BEYOND CARRIER PROTEINS

Structure and regulation of expression of the mouse GH receptor

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Abstract

GH-binding protein (GHBP) in the mouse consists of a ligand-binding domain, which is identical to the extracellular portion of the GH receptor (GHR), and a hydrophilic C-terminal domain, in place of the transmembrane and intracellular domains of the GHR. The two proteins are encoded by separate mRNAs which are derived from a single gene by alternative splicing. Determination of the gestational profiles of GHR and GHBP mRNA expression in mouse liver and placenta shows that in the liver, the 1·4 kb mRNA corresponding to the mouse GHBP increases approximately 20-fold between non-pregnant and late pregnant mice, whereas the relative increase in the expression of the 4·2 kb mouse GHR was 8-fold. The rise in the steady-state levels of both mRNAs began on day 9 of gestation. Mouse GHBP mRNA levels continue to rise until day 15 of pregnancy, while GHR mRNA abundance reaches a plateau by day 13. By elucidating the temporal changes in GHR and GHBP mRNA abundance during pregnancy and lactation in multiple maternal tissues and by assessing the ontogeny of these mRNAs in fetal and early postnatal mouse liver, our studies have demonstrated that the alternative splicing of mouse GHR/GHBP mRNA precursor is regulated in a tissue-, developmental stage- and physiological state-specific manner. *In vitro* studies using hepatocytes in culture have begun to elucidate the hormonal factor(s) involved in the gestation control of the expression of GHR and GHBP. Treatment of hepatocytes with GH or estradiol (E2) alone did not have any effect on the cellular concentrations of GHBP and GHR. However, the combination of E2 and GH up-regulated the cellular concentrations of GHBP and GHR 2- to 3-fold. GHBP and GHR mRNA concentrations were also up-regulated 2- to 3-fold. ICI 182–780, a competitive inhibitor of E2 for the estrogen receptor (ER), at different concentrations inhibited the E2- and GH-induced stimulation of GHBP and GHR. Furthermore, ER concentrations increased 5- to 7-fold in hepatocytes treated with E2 and GH compared with those in untreated cells or cells treated with either E2 or GH alone. Our studies in the mouse suggest that GHBP is an important cell-surface receptor for GH in the liver. These studies postulate that an arginine-glycine-aspartic acid sequence found on 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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Growth hormone (GH) is synthesized and secreted by the anterior pituitary gland in a pulsatile manner under the co-ordinate control of two hypothalamic neuropeptide hormones, GH-releasing factor and somatostatin (somatotropin release-inhibiting factor). GH is a polypeptide hormone that exhibits sequence and structural homology between species and with the related hormones such as prolactin. The somatogenic and metabolic effects of GH are mediated via two proteins, GH receptor (GHR) and GH-binding protein (GHBP). GHR is a transmembrane protein that binds GH with high affinity and specificity. Expression of the receptor is a requirement for cellular responsiveness to GH. The primary transcript that generates GHR mRNA can undergo alternative processing to produce several related mRNAs, including transcripts that encode the circulating GHBP and a truncated GHR that inhibits normal cellular responses to GH *in vitro* (Edens & Talamantes 1998).

GHR is a member of the cytokine/hematopoietin receptor superfamily. Binding of GH to GHR induces receptor dimerization and activation of the tyrosine kinase, JAK2, which in turn activates signaling molecules, including Stat (signal transducer and activator of transcription) factors, Src homologous collagen protein and insulin

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receptor substrates 1 and 2. Downstream events include release of second messengers, including diacylglycerol, calcium and nitric oxide, and activation of enzymes, including mitogen-activated protein kinase, protein kinase C, phospholipase A2 and phosphatidylinositol 3-kinase, which affect the function of the target cell (Carter-Su *et al.* 1996). Based on the deduced amino acid sequences, GHR is predicted to be a single-pass transmembrane protein approximately 620 amino acids in length; the exact number of amino acids varies slightly from species to species. The receptor is initially synthesized as a preprotein roughly 640 amino acids in length, containing a short signal peptide at its N-terminus for direction of the receptor to the cell surface. The mature receptor (after removal of the signal peptide) has an extracellular hormone-binding domain of approximately 245 amino acids (depending on the species) at its N-terminus, a hydrophobic transmembrane domain of 24 amino acids, and an intracellular signaling domain of approximately 350 amino acids at its C-terminus.

GHBP in the mouse consists of a ligand-binding domain, which is identical to the extracellular portion of GHR and a hydrophilic C-terminal domain, in place of the transmembrane and intracellular domains of GHR. The two proteins are encoded by separate mRNAs, which are derived from a single gene by alternative splicing (Edens & Talamantes 1998). The chemical nature and variation of GHBP among species such as humans, laboratory rodents and rabbits are relatively well described (Davis *et al.* 1992, Baumann 2001). However, the ultimate biological role and physiological significance of GHBP remain elusive, even among the most studied species (Baumann 2001). GHBP appears to be evolutionarily well conserved through vertebrates; however, the fact that GHBP is generated by either alternative splicing (i.e. rodents) or proteolytic cleavage (i.e. humans and rabbits) suggests evolutionary divergence in the mechanism of GHBP generation (Dastot *et al.* 1998). This idea is emphasized further by data in the rhesus monkey suggesting that both mechanisms for GHBP generation may co-exist (Martini *et al.* 1997). If so, the presence of variable generation mechanisms among primates may suggest that evolutionary divergence occurred within a relatively short period. Comparative studies of GHBP may help shed light on the evolution of GHBPs, on the significance of variable mechanisms of generation, and on their physiological function.

To date, aside from humans, rodents and rabbits, GHBP has been identified in a number of vertebrate species. Those species include cow, horse, pig, sheep, goat, chicken (Davis *et al.* 1992, 1994, Bingham *et al.* 1994, Jammes *et al.* 1996, Amit *et al.* 1997), turtle (Sotelo *et al.* 1997) and trout (Sohm *et al.* 1998). With the exception of trout, most of these vertebrates possess a high-affinity moiety in the range of 50–70 kDa. Porcine GHBP appears to be produced by a mechanism different from that for the rat (Bingham *et al.* 1994) and may be derived from tissues other than the liver (i.e. skeletal muscle) in fetal pigs (Schnoebelen-Combes *et al.* 1996).

Determination of the gestational profiles of GHBP and GHR mRNA expression in mouse liver and placenta shows that in the liver the 1·4 kb mRNA corresponding to the mouse GHBP increases approximately 20-fold between non-pregnant and late pregnant mice, whereas the relative increase in the expression of the 4·2 kb mouse GHR was 8-fold. The rise in the steady-state levels of both mRNAs begins on day 9 of gestation. Mouse GHBP mRNA levels continue to rise until day 15 of pregnancy, while GHR mRNA abundance reaches a plateau by day 13. Serum GHBP concentrations during the last half of gestation reflect the change in mRNA levels. By elucidating the temporal changes in GHR and GHBP mRNA abundance during pregnancy and lactation in multiple maternal tissues and by assessing the ontogeny of these mRNAs in fetal and early postnatal mouse liver, studies have demonstrated that the alternative splicing of mouse GHR/GHBP mRNA precursor is regulated in a tissue-, developmental stage- and physiological state-specific manner (Ilkbahar *et al.* 1995).

In humans, there are conflicting data regarding the role of GH as a regulator of serum GHBP. GH deficiency has been associated with low or normal serum GHBP concentrations (Baumann 1993). In cases of acromegaly, serum GHBP concentrations are either normal (Baumann 1993) or lower than normal (Mercado *et al.* 1993). In children with idiopathic short stature (Ponloura *et al.* 1992) and in GH-deficient children (Baumann 1993), serum GHBP concentrations were significantly increased after GH treatment. In pregnant mice, hypophysectomy lowered GHBP concentrations, and subsequent GH treatments partially restored GHBP concentrations (Sanchez-Jimenez *et al.* 1989). GH regulation of GHBP secretion is supported by studies in the GH-deficient dwarf rat, where continuous GH infusion increased serum GHBP concentrations to levels similar to those in normal rats (Gatford *et al.* 1998). Circulating concentrations of GHBP are greater in females than in males in both rats and humans, and may decrease the availability of circulating GH in females.

The effects of steroids on GHBP are species specific and differ between the nature of the steroid and physiological and pharmacological doses utilized in the studies. Physiological doses of androgens do not affect concentrations of GHBP in rats (Carmignac *et al.* 1993) or men (Ip *et al.* 1995). However, pharmacological doses of testosterone decrease serum GHBP concentrations in hypogonadal men, independently of GH status (Ip *et al.* 1995). Estrogens increase hepatic concentrations of GHBP mRNA and circulating concentrations of GHBP in rats (Carmignac *et al.* 1993, Gabrielsson *et al.* 1995, Bennett *et al.* 1996). In humans, circulating GHBP concentrations are generally negatively correlated with endogenous

estrogen concentrations and are reduced by physiological doses of estrogen (Massa *et al.* 1993, Kobayashi *et al.* 1994, Tato *et al.* 1995), although another study (Klein *et al.* 1996) did not find that plasma GHBP concentrations differed with stage of the ovulatory cycle in women. Conversely, pharmacological doses of estrogen increase plasma GHBP concentrations (Weissberger *et al.* 1991, Ho *et al.* 1993, Kelly *et al.* 1993, Massa *et al.* 1993, Rajkovic *et al.* 1994).

In adult rodents, GH pulsatility is sexually dimorphic (Wehrenberg & Guistana 1992). Male rodents exhibit GH secretory peaks lasting 1–1·5 h every 3–4 h, followed by distinct trough periods of low GH concentrations. In female rats, GH pulses are more random, the peaks of GH pulses are substantially lower, and trough GH concentrations between peaks are higher. However, these two distinct GH pulse patterns do not become evident until the onset of puberty, and sex steroids play a major role in these changes. Estrogen treatment of male rats resulted in a female-like GH pulse pattern (Wehrenberg & Guistana 1992). Estrogen-deficient female rats expressed a partial masculinization pattern of GH secretion (Wehrenberg & Guistana 1992). Serum GHBP concentrations are also sexually dimorphic. The more continuous female GH pulse pattern is associated with elevated serum GHBP (Gatford *et al.* 1998). The stimulatory effect of estrogen on hepatic GHR and serum GHBP is believed to be mediated by the GH pulse pattern (Gatford *et al.* 1998). Therefore, any direct effects estrogen may have on the synthesis of hepatic GHR and serum GHBP may be masked by the effect estrogen has on the GH pulse pattern. Studies in rodents have shown that the estradiol (E2) up-regulation of the liver GHR/GHBP is mediated by E2 regulation of the GH pulse pattern.

To circumvent the complexities of *in vivo* studies, studies using cultured hepatocytes from female mice have been used to determine whether E2 and GH have direct effects on hepatic GHBP production. It was shown in these studies that GHBP was significantly up-regulated when GH and E2 were used in combination. These studies demonstrated a synergism between E2 and GH to up-regulate the hepatic GHBP production in cultured mouse hepatocytes (Contreras & Talamantes 1999). The involvement of the hepatic estrogen receptor (ER) in the stimulatory effect of E2 and GH was suggested by the inhibitory effect of ICI 182–780 on the up-regulation by E2 and GH of the production of GHBP. It is possible that the inhibitory effects were accomplished by reducing the intracellular concentrations of ER, by blocking the transport of ERs into the nucleus, or by blocking ER dimerization, thus inhibiting DNA binding (White & Parker 1998). The inhibitory effects of ICI 182–780 coupled with the increase in ER concentrations in cells treated with E2 and GH suggest that ER is involved in the E2 and GH up-regulation of GHR and GHBP in cultured hepatocytes. How ER is involved is unknown. The present study indicates that E2 and GH up-regulate hepatic GHBP production by acting directly on the liver or perhaps by E2 serving a permissive role for the effects of GH regulating its own receptor. It is possible that ER could be involved in a direct manner, at the transcriptional level. Nuclear proteins such as ER regulate transcription of genes lacking estrogen response elements by modulating the activity of other transcription factors, such as activating protein-1 and nuclear factor-B (Katzenellenbogen *et al.* 1996), or by interacting with other general transcription factors, such as transcription factor IIB and transcription factor IID (Katzenellenbogen *et al.* 1996).

The GHR/GHBP gene has not been fully characterized in any species, making it difficult to determine how E2, GH and ER are involved in the regulation of GHR/GHBP. In the liver, alternative splicing of the 5′-untranslated region (UTR) of the GHR/GHBP gene results in heterogeneity in primary transcripts. Five 5′-UTRs have been reported in the rat, and four in the mouse (L1–L4) (Edens & Talamantes 1998). In the mouse, L1 and L2 are homologs of GHR1 and GHR2 of the rat GHR/GHBP gene respectively. The transcription of L1 and L2 is regulated by their own promoters. *In vivo* studies showed that although GHR1 transcription is regulated by GH, transcripts with the other GHR 5′-UTRs are not affected (Baumbach & Brendan 1995). In hepatocytes cultured with a combination of GH, E2 and dexamethasone, a 1·5- to 2-fold induction of mRNA for GHR1 has been reported (Ahlgren *et al.* 1995). In pregnant mice, L1 is responsible for the increased hepatic GHBP expression (Southard *et al.* 1995). It is possible that the E2 plus GH regulation of the expression of GHBP in cultured liver cells from female mice involves the L1 promoter.

In the liver, GH elevates Jun and Fos concentrations (Gronowski & Rotwein 1995). GH also regulates the concentrations of hepatocyte nuclear factor (HNF-1) (Legraverend *et al.* 1994), HNF-3 (Legraverend *et al.* 1994), HNF-6 (Lahuna *et al.* 1997), the CAAT enhancer-binding proteins (C/EBPs) (Clarkson *et al.* 1995), and the GH-regulated nuclear factor. GH-regulated nuclear factor is enriched in adult female rats (Waxman *et al.* 1996). GH also activates the translocation of Stat3 and Stat5 into the nucleus, which, in turn, bind to interferon-activation site-like cis elements to activate transcription (Carter-Su *et al.* 1996). To date, no interferon-activation site-like cis elements have been identified in the L1 and L2 promoters, whereas a C/EBP cis element has been identified in the L1 promoter (Menon *et al.* 1995). However, a computer analysis of the promoter and upstream regions of mouse L1 and L2 points to several potential activating protein-1- and C/EBP-binding sites, whereas no estrogen response elements have been identified (Edens & Talamantes 1998).

Interactions of GH with cell-surface receptors are critical steps in carrying out the biological functions of the hormone. Studies in the rat (Frick *et al.* 1998) and mouse

(Cramer *et al.* 1992, Camarillo *et al.* 1998), demonstrated that in order to fully understand these interactions it is not sufficient to consider GHRs to be synonymous with the protein designated GHR. It is highly probable that when GH reaches its target cells such as adipocytes and hepatocytes, it may be more likely to bind GHBP on the cell surface than to GHR. Binding of GH to GHBP would result in signal transduction independent from the known pathways initiated by GH-induced dimerization of the GHR.

It has previously been demonstrated that the large increase in GH-binding capacity of mouse liver microsomes during pregnancy is due largely to an increase in the amount of GHBP, with a more modest increase in GHR. Recent studies have demonstrated that mouse liver GHBP is predominantly present as a membrane-associated protein structurally distinct from the soluble form of GHBP present in serum (Cerio *et al.* 2002). 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 proteins, but not with GHR. Covalent crosslinking provides evidence for several GHBP-associated membrane polypeptides, with molecular masses ranging from 58 to over 200 kDa.

The studies in the mouse and the rat suggest that GHBP is an important cell-surface receptor for GH in the liver of these species. These studies 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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