, as determined by the inclusion of a small amount of 125 I-hGH in the original reaction mixture. Cytosol (4 ml, 10–20 mg of protein/ml) was applied to a column (0.7 cm $\times$ 25 cm) of hGH-Affigel 15 and allowed to interact for 1 h at 21–22°C. The column was then washed with 25 mM-Tris/HCl (pH 7.4) containing 40 mM-CaCl₂, 0.02% (w/v) NaN₃ and Trasylol (1000 k.i.u./ml) and the bound cytosolic protein(s) were eluted with 4 M-urea and 4 M-MgCl₂, either alone or sequentially, as described by Waters & Friesen (1979). The 'bound' fractions were dialysed against 25 mM-Tris/HCl, pH 7.5, containing 40 mM-CaCl₂ and then assayed as above for 125 I-hGH binding.

Results and discussion

Four peaks of radioactivity were routinely observed following ACA34 gel filtration of an

incubation mixture of ^{125}I -hGH and rabbit liver cytosol (Fig. 1). Peak 1 was small, was eluted near the void volume, was not readily displaced by excess unlabelled hGH, and therefore, probably represents a non-specific binding peak. Peak 3 was eluted in the same position as monomer hGH and represents free unbound ^{125}I -hGH. Peak 4 was eluted at the V_i of the column and represents free ^{125}I . Peak 2, always a major peak, eluted with a mean M_r 121 000 and was essentially totally displaced by excess unlabelled hGH. This peak thus represents a specific hGH-binding protein and for five separate cytosolic preparations (each gel chromatographed at 2 mg/ml) gave a mean ($\pm$ S.E.M.) specific binding of $40.2 \pm 2.6\%$. Formation of this peak was inhibited by incubation with 1 μg of bGH or oGH (87% and 75% displacement respectively) and to a lesser extent by oPRL (63%). rGH displayed typical behaviour for rabbit liver systems and competed poorly with ^{125}I -hGH (16% displacement). However, hPRL, rPRL human placental lactogen, rat LH or FSH or porcine insulin did not displace ^{125}I -hGH from this binding protein (results not shown). The effect of oPRL in this system may be attributable to the known cross-reactivity of oPRL with the true somatotrophic receptor of rabbit liver (Waters & Friesen, 1979), to the presence of up to 0.5% (w/w) contamination of the oPRL with oGH (NIADDKD Information Sheet), or to both. Nevertheless, it would appear that the binding in peak 2 showed a greater specificity for somatotrophic than lactogenic hormones, a situation typical of GH receptors in

rabbit liver. This same cytosolic hGH binding protein has also been observed in liver cytosols prepared from male and non-pregnant female rabbits as well as at much lower concentration in rabbit kidney and heart cytosols (results not shown).

If one assumes an hGH:binding protein stoichiometry of 1:1 and a M_r of 21 000 for hGH, then the hGH-binding protein present in the cytosolic fraction has an M_r of approx. 100 000. This value was confirmed by a separate series of experiments where the cytosol alone was gel chromatographed and the hGH-binding profile subsequently determined by incubation of individual column fractions with ^{125}I -hGH followed by charcoal separation (Fig. 2). Maximum specific hGH-binding activity corresponded to M_r 92 000. The M_r determined by either of these methods corresponds with the value of 100 000 determined by gel filtration for an hGH-binding protein solubilized by detergents or aqueous extraction from rabbit liver membrane preparations (Ymer & Herington, 1984). Other similarities between the cytosol and membrane-solubilized M_r 100 000 binding proteins exist. In particular, neither the cytosolic binding protein (results not shown) nor the membrane-solubilized binding protein (Ymer & Herington, 1984) are detectable by the widely used poly(ethylene glycol) method (Cuatrecasas, 1972; Shiu & Friesen, 1974; Herington & Veith, 1977). This provides an explanation for previous failures to detect such a binding protein. It is for this reason that the charcoal separation method was established and used in the present studies. In control

Fig. 1. Representative gel filtration profile on Ultrogel ACA34 of ^{125}I -hGH-binding protein complex formed in the absence (●, total binding) and presence (○, non-specific binding) of excess (1 μg) unlabelled hGH. M_r markers ($\times 10^{-3}$) are shown by the arrows. Very similar data were obtained in more than ten such experiments.

Fig. 2. Representative gel filtration profile of a 200000g cytosol preparation from rabbit liver on Ultrogel ACA34 □, A₂₈₀; ●, % specific binding of ¹²⁵I-hGH as determined by the charcoal-separation method on 100 μl aliquots of each column fraction. M_r markers (× 10⁻³) are shown by the arrows. Similar binding profiles were obtained in three such experiments.

Fig. 3. Effect of increasing cytosol protein concentration on the specific binding of ¹²⁵I-hGH (a) and time course of association (●—●) and dissociation (●---●) of ¹²⁵I-hGH and rabbit liver cytosol binding protein (b)

Each data point represents the mean of duplicates which differed by less than 5% in all cases. In (b) binding was performed at 23°C and dissociation was initiated in one set of tubes by the addition of an excess (2 μg) of unlabelled hGH in a small volume (10 μl) to avoid any dilution effects. Only specific binding, expressed as a % of total ¹²⁵I-hGH added, is shown.

experiments, reasonable agreement was observed for specific hGH binding by the cytosolic preparation by using either the charcoal or gel-filtration methods (38% versus 48% respectively).

The binding of ¹²⁵I-hGH to the cytosolic binding protein was also dependent on the Ca²⁺ or Mg²⁺ concentration of the incubation buffer, rising from 2.2 ± 1.4% specific binding (mean ± S.E.M., n = 3) in the absence of cation, up to a maximum of 42.3 ± 2.3% (n = 3) at 40 mM-Ca²⁺ or Mg²⁺. This dependence is a typical feature of the well-characterized particulate or membrane-

bound GH receptor (Tsushima, 1978; Tsushima *et al.*, 1980). Several other binding characteristics of the GH cytosolic binding protein are similar to those of the particulate or membrane-bound receptor. There was a linear relationship between the specific binding of ¹²⁵I-hGH and cytosolic protein concentration up to 200 μg per tube (Fig. 3a). Routinely 200 μg of cytosolic protein was used in each experiment, yielding 30–45% binding of labelled hGH. The kinetics of association and dissociation at 23°C were typical of membrane-bound receptors (Fig. 3b) with a binding equilibri-

um being reached in 2 h and a half-time for dissociation (following addition of an excess of unlabelled hormone) of 4 h. However, not all bound ^{125}I -hGH was dissociable from the cytosolic binding protein.

Binding affinity and capacity of the cytosolic binding protein were estimated by Scatchard analysis (Scatchard, 1949) of dose-response curves for the displacement of ^{125}I -hGH by increasing concentrations of unlabelled hGH. Binding data were determined by using either the charcoal or gel-chromatography separation methods, both of which gave very similar results. The linear Scatchard plots (Fig. 4) were consistent with data obtained previously and gave a mean binding affinity $[(2.56 \pm 0.37) \times 10^9 \text{ M}^{-1}]$ (mean $\pm$ S.E.M., $n = 15$) which was similar to, although slightly higher than, that for either particulate, detergent- or aqueous-solubilized GH receptors (Herington & Veith, 1977; Waters & Friesen, 1979; Tsushima *et al.*, 1980; Herington *et al.*, 1981). The binding capacity for the cytosolic preparations ($515 \pm 90 \text{ fmol/mg}$ of protein; mean $\pm$ S.E.M., $n = 15$) was equivalent to that for the corresponding membrane preparations ($502 \pm 56 \text{ fmol/mg}$, $n = 5$). However, when the total amount of protein/preparation was taken into account the total number of binding sites in the cytosol compared with the membrane fraction was 1–2 times higher. Thus the cytosol represents a major, hitherto unrecognized, source of naturally soluble GH receptor. The advantages of this for purification studies are obvious.

The rabbit liver cytosolic binding protein has also been shown to interact with a variety of

affinity chromatography systems. Passage of cytosol through a column of hGH (or bGH) covalently attached to Affigel 15 resulted in essentially complete retention of GH-binding activity. The elution of bound proteins with 4M-MgCl₂ (e.g. see Fig. 5) effected a 200–300-fold purification as assessed by subsequent Scatchard analysis, which indicated a markedly increased hGH binding capacity (115000 fmol/mg of protein) and an unaltered binding affinity ($1.53 \times 10^9 \text{ l/mol}$). Such behaviour on GH affinity columns strongly favours the contention that rabbit liver cytosol does indeed contain a high affinity GH binding protein. Since we have no evidence for the presence of classical PRL receptors in the cytosolic preparation, there was no advantage in sequential elution of the affinity column with 4M-urea and 4M-MgCl₂, as described by Waters & Friesen (1979).

A similar result has been achieved by using a different type of affinity column, i.e. concanavalin A-Sepharose. Again the GH-binding activity of rabbit liver cytosol was bound to the column (results not shown), indicating that it was associated with a glycoprotein species. Elution of activity was achieved with 0.1M- α -methyl manno-pyranoside. These observations are also typical of those seen with solubilized membrane GH receptors (Tsushima, 1978; Herington *et al.*, 1981).

The question remains as to the nature, relevance and source of this cytosolic binding protein. Confirmation that it is a truly soluble binding protein preparation comes from the inability to sediment the binding activity at 200000g and its free passage through a 0.22 μm Millipore filter. It is highly unlikely that it represents a proteolytic

Fig. 4. Representative Scatchard plot for the binding of ^{125}I -hGH to rabbit liver cytosol

The mean binding parameters for 15 such experiments were: affinity, $2.56 \pm 0.37 \text{ l/mol}$; capacity, $515 \pm 90 \text{ fmol/mg}$, and were determined from dose-response curves for the displacement of ^{125}I -hGH by increasing concentrations of unlabelled hGH.

Fig. 5. Representative protein (●) and GH-binding (▨) profiles following affinity chromatography of rabbit liver cytosol on hGH-Affigel 15

The experiment was carried out as described in the Experimental section and is one of ten such studies performed.

degradation product of the membrane receptor, since all studies have been carried out in the presence of the proteinase inhibitors PMSF and Trasylol. A number of possibilities exist to explain its presence in the cytosol. First, it may be newly synthesized and/or spare receptor that is being stored within the cell prior to its transport to, and incorporation into, the cell membrane. Certainly this interpretation has been applied to GH receptors that have been reported previously in Golgi fractions of the cell (Bergeron *et al.*, 1978). Second, the cytosolic binding proteins may result from extraction from the membrane during the cell homogenization process. This would imply that the GH receptor is not an integral membrane protein but rather a peripheral protein, loosely associated with the cell membrane. However, very gentle hand homogenization of rabbit liver, as opposed to the more vigorous mechanical method normally used, had no effect in reducing the amounts of cytosolic GH binding protein obtained (results not shown). Finally, it remains an intriguing possibility that the cytosolic GH binding protein is a quite different and separate binding protein from the membrane-bound receptor. If this is the case then crucial questions arise as to its potential biological role and to possible reassessment of the manner in which GH might exert its multiple intracellular biological actions.

Further studies on the purification and structural characterization of both the cytosolic and membrane-bound GH binding proteins are in progress to determine the answers to some of these critical questions. Nevertheless, the data presented have shown for the first time that large quantities of a GH binding protein, similar to the 'classical' membrane GH receptor, are present in the cytosolic fraction of rabbit liver. Previous failures to detect this binding protein are due to the inability of the widely used poly(ethylene glycol) method to precipitate effectively this cytosolic GH-binding protein. This observation suggests that alternative detection methods should be used

by others to re-question the presence or absence of cytosolic receptors for other protein and peptide hormones. Utilization of this GH-binding-protein preparation for purification and structural studies will obviate the need for artificial methods of receptor solubilization which may well alter some of the native protein characteristics.

These studies were supported by the National Health and Medical Research Council of Australia. We thank Sue Smith and Anne Saunders for preparation of the manuscript and figures.

References

- Bergeron, J. J. M., Posner, B. I., Josefsberg, Z. & Sikstrom, R. (1978) *J. Biol. Chem.* **253**, 4058–4066
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Cuatrecasas, P. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 318–322
- Herington, A. C. & Veith, N. M. (1977) *Endocrinology* **101**, 984–987
- Herington, A. C., Elson, S. D. & Ymer, S. I. (1981) *J. Receptor Res.* **2**, 203–220
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Salacinski, P. R. P., McLean, C., Sykes, J. E. C., Clement-Jones, V. V. & Lowry, P. J. (1981) *Anal. Biochem.* **117**, 136–146
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
- Shiu, R. P. C. & Friesen, H. G. (1974) *J. Biol. Chem.* **249**, 7902–7911
- Tsushima, T. (1978) in *Proc. VIth Asia Oceania Congr. Endocrinol.* (Seng, C. J., Pin, L., Tambyah, J. A., Shanmugaratnam, S., Boon, P. Y. P., Ark, C. N. S., Foo, K. N. K. & Tau, N. T., eds.), pp. 365–373, Stamford College Press, Singapore
- Tsushima, T. & Friesen, H. G. (1973) *J. Clin. Endocrinol. Metab.* **37**, 334–337
- Tsushima, T., Sasaki, N., Imai, Y., Matsuzaki, F. & Friesen, H. G. (1980) *Biochem. J.* **187**, 479–492
- Waters, M. J. & Friesen, H. G. (1979) *J. Biol. Chem.* **254**, 6815–6825
- Ymer, S. I. & Herington, A. C. (1984) *Endocrinology*, in the press