Structural studies on membrane-bound and soluble growth-hormone-binding proteins of rabbit liver

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Covalent cross-linking techniques have been used to investigate the structural characteristics of the growth-hormone (GH) receptor in a variety of rabbit liver cell membrane preparations (particulate and soluble). Two classes of GH-binding protein have been identified which differ in their M_r by gel filtration and susceptibility to precipitation with poly(ethylene glycol) (PEG). The first, a PEG-precipitable ($M_r \sim 300000$) protein, contained M_r -65000 and M_r -40000 binding proteins linked by disulphide bonds. It was present in aqueous extracts derived from microsomal membranes but was not present in cytosol preparations. The second, a PEG-non-precipitable protein ($M_r \sim 100000$) was composed of a non-disulphide-linked primary GH-binding subunit of M_r 60000–66000. This binding protein was present in all rabbit liver cell fractions and/or preparations. Both binding-protein classes contained intramolecular disulphide bonds. It is not clear whether the M_r - ~ 100000 form, or perhaps higher- M_r species which have not been identified by cross-linking studies, represents the native, endogenous, form of the GH receptor present in particulate microsomal or plasma membranes. Accordingly, although these data have identified two classes of GH-binding protein, especially a primary GH-binding subunit of M_r 60000–66000, they indicate that, unlike studies on the insulin receptor, covalent cross-linking techniques alone are not sufficient to delineate the complete subunit structure of the native and endogenous form of the GH receptor.

INTRODUCTION

Covalent cross-linking techniques have been used widely in recent years to study structural features of hormone receptors or binding proteins. Relatively few such studies (Tsushima *et al.*, 1982; Haeuptle *et al.*, 1983; Hughes *et al.*, 1983a; Donner, 1983; Ymer & Herington, 1984; Gorin & Goodman, 1984; Carter-Su *et al.*, 1984) on the GH receptor in various tissues/species have been carried out, however, and no definitive interpretation and/or analysis of GH-receptor structure is available.

Binding proteins for GH are associated with both plasma-membrane and crude intracellular membrane fractions (microsomes, Golgi, lysosomes) of various target tissues (Hizuka *et al.*, 1982; Picard & Postel-Vinay, 1984; Gorin *et al.*, 1984). Microsomal membranes from the livers of pregnant rabbits are a rich source of specific GH-binding proteins, and hence most isolation and purification work has utilized detergent- or aqueous-solubilized preparations of this fraction (Herington & Veith, 1977; Waters & Friesen, 1979; Herington *et al.*, 1981). Recently we have shown that aqueous extracts of microsomal membranes contain two GH-binding protein complexes ($M_r \sim 300000$ and ~ 120000) which were precipitable and non-precipitable by PEG respectively (Ymer & Herington, 1984). Chemical cross-linking of ^{125}I -hGH to the entire aqueous extract identified the presence of a principal GH-binding subunit of M_r 57000, with no evidence for involvement of disulphide bonding. This observation (Ymer & Herington, 1984) agreed closely with those of others (Tsushima *et al.*, 1982; Hughes *et al.*, 1983a), but was somewhat different from that of Haeuptle *et al.* (1983), who reported a

slightly-higher- M_r (67000) band that was derived, by disulphide-bond reduction, from a much larger species. Our studies on aqueous extracts also detected two other specific binding proteins, of M_r 109000 and 35000, with the latter protein being poorly cross-linked (Ymer & Herington, 1984). It was not possible at that time, however, to describe the particular binding subunit arrangement for the native form(s) of hepatic GH-binding proteins.

Therefore, in an attempt to further characterize and elucidate the subunit structure of solubilized GH-binding proteins and to determine their relationship to the membrane-bound GH receptor, we have carried out a comprehensive covalent-cross-linking study on rabbit liver preparations of aqueous-solubilized microsomal membranes, particulate microsomal and plasma membranes and the recently identified, apparently naturally soluble, cytosolic, GH-binding proteins (Ymer *et al.*, 1984).

EXPERIMENTAL

Materials

Human GH (NIAMDD-hGH-I-1), kindly provided by the National Hormone and Pituitary Program of the NIADDK (Bethesda, MD, U.S.A.), was iodinated to specific radioactivities of 30–50 $\mu\text{Ci}/\mu\text{g}$ by the Iodogen method (Salacinski *et al.*, 1981) as described previously (Ymer *et al.*, 1984; Ymer & Herington, 1985). The labelled ligand was purified by gel filtration on a column (1 cm $\times$ 55 cm) of Ultrogel AcA 54 in the absence of any bovine serum albumin. Elution was with

Abbreviations used: GH, growth hormone (the prefix h refers to the human hormone); PEG, poly(ethylene glycol); DSS, disuccinimidyl suberate; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride.

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50 mM-Tris/HCl buffer, pH 7.4, and fractions were collected in silicone-treated glass tubes. Immediately before covalent cross-linking studies the ^{125}I -hGH was repurified on a 5 ml Ultrogel AcA 202 column or Sephadex G-50 column equilibrated and eluted with 50 mM-sodium phosphate buffer, pH 7.4, containing 0.02% merthiolate. This buffer exchange was necessary, since Tris inhibits the cross-linking reaction. Unlabelled preparations of hGH were obtained from the Commonwealth Serum Laboratories, Melbourne, Australia. All other reagents, including M_r markers for polyacrylamide-gel electrophoresis, were obtained as reported previously (Ymer & Herington, 1984; Ymer *et al.*, 1984; Ymer & Herington, 1985; Herington *et al.*, 1986).

Preparation of rabbit liver fractions and detection of GH-binding protein

All preparations were made from livers of 25–28-day-pregnant rabbits in the presence of PMSF (1 mM) and 1000 kallikrein-inhibitory units of Trasylol/ml. After gentle homogenization using a few strokes in a Dounce glass homogenizer with a loose-fitting glass pestle, plasma membranes were prepared on a discontinuous sucrose gradient essentially as described by Neville (1968). Microsomal membranes (100 000 g) were prepared by differential centrifugation (Herington & Veith, 1977; Ymer & Herington, 1984). Aqueous extracts of microsomal membranes were obtained at pH 9 in 1 mM-Tris buffer without detergents (Herington *et al.*, 1981; Ymer & Herington, 1984). Rabbit liver cytosol was prepared as a 200 000 g supernatant from the preparation of microsomal membranes as described recently (Ymer *et al.*, 1984).

The binding-protein preparations were assayed as reported previously (Herington & Elson, 1979; Ymer & Herington, 1984, 1985; Ymer *et al.*, 1984), except that binding protein and ^{125}I -hGH ($> 400\,000$ c.p.m.; 400 pM) were incubated in 25 mM-sodium phosphate buffer, pH 7.5, containing 0.02% merthiolate and 10 mM-MgCl₂. The whole system was free of BSA and was operated in silicone-treated glass tubes. In some experiments using particulate membrane preparations, bound and free ^{125}I -hGH were separated by centrifugation before covalent cross-linking. Total binding and non-specific binding were determined in all cases by incubation in the absence and presence of excess unlabelled hGH (0.1 μM) respectively. Specific binding for the different receptor preparations was as follows: aqueous microsomal membranes, 30–40% binding for 350 μg of protein; cytosol, 20–30%/200 μg ; microsomal membranes, 20–30%/200 μg ; plasma membrane, 10–15%/100 μg . Covalent cross-linking is known to be relatively inefficient and $< 20\%$ of bound hormone is generally cross-linked. This is reflected in the amount of radioactivity present at the dye front of most gels and which represents free ^{125}I -hGH.

Covalent cross-linking of ^{125}I -hGH to liver fractions

All cross-linking studies have been carried out, essentially as described previously (Ymer & Herington, 1984), by using DSS (0.05 or 0.5 mM final concn.). After cross-linking for 15 min at 21–23 °C, 50 μl aliquots were removed and the reaction stopped by boiling (3 min) in the presence of 2% SDS $\pm$ 100 mM-DTT (final concns.). For the aqueous extract of microsomal membranes, cross-linking was carried out on (i) the whole extract and

(ii) partially purified preparations of the PEG- and non-PEG-precipitable components, which had been separated, either as complexes with ^{125}I -hGH (see Fig. 2 below) or before incubation with ^{125}I -hGH (results not shown), by gel filtration of aqueous extract on Ultrogel AcA 44 mini-columns (10 ml). These mini-columns were not able to be precisely calibrated, but M_r assignments of the two binding-protein forms were made on the basis of their known molecular size as determined by gel filtration on larger (1 cm $\times$ 90 cm), carefully calibrated, Ultrogel AcA 34 columns (Ymer & Herington, 1984). Cross-linking was performed on the 'peak' tubes of each complex.

SDS/polyacrylamide-gel electrophoresis on vertical slab gels [0.5 mm thick, 10% (w/v) acrylamide] was performed by the method of Laemmli (1970). In several experiments (not reported here), 5%, 7.5% and 15% gels were also run. The stacking gel in all cases was 3% acrylamide. Samples were run in both the presence and the absence of 100 mM-DTT, with 50–100 000 c.p.m. being loaded on to each sample lane. The gels were run, calibrated, stained, dried and then subjected to autoradiography at -70°C for various times using Kodak AR-5 film and du Pont Lightning Plus intensifying screens as previously reported (Ymer & Herington, 1984).

RESULTS

Covalent cross-linking of ^{125}I -hGH to aqueous extracts of rabbit liver membranes

An aqueous extract of rabbit liver was incubated with ^{125}I -hGH and then covalently cross-linked with DSS. As shown in Fig. 1, in the absence of reducing agent (lanes A and C, $-\text{DTT}$) ^{125}I -hGH-protein complexes were present at M_r 120 000, 78 000 and 52 000. In three such experiments the mean M_r ($\pm$ S.E.M.) for these bands was $127\,000 \pm 3600$, $80\,000 \pm 2000$, and $54\,000 \pm 1000$. In addition, in all experiments, there were some very-high- M_r ($> 500\,000$) cross-linked species at the stacking-gel/running-gel interface. All bands were suppressed by the presence of excess unlabelled hGH (lanes B and D) and therefore represent specific hGH-binding species. No fundamental differences in the autoradiographic pattern were observed at the two cross-linker concentrations used (0.05 mM or 0.5 mM), although the intensity of the specific bands was increased at 0.5 mM-DSS.

In the presence of DTT (lane E), specifically labelled complexes were detected at M_r 133 000, 72 000–89 000, 60 000 and 54 000, as well as at the gel interface. Closer inspection of the broad band at M_r 72 000–89 000, on this and other gels, revealed three ^{125}I -hGH-binding-protein complexes at M_r 87 000, 79 000 and 72 000. The M_r -60 000 band was observed only under reducing conditions. Some of these additional bands may be derived by disulphide-bond reduction from the very-high- M_r complex(es) ($> 500\,000$), since there was a decrease in the intensity of the bands at the gel interface (lane C) after treatment with DTT (lane E). In order to allow an estimation of the M_r of the binding proteins themselves (i.e. after subtraction of the M_r of GH), the molecular size of free ^{125}I -hGH and its behaviour under cross-linking conditions were ascertained. This was done by running DSS-treated and -untreated ^{125}I -hGH in the absence of any binding protein on 15% polyacrylamide gels $\pm$ DTT. DSS treatment did not in itself affect the size

Fig. 1. Autoradiograph of SDS/(10%)-polyacrylamide-gel-electrophoretic patterns of ^{125}I -hGH covalently cross-linked to an aqueous extract of rabbit liver microsomal membranes

Lanes A–D, without 100 mM-dithiothreitol (–DTT); lanes E and F, with 100 mM-dithiothreitol (+DTT); lanes A, C and E, total binding; lanes B, D and F, non-specific binding. Two doses of cross-linking agent (DSS) were used: lanes A and B, 0.05 mM; lanes C–F, 0.5 mM. M_r markers are indicated between the gels by the horizontal lines; the M_r values for the specifically labelled ^{125}I -hGH-binding protein complexes are shown by the arrows. The values in parentheses represent the M_r values for the binding proteins themselves after subtraction of the M_r contribution of ^{125}I -hGH (M_r 20000 non-reduced; M_r 22000 reduced) as determined in separate experiments (results not shown). A similar banding pattern was seen on three different gels (10 and 7.5% acrylamide). Abbreviation used: xs hGH, excess unlabelled hGH.

or behaviour of ^{125}I -hGH, which ran at M_r 20000 (non-reduced) and M_r 22000 (reduced) (results not shown). These M_r values have been used routinely to assess the M_r values for the binding proteins themselves (shown in parentheses in Fig. 1) under non-reduced or reduced conditions respectively. Thus it appears that the small increase in M_r of the labelled complexes under reducing conditions is due to intramolecular disulphide bonds within the binding protein, hGH, or both.

Gel filtration of aqueous extracts of rabbit liver yields two distinct proteins which specifically bind ^{125}I -hGH: a high- M_r (~ 300000) complex which was precipitable by PEG and a lower- M_r (~ 120000) complex which was not PEG-precipitable (Ymer & Herington, 1984). In an attempt to determine (a) the structural relationship between the two binding proteins and (b) the origin of the cross-linked bands observed in Fig. 1, a second series of cross-linking experiments was performed on the PEG-precipitable and non-precipitable ^{125}I -hGH complexes, first separated from each other by gel filtration on Ultrogel AcA44 mini-columns (Fig. 2). The autoradiographic profiles of SDS/polyacrylamide gels of ^{125}I -hGH covalently linked to these binding proteins are shown in Fig. 3.

Under non-reducing conditions the $M_r \sim 300000$ PEG-precipitable material (Fig. 3, lanes A and B) gave a very-high- M_r ^{125}I -hGH-binding protein complex that remained at the stacking gel/running gel interface [even on lower-percentage gels (e.g. 5%); results not shown]. This suggested that the cross-linking procedure led to the formation of the very-high- M_r (≥ 500000) aggregates or cross-linked multimers of the M_r -300000 PEG-

precipitable binding-protein complex. This band was markedly diminished when excess unlabelled hGH was included in the initial binding reaction (Fig. 3, lane B). In the presence of DTT (Fig. 3, lanes C and D) the intensity of the band at the interface decreased slightly and two new specific bands appeared at M_r 87000 and 62000. Although these bands were also occasionally present as very faint components under non-reducing conditions, they always increased in intensity under reducing conditions, even though the same total amount of radioactivity per lane was loaded on the gels. This observation suggests that the high- M_r PEG-precipitable complex is composed of GH-binding subunits held together by intermolecular disulphide bonds.

A representative autoradiographic profile for the lower- M_r non-PEG-precipitable ^{125}I -hGH complex is shown in lanes E–H (Fig. 3). A broad, specifically labelled, band was seen at M_r 78000 (lane E; –DTT), which increased to M_r 83000 in the presence of DTT (lane G), suggesting the presence of intramolecular disulphide bonds. High- M_r species or random aggregates were never observed. Thus DSS failed to covalently cross-link together all subunits of the native GH-binding protein(s): cross-linking of the PEG-precipitable ($M_r \sim 300000$) species revealed very-high- M_r material as well as, under reducing conditions, two disulphide-linked proteins of M_r 65000 and 40000, and cross-linking of the PEG-non-precipitable ($M_r \sim 100000$) protein resulted in the identification of a smaller binding subunit only (M_r 58000). This was true for a range of cross-linking conditions [DSS concentrations (0.05–0.5 mM), time and temperature, and gel concentrations (5, 7.5 and 10%)].

Fig. 2. A representative gel-filtration profile of ^{125}I -hGH bound to aqueous extract of rabbit liver microsomal membranes

Gel filtration was carried out on an Ultrogel AcA44 column (0.8 cm $\times$ 21 cm). Each collected fraction was 250 μl . Total binding ($\bullet$ — $\bullet$) and non-specific binding ($\circ$ — $\circ$) curves are shown, as are the elution positions of free ^{125}I -hGH (M_r 22000) and the two specific binding peaks (300 and 120 kDa) known to have M_r values of 300000 and 120000 as determined on larger, carefully calibrated, Ultrogel AcA 34 columns (results from Ymer & Herington, 1984).

Furthermore, it is apparent from the amount of ^{125}I -hGH present at the bottom of all gels, relative to the amount specifically cross-linked to binding protein, that the efficiency of cross-linking was relatively low.

Cross-linking of ^{125}I -hGH to rabbit liver cytosol

Previous studies from this laboratory (Ymer *et al.*, 1984) have described and characterized a soluble, specific, binding protein for hGH in 'high-speed' cytosol preparations of rabbit liver. Covalent cross-linking of ^{125}I -hGH to this preparation, followed by analysis on SDS/polyacrylamide gels revealed a major, broad, specifically labelled band with an M_r of 82000 (Fig. 4, lane A). The M_r of this band increased to 88000 in the presence of DTT (lane C). On occasions, as faintly discernible in this autoradiograph, a specific band was also present at M_r 65000 (non-reduced) and 71000 (reduced). When the DSS (cross-linker) concentration was increased 10-fold (to 0.5 mM) (lanes E–H), all bands increased in intensity and broadened significantly, and a small amount of very-high- M_r material was now observed at the top of the gel. The generalized broadening of the cross-linked hGH complexes may be due to the presence of more than one similarly sized GH-binding component or perhaps to the glycoprotein components of the GH-binding protein (Podulso, 1981; Ymer & Herington, 1984).

Cross-linking of ^{125}I -hGH to rabbit liver plasma membrane

Since the initial interaction of GH with target tissues is presumed to occur at the cell surface, the GH-binding proteins present in particulate preparations of plasma membrane were incubated with ^{125}I -hGH. The whole mixture was then either cross-linked with DSS (Fig. 5,

Fig. 3. Autoradiograph of SDS/(10%) polyacrylamide-gel electrophoretic patterns of ^{125}I -hGH covalently cross-linked (with 0.05 mM-DSS) to PEG-precipitable (lanes A–D) and PEG-non-precipitable (lanes E–H) ^{125}I -hGH-binding protein complexes partially purified (by gel filtration) from aqueous extracts of rabbit liver microsomal membranes.

Total binding and non-specific binding lanes are marked – and + excess unlabelled (xs) hGH respectively. Samples were run in the reduced (+ DTT) or non-reduced (– DTT) state. M_r markers (—) and M_r values for specifically cross-linked bands (→) are as described for Fig. 1. Similar data were obtained on three different (10 or 7.5%) gels.

Fig. 4. Autoradiograph of SDS/polyacrylamide-gel electrophoretic patterns of ^{125}I -hGH covalently cross-linked to a rabbit liver cytosol preparation

Cross-linking was carried out with 0.05 mM-DSS (lanes A–D) or 0.5 mM-DSS (lanes E–H). The designations for total and non-specific binding, M_r markers and presence of DTT are as described for Figs. 1 and 3. Similar data were obtained on at least four (10 or 7.5%) gels. Abbreviation: xs, excess unlabelled.

lanes A–D) or was separated into bound ^{125}I -hGH and free ^{125}I -hGH by centrifugation immediately before cross-linking of the membrane-bound ^{125}I -hGH (Fig. 5, lanes E–H). Essentially the same autoradiographic pattern was observed in each case, apart from the lower

Fig. 5. Autoradiograph of SDS/polyacrylamide-gel electrophoretic patterns of ^{125}I -hGH covalently cross-linked (with 0.05 mM-DSS) to rabbit liver plasma membranes, either before (lanes A–D) or after (lanes E–H) separation of membrane-bound from free ^{125}I -hGH by centrifugation

Designations for total and non-specific binding, presence of DTT, and M_r markers are as described for Figs. 1 and 3. Similar data were obtained on three different (10 or 7.5%) gels. Abbreviation: xs, excess unlabelled.

Fig. 6. Autoradiograph of SDS/polyacrylamide-gel electrophoretic patterns of ^{125}I -hGH covalently cross-linked (with 0.05 mM-DSS) to microsomal membranes of rabbit liver, after separation of membrane-bound from free ^{125}I -hGH

Designations for total and non-specific binding, presence of DTT and M_r markers are as described for Figs. 1 and 3. Similar data were obtained on four different (10 or 7.5%) gels. Abbreviation: xs, excess unlabelled.

background and the smaller amounts of material at the bottom of the gel in lanes E–H attributable to prior removal of much of the unbound ^{125}I -hGH by centrifugation.

Routinely, a broad, strongly cross-linked and specifically labelled, band was observed at M_r 79000 (non-reduced; Fig. 5, lane A) and 87000 (reduced, lane C). A similar result was obtained when different DSS concentrations (0.05, 0.25 and 0.5 mM) were used for cross-linking (not shown) or when cross-linking was performed after separation of free ^{125}I -hGH from membrane-bound hormone (lanes E and G). A very faint hGH-specific band was also present at M_r 141000 (non-reduced), increasing slightly to 144000 under reducing conditions. This band, which appears to be poorly cross-linked, ranged between M_r 120000 and 140000 (non-reduced) and was present in various amounts in different preparations of rabbit liver plasma membrane. In all cases, some very-high- M_r material was present, which was partially displaced by excess unlabelled hGH. Varying the conditions of cross-linking (DSS concentrations, time and temperature) did not seem to increase significantly the presence/detection of these minor bands. High concentrations (0.5 mM) of DSS, in fact, seemed only to decrease the amount of the 120000–140000- M_r band relative to the 87000- M_r band.

Cross-linking of ^{125}I -hGH to rabbit liver microsomal membranes

Fig. 6 is a typical autoradiographic profile of an SDS/polyacrylamide-gel of ^{125}I -hGH covalently cross-linked to rabbit liver microsomal membranes. Similar patterns were seen whether cross-linking was carried out on the whole incubation mixture (results not shown) or after separation of bound and free ^{125}I -hGH (shown in Fig. 6). Routinely, three specific and strongly cross-linked bands were observed with M_r 58000, 80000 and 140000 (lane A, non-reduced); these bands increased in M_r under reducing conditions to 65000, 86000 and 150000 respectively (lane C). Some very-high- M_r complexes were also present at the stacking gel/running gel interface as well as two less strongly cross-linked bands of quite low M_r (< 34000), which again increased slightly in the presence of DTT. All of these bands were specifically decreased in intensity by the presence of excess unlabelled hGH (lanes B and D).

DISCUSSION

These studies have investigated the nature and inter-relationships of specific hGH-binding proteins in a variety of soluble and particulate membrane fractions from rabbit liver. The data are summarized in Table 1 and indicate that the principal GH-binding subunit, which is present as a major component in all preparations, has an M_r of 58000–62000 and 61000–66000 under non-reducing and reducing conditions respectively. This is in reasonable agreement with the existence of a 67000- M_r subunit reported in rabbit liver by Haeuptle *et al.* (1983), who used photoaffinity and protein-blotting techniques. These values are slightly higher than those (~60000) reported in other cross-linking studies (Tsushima *et al.*, 1982; Hughes *et al.*, 1983a; Ymer & Herington, 1984).

In addition, a smaller binding species (M_r 34000–38000 non-reduced; M_r 33000–43000 reduced)

Table 1. A summary of M_r values for specifically labelled ^{125}I -hGH-binding protein complexes observed on 10% polyacrylamide/SDS gels for several rabbit liver membrane and soluble fractions

The data are given as the mean M_r ($\times 10^{-3}$) (for two or three preparations in each case) for the ^{125}I -hGH complex and for the 'free' binding protein, assuming a 1:1 binding stoichiometry and an M_r of 20000 (non-reduced) and 22000 (reduced) for hGH, as determined experimentally (results not shown). Each band is classified as major or minor according to the intensity of labelling on each gel.

Preparation	Specifically-labelled bands ($M_r \times 10^{-3}$)			
	Non-reduced gels		Reduced gels	
	Complex	Free binding protein	Complex	Free binding protein
Plasma membrane	82 (major) 120–140 (minor)	62 100–120	87 (major) 145 (minor)	65 123
Cytosol	82 (major) 65 (minor)	62 45	88 (major) 71 (minor)	66 49
Microsomal membrane	58 } 80 } (major) 140 } 126 } (minor) 154 }	38 60 120	65 } 86 } (major) 150 } — } (minor) 154 }	43 64 128
Aqueous extract				
Whole	54 } 80 } (major) 127 }	34 60 107	55 63 72, 79, 87 133	33 41 50, 57, 65 111
PEG-precipitable M_r 300000	≥ 300 (major) (gel interface)		≥ 300 (diminished) 87 } (minor) 62 }	65 40
PEG-non-precipitable M_r 100000	78 (major)	58	83 (major)	61

was observed as a major band in microsomal membranes, as a minor band in aqueous extracts of microsomal membranes, but only infrequently in plasma membranes. This may represent the lactogen receptor known to be present in rabbit liver microsomal membranes (Waters & Friesen, 1979), since it has a similar M_r to that (32000–35000) of the classical prolactin receptor subunit of rabbit mammary gland (Haeuptle *et al.*, 1983; Katoh *et al.*, 1985). This can be determined unequivocally only by the use of antisera raised against purified lactogen receptor. In cytosol preparations a slightly higher M_r (45000–49000; $\pm$ DTT) binding protein was occasionally observed. The plasma membrane, microsomal membrane and aqueous extracts also contained a variably cross-linked species of M_r 100000–120000. The irregular presence of these forms may simply reflect that they are generally poorly cross-linked. It is noteworthy that, in studies in other species and/or tissues, GH-binding proteins of $M_r \sim 100000$ were found as the major component [e.g. human IM9 lymphocytes (Hughes *et al.*, 1983a), rat liver membranes (Donner, 1983) and rat adipocytes (Gorin & Goodman, 1984; Carter-Su *et al.*, 1984)]. Only in rat adipocytes, however, was a rabbit-like GH-binding subunit of M_r 58000–62000 (non-reduced) also observed (Gorin & Goodman, 1984).

In contrast with the general agreement as to the relative size of the primary GH-binding subunit ($M_r \sim 60000$), there remains some inconsistency regarding the involvement of disulphide bonds in the rabbit liver GH-receptor structure. Most studies, including our own (Tsushima *et al.*, 1982; Hughes *et al.*, 1983a; Ymer &

Herington, 1984), have revealed no change in the M_r of the cross-linked ^{125}I -hGH complex in the presence of DTT. However, our present data consistently suggest at least some involvement of intramolecular disulphide bonds within the GH-binding subunit. We have no adequate explanation for these apparent discrepancies unless, under particular circumstances, the GH-binding protein is readily susceptible to reduction even in the absence of DTT. There is little evidence in rabbit liver for disulphide-linked multimers of the primary ($M_r \sim 60000$) binding subunit, although Haeuptle *et al.* (1983) did suggest the presence of such higher- M_r forms. Our present data argue for the presence of both disulphide-bond-dependent and -independent subunit species. Classification of hepatic-GH binding proteins into two classes has been suggested previously, on the basis of differing disulphide-bond involvement (Kover *et al.*, 1985; Herington, 1986), differences in binding of rat GH and the 20 kDa and 22 kDa variants of hGH (Hughes *et al.*, 1983b) and of data suggesting that anti-(GH receptor) antibodies bind to different subsets of GH receptors (Barnard *et al.*, 1984). Our own previous studies (Ymer & Herington, 1984) also showed that aqueous extracts of microsomes contain two specific GH-binding proteins that differ in their native size and their PEG-precipitability.

We have now partially purified and covalently cross-linked ^{125}I -hGH to these two binding proteins and find that they differ in their subunit make-up (see Fig. 3). The non-PEG-precipitable protein, which was eluted from an AcA 34 gel-filtration column with an M_r of

~ 100 000, gave only a GH-binding subunit of M_r 58 000 and 61 000 (– or + DTT respectively). The cytosol binding protein, also non-PEG-precipitable and eluted from the AcA 34 columns at an M_r ~ 100 000, gave very similar results. These studies indicate that the native molecule (M_r ~ 100 000) consists of at least one GH-binding subunit of M_r ~ 60 000 and that the whole complex is not cross-linked together. The nature of the other subunit(s) comprising the native form is not known at this time.

Semi-purified PEG-precipitable binding protein (M_r ~ 300 000), on the other hand, appears to consist of two GH-binding subunits of M_r 65 000 and 40 000 under reduced conditions. Under non-reducing conditions, very-high- M_r ($\geq$ 500 000) aggregates were observed. These data clearly suggest involvement of interunit disulphide bonds. It is not yet possible to discern the stoichiometry or disulphide-bond arrangement of these two subunits in forming the higher- M_r PEG-precipitable form of the binding protein.

The exact relationship, if any, between the PEG-precipitable and -non-precipitable forms is not known. We have little evidence to suggest that one is derived from the other, except for the similarity of primary GH-binding subunits produced under reducing conditions. The high- M_r PEG-precipitable binding proteins observed on gel filtration and on cross-linking of aqueous extracts may represent the native form of the receptor or simply aggregated forms of the M_r ~ 100 000 binding protein, which may itself be the true GH receptor. An alternative view, which is consistent with the major occurrence of a M_r -100 000 binding protein (containing the M_r -60 000 non-covalently linked subunit) in tissue cytosol preparations (Ymer *et al.*, 1984; Herington *et al.*, 1986), in aqueous buffer extracts of membranes, and in rabbit serum (Ymer & Herington, 1985), is that the smaller (M_r ~ 100 000) PEG-non-precipitable protein may be non-covalently and loosely attached to the cell membrane but associated with a larger non-GH-binding portion which may be embedded in, and therefore less easily extracted from, the plasma membrane. This entire complex may represent the higher- M_r PEG-precipitable form.

Unfortunately the cross-linking studies with microsomal and plasma membranes have been unable to elucidate unequivocally the native structure of the GH receptor. On the basis of previous gel-filtration studies on solubilized receptors (Herington & Veith, 1977; Waters & Friesen, 1979; Tsushima *et al.*, 1980; Herington *et al.*, 1981) it is generally believed that the rabbit liver GH receptor has a high M_r (~ 300 000). However, neither we (the present study and Ymer & Herington, 1984) nor others (Tsushima *et al.*, 1982; Hughes *et al.*, 1983a) have been able, under various experimental conditions, to cross-link such a binding species, the only high- M_r cross-linked material being M_r $\geq$ 500 000, since it does not enter 5% or 10% polyacrylamide gels. This inability may be because a 'native' receptor of M_r 300 000 does not in fact exist, the M_r from gel filtration being grossly overestimated owing to the association of detergent with binding protein (Tsushima *et al.*, 1982), simple aggregation or the known anomalous behaviour of glycoproteins on gel-filtration columns (Alhadeff, 1978). Alternatively, the inability may be related to the nature of the proteins in question. It is known that cross-linking with agents such as DSS is dependent on a variety of factors (Middaugh *et al.*, 1983), not the least being the close

proximity and availability of free α -amino groups. The GH-binding protein is at least partially glycosylated (Herington & Elson, 1979; Tsushima *et al.*, 1980), and the spatial arrangement of glycosylated subunits in the native complex may prevent adequate exposure or proximity of appropriate free α -amino groups. The lack of extensive intersubunit disulphide-bonding in the binding-protein structure may add to the difficulty of identifying a larger native form; the insulin receptor, for example, has been easily identified in its native form (M_r ~ 350 000) because of its disulphide-linked construction; only under reducing conditions can one dissociate the insulin receptor into its component primary subunits (Czech, 1985). However, even in this extensively studied situation, there is little if any DSS-induced covalent cross-linking together of subunits.

In conclusion, the present studies have indicated that at least two different classes of GH-binding protein exist in rabbit liver. However, covalent-cross-linking studies are not sufficient in this instance to elucidate the native, endogenous, form(s) of the GH receptor. The recent development of monoclonal antibodies against the rabbit liver membrane GH receptor (Simpson *et al.*, 1983; Barnard *et al.*, 1984), together with such techniques as cell-surface-receptor iodination and biosynthetic labelling, should permit, as has been possible for the insulin receptor (Van Obberghen *et al.*, 1981; Kasuga *et al.*, 1982), new studies to delineate further the true nature of the native GH-receptor complex.

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