Structurally distinct membrane-associated and soluble forms of GH-binding protein in the mouse

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Abstract

It has previously been shown that the large increase in GH-binding capacity of mouse liver microsomes during pregnancy is due largely to an increase in the amount of GH-binding protein (GHBP), with a more modest increase in GH receptor (GHR). Here we show that mouse liver GHBP is predominantly present as a membrane-associated protein structurally distinct from the soluble form of GHBP present in serum. Liver GHBP is associated with both intracellular membranes and the plasma membrane. Membrane-associated GHBP and soluble GHBP appear to be identical polypeptides distinguished by the addition of different N-glycans to asparagine residues. The pattern of release of GHBP from membranes by various treatments indicates that GHBP

associates with membranes through noncovalent interactions with one or more membrane protein, but not with GHR. Covalent crosslinking provides evidence for several GHBP-associated membrane polypeptides, with molecular masses ranging from 58 kDa to over 200 kDa.

These studies in the mouse and similar studies in the rat suggest that GHBP is an important cell-surface receptor for GH in the liver of these species. We postulate that an arginine-glycine-aspartic acid sequence found on rat and mouse GHBP but absent in other species is responsible for the association of GHBP with the plasma membrane by binding to one or more integrins on the surface of liver cells.

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Introduction

Growth hormone (GH) is a polypeptide hormone with diverse physiological functions (Thomas 1998, Waters *et al.* 1999). Vertebrates examined to date appear to contain two distinct but related proteins capable of binding GH with high affinity: a GH receptor (GHR) and a GH-binding protein (GHBP) (see Herrington & Carter-Su (2001) and Baumann (2001) for recent reviews). GHR is a single-pass transmembrane protein. GHBP consists of the extracellular (hormone-binding) domain of GHR with, in some cases, a short carboxyl-terminal extension having an amino acid sequence not present in GHR. GHBP may be produced by two independent mechanisms: specific proteolysis of GHR (demonstrated to occur in human and rabbit) and alternative splicing of RNA transcripts from the GHR/GHBP gene (demonstrated to occur in mouse and rat). There is some evidence that the two mechanisms may occur in the same organism (Martini *et al.* 1997). The protein we designate GHBP here is sometimes referred to as a short form of GHR (GHR_s). This nomenclature has its merits,

but risks confusion with other short forms of GHR that contain a transmembrane domain and truncated cytoplasmic domains of various lengths. In this report, GHBP refers to proteins that contain the extracellular domain of GHR but lack both the transmembrane and cytoplasmic domains.

The localization of GHR to cell membranes has been demonstrated in numerous studies. GHBP lacks the hydrophobic transmembrane domain of GHR and thus is predicted to be a soluble protein. Consistent with this prediction, GHBP has been detected as a soluble protein in blood and in the cytosol of several tissues. Recombinant human GHBP has been extensively studied as a soluble analog of GHR. It is not surprising therefore, that GHR has come to be considered as *the* GHR, the only protein in cell membranes capable of specific, high-affinity interaction with GH. GHBP is usually considered as a circulating hormone-binding protein whose functions, while not completely understood, do not include acting as a cell-surface receptor. There is, however, convincing evidence that this distinction of roles for GHR and GHBP is an oversimplification.

Studies in mouse and rat have taken advantage of the unique carboxyl-terminal extension (or 'hydrophilic tail') of GHBP produced by alternative splicing to specifically detect GHBP using 'anti-tail' antibodies that do not recognize GHR. Frick *et al.* (1994) used immunoprecipitation with GHR- and GHBP-specific antibodies to show that GHBP accounts for about 50% of the GH-binding capacity on the surface of freshly isolated rat adipocytes. GHBP in rat liver, muscle and fat extracts is found predominantly in the microsomal fraction and accounts for at least half of the GH-binding capacity in detergent extracts of these tissues (Frick *et al.* 1998). In the mouse, there are large increases in the GH-binding capacity of liver microsomes and serum during pregnancy (Cramer *et al.* 1992). In fact, late-pregnant mouse liver probably has the highest concentration of microsomal binding sites for GH of any tissue examined in any species, making it convenient for analysis of these sites. Camarillo *et al.* (1998) measured GH-binding capacity (by radioreceptor assay) and GHR and GHBP concentrations (by RIA) for microsomes from late-pregnant mouse liver. The amount of GHR present is much too low to account for the GH-binding capacity. Most of the GH that binds to the microsomes binds to GHBP.

The interactions of GH with cell-surface receptors are critical steps in the elaboration of the physiological functions of the hormone. The rat and mouse studies cited above indicate that in order to fully understand these interactions it is not sufficient to consider GHRs to be synonymous with the protein designated GHR. Indeed, GH reaching such important target cells as adipocytes and hepatocytes may be more likely to bind to GHBP on the cell surface than to GHR. Binding of GH to GHBP could result in signal transduction independently of the known pathway initiated by GH-induced dimerization of GHR. Alternatively, GHBP on the cell surface could act as a nonsignaling competitor with GHR for binding to GH. The studies reported here focus on GHBP in mouse liver, an organ that is both a central target tissue for GH and the major site of synthesis for the soluble, circulating GHBP.

Materials and Methods

Reagents

Polyclonal antisera specific for mouse GHBP and mouse GHR have been described previously (Cramer *et al.* 1992, Camarillo *et al.* 1998). These antisera were raised in rabbits to synthetic peptides corresponding to the hydrophilic tail of mouse GHBP and to a portion of the intracellular domain of mouse GHR. Peroxidase-linked anti-rabbit immunoglobulin F(ab')₂ fragment and chemiluminescent detection reagents (ECL+) were from Amersham Pharmacia Biotech, Piscataway, NJ, USA. Peptide:N-glycosidase F (PNGase F) was from New England Biolabs, Beverly, MA, USA. Water-soluble, primary

amine-reactive crosslinking reagent, bis(sulfosuccinimidyl)suberate (BS³) and protein assay reagents (BCA protein assay kit) were from Pierce, Rockford, IL, USA.

Tissues

Timed-pregnant (plug found = day 0 of pregnancy) and virgin female Swiss-Webster mice were obtained from Hilltop Lab Animals, Scottsdale, PA, USA and were maintained in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Procedures were approved by the Animal Care and Use Committee of Indiana University of Pennsylvania. Animals were killed by decapitation under halothane anesthesia. Livers were excised, rinsed briefly in ice-cold saline and immediately frozen on dry ice. Serum was prepared from trunk blood. Liver and serum samples were stored at -80 °C.

Preparation of liver cell fractions

All procedures were carried out at 0–4 °C. Protein concentrations were determined by the BCA method in the presence of 1% SDS to solubilize membrane proteins. Crude microsomes were prepared by a modification of the calcium aggregation method (Schenkman & Cinti 1978, Smith & Talamantes 1988). The tissue was homogenized with a rotor/stator device (Tissue Tearor; Biospec Products, Bartlesville, OK, USA) in 0.36 M sucrose, 50 mM Hepes, 1 mM phenylmethylsulfonyl fluoride, 10 µM soybean trypsin inhibitor, pH 8.0 (2 ml/g tissue). The supernatant after centrifugation at 10 000 *g* for 30 min (soluble fraction) was mixed with an equal volume of 20 mM Hepes, 20 mM CaCl₂, pH 8.0 and centrifuged at 15 000 *g* for 1 h. The pellet was resuspended in 10 mM Hepes, 10 mM CaCl₂, pH 8.0 (5 ml/g tissue) and centrifuged at 10 000 *g* for 15 min. The resulting pellet was again resuspended in 10 mM Hepes, 10 mM CaCl₂ (1 ml/g tissue) to give the crude microsomes. A plasma membrane-enriched fraction was prepared by the method of Hubbard *et al.* (1983). The fractions obtained were assayed for alkaline phosphodiesterase (marker for plasma membrane) and for NADH-cytochrome c reductase (marker for endoplasmic reticulum) as described (Hubbard *et al.* 1983).

Treatments of crude microsomes

Magnesium chloride treatment This treatment (Gerasimo *et al.* 1979) has been shown to dissociate GH from liver GH-binding sites. Crude microsomes (3–4 mg protein/ml) were mixed with 4 volumes of 3.8 M MgCl₂ and incubated for 15 min at room temperature. The treated microsomes were recovered by centrifugation (5000 *g* for 15 min) after addition of 25 mM Hepes, pH 7.4 (3 ml/ml 3.8 M MgCl₂). The microsomes were

washed with 25 mM Hepes, pH 7.4 and then washed and resuspended in 10 mM Hepes, 10 mM CaCl_2 , pH 8.0 to give the MgCl_2 -treated microsomes.

Sodium carbonate treatment This treatment (Fujiki *et al.* 1982, Castle 1995) has been shown to convert microsomal vesicles to open sheets. The vesicle contents and peripheral membrane proteins are removed while integral membrane proteins and lipid-anchored proteins are retained. Crude microsomes were diluted 50-fold with ice-cold 0.1 M Na_2CO_3 and incubated for 30 min on ice. Microsomes were recovered by centrifugation at 100 000 g for 1 h and resuspended in 10 mM Hepes, 10 mM CaCl_2 , pH 8.0 to give the Na_2CO_3 -treated microsomes.

CHAPS treatment The procedure for treatment with this zwitterionic detergent was developed for solubilization of GH-binding sites from mouse liver microsomes (Smith *et al.* 1988). Microsomes were incubated in the presence of 0.75% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) followed by centrifugation at 100 000 g for 1 h to separate the soluble CHAPS extract and the insoluble fraction. The insoluble fraction was resuspended in 10 mM Hepes, 10 mM CaCl_2 , pH 8.0 to give the CHAPS-treated microsomes.

Enzymatic deglycosylation

Microsomes and serum were incubated with PNGase F to remove asparagine-linked carbohydrates from glycoproteins. Samples were first denatured by heating to 100 °C for 10 min in the presence of 0.5% SDS and 1% 2-mercaptoethanol. Reaction conditions were as recommended by the manufacturer. For complete deglycosylation, samples were incubated with 10 U PNGase F/ μg protein at 37 °C for 1 h. For partial deglycosylation, the enzyme concentration was reduced to 0.08 U/ μg protein for microsomes or 0.15 U/ μg protein for serum.

Covalent crosslinking

Conditions for crosslinking were similar to those used previously for mouse liver microsomes (Smith & Talamantes 1987). Microsomes (100 μg protein) in 25 μl 20 mM Hepes, 10 mM CaCl_2 , 0.1% BSA, pH 8.0 were crosslinked by addition of 2.5 μl 10 mM BS^3 in 5 mM citrate, pH 5.0 and incubation for 30 min at 22 °C. The reaction was terminated by the addition of 50 μl 1.0 M Tris-HCl, pH 8.0. Microsomes were recovered by centrifugation, washed with 10 mM Hepes, 10 mM CaCl_2 , pH 8.0, and resuspended in SDS sample buffer.

Immunoblotting

Proteins were separated by SDS-PAGE and transferred to PVDF membranes. Following a brief wash in 20 mM Tris,

140 mM NaCl, 0.1% Tween-20, pH 7.6 (TBST), unbound sites were blocked by incubation in TBST containing 3% dry milk and 3% bovine serum for 1 h. After washing in TBST, blocked membranes were incubated with anti-mouse GHBP or anti-mouse GHR antiserum in blocking solution for 1 h. Membranes were washed again in TBST and incubated with peroxidase-conjugated second antibody in blocking solution for 1 h. After a final washing, bound antibody was visualized with the ECL+ luminescent detection reagent following the manufacturer's instructions.

Molecular structure modeling

Theoretical model structures of mouse GHBP were constructed by comparative modeling based on the available X-ray crystal structures for human GHBP. Models were produced using the SWISS-MODEL protein modeling server from ExPASy (Guex *et al.* 1999). Four different models were generated. A composite model was based on five structure templates selected by automated database searching. The templates used were human GHBP structures from both the 1:1 GH:GHR complex (pdb code 1 HWH (Sundstrom *et al.* 1996), and pdb code 1A22 (Clackson *et al.* 1998)) and the 1:2 GH:GHBP complex (pdb code 3 HHR (de Vos *et al.* 1992), and pdb code 1 HWG (Sundstrom *et al.* 1996)). Three additional models were generated based on either: (i) GHBP in the 1:1 complex; (ii) GHBP bound to site 1 of GH in the 1:2 complex (GHBP1); or (iii) GHBP bound to site 2 of GH in the 1:2 complex (GHBP2). The theoretical models do not include the hydrophilic tail (residues 246–273) of mouse GHBP. The models were visualized using RasMol (Sayle & Milner-White 1995).

Results and Discussion

Liver GHBP is a membrane-associated protein structurally distinct from serum GHBP

The up-regulation of GHBP and GHR during pregnancy in the mouse affords an enriched source for characterization of these proteins. GHBP was readily detectable by immunoblotting of both serum and liver microsomes from late-pregnant mice (Fig. 1A). Two major bands were detected in each sample. The apparent molecular masses are 49 and 44 kDa for serum GHBP and 43 and 40 kDa for microsomal GHBP. In both cases, the lower molecular mass component is the most abundant. The same bands were detectable in serum and microsomes from virgin female mice upon longer exposure of the film to the luminescent signal. As shown in Fig. 1B, immunoblotting for GHR revealed the presence of a single, 110 kDa band in microsomes from late-pregnant mice. This band could also be detected in microsomes from virgin mice after

Figure 1 Detection of GH-binding protein (GHBP) and GH receptor (GHR) by immunoblotting. Equal amounts of serum (0.25 μ l) or crude liver microsomes (10 μ g protein) from virgin and day 16 pregnant mice were analyzed using antibodies for GHBP (A) or GHR (B). Films were exposed for 1 min (top) or 20 min (bottom).

longer exposures. On some blots, GHR was resolved into two bands with molecular masses of 112 and 107 kDa (for example, see Fig. 3B).

The large differences in amount of GHBP and GHR detected in virgin and pregnant samples are consistent with the gestational profiles determined by RIA (Cramer *et al.* 1992, Camarillo *et al.* 1998). Immunoblotting confirms the high concentration of GHBP in late-pregnant liver microsomes detected by RIA (Camarillo *et al.* 1998) and shows that microsomal GHBP is structurally distinct from serum GHBP. Both serum and microsomal forms of GHBP (and GHR also) are heterogeneous with two major forms present in each case. The apparent molecular masses for GHBP and GHR are considerably higher than predicted from their amino acid sequences (31.6 kDa for GHBP, 70.2 kDa for GHR) but are consistent with previous estimates from crosslinked complexes containing 125 I-GH (Smith & Talamantes 1987, 1988).

Microsomes were prepared by vigorous homogenization so it is conceivable that the GHBP detected in this fraction represents soluble, cytosolic GHBP trapped within microsomal vesicles produced during homogenization. We therefore compared the microsome fraction with the soluble fraction produced by initial low-speed centrifugation of the liver homogenate. The concentration of GHBP relative to total protein is much higher in crude microsomes than in the soluble fraction (Fig. 2A), demonstrating that the GHBP in the microsome fraction is not trapped, soluble GHBP. The GHBP in the microsomes could also be soluble GHBP enclosed within lipid vesicles in liver cells. However, the microsomes were prepared from frozen tissue by a method expected to release soluble

A: Soluble and microsome fractions

B: Crude microsome and plasma membrane fractions

Figure 2 Distribution of GHBP and GHR in soluble and membrane fractions from mouse liver. (A) Equal amounts (50 μ g protein) of the soluble fraction (low-speed supernatant) and crude microsome fraction of livers from virgin and day 14 pregnant mice were immunoblotted for GHBP. (B) Equal amounts (5 μ g protein) of crude microsomes and a plasma membrane-enriched fraction from day 16 pregnant mice were immunoblotted for GHBP (left) and GHR (right).

contents from lipid vesicles (vigorous homogenization in a hypertonic medium followed by repeated resuspension in a hypotonic buffer). When the final microsomal suspension is repeatedly frozen, thawed and centrifuged, essentially all of the GHBP is recovered in the membrane pellet. Thus, GHBP present in liver is fundamentally different from the circulating serum GHBP. It is stably associated with cell membranes with very little soluble GHBP present.

A plasma membrane-enriched membrane fraction was prepared in order to determine whether membrane-associated GHBP in liver might potentially compete with GHR for binding to extracellular GH. Based on marker enzyme analysis, the plasma membrane fraction was enriched 13-fold for plasma membrane but also contained a considerable amount of endoplasmic reticulum (and potentially other membranes as well). Both GHR and GHBP are present in the plasma membrane fraction (Fig. 2B). As expected given the role of GHR in

binding extracellular GH, the plasma membrane fraction is significantly enriched in GHR compared with the crude microsomes. The plasma membrane fraction is also enriched in GHBP, but to a lesser extent than for GHR. This lower enrichment for GHBP suggests that while GHBP is present in the plasma membrane, significant amounts of GHBP are also associated with other membrane components. The enrichment of the larger form of membrane-associated GHBP (43 kDa) in the plasma membrane fraction compared with the crude microsomes is comparable with that observed for GHR, indicating that the 43 kDa form of GHBP is primarily associated with the plasma membrane while the smaller (40 kDa) form is mostly localized to other membrane components.

Camarillo *et al.* (1998) found that the amount of GHR present in microsomes from late-pregnant mouse liver is only about one-tenth of the total GH-binding capacity. Membrane-associated GHBP thus accounts for most of the GH bound to liver microsomes *in vitro*. The 43 kDa membrane-associated GHBP is present on the plasma membrane of liver cells and thus may potentially bind extracellular GH *in vivo*. It is relevant in this regard that all of the GH-binding sites on a plasma membrane fraction from rat liver appear to be present on the extracellular surface (Bergeron *et al.* 1978). Also, membrane-associated GHBP accounts for about 50% of the GH-binding capacity on the surface of rat adipocytes (Frick *et al.* 1994). Studies with mouse or rat hepatocytes would be useful to define the role of membrane-associated GHBP as a receptor for extracellular GH in liver.

A decade prior to the purification of GHR and GHBP it was shown that there is a higher concentration of GH-binding sites in Golgi fractions from rat liver than in plasma membrane fractions (Bergeron *et al.* 1978). Subsequently, it was shown that some GH-binding sites in Golgi and endoplasmic reticulum fractions from rat liver have estimated molecular masses (33–43 kDa) consistent with those for GHBP (Husman *et al.* 1989). In rat adipocytes (Frick *et al.* 1994), GHBP is present on the extracellular surface of the plasma membrane, but the majority of GHBP is associated with intracellular membranes. All these findings and our analysis of GHBP in mouse liver indicate that most of the GHBP in mouse and rat tissues is present on intracellular membranes, notably in the endoplasmic reticulum and the Golgi complex. In mouse liver one particular form of GHBP, with an apparent molecular mass of 40 kDa, is most prominent on intracellular membranes while a 43 kDa form of lower overall abundance in the liver is enriched in the plasma membrane fraction.

Membrane-associated GHBP and serum GHBP are differentially glycosylated

Previous studies of ¹²⁵I-GH crosslinked to mouse liver GH-binding sites indicate that both GHBP and GHR contain asparagine-linked carbohydrate (Smith &

Figure 3 Removal of Asn-linked carbohydrates from GHBP and GHR. For complete deglycosylation (A, B), serum and liver microsomes from day 16 pregnant mice were treated with a high concentration of peptide:N-glycosidase F (PNGase F) and blotted for GHBP (A) or GHR (B). For partial deglycosylation (C), samples were treated with a lower concentration of enzyme. Aliquots were removed at intervals, heated in SDS sample buffer, and blotted for GHBP.

Talamantes 1987). Since N-glycans are known to be important sorting signals for both secreted (Scheiffele *et al.* 1995) and membrane (Gut *et al.* 1998) proteins, differential glycosylation of GHBP in mouse liver might play a role in determining whether the protein is secreted into the circulation or targeted to different cellular membranes.

Asparagine-linked carbohydrates were completely removed from GHBP by incubation of serum or liver microsomes with high concentrations of PNGase F after denaturation in SDS. As shown in Fig. 3A, differences in N-glycan composition account for all of the difference in molecular mass for membrane-associated and serum GHBP. Deglycosylation produces a single 32 kDa band in each case. The multiple bands detected in both serum and liver microsomes therefore reflect different amounts of asparagine-linked carbohydrate on GHBP. The deglycosylated molecular mass is close to the 31.6 kDa predicted from the amino acid sequence, suggesting that no other carbohydrate or large groups are present on liver or serum GHBP. Liver GHR also contains asparagine-linked carbohydrate (Fig. 3B) and based on the difference in mobility on SDS-PAGE, appears to be more highly glycosylated than either form of GHBP. The differences between intact and deglycosylated molecular masses are

23 kDa for GHR, 12 kDa for the major GHBP band in serum and 8 kDa for the major GHBP band in liver. The apparent molecular mass of the deglycosylated liver GHR (87 kDa) is higher than predicted from the amino acid sequence (70.2 kDa) indicating the presence of other groups. This difference may be due to the conjugation of ubiquitin to GHR, which has been shown to be required for ligand-induced internalization of human GHR (Govers *et al.* 1999).

Our results showing differential glycosylation on asparagine residues of GHBP in mouse liver and serum are similar to previous results for rat GHBP (Sadeghi *et al.* 1990, Frick *et al.* 1998). Different asparagine-linked carbohydrates account for two major forms of rat GHBP in both liver microsomes and serum, with the serum forms having higher molecular masses. In the rat, serum GHBP (but not liver GHBP) contains sialic acid while liver GHBP (but not serum GHBP) contains high-mannose-type carbohydrate (Frick *et al.* 1998). Given the observed similarities in glycosylation patterns for mouse and rat GHBP, it is likely that the structural differences in N-glycans on soluble GHBP in serum and membrane-associated GHBP in rat liver occur in the mouse as well.

We chose to address a different question regarding the structure of the asparagine-linked carbohydrates on mouse GHBP: how many asparagines are glycosylated *in vivo*? Incubations of serum and liver microsomes with lower concentrations of PNGase F for various times was used to visualize intermediates formed by stepwise removal of each N-glycan from serum and liver GHBP (Fig. 3C). For serum, two intermediate bands are found between the completely deglycosylated 32 kDa band and the major 44 kDa intact band. This major form of GHBP in serum therefore is glycosylated on three asparagines. Only one intermediate is observed below the major 40 kDa intact band for liver microsomes, so the major form of membrane-associated GHBP is only glycosylated on two sites. The 43 kDa form of membrane-associated GHBP (enriched in the plasma membrane fraction) is either glycosylated at an additional site (three sites as for the major serum form) or contains more carbohydrate on the two sites glycosylated in the 40 kDa microsomal GHBP.

The evolutionary conservation of potential asparagine-linked glycosylation sites (Asn-X-Ser/Thr) in the extracellular domain of GHR has been noted previously (Harding *et al.* 1994). Mouse GHR and GHBP contain five such sites. Identical or similar sites are present in 12 of the 13 other mammalian GHR sequences present in public databases (one site is absent in gray opossum GHR). In contrast, only one of these five potential glycosylation sites is present in the mouse prolactin receptor. Although many studies indicate that GHR is a glycoprotein, little is known regarding the actual use of each of the five potential sites on GHR or GHBP of any species. Recombinant porcine GHR expressed in mouse L cells was apparently glycosylated on three asparagine residues

(Harding *et al.* 1994). Elimination of any one or all of these sites by *in vitro* mutagenesis did not prevent GH signaling in these cells, but in some cases the GH-binding affinity and receptor internalization kinetics were altered. Our results indicate that the major forms of soluble and membrane-associated GHBP in the mouse also are glycosylated at no more than three sites. We were unable to sufficiently resolve the deglycosylation intermediates for liver GHR (data not shown). The results reported here, together with previous results, indicate an important role for N-glycans in the targeting of GHBP to different destinations in rodents. Whether this targeting involves use of different sites or different processing of N-glycans on the same sites is unclear.

Since the liver is thought to be the major site of synthesis of circulating, soluble GHBP, it might be argued that the membrane-associated forms of GHBP in this tissue are all intermediates in the secretory pathway for serum GHBP. While intermediates in this pathway must be present in the liver, it is not clear that the membrane-associated forms represent these intermediates. Membrane-associated forms of GHBP apparently identical to the liver forms are present in rat adipose tissue, but kinetic evidence indicates that adipose tissue does not secrete significant amounts of GHBP (Frick *et al.* 1998). In the mouse, the difference in electrophoretic mobility between the major form of serum GHBP (44 kDa) and the liver GHBP enriched in plasma membrane (43 kDa) is small but was consistently observed in many gels with various protein loads and sample buffer compositions. Thus, if the 44 kDa serum GHBP is derived from the 43 kDa plasma membrane-associated form, there must be some modification made to the N-glycan portion of the molecule on the exterior of the cell. A significant modification would be required if the 49 kDa serum GHBP is derived by this mechanism.

It seems more likely that there are at least two pathways for newly synthesized GHBP in the liver, one leading to secretion of soluble GHBP into the serum (possibly involving processing of the N-glycans of the 40 kDa liver GHBP to yield the 44 and 49 kDa serum forms) and one leading to the association of the 43 kDa GHBP with the plasma membrane (possibly by alternative processing of the N-glycans on the 40 kDa GHBP). Support for this scenario comes from glycosidase experiments on rat GHBP. Frick *et al.* (1998) found that the 52 and 44 kDa rat serum GHBP contain sialated N-glycans while the 42 and 38 kDa liver GHBP do not. They also digested serum and liver samples with endoglycosidase H, which cleaves the high-mannose N-glycans initially added to asparagines in the endoplasmic reticulum. While Frick *et al.* state that this enzyme degrades liver GHBP but not serum GHBP, this statement oversimplifies the data they present (Figure 4c in their report). A strong band is still visible for the higher molecular mass liver GHBP (42 kDa) after endoglycosidase H treatment, while the lower molecular

Figure 4 Postulated processing pathways for liver GHBP in rodents based on characterization of mouse GHBP (this report) and rat GHBP (Frick *et al.* 1998). Apparent molecular masses are given for the two major forms of serum GHBP (a and b), the liver form enriched in plasma membrane, and the putative processing intermediate. Where two molecular masses are given, the first is for the mouse protein. Except for molecular masses, the structural information shown has been determined only in mouse or rat, not in both (see text for details).

mass liver GHBP (38 kDa) is absent. These data indicate that only the 38 kDa liver GHBP (equivalent to the 40 kDa mouse liver GHBP) contains high-mannose-type N-glycans characteristic of partially processed or immature N-glycans.

We hypothesize that, in the mouse, the 40 kDa liver GHBP is a membrane-associated processing intermediate, localized in the endoplasmic reticulum. Alternative processing of this intermediate leads to formation of the 49 and 44 kDa GHBP that are secreted or to formation of the 43 kDa GHBP that is translocated to the plasma membrane. The pathway from the 40 kDa GHBP to the serum forms involves the glycosylation of at least one more asparagine in the endoplasmic reticulum and additional processing including sialation in the Golgi. The pathway to the plasma membrane-associated form involves additional processing (including the possible use of a new site) but not sialation. Figure 4 presents this hypothesis in a more general form for both mouse and rat GHBP.

GHBP associates with membranes by noncovalent interactions with other proteins

GHBP is predicted from its amino acid sequence to be a soluble protein, and has been clearly demonstrated to be a soluble protein in blood and within cells. Thus, the means by which mouse GHBP associates with liver membranes is unclear. GHBP does not have a hydrophobic segment long enough to span a lipid bilayer. Covalent attachment of lipids could serve to anchor GHBP to the membrane. However, mouse GHBP does not contain an amino-terminal glycine or carboxyl-terminal cysteine required

Figure 5 Effect of membrane treatments on GHBP and GHR. Equal amounts (20 µg protein) of crude and treated microsomes and the soluble CHAPS extract from day 16 pregnant mice were immunoblotted for GHBP (top) and GHR (bottom).

for addition of myristoyl or farnesyl groups respectively. The addition of palmitoyl groups to cysteine residues is possible. In contrast to these lipid additions, which are found for proteins associating with the cytoplasmic face of the plasma membrane, glycosyl phosphatidylinositol addition occurs in proteins associating with the extracellular face. Standard methods for detection of glycosyl phosphatidylinositol-anchored proteins (phospholipase C digestion, Triton X-114 partitioning) provided no evidence for this membrane anchor on mouse GHBP (data not shown). Additional evidence against a lipid anchor for mouse GHBP is described below.

As an alternative to covalent attachment of lipid to GHBP, noncovalent interactions with one or more other membrane protein could explain its association with membranes. Since GH contains two sites able to bind either GHBP or GHR, the formation of a 'mixed' GHR-GH-GHBP complex on the cell surface might be responsible for the association of mouse GHBP with membranes. In this case, membrane association of GHBP should be GH-dependent as GHBP and GHR can interact only via the bound ligand, GH. As shown in Fig. 5, removal of endogenous GH from liver microsomes by treatment with 3.0 M MgCl_2 does not reduce the association of GHBP with the microsomal membranes. This finding, taken together with the quantitation of GHBP and GHR in pregnant mouse liver microsomes (Camarillo *et al.* 1998), shows that, while GHR-GH-GHBP complexes may conceivably be present, they cannot account for a significant fraction of the GHBP associated with microsomal membranes.

Treatment of membrane fractions with sodium carbonate (Fujiki *et al.* 1982) has been used to remove

Figure 6 Covalent crosslinking of membrane-associated GHBP. (A) Crude microsomes (50 µg protein) and products from crosslinking of microsomes (50 µg protein) from day 16 pregnant mice run on an 8% gel and immunoblotted for GHBP. (B) Crosslinking products from equal amounts (50 µg protein) of crude microsomes from virgin mice and crude and treated microsomes from day 16 pregnant mice run on a 6% gel and immunoblotted for GHBP.

extrinsic proteins associated with the membranes by noncovalent interactions without affecting intrinsic, membrane-spanning proteins or lipid-anchored proteins (Castle 1995). This treatment, as well as treatment with a relatively mild zwitterionic detergent (CHAPS), effectively removes GHBP from the microsomal membranes (Fig. 5). GHR, a transmembrane protein, was not released from the membranes by any of these treatments. These results indicate that liver GHBP is not a transmembrane or lipid-anchored protein. With these possibilities eliminated, it appears that GHBP probably associates with membranes via noncovalent interactions with one or more membrane proteins other than GHR.

Covalent crosslinking was utilized to examine whether GHBP specifically interacts with other membrane proteins. The amine-reactive crosslinker BS³ was used to crosslink interacting proteins through nearby amino-terminal or lysine sidechain groups. Three high molecular mass GHBP-immunoreactive bands were consistently observed in several crosslinking experiments (Fig. 6). The three bands have apparent molecular masses of 129, 107 and 98 kDa. Assuming that each represents a complex containing a single molecule of GHBP and subtracting the molecular mass of the major form of microsomal GHBP (40 kDa), gives molecular masses of 87, 69 and 58 kDa for the putative GHBP-interacting polypeptides. GHBP immunoblots of crosslinked microsomes also consistently showed immunoreactivity at higher molecular masses (over 200 kDa); however, this reactivity was somewhat

diffuse and distinct bands could not reproducibly be distinguished. No corresponding bands were detected for control samples of crosslinked microsomes with little or no GHBP present (virgin, sodium carbonate-treated and CHAPS-treated microsomes; Fig. 6B). The same pattern of high molecular mass GHBP immunoreactivity was observed when GH-depleted (MgCl₂-treated) microsomes were crosslinked (Fig. 6B), indicating that GH is not a component of these complexes and that the complexes are stable in the absence of GH.

These crosslinking experiments provide evidence for the specific interaction of membrane-associated GHBP in mouse liver with three or more polypeptides present on liver membranes. The interaction of GHBP with more than one protein might be expected, since GHBP is present in multiple membrane types. However, further studies will be required to confirm which if any of the crosslinked polypeptides represent authentic GHBP-associated membrane proteins.

A search for known protein-interaction domains within the mouse GHBP sequence suggests one possibility for a GHBP-interacting protein. Mouse GHBP contains the sequence arginine-glycine-aspartic acid (RGD), a recognition sequence for integrins (Ruoslahti 1996). Integrins (reviewed by Plow *et al.* 2000) are a family of ubiquitously expressed, transmembrane proteins localized to the plasma membrane. They contain binding sites for multiple ligands and are involved in signaling across the plasma membrane in both directions. Different α and β chains can be combined to form over 20 different heterodimeric integrins in mammals. At least eight different integrins bind to extracellular ligands containing the RGD sequence. Insulin-like growth factor-binding protein-1 (IGFBP-1) provides a relevant example of an integrin ligand. IGFBP-1 contains an RGD sequence and binds to an integrin (integrin $\alpha_5\beta_1$) on cell surfaces through this sequence (Jones *et al.* 1993). This binding protein-integrin interaction is involved in controlling the migration of human placental trophoblast cells (Gleeson *et al.* 2001).

The three-dimensional structure of mouse GHBP was modeled in order to determine the accessibility of the RGD sequence for interactions with other proteins. Mouse GHBP (excluding the hydrophilic tail) has 65% sequence identity with human GHBP, allowing a reliable (Guex *et al.* 1999) prediction of its structure based on the known X-ray crystal structures for human GHBP. It should be noted that while GHBP in mouse liver is glycosylated and associates with membranes in the absence of GH, all the human GHBP structures available are from complexes composed of nonglycosylated GHBP bound to GH and, in some cases, a second molecule of GHBP. Thus, while comparative modeling is expected to give a reliable prediction of the overall architecture of mouse GHBP, important structural details may not be accurately represented in the models. As shown in Fig. 7, the RGD sequence of mouse GHBP (residues 152–154) is predicted

Figure 7 Location of the arginine-glycine-aspartic acid (RGD) domain of mouse GHBP. (A) Peptide backbone trace for the composite theoretical model of mouse GHBP showing the location of the RGD sequence. (B) Identical view of one of the templates used to construct the composite model, human GHBP from the 1:1 GH:GHBP complex (pdb code 1 HWH). The dotted line approximates the region of interaction with GH in the complex. (C) Space-filling view of mouse GHBP theoretical models showing surface exposure of the RGD sequence and proximity of a potential glycosylation site, asparagine 145 (N). The three models were based on human GHBP in the 1:1 complex (left), GHBP1 in the 1:2 complex (middle), and GHBP2 in the 1:2 complex (right).

to be located far from the GH-binding site and close to the point where, in GHR, the transmembrane domain anchors the protein to the membrane. In all four theoretical models generated, the RGD sequence is exposed on the surface of GHBP, with arginine 152 being the first residue in a β -strand following a loop from residues 146 or 147 to 151. One of the five potential sites for the addition of N-glycans to mouse GHBP (asparagine 145) is also exposed on the surface in close proximity to the RGD sequence. A protein bound to the RGD sequence would not directly affect the GH-binding site of GHBP, but would interfere with GH-induced dimerization of GHBP since the corresponding residues of human GHBP (HAD) and rat GHBP (RGD) are in the contact region between the two GHBPs in the 1:2 GH:GHBP complex (Esposito *et al.* 1998).

While integrins are plausible candidates for a GHBP-associated protein, there is no direct evidence for this interaction. Integrin subunits typically have molecular masses of 130–200 kDa, so they are too large to be part of the 98–129 kDa GHBP-containing complexes obtained by crosslinking of microsomes. They could be responsible for the somewhat diffuse, higher molecular mass immunoreactivity detected in these samples. Alternatively, some integrins are sensitive to specific proteolysis that can be important in modifying their actions. For example, during T-cell activation a 150 kDa α integrin subunit is cleaved to give 80 kDa and 66 kDa fragments (Blue *et al.* 1993). Thus the lower molecular mass GHBP-containing complexes could contain such integrin fragments.

It may be a coincidence that of the 19 species for which GHR extracellular domain sequences are available, only

mouse and rat have the RGD sequence. The corresponding sequence is histidine-alanine-aspartic acid in all other mammals except guinea pig. Of all these species, only mouse and rat have been shown to have membrane-associated forms of GHBP. Interestingly, while the aspartic acid residue in this sequence is highly conserved in GHR, being present in all known sequences, it has very different functional roles in rat and human. Mutation of this aspartic acid to histidine in human GHR leads to GH insensitivity (Laron syndrome). The mutant human GHR is characterized by defects in expression and activity. The same mutation introduced into rat GHR has no effect on expression or activity of rat GHR (Esposito *et al.* 1998). One possible interpretation of this finding is that in the rat (and the mouse), this highly conserved residue is dispensable for GHR but required for proper function of GHBP. Clearly, this residue has no direct role in the binding of GH by GHBP; it may serve some other role. If an integrin does bind to the RGD sequence on GHBP to tether the GHBP to the membrane, then the addition of carbohydrate to asparagine 145 would be expected to have a significant effect on this interaction. Thus, RGD-dependent binding of GHBP to an integrin could explain how differential glycosylation of GHBP results in either association of GHBP with the plasma membrane or secretion into the blood.

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