Evidence from the use of monoclonal antibody probes for structural heterogeneity of the growth hormone receptor

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1. We describe the use of four monoclonal antibodies (MAbs) to the rabbit liver growth hormone (GH) receptor and one raised against purified rat liver GH receptor to characterize liver receptor subtypes which differ in their hormone-binding regions. 2. The anti-(rat liver GH receptor) MAb both inhibited and precipitated rat and rabbit GH receptors, but only one-half of ^{125}I -oGH (ovine GH) binding to liver microsomes could be inhibited by excess antibody. Conversely, only one-half of ^{125}I -anti-(rat GH receptor) MAb binding was inhibited by excess oGH and Scatchard plots for this MAb exhibited two components. Although only 50% of ^{125}I -oGH binding to membranes was inhibited by this MAb, all solubilized receptor could be immunoprecipitated. 3. We postulate two epitopes for the anti-(rat GH receptor) MAb, one located at the hormone-binding site (inhibitory site) and one elsewhere (immunoprecipitating site). 4. A second, rabbit-specific antibody (MAb 7) inhibited 85% of hormone binding but only 30% of ^{125}I -anti-(rat GH receptor) MAb binding to rabbit liver microsomes. A combination of this MAb with the anti-(rat GH receptor) MAb totally inhibited ^{125}I -oGH binding. MAb 7 alone totally inhibited ^{125}I -rat GH binding to rabbit liver microsomes, as it did with ^{125}I -oGH binding to purified receptor. 5. On the basis of these results and others we postulate three types of GH receptor in rabbit liver membranes and ascribe approximate extents of ^{125}I -oGH binding to each. 6. A cytosolic 'GH receptor' which is not poly(ethylene glycol)-precipitable is shown to share five epitopes with 'type 2' microsomal receptors. 7. Purified plasma membrane and endoplasmic reticulum fractions derived from a rabbit liver microsomal preparation have identical antigenic characteristics with respect to the GH-binding region, indicating that the heterogeneity we describe is not related to receptor processing. 8. Of the three types of GH receptor in the plasma membrane of the rabbit (and possibly rat) we postulate that one (type 1) corresponds to the GH receptor involved in stimulating growth and possesses all of the epitopes studied here. A second (type 2) appears to be identical with the cytosolic 'GH receptor' and lacks the epitope for the anti-(rat GH receptor) MAb in the hormone binding site region. A third (type 3) does not possess the epitope for the inhibitory anti-(rabbit GH receptor) MAb, appears not to bind rat GH and is lost during purification. 9. The availability of type-specific MAbs will facilitate assignment of specific functions to liver receptor subtypes which mediate the multiple functions of GH.

INTRODUCTION

It is now well established that GH in several species exists not as a single peptide but as a family of peptides which differ in their bioassay and immunological properties (Russell *et al.*, 1978; Moore *et al.*, 1982; Baumann & Abramson, 1983; Chawla *et al.*, 1983; Paladini *et al.*, 1983; Hart *et al.*, 1984). Some of this variation arises at the transcriptional level, and some results from selective proteolysis of the mature, or 22 kDa, form of the hormone (Chawla *et al.*, 1983; Paladini *et al.*, 1983). The functional importance of these variants is not clear, but in the human the 20 kDa and 22 kDa forms are found both in the pituitary and in the circulation (Baumann & Abramson, 1983), and these differ in their effects on carbohydrate metabolism (Frigeri *et al.*, 1979).

Individual members of this peptide family could

produce different effects on target organs either by interacting differently with a single class of receptor or by binding preferentially to particular types of receptor which mediate specific actions of GH. The existence of receptor subtypes could also explain the differences in dose-response curves, time courses, sensitivity to refractoriness and other phenomena described by Grichting *et al.* (1983) in their comparison of the different actions of GH *in vitro*.

Evidence has recently been presented for the existence of tissue-specific prolactin receptors which may mediate the various actions of prolactin (Waters *et al.*, 1984), and in that study evidence for 'iso-receptors' of other peptide hormones was summarized. This report describes the use of a panel of monoclonal antibodies to the GH receptor [recently developed in our laboratory (Barnard *et al.*, 1984)] to reveal receptor heterogeneity in the rabbit and rat liver.

Abbreviations used: GH, growth hormone (somatotropin); PRL, prolactin; the prefixes h, o and r refer to the human, sheep and rat hormones respectively; MAb, monoclonal antibody; PEG, poly(ethylene glycol).

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MATERIALS AND METHODS

Materials

GH (oGH S-12 and I-3), GH (hGH HS 2019G), rGH (I-4), and PRL (oPRL S-15) were gifts from the National Pituitary Agency (Baltimore, MD, U.S.A.). GH for receptor preparation was kindly provided by the Human Pituitary Advisory Committee of Australia.

All hormones except rGH were iodinated by the lactoperoxidase method of Thorell & Johansson (1971) as described previously (Waters & Friesen, 1979). Rat GH was iodinated by the Iodogen method of Salacinski *et al.* (1981). Hormones were fractionated on a Sephadex G-100 column (1.7 cm $\times$ 50 cm) after iodination. MABs were iodinated by the lactoperoxidase or Iodogen methods and were similarly fractionated. Specific radioactivities of iodinated MABs were in the range 50–150 μ Ci/ μ g.

Rabbit anti-(mouse immunoglobulins) were purchased from DAKO Immunoglobulins (Copenhagen, Denmark). Anti-isotype antibodies were obtained from Miles Laboratories (Elkhart, IN, U.S.A.).

Reagents for enzyme assays were obtained from Sigma or BDH.

Reagents for hybridoma production

RPMI-1640 medium was purchased from Flow Laboratories, HB101 medium was obtained from Hana Biologicals (Berkeley, NJ, U.S.A.), all other reagents were obtained from Sigma. Costar 24-well plates were purchased from Costar (Cambridge, MA, U.S.A.) and flexible 96-microwell poly(vinyl chloride) microtiter plates were obtained from Dynatech Laboratories (Alexandria, VA, U.S.A.).

Preparation of receptor-containing fractions

The GH receptor preparations used in this study are derived from three stages in the purification method for rabbit GH receptor, as described previously (Waters & Friesen, 1979). The majority of the studies involving inhibition of 125 I-hormone binding were done with crude microsomal (100 000 *g*, 60 min) fractions of late pregnant rabbit liver or rat liver. Immunoprecipitation studies were undertaken with either Triton X-100-solubilized (1% v/v) extracts of the microsomal membranes (300 000 *g*, 90 min supernatants), or with rabbit liver GH receptor partially purified from such supernatants by differential affinity chromatography on an hGH affinity column (Waters & Friesen, 1979). Inhibition studies were also performed with this partially purified GH receptor. Affinity-purified rat GH receptor was used in the hybridoma screening procedure described below. Rabbit liver cytosol (100 000 *g*, 60 min, supernatant filtered through Whatman no. 541 paper to remove fat) was also used for some precipitation and inhibition studies.

Receptor-binding assays were carried out according to the method of Barnard *et al.* (1984) using 125 I-oGH, 125 I-hGH or 125 I-rGH. Inhibition and precipitation assays with rabbit liver cytosol were carried out by standard methods (Waters & Friesen, 1979) except that 100 μ l of precipitating MAB 1 [at an added dilution of 1:1000 in RRA buffer (Waters & Friesen, 1979)] was incorporated into the preincubation step of the inhibition assay. This enables PEG precipitation of receptor-hormone complexes which are otherwise PEG-soluble (Barnard *et al.*, 1984). All data has been plotted as

a percentage of specific binding, i.e. non-specific binding has been subtracted. Specific, ligand-displaceable binding is determined in binding control tubes with parallel, serial dilutions of an anti-*Brucella* MAB of the same isotype (IgG $_{1\kappa}$) as the test MAB.

The fluorescence method of Stowell *et al.* (1978) was used to determine the protein concentration of affinity-purified GH receptor preparations.

Immunodepletion studies

Affinity-purified GH receptor or rabbit liver cytosol (400 μ l; at four times the concentration required for maximum specific binding of 125 I-GH in the radioreceptor assay) was incubated with 400 μ l of test MAB (at 1:1000 dilution in RRA buffer) for 3 h at 4 °C. Rabbit anti-(mouse globulins) (0.4 ml at the appropriate dilution) and normal mouse serum (at 1:100 dilution in RRA buffer, 0.4 ml) were then added and incubation was continued for 90 min at room temperature. The reaction mixture was centrifuged at 1600 *g* for 25 min at 4 °C and the supernatant was tested (in parallel with undepleted GH receptor) for residual GH binding activity in a standard radioreceptor assay with added MAB 1 to detect residual PEG-soluble GH receptor.

Inhibition of 125 I-MAB binding to GH receptor by GH

This was assayed in the same manner as the standard inhibition assay (Waters & Friesen, 1979), but using radiolabelled antibody and an appropriate dilution of membranes (Barnard *et al.*, 1984).

Determination of affinity constants (K_d)

Binding affinities of MABs were estimated by Scatchard analysis (Scatchard, 1949) of 125 I-MAB binding to pretitrated rabbit liver or rat liver membranes in the presence of increasing concentrations of corresponding unlabelled MAB (Barnard *et al.*, 1984). Curves were fitted with the Rodbard-Munson program SCAFIT provided by the Biomedical Computing Technology Information Center (Vanderbilt, TN, U.S.A.).

Immunization and hybridoma production

Production of rabbit-specific MABs. The immunization protocol, screening procedure and characterization of MABs 1, 2, 5 and 7 has been described in detail previously (Barnard *et al.*, 1984).

Production of MAB 263 which recognizes a cross-species determinant. hGH affinity-purified rat GH receptor was prepared by the method of Waters & Friesen, (1979). The purified receptor had a specific activity of 900 fmol/mg. BALB/c mice (previously immunized with purified rabbit GH receptor) were given a series of subcutaneous injections of 0.2 ml of rat GH receptor (0.8 mg) in Freund's complete adjuvant at 2 week intervals. This was followed by an intraperitoneal boost of 0.4 mg in normal saline. At 4 days after the boost injection, serum from these mice inhibited 50% of 125 I-oGH binding to rat membranes at a final dilution of 1:400.

At 3 days after the boost injection, splenic lymphocytes from three mice were pooled and fused with NS-1 myeloma cells, as described by Barnard *et al.* (1984).

Screening of hybridoma supernatants

Screening procedures were identical to those described by Barnard *et al.* (1984), except that affinity-purified rat

Fig. 1. Scatchard analysis of ^{125}I -Mab 263 binding to (a) rabbit and (b) rat liver microsomes

Specific binding of added radioactivity was 4% to rat liver membranes and 5% to rabbit liver membranes. Points represent means of triplicate determinations for one membrane preparation. Very similar results were obtained with a second preparation. Curves were fitted with the Rodbard-Munson SCAFIT program. B/F , bound/free ratio. Non-specific binding to rat and rabbit liver membranes was 8% and 3% respectively of radioactivity added. Specific activity of ^{125}I -Mab 263 was 112 $\mu\text{Ci}/\mu\text{g}$.

GH receptor or rat liver microsomes were substituted for rabbit GH receptor or microsomes in the initial screening assays. Of 384 hybridoma supernatants screened, one was positive for precipitation of ^{125}I -hGH affinity-labelled rat GH receptor and also inhibited ^{125}I -oGH binding to rat liver microsomes. The parent line was cloned by limiting dilution and the strongest stable secretor was grown in pristane-primed BALB/c mice to produce ascitic fluid. The hybridoma continued to produce MAb as an ascitic tumour. The MAb was purified from ascitic fluid by $(\text{NH}_4)_2\text{SO}_4$ precipitation followed by dialysis or gel filtration on Sephacryl S-300. Estimates of ^{125}I -Mab purity and isotyping of Mab 263 was carried out as described by Barnard *et al.* (1984). Electrophoresis of ascites fluids showed the MAbs to be intact and homogeneous.

Fig. 2. Inhibition of ^{125}I -oGH binding to rat and rabbit liver microsomes by Mab 263

Rat liver microsomes (○) were used at a final GH receptor concentration of 45 fmol/ml and rabbit liver microsomes (●) at a final GH receptor concentration of 45 fmol/ml. Points represent means of quintuplicate (○) or triplicate (●) determinations with S.E.M. values shown. Parallel dilutions of unrelated ascites fluids were used as binding controls. Specific binding to rat and rabbit liver microsomes was 11% and 18%, respectively, of added radioactivity. Non-specific binding was respectively 10% and 9% of added radioactivity.

Fig. 3. Scatchard analysis of ^{125}I -hGH binding to rabbit liver microsomes in the presence and absence of Mab 263

Rabbit liver microsomes were added at 400 fmol/ml (●; membrane preparation 1) or 600 fmol/ml (▲; preparation 2). Scatchard analysis was performed by the standard method (see the Materials and methods section) except Mab 263 at a final dilution of 1:500 was co-incubated with ^{125}I -hGH and membranes overnight at 4 °C. ○, Membrane preparation 1 with Mab 263; △, membrane preparation 2 with Mab 263. Points represent means of triplicate determinations. B/F , bound/free ratio. Maximum specific binding was 11% of radioactivity added. Non-specific binding was 4.5%.

Mab 263 did not possess anti-hormone activity and did not inhibit ^{125}I -insulin or ^{125}I -PRL binding to insulin or prolactin receptors in rabbit or rat liver microsomes. It also did not inhibit ^{125}I -PRL binding to rabbit mammary microsomes.

Fig. 4. Inhibition of ^{125}I -MAb 263 binding to rat (○) and rabbit (●) liver membranes by unlabelled oGH

Rabbit microsomes were at a final GH receptor concentration of 45 fmol/ml. Rat microsomes were at a final GH receptor concentration of 90 fmol/ml. Specific binding of ^{125}I -MAb 263 was 4% of radioactivity added to both rat and rabbit microsomes, determined by displacement with excess unlabelled MAb. Points represent means of triplicate determinations, with s.e.m. values indicated. The curve for inhibition of MAb 263 binding to rabbit microsomes (●) differs significantly where indicated from the curve of Fig. 7. These points were paired for statistical comparison on the basis of equimolar addition of oGH and MAb 7; * $P < 0.025$, ** $P < 0.01$. Non-specific binding was 12% and 4% of radioactivity added to rat and rabbit liver microsomes respectively.

Isolation of blood sinusoidal rabbit plasma membranes

For subfractionation studies, plasma membranes were prepared using a modification, by Wisher & Evans (1975), of the method of Touster *et al.* (1970). Unperfused liver (30 g) from a fasted rabbit was dispersed in 0.25 M-sucrose/5 mM-Tris/HCl, pH 8.0, using two strokes of a loose-fitting Dounce homogenizer. Further homogenization was performed with two up-and-down strokes in a Potter homogenizer with a Teflon pestle rotating at 300 rev./min. A microsomal fraction was prepared and then fractionated on a discontinuous sucrose gradient as described by Touster *et al.* (1970). Upper and lower sucrose layers (0.25 M and 57%, w/v, respectively) were the same as those used by Touster *et al.* (1970). The sucrose concentration in the middle layer was varied from 24% to 34% (w/v) in 2% increments to ascertain the best conditions for enrichment of the plasma membrane fraction and separation from endoplasmic reticulum, with the conclusion that for rabbit liver membranes the 34% concentration used by Touster *et al.* (1970) was optimal.

The degree of purity of the plasma membrane fractions was estimated by comparing relative specific activities of marker enzymes in homogenates and membrane fractions. 5'-Nucleotidase was used as the plasma membrane marker, succinate:cytochrome *c* reductase for mitochondria, and NADPH:cytochrome *c* reductase or glucose-6-phosphatase for endoplasmic reticulum. Glucose-6-phosphatase appeared to be a better marker for endoplasmic reticulum than NADPH:cytochrome *c*

Fig. 5. Inhibition of ^{125}I -MAB 263 binding to rabbit liver microsomes by MAb 2

Rabbit liver microsomes were used at a final GH receptor concentration of 45 fmol/ml. Specific binding of ^{125}I -MAB 263 was 6% of radioactivity added. Points represent means of triplicate determination with s.e.m. values shown. Parallel dilutions of unrelated ascites fluid were used as binding controls. Non-specific binding was 9% of radioactivity added.

reductase in rabbit liver, because glucose-6-phosphatase activity decreased in inverse proportion to the enrichment of 5'-nucleotidase in plasma membrane fractions.

5'-Nucleotidase activity was estimated in liver homogenates and fractions using the method of Emmelot & Bos (1966), except that sodium potassium tartrate at 10 mM was included as an inhibitor of acid phosphatases (Michell & Hawthorne, 1965). Glucose 6-phosphatase was determined in 0.1 mM-Tris/maleate buffer, pH 6.2, containing 4 mM-EDTA and 1 mM NaF (a modification of the original method of Swanson, 1955). The activity of non-specific phosphatases in both 5'-nucleotidase and glucose-6-phosphatase assays was estimated using 5 mM-phenyl disodium orthophosphate as substrate without 5'-AMP or glucose-6-phosphate (Pass *et al.*, 1981). Inorganic phosphate liberated in the above assays was estimated by the method of King (1932).

NADPH and succinate:cytochrome *c* reductases were estimated by the method of de Duve *et al.* (1955). The concentration of protein in liver homogenates and fractions was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

RESULTS

Properties of anti-(GH receptor) MABs

We previously reported the production and characterization of four MABs (1, 2, 5 and 7) which react exclusively with GH receptors in rabbit tissue (Barnard *et al.*, 1984). MAB 263 precipitates affinity-purified rabbit and rat GH receptor and inhibits GH binding to both rat and rabbit liver membranes. A titre of 1:20000 [defined by Barnard *et al.* (1984)] was obtained with ascitic fluid at protein concentration of 3.2 mg/ml. MAB 263 was isotypized as an IgG_{1κ}. Scatchard analysis of ^{125}I -MAB

Fig. 6. Inhibition of ^{125}I -oGH binding to rabbit liver microsomes by MAb 263 and/or MAb 7

Inhibition assays were performed by the standard method (see the Materials and methods section). MAb concentrations were standardized to 5 mg/ml before serial dilution. Specific binding of ^{125}I -oGH was determined by addition of excess unlabelled oGH to parallel dilutions of unrelated ascites fluid (anti-brucella) in binding controls. The curve showing inhibition of oGH binding by MAb 7 (○) differs significantly from the curve showing inhibition by a combination of MAbs 263 and 7 at the same total concentration (■) where indicated. The upper curve (●) shows inhibitions by MAb 263 alone. Points are means of triplicate determinations with S.E.M. value indicated. * $P < 0.05$; ** $P < 0.01$. Specific binding of radioactivity added was 11%. Non-specific binding was 6% of radioactivity added.

263 binding to rat and rabbit liver microsomes showed two classes of binding site (Fig. 1). Use of Munson's curve-fitting program gave affinity constants of $14.0 (\pm 4.0) \times 10^{10} \text{ M}^{-1}$ and $4.5 (\pm 2.0) \times 10^9 \text{ M}^{-1}$ in the rabbit and $9.0 (\pm 3.0) \times 10^9 \text{ M}^{-1}$ and $2.5 (\pm 1.0) \times 10^8 \text{ M}^{-1}$ in the rat.

Evidence for receptor heterogeneity in microsomal membranes

MAb 263 inhibits 50% of oGH binding to rat and rabbit liver microsomes (Fig. 2). Scatchard analysis of ^{125}I -GH binding to rabbit liver microsomes in the presence or absence of MAb 263 showed inhibition by reduction in number of receptor sites, with unblocked sites having unchanged affinity for GH (Fig. 3), as for MAb 7 (Barnard *et al.*, 1984). Conversely, ^{125}I -MAb 263 binding to rat or rabbit liver microsomes is only 50% inhibited by excess oGH (Fig. 4). This shows the presence of MAb 263 epitopes not within 3.5 nm (35 Å) of the hormone-binding sites (Tzartos *et al.*, 1981). The existence of different types of MAb 263 binding site is also evidenced by other observations. Firstly, Scatchard plots of MAb 263 binding to rabbit liver microsomes are biphasic (Fig. 1). Secondly, inhibition of ^{125}I -MAb 263 binding to rabbit liver microsomes by MAb 2, a

Fig. 7. Inhibition of ^{125}I -MAb 263 binding to rabbit liver microsomes by MAb 7

Rabbit microsomes at a final GH receptor concentration of 45 fmol/ml were incubated overnight at 4 °C with serial dilutions of MAb 7. ^{125}I -MAb 263 was then added and incubated for 4 h at 22 °C. Specific binding of ^{125}I -MAb 263 was 5% of radioactivity added, determined by displacement with excess unlabelled MAb (10 µg/ml). Points represent means of triplicate determinations with the S.E.M. values indicated. Parallel dilutions of unrelated ascites fluid were used as binding controls. Non-specific binding was 4% of added radioactivity. The curve differs significantly from the corresponding curve in Fig. 4 (inhibition of MAb 263 binding to rabbit liver microsomes by oGH) where indicated. The points were paired for statistical comparison on the basis of equimolar addition of oGH and MAb 7; * $P < 0.025$; ** $P < 0.01$.

precipitator only (Barnard *et al.*, 1984), also shows two components with total inhibition (Fig. 5).

Although MAb 7 inhibits 80–90% of GH binding to rabbit liver microsomes [Fig. 6 and Barnard *et al.* (1984)], at high concentration (25 µg of MAb protein/ml) it inhibits only 30% of ^{125}I -MAb 263 binding to the same microsomes (Fig. 7). oGH is capable of inhibiting only 50% of MAb 263 binding to these microsomes (Fig. 4). Thus, MAb 7 is less effective than oGH in inhibiting MAb 263 binding despite the much higher affinity of MAb 7 for the GH binding site [MAb 7, $6.4 \times 10^{10} \text{ M}^{-1}$; oGH, $1 \times 10^9 \text{ M}^{-1}$ (Barnard *et al.*, 1984)].

These results indicate that in microsomes only 30% of MAb 263 binding sites are within 3.5 nm (35 Å) of the MAb 7 epitope and that some GH binding sites closely associated with the MAb 263 epitope are not associated with the MAb 7 epitope. This is borne out by the observation that a combination of MAbs 263 and 7 is more effective in inhibiting oGH binding to rabbit liver microsomes than either antibody alone (Fig. 6).

Inhibition of rat GH binding by MAbs

Although MAb 7 inhibits a maximum of 85% of ^{125}I -oGH binding to rabbit liver microsomes, it completely inhibits ^{125}I -rGH binding to the same microsomes (Fig. 8). MAb 263 inhibits a maximum of 60% of ^{125}I -rGH specific binding. Thus, rGH does not occupy all of the receptors which bind oGH (see discussion and Fig. 14).

Fig. 8. Inhibition of ¹²⁵I-rGH binding to rabbit liver microsomes by MAb 263 (●) and MAb 7 (▲)

Concentration of MAbs was standardized to 5 mg/ml before serial dilution. Final concentration of oGH binding sites in microsomes was 800 fmol/ml; 5% of ¹²⁵I-rGH added was specifically bound. Unlabelled rGH was used to determine specific binding. Non specific binding was 9% of added radioactivity. Points represent the means of triplicate determinations with the S.E.M. values indicated. Parallel dilutions of unrelated ascites fluid were used as binding controls. The curves differ significantly as indicated: **P* < 0.01, ***P* < 0.005.

These observations provide additional evidence for a class of oGH binding sites not associated with the MAb 7 epitope.

Reactivity of MAbs with affinity-purified receptor

We previously reported that GH affinity-purified rabbit GH receptor consisted of both PEG-precipitable

Fig. 9. Titration of precipitating MAbs against liver cytosolic 'GH receptor'

Final concentration of GH binding sites was 63 fmol/ml. The ordinate shows the antibody-precipitable specific binding as a percentage of the maximum binding precipitated by MAb 5. Maximum specific binding of ¹²⁵I-hGH added was 9%. Non-specific binding was 18% of radioactivity added. Points represent means of triplicate determinations, with the S.E.M. values indicated. ○, MAb 5 (8 μg/ml at 1:5000); ●, MAb 1 (4 μg/ml at 1:5000); ▲, MAb 2 (4 μg/ml at 1:5000).

and PEG-soluble components (Barnard *et al.*, 1984). Both components were precipitable by MAbs 1, 2 and 5, enabling detection of additional specific GH binding (Barnard *et al.*, 1984). Both components of GH binding were also completely immunodepleted by MAb 263. However, MAb 263 does not inhibit hGH binding to the affinity-purified rabbit GH receptor, so that the epitope, although present, is not within 3.5 nm (35 Å) of the hormone-binding sites (Tzartos *et al.*, 1981).

On the other hand, MAb 7 completely inhibits GH

Table 1. Enzyme activities measured in liver fractions used for inhibition studies

Values are means of triplicate determinations on liver fractions used in subsequent inhibition studies; S.E.M. is given in parentheses.

Enzyme	Enzyme activity in:					
	Homogenate (30 mg/ml) (A)	Plasma membrane fraction (0.8 mg/ml) (B)	Microsomal pellet (5.0 mg/ml) (C)	Endoplasmic reticulum (3.8 mg/ml) (D)	Relative specific activity (B/A)	Relative specific activity (B/C)
5'-Nucleotidase (μmol of P _i /h per mg of protein)	1.3 (±0.1)	26 (±0.3)	4.6 (±0.1)	5 (±0.8)	20±2.0	5.7±0.2
Glucose 6-phosphatase (μmol of P _i /h per mg of protein)	0.58 (±0.1)	0.58 (±0.1)	3.9 (±0.3)	1.4 (±0.2)	1.0±0.4	0.15±0.2
NADPH:cytochrome <i>c</i> reductase (nmol/min per mg of protein)	17 (±1.0)	34 (±1.0)	69.5 (±5.0)	70 (±1.0)	2.0±0.1	0.49±0.4
Succinate:cytochrome <i>c</i> reductase (nmol/min per mg of protein)	10 (±1.0)	0	0	0	0	—

Fig. 10. Inhibition of ^{125}I -hGH binding to liver cytosolic GH receptor by MAb 7

GH receptor was added at a concentration of 317 fmol/ml; 5% of ^{125}I -hGH was specifically bound. Unlabelled oGH was used to determine specific binding. Precipitating MAb 1 (100 μl) was added at 1:1000 dilution in the pre-incubation step to enable PEG precipitation of the cytosolic receptor (see the Materials and methods section). Points represent the means of triplicate determinations with the S.E.M. values indicated. Parallel dilutions of unrelated ascites fluid were used as binding controls.

binding to the affinity-purified rabbit GH receptor (Barnard *et al.*, 1984). Thus GH binding sites specifically inhibitable by MAb 263 have apparently not been recovered during the affinity-purification procedure for rabbit GH receptor (see the Discussion). The situation for rat GH receptor is analogous. Although MAb 263 inhibits GH binding to rat liver microsomes and immunoprecipitates affinity-purified rat GH receptor, it does not inhibit hGH binding to the latter.

Antigenic characterization of the liver cytosolic GH binding protein

Ymer *et al.* (1984) recently reported the existence of a PEG-soluble GH binding protein in rabbit liver cytosol which was detectable by gel filtration. We have identified a similar binding protein in our affinity-purified rabbit GH receptor preparations (Barnard *et al.*, 1984). The three rabbit-specific precipitating MAbs, 1, 2 and 5, were all strongly reactive with the cytosolic component (Fig. 9). MAb 7 completely inhibits ^{125}I -GH binding to this protein (Fig. 10). Although immunodepletion studies showed that MAb 263 precipitated this protein, it did not inhibit GH binding, similar to the affinity-purified receptor. Thus, the cytosolic protein shares at least five epitopes with the membrane-associated GH binding proteins.

Scatchard analysis (Fig. 11) shows that there is approx. 7 times more GH binding capacity/g of tissue homogenized in liver cytosol relative to membranes, irrespective of whether homogenization is obtained by Polytron or Dounce homogenization.

Fig. 11. Scatchard analysis of ^{125}I -hGH binding to rabbit liver microsomes and cytosol prepared from the same liver

Final concentration of GH binding sites in liver microsomes added at 1:16 (w/v) dilution was 63 fmol/ml. Final concentration of GH binding sites in cytosol added at 1:160 (w/v) dilution was 42 fmol/ml. Curves were fitted by the Rodbard-Munson SCAFIT program. ●, Liver microsomes; ▲, liver cytosol. B/F, bound/free ratio. Maximum specific binding to liver microsomes was 8% of radioactivity added. Maximum specific binding to cytosol was 9% of radioactivity added. Non-specific binding to microsomes and cytosol was 3% and 9% respectively.

Fig. 12. Inhibition of ^{125}I -oGH binding to plasma membrane and endoplasmic reticulum enriched fractions (prepared as described in the Materials and methods section) by MAb 7

Endoplasmic reticulum and plasma membrane fractions were added at a dilution which gave 4% specific binding of radioactivity added. Points represent means of triplicate determinations with the S.E.M. values indicated. (●); Endoplasmic reticulum fraction added at 3.8 mg/ml; (○), plasma membrane fraction added at 0.8 mg/ml. The curves do not differ significantly at any of the points ($P > 0.1$). Parallel dilutions of unrelated ascites fluid (anti-*Brucella*) were used as binding controls. Non-specific binding was 9% of radioactivity added to plasma membranes and 10% of radioactivity added to endoplasmic reticulum fractions.

Fig. 13. Inhibition of ^{125}I -oGH binding to plasma membrane and endoplasmic reticulum enriched fractions by MAb 263

Endoplasmic reticulum and plasma membrane fractions were added at a dilution which gave 4% specific binding of radioactivity added. Points represent means of triplicate determinations with the S.E.M. values indicated. ●, Endoplasmic reticulum fraction added at 3.8 mg/ml; ○, plasma membrane fraction added at 0.8 mg/ml. The curves are not significantly different ($P > 0.1$). Parallel dilutions of unrelated ascites fluid were used in binding controls.

Close antigenic similarity of plasma membrane and endoplasmic reticulum GH receptors

The microsomal pellet used in most of our inhibition studies contains vesicles derived from both the blood sinusoidal microvillar region of the hepatocyte plasma membrane and the endoplasmic reticulum (Evans, 1978). These may differ in their reactivity with our MAbs, giving the appearance of heterogeneity which is not seen by circulating GH. Hence we prepared enriched plasma membranes and endoplasmic reticulum from our microsomal pellets (see the Materials and methods section). Enzyme activities measured in the liver fractions used for inhibition studies are shown in Table 1. The absolute values and relative specific activities compare favourably with those reported by Touster *et al.* (1970) and Pass *et al.* (1981), in their rat liver fractionation studies. The plasma membrane marker enzyme 5'-nucleotidase has been enriched 20-fold over the levels measured in the crude liver homogenate and close to 6-fold over the microsomal pellet. Plasma membrane contamination of the endoplasmic reticulum was a maximum of 19% based on the relative specific activities of 5'-nucleotidase in these fractions. Although endoplasmic reticulum contamination of the plasma membrane fraction appears to be around 50%, since the specific binding of ^{125}I -GH to endoplasmic reticulum is so much less per mg of membrane protein (10%/mg for endoplasmic reticulum, 50%/mg for plasma membrane), it can be readily calculated that any endoplasmic reticulum contribution to ^{125}I -oGH binding in the plasma membrane fraction would account for less than 20% of total specific binding.

Inhibition of ^{125}I -oGH binding to plasma membrane-

Fig. 14. Proposed model for rabbit liver growth hormone receptors

Numbered areas represent antibody binding 'regions' (as defined by Tzartos *et al.* 1981) recognized by the designated MAb. Approximate percentage of total oGH binding to each component is indicated in brackets.

and endoplasmic reticulum- enriched fractions was virtually identical (Figs. 12 and 13), with either MAb 263 or MAb 7 in two separate membrane preparations. Thus, very similar receptor associated antigens are found in the plasma membrane and endoplasmic reticulum fractions which together make up the microsomal fraction. We conclude that the antigenic variation which we have shown to be associated with liver microsomes is present in the plasma membrane and does not result from subcellular heterogeneity.

DISCUSSION

The available data in this and our previous study (Barnard *et al.*, 1984) can be accommodated by the model for rabbit GH receptors shown in Fig. 14. Of the three types of GH binding proteins, Type 1 contains both MAb 7 and hormone-binding-site-located MAb 263 epitopes, whereas types 2 and 3 contain only MAb 7- or hormone-binding-site-located MAb 263 epitopes, respectively. This classification is based on the competition studies of Figs. 2-8. Accordingly, the model predicts that receptor occupancy by oGH will inhibit 50% of the maximum possible binding of MAb 263. Conversely, it predicts that excess MAb 263 will inhibit 50% of oGH binding. This is consistent with data presented in Figs. 2 and 4. The model predicts 30% inhibition of MAb 263 binding by MAb 7. This is in agreement with data

presented in Fig. 7. The model proposes the existence of two kinds of MAb 263 epitope (those associated with the GH binding site and those not so associated). This is compatible with the data depicted in Figs. 1(a) and 5. It is possible, however, that the high-affinity MAb 263 epitope corresponds to the epitope found on either type 1 or type 3 receptors. In the latter case, the high-affinity to low-affinity site ratio predicted by the model would be 20:80; in the former case, 30:70. The data presented in this report do not permit a choice between these two alternatives. The model predicts that inhibition of oGH binding by MAb 7 will be augmented by the combination of MAbs 7 and 263. This is observed (Fig. 6).

We cannot exclude the possibility of inter-receptor interactions or receptor-membrane interactions contributing in some way to the heterogeneity of receptor-MAb interactions. Further studies are being undertaken to investigate the possible role of such interactions.

The proposed model is based on MAb binding characteristics which are reasonably constant in a number of membrane preparations, prepared by diverse means. For six different preparations, the mean level of inhibition by MAb 263 was $48 \pm 6\%$ (S.E.M.). These included preparations from frozen liver, from fresh liver, a plasma membrane fraction and two endoplasmic reticulum fractions (the preparation of which involves prolonged ultracentrifugation at 4 °C). The average intra-assay variation from the mean level of inhibition in each of these preparations was 4%. The relatively small variation between preparations contra-indicates significant proteolytic effects.

We do not believe that the biphasic Scatchard plot (Fig. 1) and biphasic inhibition curve (Fig. 5) can be explained by heterogeneity of MAb 263 rather than heterogeneity of MAb binding sites. Our evidence shows that our iodinated or uniodinated MAb preparations do not contain mixtures of mono- and bivalent MAb fragments. Electrophoresis of monoclonal ascites fluids has shown only a single protein band characteristic of intact IgG_{1k}. Moreover, inhibition of GH binding to cytosol (Fig. 10) or to affinity-purified GH receptor (Barnard *et al.*, 1984) by MAb 7 exhibits a single component, so the biphasic inhibition of binding to microsomes is not a product of MAb heterogeneity, in the case of this MAb at least. Greater than 89% of iodinated MAb 263 is PEG-precipitable, demonstrating a high degree of structural integrity of the MAb [free light chains not being precipitable in 30% (w/v) PEG] despite the high specific radioactivity of iodination (112 $\mu\text{Ci}/\mu\text{g}$ for MAb 263). Under these conditions all of the MAb molecules will be multiply iodinated (between 6 and 8 atoms of iodine/MAb molecule), rendering the MAb population relatively uniform, compared with an MAb population iodinated to a low specific radioactivity. The observation that hormone-binding-site MAb 263 epitopes are not present in either hGH-affinity-purified rabbit liver GH receptor or in the PEG-soluble cytosolic form leads one to suspect that these two may be similar, and this is supported by strong reactivity of precipitating MAbs 1, 2 and 5, as well as inhibitory MAb 7 with both cytosolic and affinity-purified receptors. The finding that both receptors can be immunodepleted by MAb 263 further strengthens this concept. Our results find a parallel in the work of Amit *et al.* (1984), who have described an inducible cytosolic PRL receptor which cross-reacts with the membrane-bound PRL receptor. On the basis of their induction

experiments, Amit *et al.* (1984) proposed that the cytosolic proteins may be intermediates in receptor synthesis prior to membrane insertion. Further work is necessary to test the applicability of this proposal to the cytosolic GH binding protein.

Immunodepletion of both cytosolic and affinity-purified GH receptors by MAb 263 also leads us to postulate a second MAb 263 epitope not associated with the hormone-binding site. MAb 2 will displace all ^{125}I -MAb 263 binding (Fig. 5) to rabbit liver microsomes, but shows a biphasic inhibition curve, in agreement with the idea of two types of MAb 263 epitope, as is the biphasic Scatchard plot for ^{125}I -MAb 263 binding. Finally, only 50% of ^{125}I -MAb 263 binding can be inhibited by excess oGH, so the remainder must be located away from the hormone-binding site. Figs. 2 and 4 provide evidence for two kinds of binding site for oGH and for MAb 263 in rat liver microsomes, and these results may be a reflection of the presence of type, 1, 2 and 3 receptors. It is not possible to establish this at present owing to the species specificity of the other MAbs. It is of considerable interest that none of the six MAbs we have raised against hGH-affinity-purified rabbit GH receptor (type 2 receptor) showed any reactivity against rat liver GH receptor. A similar finding was reported by Simpson *et al.* (1983) with their anti-(rabbit GH receptor) MAb. On the other hand, the MAb raised against purified rat receptor cross-reacts with types 1 and 3 of rabbit liver GH receptor, and with human GH receptors in IM9 lymphocytes (P. Gorden, personal communication). Thus the hormone-binding-site MAb 263 epitope would appear to be closely associated with the *bona fide* GH receptor, as opposed to the type 2 binding protein. The data of Fig. 8 implies that rat GH (which is presumably growth active in the rabbit) does not bind to type 3 receptors, so it seems likely that our type 1 receptor corresponds to the subset of receptors common to rabbit and rat liver which Hughes *et al.* (1983) have suggested mediate the 'growth' actions of GH. These receptors have high affinity for rat GH and 20 kDa hGH, but are not distinguishable from the second class in terms of oGH binding. We estimate type 1 receptors make up approx. 30% of the total ^{125}I -oGH binding to rabbit liver microsomes.

While the MAbs described here should provide useful tools for defining the functional significance of the three types of receptor, it is important to show that the receptor types are present in the plasma membrane, and not simply a product of receptor processing. In this study we have demonstrated that there is no difference between the antigenic characteristics of purified plasma membrane, endoplasmic reticulum or original microsomal membrane preparations with MAb 263 and MAb 7. Hence these three receptor types (including type 2, which is antigenically identical to the cytosolic receptor using the probes described here) are all present in the plasma membrane, and all may mediate biological action(s) of GH. The correspondence between receptor type and biological action as well as the subunit sizes of the receptor types are the subjects of our current investigations. Once these have been established it should be possible to clone each receptor subtype separately and determine the extent of their sequence homologies. This may be of value in determining the role of the abundant cytoplasmic 'receptor', the function of which is presently enigmatic.

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