Characterization of hepatic lactogen receptor

Subcellular distribution and characterization of *N*-linked carbohydrate chains

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The types of carbohydrate chains present in a rat liver lactogenic hormone-binding receptor species with an M_r of 82000, and in its hormone-binding subunits with M_r values of 40000 and 35000, were characterized using carbohydrate-chain-cleaving enzymes and affinity cross-linking. The subcellular distribution of lactogenic hormone-binding species was studied in organelle-enriched fractions. The monomeric M_r -40000 and M_r -35000 species contain *N*-linked tri- or tetra-antennary complex and high-mannose chains respectively. The M_r -82000 species exists in two forms, where the M_r -40000 and M_r -35000 subunits are each combined with unglycosylated and, with the technique used, unlabelled subunit(s). Studies with organelle-enriched fractions revealed that the M_r -35000 species was found in an endoplasmic reticulum-enriched fraction. The M_r -40000 species was the predominant monomeric binding species in Golgi/endosome- and plasma membrane-enriched fractions. It is suggested that the M_r -35000 species is a precursor to the M_r -40000 species. In lysosome/endosome- or lysosome-enriched fractions, a broad distribution in M_r (35000–40000) was characteristic of the hormone-binding species. The M_r -82000 species was only found in a Golgi/endosome-enriched fraction. Labelling of endosome lactogen receptor by injection of ^{125}I -labelled ovine prolactin *in vivo* and cross-linking yielded only the M_r -40000 species. Thus, the M_r -40000 and M_r -35000 lactogenic hormone-binding species each appear to be combined with the unglycosylated receptor subunit(s) in the Golgi complex to form M_r -82000 heterodimeric complexes.

INTRODUCTION

In the livers of several species, a membrane receptor is found for prolactin (Posner *et al.*, 1974; Gertler *et al.*, 1984; Waters *et al.*, 1984; Haro & Talamantes, 1985). Previous studies in our laboratory indicated the presence of a lactogenic hormone-binding species in detergent-solubilized crude membrane fractions from female rat liver with an M_r of about 80000–90000 (Haldosén & Gustafsson, 1987). This molecular species was shown to contain M_r -40000 and -35000 hormone-binding subunits. From those studies we could not determine whether the subunits were combined with each other or with additional subunit(s) to make up the M_r -80000–90000 species.

Ovary and Leydig cell preparations from rat both contain a lactogenic hormone-binding entity with an M_r of about 80000 (Bonifacino & Dufau, 1984, 1985). In both tissues this molecular species was shown to contain a hormone-binding subunit with an M_r of about 40000. Three binding species with M_r values of 95000, 86000 and 43000 have been identified in rat liver for the prolactin-related hormone, growth hormone (Husman *et al.*, 1988). The M_r -43000 species was found to be a constituent of the M_r -86000 species. In mouse liver, three immunologically related growth-hormone-binding species with M_r values of 101000, 40700 and 37500

have been identified (Smith *et al.*, 1988). Recently Boutin *et al.* (1988) have cloned cDNA encoding the rat liver M_r -41000 prolactin receptor. The amino acid sequence deduced from the cDNA sequence was shown to have strong localized sequence identity with the earlier published amino acid sequence of the rabbit and human growth hormone receptors (Leung *et al.*, 1987).

Specific lactogenic binding activity has been found in plasma membrane-enriched, Golgi-enriched and lysosome-enriched fractions prepared from rat liver (Norstedt *et al.*, 1984; Borst *et al.*, 1985). Affinity cross-linking studies revealed the presence of an M_r -36000 lactogenic hormone-binding entity in plasma membrane- and Golgi-enriched fractions (Borst *et al.*, 1985).

Rat or mouse liver receptor and rat ovarian receptor have been shown to be retained on and specifically eluted from lectin-chromatography gel (Costlow & Gallagher, 1979; Bhattacharya & Vonderhaar, 1981; Kusuda & Dufau, 1986; Mitani & Dufau, 1986). Furthermore, the inhibition by lectins of the binding of lactogenic hormones to rat and mouse liver receptors (Costlow & Gallagher, 1977, 1979; Bhattacharya & Vonderhaar, 1981) indicate that carbohydrate chains are linked to the receptor. The deduced amino acid sequence from the recently cloned prolactin receptor cDNA revealed three potential asparagine glycosylation sites (Boutin *et al.*, 1988). By treating rats with the glycosylation inhibitor

Abbreviations used: AP preparation, acid-precipitated preparation; bGH, bovine growth hormone; CMF, crude membrane fraction; DSS, disuccinimidyl suberate; Endo F and H, endo- β -*N*-acetylglucosaminidase F and H respectively (EC 3.2.1.96); hGH, human growth hormone; IMF, intermediate fraction; LDMF, low-density membrane fraction; M, membrane preparation; *N*-glycanase, peptide *N*-glycosidase F (EC 3.2.2.18); *O*-glycanase, endo- α -*N*-acetyl-galactosaminidase (EC 3.2.1.97), oPRL, ovine prolactin; PMF, plasma membrane fraction; RMF, residual microsome fraction; PAGE, polyacrylamide-gel electrophoresis.

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tunicamycin, it has been shown that *N*-linked oligosaccharide chains are important for the insertion of functional prolactin receptor into plasma membranes of rat liver (Savoie *et al.*, 1986).

In an attempt to elucidate the type(s) of carbohydrate chains linked to the different lactogen hormone-binding species in rat liver, we have undertaken an investigation involving analysis of glycosidase-treated, covalently cross-linked ^{125}I -labelled human growth hormone (hGH)-receptor complex on SDS/polyacrylamide-gel electrophoresis (PAGE). Subcellular fractions of rat liver have also been investigated with respect to presence and type of ^{125}I -hGH-binding species.

MATERIALS AND METHODS

Materials

Virgin and late pregnant female Sprague-Dawley rats (Anticimex, Stockholm, Sweden), 8–10 and 11–13 weeks of age respectively, were used throughout the study. Virgin rats were injected with 17β -oestradiol-valerate in order to increase the concentration of liver lactogen receptor (Haldosén & Gustafsson, 1987). In some experiments, rats were injected with ^{125}I -labelled ovine prolactin (oPRL) (see legend to Fig. 6). Biosynthetic methionyl somatotropin (Somatonorm; 2 units/mg) was a gift from AB Kabi (Stockholm, Sweden). Ovine prolactin (NIADDK-oPr1-16) and bovine growth hormone (bGH) (NIH-GH-B-14) were obtained from the Hormone Distribution Program (National Institute of Arthritis, Metabolism and Digestive Diseases, NIH, Bethesda, MD, U.S.A.). ^{125}I -hGH and ^{125}I -oPRL were prepared by the Iodogen method (Fraker & Speck, 1978). For a detailed description see Haldosén & Gustafsson (1987). A specific radioactivity of 30–40 $\mu\text{Ci}/\mu\text{g}$ of hormone was repeatedly obtained using this method. Endo- β -*N*-acetylglucosaminidase F (Endo F; EC 3.2.1.96) containing an undefined amount of glycopeptide *N*-glycosidase F (*N*-glycanase; EC 3.2.2.18), endo- β -*N*-acetylglucosaminidase H (Endo H; EC 3.2.1.96) and neuraminidase were purchased from Boehringer Mannheim Biochemica (Penzberg, Germany). Endo F and endo- α -*N*-acetylgalactosaminidase (*O*-glycanase; EC 3.2.1.97) were obtained from Genzyme Corp. (Boston, MA, U.S.A.). Ovalbumin (for Endo H and Endo F) and RNAase B (for *N*-glycanase) were used to test the activity of the glycosidases (results not shown). For sources of other chemicals used in this study, see Haldosén & Gustafsson (1987).

Preparation of crude membrane fraction and hepatic subcellular fractions

Crude membrane fraction (CMF) was prepared as described earlier (Haldosén & Gustafsson, 1987). This membrane preparation has been characterized by enzyme-marker analysis (Andersson *et al.*, 1978). It is slightly enriched in microsomes and Golgi vesicles as compared with total particulate fraction of rat liver. It is depleted of mitochondria, lysosomes and plasma membranes. The residual microsome (RMF), intermediate (IMF) and low-density membrane fraction (LDMF) were isolated as described by Eriksson *et al.* (1986). These fractions have been defined by marker-enzyme analysis and ultrastructural morphology (Andersson & Eriksson, 1981; Khan *et al.*, 1981; Ferland *et al.*, 1984; Eriksson *et al.*, 1986). In brief, the RMF predominantly consists of

vesicles derived from the rough and smooth endoplasmic reticulum and exhibits a 2–3-fold relative enrichment (on a protein basis) of the endoplasmic reticulum marker NADPH-cytochrome *c* reductase compared with a total particulate fraction. The enrichment values for fluoride-stimulated adenylate cyclase (blood-sinusoidal plasma membrane), tartrate-sensitive acid phosphatase (lysosomes) and UDP-galactosyltransferase using *N*-acetylglucosamine as acceptor (Golgi complex) in the RMF are 1.3-, 0.6- and 1.0-fold respectively. Thus, a minor contamination of plasma membrane-derived vesicles in this fraction cannot be excluded. Marker-enzyme analysis of IMF demonstrates a 3-, 6- and 16-fold enrichment of NADPH-cytochrome *c* reductase, adenylate cyclase and galactosyltransferase respectively, with no enrichment (< 1) of acid phosphatase. The LDMF is a pool of Golgi cisternae and vesicles in addition to secretory vacuoles (Eriksson *et al.*, 1986) and endocytic vesicles (Engfeldt & Andersson, 1987). This fraction exhibits a 40–50-fold enrichment of galactosyltransferase activity, a 10-fold increase in adenylate cyclase activity and no enrichment in acid phosphatase or NADPH-cytochrome *c* reductase activity. Plasma membrane-fraction (PMF), mainly consisting of the bile canalicular domain but also the blood-sinusoidal domain, were prepared by the method of Song *et al.* (1969). The enrichment of fluoride-stimulated adenylate cyclase is about 20-fold compared with the homogenate. The lysosomal fractions 1 and 2 (L1, L2) were isolated by the method of Wattiaux *et al.* (1978). Morphological analysis of the L1 fraction shows a large proportion of lipoprotein-filled endocytic vacuoles, in addition to typical secondary lysosomes/dense bodies (Khan *et al.*, 1981; Ferland *et al.*, 1984). Marker-enzyme analysis demonstrates a 2.4-, 10- and 2.5-fold enrichment of galactosyltransferase, acid phosphatase and adenylate cyclase activities respectively. The L2 fraction contains mature or secondary lysosomes and is 20-fold enriched in acid phosphatase, with no enrichment of galactosyltransferase or adenylate cyclase activities.

Solubilization of hepatic membranes and precipitation of solubilized crude membrane proteins

For a detailed description of solubilization of hepatic membranes and preparation of acid-precipitated solubilized crude membrane proteins (AP preparation) see Haldosén & Gustafsson (1987).

Binding and cross-linking of iodinated hormone to membranes and to solubilized and redissolved acid-precipitated extracts

Aliquots of membranes and of AP preparation were incubated with ^{125}I -hGH for 18–20 h at 20 °C in the absence or presence of unlabelled hGH. Detailed information concerning incubation parameters is found in legends to Figures. Cross-linking was performed with disuccinimidyl suberate (DSS) as earlier described (Haldosén & Gustafsson, 1987).

N-Glycanase F treatment

Aliquots of ^{125}I -hGH-incubated and cross-linked CMF containing 150 μg of protein were pipetted into Eppendorf minifuge tubes. After centrifugation at 10000 *g* for 20 min in an Eppendorf minifuge centrifuge, the pellet was resuspended with 75 μl of a solution containing 0.5% (w/v) SDS and 1% (v/v) β -mercaptoethanol. After heating at 100 °C for 3 min, 150 μl of Tris buffer

(0.1 M, pH 9.0) containing 75 mM-EDTA and 1% (v/v) β -mercaptoethanol was added. Endo F/*N*-glycanase F was added (final concentration, 2 units of Endo F activity/mg of protein) and the samples were incubated for 16–20 h at 37 °C.

An aliquot (50 μ l) of 125 I-hGH-incubated and cross-linked AP preparation containing 60 μ g of protein was mixed with 25 μ l of a solution containing 1% (w/v) SDS and 3% (v/v) β -mercaptoethanol. After heating at 100 °C for 3 min, 50 μ l of Tris buffer (0.1 M, pH 9.0) containing 75 mM-EDTA and 1% (v/v) β -mercaptoethanol was added. Endo F/*N*-glycanase F was added (final concentration 2 units of Endo F activity/mg of protein) and the samples were incubated for 16–20 h at 37 °C.

Endo F treatment

Aliquots of incubated and cross-linked CMF containing 100 μ g of protein were pipetted into Eppendorf minifuge tubes. After centrifugation at 10000 *g* for 20 min in an Eppendorf minifuge centrifuge, the pellet was resuspended with 50 μ l of a solution containing 0.5% (w/v) SDS and 0.1 M- β -mercaptoethanol. After heating at 100 °C for 3 min, 35 μ l of sodium acetate buffer (0.44 M, pH 6.0), 25 μ l of 7.5% (v/v) Nonidet P40, 21 μ l of 0.214 M-EDTA and 16 μ l of water were added. Highly purified Endo F was added (final concentration 3 munits/mg of protein) and the samples were incubated for 16–20 h at 37 °C.

Aliquots (50 μ l) of incubated and cross-linked AP preparation containing 60 μ g of protein were mixed with 10 μ l of a solution containing 1.7% (w/v) SDS and 0.33 M- β -mercaptoethanol. After heating at 100 °C for 3 min, 10 μ l of sodium acetate buffer (0.44 M, pH 6.0), 10 μ l of 12.5% (v/v) Nonidet P40, 14 μ l of 0.214 M-EDTA and 4 μ l of water were added. Highly purified Endo F was added (final concentration 3 munits/mg of protein) and the samples were incubated for 16–20 h at 37 °C.

Endo H treatment

Aliquots (50 μ l) of incubated and cross-linked AP preparation containing 70 μ g of protein were mixed with 44 μ l of a solution containing 0.2% (w/v) SDS, 0.1 M- β -mercaptoethanol and 2 mM-phenylmethanesulphonyl fluoride. After heating the samples at 100 °C for 3 min, 44 μ l of potassium phosphate buffer (0.1 M, pH 5.7) was added. In order to remove excess SDS, the samples were left to stand overnight at 4 °C and then centrifuged in an Eppendorf minifuge centrifuge at 10000 *g* for 15 min. The supernatant was removed, mixed with Endo H preparation (final concentration 20–50 munits/mg of protein) and the samples were incubated for 16–20 h at 37 °C.

O-Glycanase treatment

Aliquots (50 μ l) of incubated and cross-linked AP preparation containing 100 μ g of protein were mixed with 50 μ l of citrate/maleate buffer (0.2 M, pH 6.0). Neuraminidase (final concentration 1 unit/ml) was added, after which the samples were incubated for 1 h at 37 °C. O-Glycanase was added (final concentration 10–20 munits/mg of protein) and the samples were incubated for 16–20 h at 37 °C.

Electrophoresis and autoradiography

For a detailed description of SDS/PAGE of cross-linked samples performed according to the method of Laemmli (1970) and autoradiography, see Haldosén & Gustafsson (1987).

RESULTS

We have used biosynthetic methionyl somatotropin (M_r -22 500), here designated hGH, for labelling of specific lactogenic binding sites in different preparations of female rat liver. hGH has earlier been shown to bind to both lactogenic and somatogenic binding sites in rat liver (Ranke *et al.*, 1976).

The crude membrane fraction of rat liver is a mixture of membranes with different subcellular origins (see the Materials and methods section) and has either been used as membrane (M), Triton X-100-solubilized or redissolved, acid-precipitated Triton X-100-solubilized (AP) preparation. With an affinity cross-linking technique, we have earlier shown the presence of specific 125 I-hGH binding entities in these preparations with apparent M_r of 40000 (M), 87000, 40000, 35000 and 32000 (Triton X-100-solubilized), and 87000, 40000 and 35000 (AP) (Haldosén & Gustafsson, 1987, 1988). The M_r -87000, -40000 and -35000 binding sites and the M_r -32000 binding site displayed specific lactogenic and somatogenic binding activities respectively. Further analysis of different membrane preparations has since yielded the following apparent M_r values for the specific 125 I-hGH binding entities: 82200 (s.d. $\pm$ 4900; *n* = 31), 40600 ($\pm$ 1700; *n* = 39), 35600 ($\pm$ 1300; *n* = 27) and 31500 ($\pm$ 1400; *n* = 8).

Fig. 1. N-Glycanase treatment of lactogen receptor- 125 I-hGH complex

Acid-precipitated solubilized proteins (560 μ g) were incubated with 125 I-hGH (4.5×10^5 c.p.m.) in the absence (—) or presence (+) of a 100-fold excess of unlabelled hGH at 20 °C for 18 h. Cross-linking and treatment of samples with N-glycanase was performed as described in the Materials and methods section. Portions were taken to SDS/PAGE, which was performed on 7.5% polyacrylamide gels under reducing conditions. The autoradiogram of the dried gel is shown. The positions of the 14 C-labelled M_r markers are shown on the left of the autoradiogram; and positions of the major labelled species are indicated on the right.

Fig. 2. Endo H treatment of lactogen receptor- ^{125}I -hGH complex

Acid-precipitated solubilized proteins (300 μg) were incubated with ^{125}I -hGH (2.5×10^6 c.p.m.) in the absence (-) or presence (+) of a 100-fold excess of unlabelled hGH at 20 °C for 18 h. Cross-linking and treatment of samples with Endo H was performed as described in the Materials and methods section. SDS/PAGE and autoradiography were carried out as described in the legend to Fig. 1.

The *N*-glycanase activity of a commercial preparation containing both EndoF and *N*-glycanase activity was used to remove all types of asparagine-linked glycans (Tarentino *et al.*, 1985). Plummer *et al.* (1984) have shown that these enzymes have different pH optima (Endo F, pH 4–6; *N*-glycanase, pH 8–9). In our hands and when working according to the instructions of the manufacturers, no Endo F activity was seen at pH 4 (results not shown). At pH 9, a clear effect on the electrophoretic mobility of cross-linked entities was seen (Fig. 1). In the AP preparation, a decrease in molecular mass was seen for all binding species. The M_r -104000, -62000 and -57000 complexes, which represent M_r -82000, -40000 and -35000 binding species respectively, were decreased by M_r -10000, -10000 and -5000 respectively. Incubated and cross-linked M preparation, subjected to *N*-glycanase treatment, showed a 10000 shift in M_r for the M_r -62000 complex (results not shown). The mean values for the shifts were: M_r -104000 complex, 10200 ($n = 3$); M_r -62000 complex, 10900 ($n = 9$) and M_r -57000 complex, 5600 ($n = 4$). From these experiments, it was concluded that the lactogenic hormone-binding entities contain *N*-linked oligosaccharide chains. The appearance of M_r -104000 and -57000 complexes in AP preparations is due to exposure of lactogenic hormone-binding sites by detergent treatment.

In order to remove biantennary complex (Endo F) and hybrid and high-mannose (Endo F, Endo H) chains (Tarentino *et al.*, 1985) which are possibly present on hormone-binding species, cross-linked AP preparation was treated with a highly purified Endo F preparation (results not shown) and Endo H (Fig. 2). A decrease in M_r of 5000 was seen for the M_r -57000 complex (M_r -35000 binding entity) (Fig. 2, lanes a and c). The complex at M_r -62000 (M_r -40000 binding entity) was not affected by Endo F or Endo H. The major part of the M_r -104000 complex (M_r -82000 binding entity) was not affected either. However, a minor part showed a decreased M_r on treatment with Endo F or Endo H. The maximal decrease

Fig. 3. Neuraminidase treatment of lactogen receptor- ^{125}I -hGH complex

CMF (lanes a–d) and AP (lanes e–h) preparations containing 750 μg of protein were incubated with ^{125}I -hGH (5×10^6 c.p.m.) in the absence (-) or presence (+) of a 200-fold excess of unlabelled hGH at 20 °C for 18 h. Cross-linking and treatment of samples with neuraminidase was performed as described in the Materials and methods section. SDS/PAGE and autoradiography were carried out as described in the legend to Fig. 1.

in M_r for this complex was calculated to be 5000. When cross-linked M preparation was treated with Endo F or Endo H, no shift of the M_r -62000 complex was seen (results not shown). Due to the effect of Endo H, and taking into consideration that hGH has an M_r of 22000, it was concluded that the M_r -35000 (M_r -57000 complex) and a minor part of the M_r -82000 (M_r -104000 complex) lactogen hormone-binding species contain high-mannose chains. From these experiments, it could also be concluded that the M_r -40000 (M_r -62000 complex) and the major part of the M_r -82000 hormone-binding species have tri- or tetra-antennary complex (and not biantennary complex), hybrid or high-mannose oligosaccharide chains linked to the protein backbone.

Since complex oligosaccharide chains often contain sialic acids (Sharon & Lis, 1982) we investigated whether this was the case with the complex chains of the M_r -40000 and the major part of M_r -82000 entities. Fig. 3 shows the results of neuraminidase treatment of cross-linked M and AP preparations (Fig. 3, lanes a–d and e–h respectively). The M_r -62000 complex (M_r -40000 binding entity) in both preparations exhibited a decrease in M_r of about 3000–4000. The M_r -104000 complex (M_r -82000 binding entity) showed a similar decrease in M_r with some radioactivity unchanged. The M_r -57000 complex (M_r -35000 binding entity) was not affected by neuraminidase treatment. These results further confirm the results presented above indicating that the M_r -40000 and the major part of M_r -82000 entities contain tri- or tetra-antennary complex chains. The absence of sialic acid on the M_r -35000 binding entity also further confirms that the oligosaccharide chains present on this entity are of the high-mannose type. Hybrid chains are often sialylated (Schachter *et al.*, 1983). Treatment of cross-linked M and AP preparations with *O*-glycanase in combination with neuraminidase did not result in any additional change in the electrophoretic mobility of any lactogenic hormone-binding entity (results not shown).

Fig. 4 shows incubated and cross-linked aliquots of

Fig. 4. Cross-linking of ^{125}I -hGH to different subcellular fractions of female rat liver

Samples containing 200 μg of protein (except the PMF, which contained 70 μg of protein) were incubated with ^{125}I -hGH (3×10^5 c.p.m.) in the absence (–) or presence (+) of unlabelled hGH at 20 °C for 18 h. Cross-linking was performed as described in the Materials and methods section. SDS/PAGE and autoradiography were carried out as described in the legend to Fig. 1. Lanes a and b, solubilized residual microsomes; lanes c and d, IMF; lanes 3 and 4, LDMF; lanes g and h, PMF.

Fig. 5. Cross-linking of ^{125}I -hGH to the lysosome/endosome- and lysosome-enriched fractions L1 and L2 respectively

Triton X-100-solubilized preparation of crude membrane preparation (lanes a and b), lysosome/endosome-enriched fraction L1 (lanes c and d) and lysosome-enriched fraction L2 (lanes e and f) containing 200 μg of protein were incubated with ^{125}I -hGH (2.6×10^5 c.p.m.) in the absence or presence of unlabelled hGH at 20 °C for 18 h. Cross-linking was performed as described in the Materials and methods section. SDS/PAGE and autoradiography were carried out as described in the legend to Fig. 1.

rat liver fractions enriched with different subcellular components and containing equal amounts of protein (200 μg), except the PMF which contained 70 μg of protein. In this experiment, all fractions were used as membrane preparations except the RMF, which was Triton X-100-solubilized prior to incubation.

Cross-linked RMF was shown to contain M_r -62000, -57000 and -54000 complexes (Fig. 4, lanes a and b). The M_r -54000 complex represents the somatogenic M_r -32000 binding species which is seen in Triton X-100-solubilized crude membrane preparations. The RMF is mainly enriched in smooth and rough endoplasmic reticulum, but a slight enrichment with plasma membranes cannot be excluded (see the Materials and methods section). In order to detect the M_r -57000 and -54000 complexes, the

Fig. 6. Cross-linking of endocytosed prolactin receptors in female rats

^{125}I -oPRL (200 ng/100 g body wt.) was injected *in vivo* into a branch of the superior mesenteric vein. Labelling proceeded for 10 min, followed by preparation of an LDMF fraction. In separate animals a 1000-fold molar excess of either unlabelled oPRL (lane b) or unlabelled bGH (lane c) was co-injected with the tracer. After fractionation, the internalized tracer was cross-linked to the receptor. SDS/PAGE and autoradiography were carried out as described in the legend to Fig. 1.

RMF had to be solubilized with Triton X-100 prior to incubation (results not shown). The M_r -62000 complex was seen in both membrane and Triton X-100-solubilized preparations of this fraction. The same autoradiographic picture was seen after SDS/PAGE under non-reducing conditions (results not shown). Cross-linked IMF, enriched in *cis*-Golgi elements (G. Andersson, A. Witte, J. Saraste & H. Schachter, unpublished work), was shown to contain a main complex with an M_r value of 62000 and a lesser amount of complexes with M_r values of 57000 and 54000. This was the case in both membrane (Fig. 4, lanes c and d) or Triton X-100-solubilized (results not shown) preparations. Detergent-solubilized and cross-linked IMF, run on SDS/PAGE under non-reducing conditions, contained an additional M_r -104000 complex (results not shown). Major complexes with M_r values of 104000 and 62000 were detected when the LDMF was analysed. Low amounts of M_r -57000 and -54000 complexes were also present. This pattern was the same whether membrane (Fig. 4, lanes e and f) or Triton X-100-solubilized (results not shown) LDMFs were analysed. In membrane (Fig. 4, lanes g and h) or Triton X-100-solubilized (results not shown) preparations of PMF, an M_r -62000 complex and, on prolonged exposure of X-ray film (results not shown), an M_r -57000 complex were detected. When SDS/PAGE was run under non-reducing conditions, no additional complex was detected.

The lysosome/endosome- and lysosome-enriched fractions L1 and L2 (see the Materials and methods section) were analysed as membrane preparation with respect to the presence of specific ^{125}I -hGH-binding species (Fig. 5).

In both fractions, a diffuse broad band representing complexes with M_r values between 62000 and 57000 was detected (Fig. 5, lanes c-f). Triton X-100-solubilized and cross-linked L1 and L2 fractions, run on SDS/PAGE under non-reducing conditions, showed no additional complexes (results not shown). Analysis of cross-linked endosomal lactogenic receptors in LDMF, prepared from rats injected with ^{125}I -oPRL and killed after 10 min, showed a complex with an M_r value of 62000 (Fig. 6, lane a). The lactogenic hormone-binding specificity of this species was controlled by co-injection with the tracer, in separate animals, of either unlabelled oPrl or unlabelled bGH (Fig. 6, lanes b and c respectively).

DISCUSSION

The deglycosylation experiments indicate that two populations of M_r -82000 species exist (Figs. 7c and 7d). The M_r value of 82000 probably represents the most abundant entity containing the M_r -40000 subunit, whereas the less abundant M_r -82000 binding species containing the M_r -35000 subunit is probably found in the lower part of the autoradiographic band. Perhaps a carefully designed gradient gel could resolve these two entities. Below we will still use the term M_r -82000 species to designate both populations of binding entities.

Fig. 7. Proposed structures of the different lactogen-binding entities in female rat liver

C, tri- or tetra-antennary complex chains; M, high-mannose chains; 35 and 40, M_r -35000 and -40000 subunits respectively; X, subunit of unknown M_r .

The deglycosylation experiments also conclude that the M_r -82000 species is not composed of two identical subunits. A homodimer of M_r -40000 species would be decreased by 20000 in M_r by the action of *N*-glycanase. A heterodimer of M_r -40000 and M_r -35000 species would decrease by 15000 in M_r . Thus, the M_r -40000 and M_r -35000 subunits each appear to be combined with a subunit(s) not visualized with affinity cross-linking (Figs. 7c and 7d). Following the action of *N*-glycanase, the decrease in M_r is 10000 for both M_r -82000 and M_r -40000 species. This indicates that the undetected subunit(s) is probably not glycosylated.

The lactogenic M_r -35000 and somatogenic M_r -32000 hormone-binding species were both detected in the RM fraction. This fraction had to be solubilized prior to incubation in order to label these species. This suggests that the binding sites are found on the luminal side of the membrane vesicles. Furthermore, the fact that the M_r -32000 species was not found in the plasma membrane-enriched fraction suggests that the M_r -35000 and -32000 species seen in the RM fraction are situated in vesicles derived from endoplasmic reticulum and not in plasma membrane vesicles contaminating this fraction. Low amounts of the M_r -40000 species were detected in membrane and detergent-solubilized preparations of the RM fraction. Thus, the binding site of this entity appears to be exposed to the exterior of the membrane vesicles. This makes it reasonable to assume that the M_r -40000 species is located on plasma membrane vesicles that are present as a contaminant in the RM fraction.

The M_r -40000 species was clearly detectable and the M_r -35000 and -32000 species weakly detectable in membrane and Triton X-100-solubilized preparations of IMF and LDMF which were enriched in Golgi vesicles. When crude membrane preparations of rat liver are analysed, the M_r -35000 and -32000 species are detected only when this preparation is solubilized by Triton X-100 prior to incubation (Haldosén & Gustafsson, 1987). An explanation for the detection of the M_r -35000 and -32000 but also the M_r -40000 species in membrane preparations of IMF and LDMF could be that vesicles are disrupted during preparation, resulting in exposure of binding sites. By use of electron microscopy, it has earlier been demonstrated that LDMF contains disrupted Golgi vesicles when this fraction is sedimented and resuspended (Andersson *et al.*, 1981). The M_r -40000 and -35000 but not the M_r -32000 species were also detected in membrane and Triton X-100-solubilized preparations of plasma membrane-enriched fractions.

The facts that (1) removal of *N*-linked oligosaccharides from the M_r -40000 and M_r -35000 species by *N*-glycanase produces only one ^{125}I -labelled species with an M_r of around 30000; (2) the M_r -35000 species contains high-mannose oligosaccharide chains; and (3) the M_r -35000 species is the only lactogenic hormone-binding subunit found in vesicles probably derived from endoplasmic reticulum, make it reasonable to propose that the M_r -35000 species is a precursor to the M_r -40000 species. A definite resolution of this issue obviously requires that both species are purified and amino acids sequenced. The M_r -40000 species contains sialylated tri- or tetra-antennary oligosaccharide chains. The α -2-6-sialyltransferase responsible for terminal sialic acid transfer is markedly enriched in *trans*-Golgi cisternae and particularly in the adjacent *trans*-Golgi reticulum (Roth *et al.*, 1985). Thus it is quite possible that the M_r -40000 species, which is

found in IMF and LDMF and which is complexed with the undetected subunit(s) (see below), is found in vesicles derived from *trans*-Golgi structures.

The finding of high-mannose-containing M_r -35000 species in Golgi- and plasma membrane-enriched fractions is intriguing. All types of *N*-linked oligosaccharide chains in mammalian cells are synthesized via a common high-mannose oligosaccharide ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$) which is co-translationally added in the lumen of the endoplasmic reticulum to the newly synthesized protein backbone (Sharon & Lis, 1982). By the action of very specific glycosidases and glycosyltransferases, the conversion of high-mannose chains to complex chains starts in the endoplasmic reticulum and proceeds further in the different Golgi compartments. The carbohydrate chain is rendered Endo H-insensitive by the action of the mannosidase II, the action of which is believed to be restricted to the medial Golgi cisternae (Dunphy & Rothman, 1985). Thus, our results indicate that a minor fraction of the M_r -35000 species escapes the action of the above-mentioned medial Golgi enzyme. Tauber *et al.* (1986) have studied the glycosylation of an M_r -80000 rat liver glycoprotein which is partly inserted into the plasma membrane and partly secreted into the serum. The serum form of this protein is glycosylated with complex-type oligosaccharides and the plasma membrane form has high-mannose glycans. Thus the Golgi complex may process one and the same glycoprotein differently.

In the endosome/lysosome (L1)- and lysosome (L2)-enriched fractions, a broad autoradiographic band was seen. The M_r values of the hormone-binding entities giving rise to this band were calculated to be between 35000 and 40000. This finding might be explained by the presence of 'binders' in different stages of degradation (Tauber *et al.*, 1983). The cross-linking of injected and endocytosed ^{125}I -oPRL *in vivo* to a single binding species with an M_r value of 40000 in LDMF and, furthermore, the short labelling time used (10 min) suggest that the M_r -40000 species, which is not complexed with undetected subunit(s) (see below) is found in early endosomes which are present in LDMF (Bergeron *et al.*, 1986; Engfeldt & Andersson, 1987).

The M_r -82000 species was only detected in the Golgi-enriched fractions IMF and LDMF. Detection of this hormone-binding entity in IMF required detergent solubilization and that SDS/PAGE was run in the absence of reductant. Surprisingly, the M_r -82000 species was detected in LDMF without prior detergent solubilization. This is in contrast to the conditions needed for detection of this species in crude membrane fraction. In our earlier study (Haldosén & Gustafsson, 1987) we hypothesized that detergent treatment either brings subunits into favourable positions for cross-linking, exposes suitable sites for cross-linking due to disruption of membrane vesicles, or removes membrane lipids surrounding the proteins which might hinder cross-linking. A likely explanation for the detection of the M_r -82000 binding entity in LDMF without prior detergent treatment could be the presence of disrupted vesicles. ^{125}I -hGH can reach the binding sites on the luminal side of disrupted vesicles and DSS may cross-link the hormone-binding subunit to the undetected subunit(s) either from the inside or the outside of the disrupted vesicles.

The following is known about the unidentified subunit(s): (1) it is not glycosylated (see the Results section); (2) it is probably not a peripheral protein since membrane

washing with 150 mM-potassium chloride and 10 mM-EDTA or exposure of the detergent-solubilized preparation to a low pH does not result in decrease or loss of the M_r -82000 species (L.-A. Haldosén & J.-Å. Gustafsson, unpublished work); (3) it is probably not a proteolytic product of the M_r -82000 species, since inclusion of protease inhibitors during membrane preparation and sample incubation does not result in a decrease or loss of the M_r -82000 species (L.-A. Haldosén, G. Andersson & J.-Å. Gustafsson, unpublished work). Furthermore, the recently published cDNA sequence of the rat prolactin-binding species (Boutin *et al.*, 1988) predicts M_r for the prolactin receptor of about 33000 (unglycosylated). This indicates that the unidentified subunit is an integral membrane protein but it could also be a peripheral protein that interacts with the hormone-binding entity in a manner resistant to dissociation by high salt, chelating agents and non-ionic and zwitterionic (L.-A. Haldosén & J.-Å. Gustafsson, unpublished results) detergents. From these experiments it is suggested that each of the M_r -40000 and M_r -35000 hormone-binding entities are combined with undetected subunit(s) in the Golgi complex. At present, we have no indications that this subunit(s) is also present in other subcellular fractions such as endoplasmic reticulum, plasma membranes, endosomes or lysosomes. If confined to the Golgi complex, the formation of an heterodimeric M_r -82000 complex appears to be a reversible process during a short period in the biosynthesis and intracellular transport of the lactogen receptor. As the functional relevance of this heterodimeric complex has to await elucidation of the function of the unidentified subunit, it may be speculated that formation of this complex might be involved in the regulation of transport and/or targetting of, or be necessary for the intracellular signal transmission from, the lactogen-binding ligand-receptor complex.

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