Serum and liver cytosolic growth-hormone-binding proteins are antigenically identical with liver membrane ‘receptor’ types 1 and 2

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INTRODUCTION

We have previously reported the use of a panel of MAbs to the presumptive GH receptor in rabbit and rat liver [1,2] to demonstrate a close antigenic and possible ontogenic relationship between membrane ‘receptor’ subtypes 1 and 2 and the cytosolic GH-binding proteins that are present in large amounts in the rabbit liver [2,3]. We also reported antigenic mapping studies with these MAb, on the basis of which we postulated the existence of three subtypes of rabbit liver GH receptor [2], one of which (type 3) was not recovered after Triton X-100 solubilization of liver microsomes (microsomal fractions), although it shared at least one antigenic determinant with the other subtypes [2].

Ymer and co-workers [4] recently reported the existence of a serum GH-binding protein in the rabbit in man [5]. In the present paper we compare salient physico-chemical and antigenic features (with respect to our MAb panel) of these serum binding proteins with the cytosolic GH-binding proteins and with presumptive receptor subtypes 1, 2 and 3 in the rabbit liver.

MATERIALS AND METHODS

Materials

oGH (S-13 and I-3), hGH (I-1), oPRL-16, rabbit GH (AFP-684C) and rat PRL (B-3) were gifts from the National Hormone and Pituitary Program (Baltimore, MD, U.S.A.). hGH for receptor purification was generously provided by the Human Pituitary Advisory Committee of Australia (Woden, A. C. T., Australia). Porcine insulin was from Connaught Laboratories (Toronto, Canada).

Hormones were iodinated by the lactoperoxidase method of Thorell & Johansson [6], as described previously [7], and fractionated on a Sephadex G-100 column. Rabbit anti-mouse immunoglobulins were purchased from DAKO immunoglobulins (Glostrup, Denmark).

Preparation of receptor and binding-protein-containing fractions

The liver receptor preparations used in the present study were derived from a three-stage purification of the GH receptor [7]. Studies on inhibition of [1^125]iodohormone binding reported previously [1,2] were carried out on crude microsomal (100000 g, 60 min) fractions of rabbit liver. The cytosolic preparations used in the present (and previous) studies [2,3] were the supernatants from the crude microsomal preparations, filtered through Whatman no. 541 filter paper to remove fat. Immunoprecipitation and inhibition experiments were also carried out with Triton X-100 (1%, v/v)-solubilized extracts of these microsomal membranes (300000 g/90 min supernatants) or with rabbit liver GH receptor partially purified from these extracts by differential affinity chromatography on an hGH affinity column [7]. GH-binding proteins were purified by the same affinity-chromatography procedure from serum,

Abbreviations used: GH, growth hormone (somatotropin); PRL, prolactin; the prefixes h and o refer to the human and sheep hormones respectively; MAb, monoclonal antibody; PEG, poly(ethylene glycol); DMSO, dimethyl sulfoxide; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; r.r.a., radioreceptor assay.
after addition of MgCl₂ to 10 mM. In this case, no Triton X-100 was added to the serum or purification buffers. Laemmli [8] gels of this material showed major bands at 52000 and 80000 Mr, and the degree of purification was 105-fold. These bands presumably contain additional protein impurities, since this preparation was less than 1% pure as assessed by specific binding activity.

**Affinity cross-linking**

This was performed with either hGH-affinity-purified serum binding protein or hGH-affinity-purified liver GH receptor. Binding protein at approx. 10 fmol/ml was incubated for 12–14 h with ¹²⁵I-hGH (300 000 c.p.m./ml) in 20 mM-Hepes buffer [containing 0.1% Triton X-100, 10 mM-MgCl₂, 0.2% bacitracin, leupeptin and pepstatin (10 µg/ml), 10 mM-benzamidine and 1 mM-PMSF] at 20 °C. Disuccinimidyl suberate (Pierce) was added as a 100-fold concentrate in DMSO, with vortex mixing, to give a final concentration of 0.5 mM. Binding protein was left 15 min at 4 °C to cross-link, then cross-linking was blocked by the addition of Tris to 20 mM, pH 7.4. The solution was centrifuged briefly to clarify, then MAb 263 was added to 1:5000 (v/v) final dilution in the Hepes cross-linking buffer. This was left 8–12 h at 4 °C, then goat anti-mouse second antibody was added to a final dilution of 1:200, followed by a further 3 h incubation at 4 °C. Cross-linked, immunoprecipitated, ¹²⁵I-labelled complexes were harvested by centrifugation (2000 g, 20 min), then solubilized in Laemmli sample buffer with 10 mM-DTT and subjected to slab-gel electrophoresis by the method of Laemmli [8]. Gels were dried and autoradiographed with intensifying screens (Lightning-Plus; du Pont).

**Serum**

Serum was obtained from the arterial blood of mature male and female rabbits. After complete clotting at room temperature and 2 h at 4 °C to allow clot retraction, the blood was centrifuged at 4 °C. Serum and cytosol preparations were stored at −20 °C. The Ca²⁺ concentration in samples was determined by atomic-absorption spectrophotometry.

**Assay methods**

The cytosolic GH binding proteins and serum GH-binding proteins are PEG-soluble [2,4]. In order to perform receptor-binding assays by the standard methods [7], a precipitating MAb (MAb 1), which does not interfere with hormone binding [1], was added to the incubation mixtures at a 1:1000 dilution in r.r.a. buffer

![Fig. 1. Titration of precipitating MAbs against (a) detergent-solubilized liver microsomes; (b) cytosolic GH-binding proteins, (c) female and (d) male serum GH-binding proteins](image)

The ordinate shows the antibody-precipitable specific binding as a percentage of PEG-precipitable specific binding. Points represent means of triplicate determinations with s.e.m. values indicated. At a final assay dilution of 1:5000 the concentrations of the MAbs were: O, MAb5 (8 µg/ml); ●, MAb43 (4 µg/ml); ●, MAb1 (4 µg/ml); ▲, MAb2 (4 µg/ml); △, MAb44 (4 µg/ml). For (a), (b), (c) and (d) respectively the final concentrations of GH-binding sites were 90, 63, 300 and 320 fmol/ml. Specific binding of ¹²⁵I-hGH was 11, 9, 24 and 30% respectively. Non-specific binding was 5, 18, 14 and 17% respectively.
Liver growth-hormone-binding proteins

The binding-protein–hormone complexes were then PEG-precipitable. Our previous work [1] has shown that at least half of the affinity-purified rabbit 'GH receptor' is PEG-soluble, so to render the complexes completely PEG-precipitable, MAb 1 was also incorporated in inhibition assays with the affinity-purified 'receptor'.

Cytosol, serum and affinity purified 'GH receptor' preparations were pre-titrated for 

Inhibition of GH binding to cytosolic or serum binding proteins

A 100 µl portion of appropriately diluted serum, cytosol or affinity-purified rabbit GH receptor was pre-incubated overnight at 4 °C with serial dilutions of the test MAb (100 µl). Parallel serial dilutions of an unrelated MAb (anti-Brucella) were used as binding controls. A 100 µl portion of MAb 1 at 1:1000 was added to both test and control tubes. A 100 µl portion of 

Precipitation of binding-protein–hormone or 'GH receptor'–hormone complexes by MAb

Titration of precipitating MAb against Triton X-100-solubilized receptor–hormone complexes was performed by the standard double-antibody method as detailed in reference [1]. Titration of precipitating MAb against cytosolic or serum binding proteins was performed by using PEG to separate bound and free binding proteins as follows. After affinity labelling the diluted preparations overnight at 4 °C with 

Determination of affinity constants

Binding affinities of serum and cytosolic GH-binding proteins were estimated by Scatchard [9] analysis of 

respectively. Non-specific binding was 23 and 21% for hGH and oGH respectively. hGH-affinity-purified receptor was added to a final concentration of 52 fmol/ml. For (b), specific binding of hGH and oGH was 9 and 8% of added radioactivity respectively. Non-specific binding was 20 and 16% respectively. Binding protein was at a final concentration of 50 or 200 fmol/ml. For (c), specific binding was 22 and 6% for hGH and oGH respectively. Non-specific binding was 17% and 20% respectively.
cytosol. The specific radioactivity of the radiolabelled hormone was calculated from the fractional incorporation of total added radioactivity into peak fractions eluted from the Sephadex G-100 column used in the iodination procedure. No correction was made for bindability of labelled hormone.

**Production and characterization of the anti-(GH receptor) MAbs**

This has been described in detail previously [1,2]. The MAbs do not cross-react with prolactin or insulin receptors in the rabbit or rat. MAbs 263, 7 and 44 inhibit components of GH binding to rabbit liver microsomes, cytosol and affinity-purified 'GH receptor' (see [1,2] and the Results section).

**Independence of epitopes recognized by MAbs**

MAbs were tested for mutual competition and for competition with GH for binding sites in rabbit liver microsomes. The inhibition assays were performed by the standard method [2,3] with either 125I-MAb or 125I-GH as ligand. In this way, epitopes recognized by MAbs, 1, 2, 5, 7, 263, 43 and 44 were shown to be different, although in some cases (detailed in [2]) the MAb-binding regions (as distinct from epitopes) overlap.

**RESULTS**

**Comparative precipitation curves**

Figs. 1(a)–1(d) show the titration curves for the panel of MAbs against solubilized liver microsomes (Fig. 1a), liver cytosol (Fig. 1b) and unpurified serum binding proteins (Figs. 1c and 1d). The curves for serum and cytosol are virtually identical. Corresponding curves are parallel, and the relative order of titres is the same for both preparations (MAb 43 > MAb 5 > MAb 1 > MAb 2 > MAb 44). The curve for solubilized microsomes is not identical with the curves for cytosol or serum binding proteins, but the order of titres is preserved for the MAbs tested. The decrease in titre observed with detergent-solubilized 'GH receptor' (Fig. 1a) is attributable to the lower sensitivity of our double-antibody method and possibly to diminished reactivity of epitopes in the presence of detergent. A similar decrease in titre is observed when the double-antibody method is used with serum or cytosol, but in these cases, where detergent is not present, the slope of the titration curves is unchanged. A similar pattern is seen with affinity-purified 'GH receptor' derived from the solubilized microsomes [1]. Figs. 1(c) and 1(d) show the identity of antigenic profiles for male and female serum binding proteins.

**Comparative inhibition curves**

MAb 7 and MAb 263 inhibit components of GH binding to rabbit liver microsomes, affinity-purified preparations from these microsomes and the cytosolic binding proteins [1,2]. Figs. 2(a)–2(c) show inhibition of 125I-hGH or 125I-oGH binding to affinity-purified rabbit 'GH receptor' (Fig. 1a), cytosolic binding proteins (Fig. 1b) and serum binding proteins (Fig. 1c) by MAbs 263 and 7. Cytosolic and serum binding proteins were rendered PEG-precipitable by the inclusion of MAb 1 in the incubation mixture (see the Materials and methods section).
Liver growth-hormone-binding proteins

Fig. 5. (a, b) \(\text{Ca}^{2+}\)-dependence of hGH binding to serum and cytosolic binding proteins, and (c, d) \(\text{Ca}^{2+}\)-independence of oGH binding to serum and cytosolic binding proteins

Mab 1 was added to a final dilution of 1:5000 (v/v). (a) Serum at 1:100 (v/v) final dilution; (b) cytosol at 1:250 (v/v) final dilution. • Specific binding of \(^{125}\text{I}-\text{hGH}\) with increasing concentrations of \(\text{Ca}^{2+}\) and without \(\text{Na}^{+}\); ▲ specific binding of \(^{125}\text{I}-\text{hGH}\) with increasing concentrations of \(\text{Na}^{+}\) and with 150 mm-\(\text{Na}^{+}\); ○ inhibition of specific binding of hGH, by MAb 263 [1:5000 (v/v) final] at each \(\text{Ca}^{2+}\) concentration in the absence of \(\text{Na}^{+}\); ■ for ○ at zero added \(\text{Ca}^{2+}\), but with the inclusion of 10 mm-EDTA. These two points (■, ○) do not differ significantly \((P > 0.10, \text{paired} \ t \text{test})\). The specific radioactivity of \(^{125}\text{I}-\text{hGH}\) was approx. 64 \(\mu\text{Ci}/\mu\text{g}\). The specific binding was determined by displacement with excess unlabelled oGH (2 \(\mu\text{g/ml}\)) for reasons given in the Results section. Points represent means of triplicate determinations, with the s.e.m. indicated. (c) Serum at 1:10 (v/v) final dilution; (d) Cytosol at 1:125 (v/v) final dilution. •. Specific binding of \(^{125}\text{I}-\text{LoGH}\) with increasing concentrations of \(\text{Ca}^{2+}\) and without \(\text{Na}^{+}\); ○ inhibition of specific binding of oGH by MAb 263 [1:5000 (v/v) final] at each concentration of \(\text{Ca}^{2+}\). The specific radioactivity of \(^{125}\text{I}-\text{oGH}\) was approx. 77 \(\mu\text{Ci}/\mu\text{g}. Specific binding was determined by displacement with excess unlabelled oGH (2 \(\mu\text{g/ml}\)).

The same inhibition pattern is seen with both MAb for all three preparations. Just as for cytosol and affinity-purified rabbit 'GH receptor', MAb 263 does not inhibit \(^{125}\text{I}-\text{hGH}\) binding, but inhibits up to 30\% of \(^{125}\text{I}-\text{oGH}\) binding to the serum binding proteins. So, with respect to the MAb 263 epitope, the serum binding proteins display the same differential binding of hGH and oGH as do the cytosolic binding proteins and the affinity-purified 'GH receptor' [3]. MAb 7 completely inhibits oGH or hGH binding to the serum binding proteins, so analogues of the postulated membrane receptor type 3 are not present in the serum, just as they are absent from cytosol (see the Discussion section).

The antigenic profiles are qualitatively the same in male and female sera, but there is a slightly greater amount of GH-binding protein in female serum (see below).

GH-binding capacity and affinity of the serum binding proteins

Scatchard analysis [9] was performed on serum from four mature male and four mature female rabbits (Fig. 3). The affinity of the serum binding protein for hGH was not significantly different \((P > 0.1)\) in males and females \([2.45(\pm 0.15) \times 10^6 \text{M}^{-1} \text{mean} \pm \text{s.e.m.}, n = 8]\). The binding capacity was marginally higher in females \([12980(\pm 1320) \text{fmol/ml of undiluted serum in females} (n = 4)]; \text{cf.} \ 9380(\pm 1070) \text{fmol/ml} \text{in males} (n = 4, P < 0.05)]\). In comparison, the binding capacity of female liver microsomes is 8000 ± 1600 fmol/g of liver homogenized and the affinity for hGH is approx. \(3 \times 10^9 \text{M}^{-1}[7,10]\).

When serum was centrifuged at 100000 \(g\) for 1 h at 4 °C, the binding capacity and affinity estimates were not decreased \((\text{paired} \ t \text{test}; P > 0.10 \text{at all points on displacement curves from which Scatchard plots were derived})\), so GH-binding sites on blood cells are not interfering in the assay systems.

Specificity of the serum-binding-protein binding sites

Fig. 4(a) shows the competition of oGH, hGH, oPRL and porcine insulin for \(^{125}\text{I}-\text{hGH}\)-binding sites precipitable from male serum by MAb 1. As for membrane-associated GH-binding sites, PRL competes with the GHs, but with approx. 10-fold lower potency than oGH \((n = 4, P < 0.05)]\). Insulin has no effect on GH binding to the serum binding proteins, even at concentrations as high as 10 \(\mu\text{g/ml}\). Fig. 4(b) shows the competition of rabbit GH and rat prolactin for \(^{125}\text{I}-\text{hGH}\)-binding sites precipitable from male rabbit serum. The homologous ligand, rabbit GH,
Ca\textsuperscript{2+}-dependence of GH binding to serum, cytosol and microsomes

hGH binds to serum (Fig. 5a) and cytosolic binding proteins (Fig. 5b) with virtually complete Ca\textsuperscript{2+}-dependence. Physiological concentrations of Na\textsuperscript{+} can largely obviate the requirement for Ca\textsuperscript{2+}, so that, in the presence of Na\textsuperscript{+}, a smaller Ca\textsuperscript{2+}-potentiated component is observed (Figs. 5a and b). By contrast, oGH binding to cytosolic or serum binding proteins is not Ca\textsuperscript{2+}-dependent or Ca\textsuperscript{2+}-potentiated (Figs. 5c and d). This is significant, since the Ca\textsuperscript{2+}-dependent hGH binding is completely displaceable by oGH. Apparently these ligands bind to the same sites, but with different cation-dependencies. hGH binding to liver microsomes is also Ca\textsuperscript{2+}-dependent, and oGH binding is not, in a manner parallel with that for serum and cytosolic binding proteins (results not shown).

Affinity labelling

Fig. 6 shows the close similarity between serum and liver-membrane-derived GH-binding proteins, in that both run at 55000 Mr (when 22000 is subtracted for the contribution of hGH), and, in both cases, the radioligand is displaceable by 5\mu g of hGH/ml included in the original incubation mixture. Although total displacement was not obtained for the membrane-derived binding protein, residual binding in the presence of excess unlabelled hormone is small relative to the uncompeted-for 77000 Mr complex. The displaceable component at 45000 Mr probably represents hGH dimer. This implies that the hGH dimer is capable of binding to membrane and serum binding proteins, since the cross-linked hGH-receptor complex is immunoprecipitated with anti-receptor antibody before electrophoresis.

DISCUSSION

The ligand-binding studies hitherto performed by numerous workers (for review, see [13]) have, with rare exception, not addressed the question of the relevance of the GH-binding sites detected to the mediation of GH actions. Griechting and co-workers [14] did not detect gross differences in binding capacity of adipocytes sensitive or refractory to the insulin-like actions of GH, but could not rule out modulation of small subsets of receptors. Baxter & Zaltsman [15] showed that Somatomedin-C levels positively correlated with 'GH receptor' number in hypophysectomized rats, but negatively correlated in intact animals. The 20000-Mr variant of hGH is equipotent with 22000-Mr hGH in stimulating growth in hypophysectomized rats [16], but is a poor inhibitor of 22000-Mr hGH binding to rabbit liver microsomes [17]. Hughes et al. [12] showed that 20000-Mr hGH was bound with high affinity only by a subset of 'receptors' in the rabbit. To understand these results, the equation of subsets of GH-binding sites with metabolic actions is essential.

The existence of a close structural relationship between subsets of membrane-associated GH-binding sites (the putative 'receptor' subtypes 1 and 2 [2]), the serum GH-binding proteins and the cytosolic GH-binding proteins dictates a reappraisal of earlier ligand-binding studies, which could not discriminate distinct subsets of binding sites.

Our previous studies revealed antigenic heterogeneity...
of the liver GH-binding sites [2], as well as selective ligand binding to the ‘receptor’ subtypes so defined [2,3]. Analogues of subtypes 1 and 2 exist in large amounts in liver cytosol [2,3]. The present work shows the existence in the circulation of corresponding proteins sharing epitopes with membrane ‘GH receptor’ subtypes 1 and 2 and cytosolic binding proteins. The common epitopes are located both in the hormone-binding region and elsewhere.

The existence of a ‘type 3’ GH receptor in rabbit liver membranes was inferred from our earlier antigenic mapping studies [2]. Inhibition of specific 125I-oGH binding to rabbit liver membranes was not total, even at high MAb 7 concentrations (although maximum inhibition obtained could be augmented with MAb 263) [2]. MAb 7 could, however, totally inhibit 125I-GH binding to rabbit liver membranes [2]. By contrast, MAb 7 totally inhibits 125I-oGH binding to serum or cytosol. These results can be interpreted as evidence against the presence of the ‘type 3’ GH receptor in serum and cytosol, although it is present in liver microsomes before solubilization and in plasma membrane or endoplasmic reticulum fractions derived from the microsomes [2,3].

The possibility emerges that the cytosolic GH-binding proteins may be newly synthesized serum binding proteins in transit to the cell membrane and hence the circulation, or even endocytosed serum binding proteins.

We have previously shown that the same antigens are associated with GH-binding sites in both endoplasmic reticulum and plasma-membrane fractions from hepatocytes. It is now apparent that antigenically identical analogues of presumptive receptor subtypes 1 and 2 exist in the endoplasmic reticulum, cytosol, hepatocyte membrane and the circulation. These findings strongly implicate the liver as a site of synthesis of the serum GH-binding proteins.

In their studies of hGH heterogeneity, Stolar et al. [18] and other groups [19–21] were unable to demonstrate binding of exogenous GH to human plasma proteins, by gel filtration of plasma/GH co-incubation mixtures. However, these studies did not rule out the possibility of non-covalent interaction between hGH and plasma proteins. Our work supports the finding of Ymer et al. [4] that GH is bound with high affinity by a specific binding protein in the rabbit and in man [5]. Furthermore, the application of MAb has enabled quantification and antigenic characterization of the serum GH-binding protein in the rabbit. In addition, we have shown that the major GH-binding subunit of both liver and serum that is immunoprecipitated by MAb 263 has an Mr of 55000.

The rabbit serum binding protein has high affinity for hGH (2.45 \times 10^8 M^{-1}) and is present in substantial amounts in the serum (~ 13 pmol/ml of undiluted female serum; cf. ~ 8 pmol of microsomal ‘receptor’/g of female liver homogenized). Given the quantity of serum binding protein and the similar affinities of membrane and serum-binding-protein binding sites for GH, the conditions exist for the controlled exchange of GH from serum binding proteins to membrane receptors.

The binding capacity we measure in male or non-pregnant female rabbits is higher than that reported by Ymer et al. [4] [our value 13 pmol/ml (female); cf. 3.8 pmol/ml [4]]. Ymer et al. [4] used a gel-filtration system at room temperature to separate bound from free ligand. This process may well involve some dissociation of bound hormone, as evidenced by the small competition of oPRL for serum binding sites reported by these workers (although Ymer et al. [22] did report substantial displacement of 125I-hGH from the cytosolic binding protein by oPRL when the gel-filtration method was used). We find substantial displacement of 125I-hGH from the serum binding proteins by oPRL, albeit with 20-fold lower potency than hGH, as for membrane-associated ‘GH receptors’ [7]. This result is consistent with the similarity of the serum binding protein to membrane-associated ‘GH receptors’ and cytosolic binding proteins in four other respects (i.e. antigenic identity of binding and other sites, similar affinity, differential ligand binding [3] and identical cation-dependence).

Our estimate of the affinity of the serum binding protein for hGH [2.45 (\pm 0.15) \times 10^8 M^{-1}] is close to values reported for membrane-associated ‘GH receptor’ [7,10] and cytosolic binding proteins [2,22].

It is significant that the serum binding proteins show the same differential binding of hGH and oGH as do the cytosolic and membrane-associated binding sites [3]. Our finding, that oGH and hGH binding to the same sites is Ca\({}^{2+}\)-independent in the former case and Ca\({}^{2+}\)-dependent in the latter case, supports our earlier observations of differential ligand binding [3]. In studies of GH-binding sites in the mouse liver, Ciccia-Torres & Dellacha [23] raised the possibility of shared binding sites that bound lactogenic and somatogenic ligands with different cation-dependencies.

Differential binding of GH variants to the serum binding proteins could control their particular availability to membrane GH receptors in target organs or the rate of clearance of different GHs from the circulation (Baumann et al. [24] have recently shown that the 20000- and 22000-Mr GHs have different metabolic half-lives). Discrimination of PRL and GH by the serum binding protein may be significant in this regard. Shin’s study with the MCF-7 and T-47D mammary-tumour-cell lines [25] showed that both hGH and hPRL bind with similar affinity to the PRL receptor in these human cells. Kleinberg & Todd [26] have found that hGH is a potent lactogen in subhuman primates. Sequestration of hGH by the circulating serum binding proteins could be necessary to prevent a permanent state of galactorrhoea. Conversely, a deficiency of serum GH-binding proteins could have a role in the aetiology of galactorrhoea.

The serum GH-binding proteins are likely to play a crucial role in regulating availability of GH to membrane-associated receptors or the cell interior.

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