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Growth hormone receptor synthesis and release in tumorous somatolactotrophs

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The growth hormone (GH) receptor (GHR) gene is expressed in pituitary somatotrophs and lactotrophs, in which GHR/GH-binding protein (GHBP) immunoreactivity is primarily located in secretory granules. The possibility that the GHR gene may be similarly expressed in GH_3 cells was therefore investigated, since these tumorous pituitary cells are used as models of GH- and prolactin-secreting cells but lack secretory granules. GHR/GHBP gene transcripts were detected in GH_3 cells and were homologous to hepatic GHR/GHBP transcripts. Immunoreactive GHRs were also detected by Western Blotting and GHBP/GHR immunoreactivity was localized by immunogold electron microscopy within each intracellular compartment. Although the immunoreactive GHR/GHBP content of these cells was less than that in rat tissues, GHBP release from GH_3 cells into incubation media (relative to tissue content) was much greater than GHBP release from rat tissue explants. These results demonstrate GHR expression in GH_3 cells, comparable with that in normal pituitary cells. These tumorous somatolactotrophs could thus provide a model for studies on GHR/GHBP synthesis and release and for studying GH effects on pituitary function. The release of GHBP from pituitary cells also indicates, for the first time, an extra-hepatic source of the plasma GHBP.

Keywords: growth hormone; growth hormone receptors; GH binding proteins; autoregulation; pituitary cells

Introduction

The pituitary gland has traditionally been thought to be resistant to the effects of growth hormone (GH), as GH is unable to autoregulate its secretion from pituitary glands of rats, mice, pigs and chickens (Richman *et al.*, 1981; Lamberts & Oosterom, 1985; Glenn, 1986; Kraicer *et al.*, 1988; de Zegher *et al.*, 1990; Harvey & Baidwan, 1990). This GH-resistance was thought to result from a lack of tissue GH receptors (Goodyer *et al.*, 1984; Fraser *et al.*, 1991). The GH receptor (GHR) gene is, however, now known to be expressed in the pituitary gland, in which GH-binding sites have been detected in plasma and nuclear membranes and in cytosolic compartments (Fraser & Harvey, 1992; Hull *et al.*, 1992; Harvey *et al.*, 1993). These binding sites may, however, reflect the ubiquitous presence of GH-binding proteins (GHBPs) rather than authentic receptors. Although GHR immunoreactivity is abundantly present in all pituitary cell types (Harvey *et al.*, 1993), much of this immunoreactivity is due to the cross-reactivity of GHBP, which is identical to the extracellular domain of the GHR (Baumbach *et al.*, 1989). Moreover, as most of the GHR immunoreactivity is located within secretory granules of adenohypophyseal cells, it is still unclear if these cells are target sites for GH action.

In contrast with normal rat somatotrophs, GH release from tumorous somatolactotrophs (GH_3 cells), that secrete both GH and prolactin, is characterized by an autoregulatory mechanism (Lapp *et al.*, 1989; Stachura *et al.*, 1990). A GH moiety may thus act in intracrine, autocrine or paracrine ways to inhibit GH secretion, although this moiety is structurally distinct from 22 kDA rat GH (Lapp *et al.*, 1989; Stachura *et al.*, 1990). These cells are also responsive to other GH action, as GH can increase IGF-I synthesis in GH_3 cells *in vitro* (Fagin *et al.*, 1987, 1989). These tumorous cells, which are commonly used as models for the study of GH and prolactin secretion (Gourdji *et al.*, 1982; Hinkle, 1984), may thus differ from normal somatotrophs in GHR gene expression.

Cloned lines of pituitary tumor cells are uniquely suited to the study of receptors, because of their homogeneity and unlimited availability (Hinkle, 1984). They are, however, an imperfect model of GH and prolactin secretion, since their interactions with some regulatory factors differ from those of normal GH- and prolactin-releasing cells. Indeed, they may lack functional receptors for dopamine and GH-releasing factor and have an overabundance of thyrotropin-releasing hormone receptors (reviewed by Gourdji *et al.*, 1982). The feedback inhibition of GH secretion by these tumorous cells could thus be anomalous and reflect a difference in GHR gene expression. Alternate transcription of the GHR gene, which normally occurs in pituitary cells and leads to the production of GHBPs may, for instance, not occur in tumorous somatolactotrophs. Since GHBPs compete with GHRs for GH and inhibit GH actions *in vitro* (Lim *et al.*, 1990; Mannor *et al.*, 1991; Amit *et al.*, 1992), a GHBP deficiency could sensitize GH_3 cells to GH action. The possibility that GHR gene expression may be anomalous in GH_3 cells was therefore addressed in the present study. The intracellular distribution of GHR immunoreactivity within these clonal cells was also determined, since they also differ from normal pituitary cells in lacking secretory granules and having a much higher nuclear/cytoplasmic volume ratio (Gourdji *et al.*, 1972). A summary of these results was recently reported (Hull *et al.*, 1994).

Results

Northern analysis

As expected, RNA moities of 4.4 kb and 1.2 kb in rat liver hybridized with the rabbit GHR cRNA probe (Figure 2). A 4.4 kb moiety was also observed in rat pituitary glands, GH_3 cells and in rat pituitary glands, although of lower abundance. A 1.2 kb transcript in GH_3 cell RNA was not evident, however, even when the blot was overexposed or when the gels were overloaded with a fivefold excess of total RNA.

Polymerase chain reaction

After 30 cycles of amplification by PCR with oligonucleotide primers for the extracellular (rat 9 and 10) or intracellular (khu 11 and 12) coding regions of the GHR (Figure 1), cDNA fragments of predicted size (435 bp and 800 bp, respectively) were generated from reverse transcribed GH_3RNA, identical to those generated from liver RNA (Figure 3). A second cycle of amplification followed by

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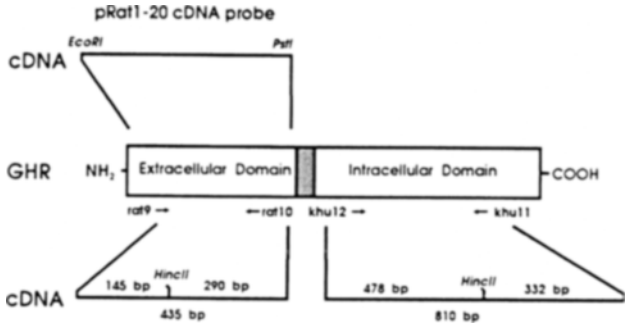

Figure 1 Sites of primer annealing and restriction enzyme digestion on the GHR cDNA (the transmembrane region is depicted by the hatched box). The portion of the coding region for the extracellular domain contained within the cDNA probe 'pRat1-20' is also shown

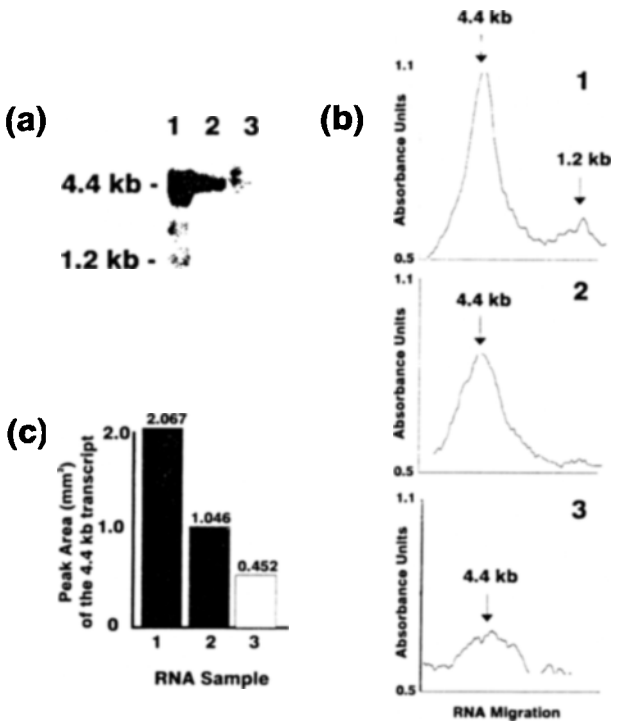

Figure 2 (**a**) Northern analysis of rat liver (lane 1), rat pituitary (lane 2) and GH_3 cell (lane 3) RNA. The membrane was incubated with a rabbit GHR cRNA probe, and the size of hybridizing moieties was determined by comparison with molecular size markers. (**b**) The intensity of hybridization was determined by laser densitometry of the autoradiograph and expressed in Absorbance units for liver (1), pituitary (2) and GH_3 cell (3) RNA. The area under the curve for the 4.4 kb transcript was subsequently calculated by integration (**c**)

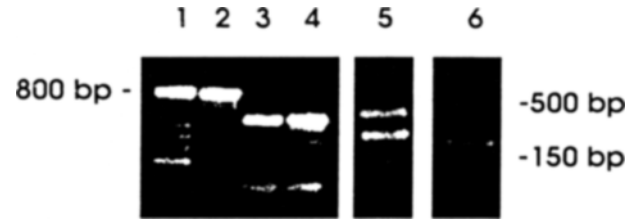

Figure 3 PCR analysis of GH_3 cell cDNA. Reverse-transcribed total RNA extracted from rat liver (lane 1) and GH_3 cells (lane 2) was amplified by the polymerase chain reaction with 3′ and 5′ oligonucleotide primers for the intracellular domain of the rat liver GHR cDNA (khu 11 and 12). HincII digests of the PCR products derived from GH_3 cDNA samples are also shown (lane 5). The hepatic (lane 3) and GH_3 cell (lane 4) cDNA was also amplified in the presence of 3′ and 5′ primers for the extracellular domain (rat 9 and 10) and digested by HincII (shown in lane 6 for GH_3 cDNA). The cDNA fragments were subjected to 1.4% agarose gel electrophoresis and were visualized by ethidium bromide staining. When Superscript was omitted from the original reverse transcription mixture, no amplification was observed with either set of primers (data not shown)

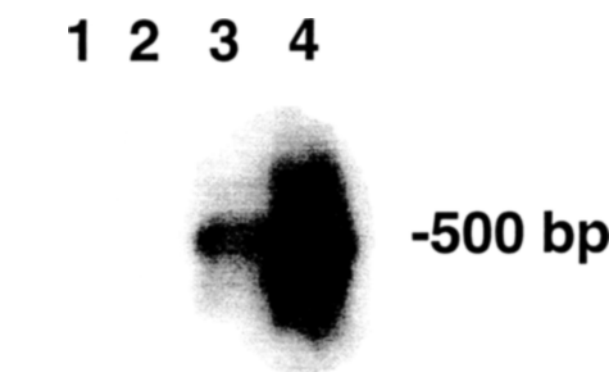

Figure 4 Southern analysis of cDNA fragments generated by the polymerase chain reaction. Moieties amplified by intracellular primers from GH_3 cell (lane 1) and rat liver (lane 2) cDNA were subjected to electrophoresis, transferred to nylon membranes and hybridized with radiolabelled pRat1-20, which encodes the extracellular domain of the rat GHR. GH_3 (lane 3) and hepatic (lane 4) cDNA amplified in the presence of extracellular primers was similarly screened for homology with extracellular GHR sequences. As expected, specific hybridization was observed with fragments amplified with extracellular, but not intracellular primers

endonuclease digestion of the GH_3 (Figure 3) and hepatic (data not shown) products with *Hinc*II resulted in smaller fragments of expected size (approximately 480 bp and 330 bp for intracellular cDNA, 150 and 300 for extracellular cDNA). The cDNA moiety amplified with khu 9 and 10 hybridized with a cDNA probe coding for the extracellular domain, whereas hybridization did not occur with fragments generated by amplification with oligonucleotide primers for the intracellular domain (Figure 4).

Western analysis

Translation of the GHR/GHBP gene transcripts was indicated by Western analysis. Proteins in GH_3 cells of 100 kDa (corresponding to the full-length GHR) and 46 kDa (corresponding to GHBP) were immunoreactive with a polyclonal antibody (p(Ab)2) raised against the rat GHBP (Figure 5). These were of identical size to proteins in rat liver that were similarly immunoreactive. In both cases, the larger proteins were not labelled after the primary antibody was preabsorbed with recombinant rat GHBP, however, labelling of the 46 kDa proteins was unaltered or enhanced in the presence of rGHBP. Neither protein, however, was labelled in the absence of the primary antibody or when this was substituted with preimmune rabbit serum (data not shown).

Immunogold cytochemistry

Immunoreactivity for the GHR/GHBP was also evident in GH_3 cells immunocytochemically stained with MAb 263 (Figure 6b). Immunoreactive gold particles were found throughout the cytoplasm and in the nucleus. Although the number of cells examined was too low to permit statistical analysis, the approximate nuclear:cytoplasmic ratio in gold particle abundance (grains cm^2 photograph) was 1:2.

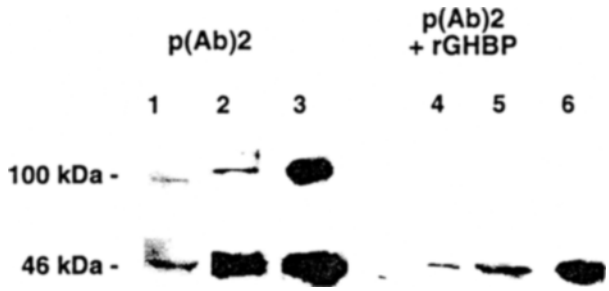

Figure 5 Western analysis showing proteins immunoreactive with p(Ab)2 antisera (raised in rabbits against recombinant rat GHBP (rGHBP) in GH_3 cells (lanes 1 and 2) and rat liver (lane 3). A replicate blot containing GH_3 cell (lanes 4 and 5) rat liver (lane 6) homogenates was also stained with p(Ab)2 that had been preabsorbed with rGHBP

Immunocytochemical staining of nuclear and cytoplasmic compartments (in a ratio of 1:1.7) was also shown using MAb 4.3 (raised against the rat GHBP hydrophilic tail) as the primary antibody (Figure 6a). In both cases staining was restricted to intracellular compartments and was not observed extracellularly. The specificity of this staining was indicated by its absence when MAb 263 or MAb 4.3 were replaced by PBS or unrelated antibodies (data now shown).

Radioimmunoassay

GHRs and/or GHBPs in GH_3 cells were also detected by radioimmunoassay. Cellular extracts and incubation media inhibited the binding of [^{125}I-]hGHBP or [^{125}I-]rGHBP to p(Ab)1 or MAb 4.3 in a manner parallel with the hGHBP and rGHBP standards (Figure 7). The amount of hGHBP/rGHBP immunoreactivity in GH_3 cells was, however, far less than that in the pituitary gland or in any extrapituitary tissue (Figure 8). Estimates of rGHBP immunoreactivity in GH_3 cells and tissue extracts using p(Ab)1 were generally comparable with p(Ab)1 immunoreactive hGHBP estimates ($r = 0.957$, $P<0.001$, $n = 11$) and hGHBP estimates are therefore not shown in Figures 8 and 9. When GH_3 cells and rat tissues were incubated in Medium 199, GHBP immunoreactivity was measurable in each incubation media, although at concentrations far less (mean = $1.7 \pm 0.3\%$) than those in tissues (Figure 8). Hepatic media contained 1.88% of the GHBP in tissue whereas pituitary media contained 3.13% of the pituitary GHBP content. In contrast, the culture media of GH_3 cells contained 5.81% of the tissue GHBP content.

Discussion

Tumorous somatolactotrophs differ from normal adenohypophyseal cells in having an overabundance or deficiency of functional receptors for many stimulatory or inhibitory factors regulating GH and prolactin release (Gourdji *et al.*, 1982). Despite these receptor anomalies, the results of this study clearly demonstrate normal expression of the GHR gene in GH_3 cells, in which immunoreactive GHRs and/or GHBPs are ubiquitously distributed.

Expression of the GHR gene in GH_3 cells may, however, be less than in liver and other GH target sites, since GHR gene transcripts were not easily detected by Northern blotting. Moreover, while the ratio of GHBP:GHR transcripts (as anlysed by a rat GHR cDNA probe) in the rat liver is >7:1 (Bingham *et al.*, 1994), Northern analysis failed to detect GHBP RNA in GH_3 cells. This apparent GHBP transcript deficiency may be due in part to the use of a rabbit GHR cRNA probe, as hybridization to the hepatic, 1.2 kb GHBP transcript was also very low (Figure 2a). The abundance of GHBP transcripts is, however, similarly low in normal pituitary cells, in which Bingham *et al.* (1994) found the abundance of GHR mRNA to be fivefold greater than GHBP transcripts. The immunoreactive GHBP content in

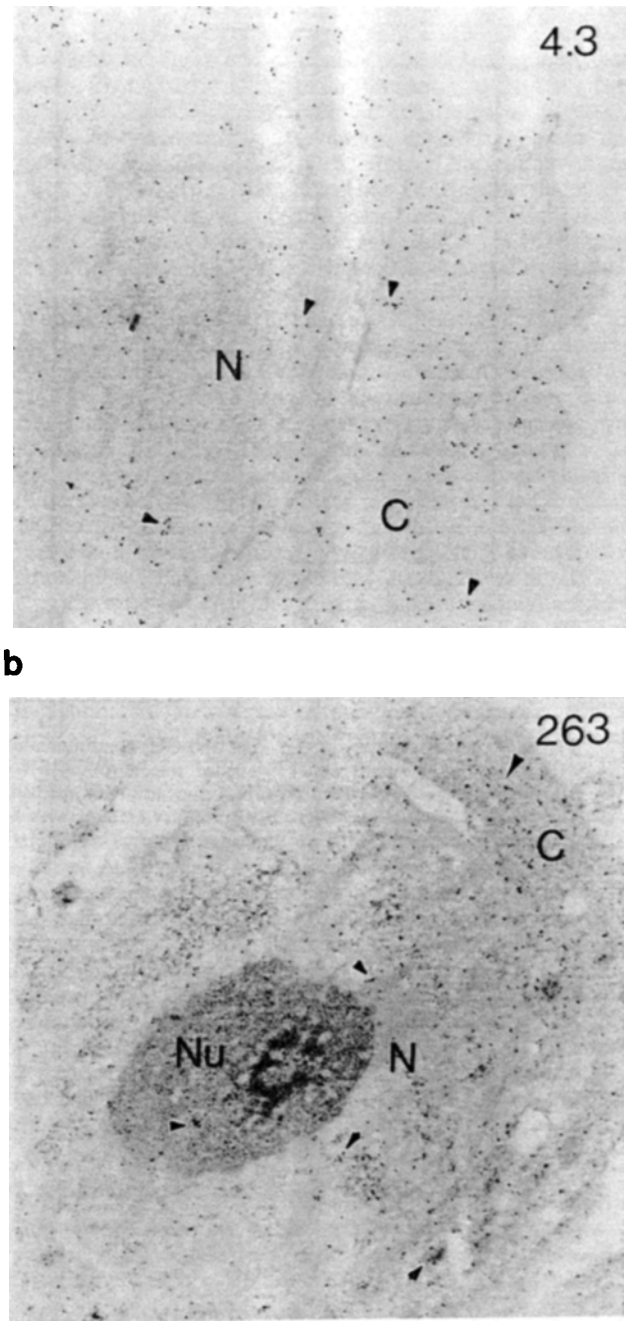

Figure 6 Immunocytochemical staining of GH_3 cells. Pelleted cells were fixed in 2% glutaraldehyde/0.2% paraformaldehyde, dehydrated and embedded in LR gold resin. Ultrathin sections were prepared on uncoated nickel grids and stained with either MAb 4.3 (**a**), raised against the hydrophilic tail of the rat GHBP, or MAb 263 (**b**), which recognizes an epitope in the GH-binding domain of the rat GHBP. GHBP-like immunoreactivity was detected by colloidal gold-labelled anti-mouse IgG antibody and cellular ultrastructure was revealed by staining with uranyl acetate and lead citrate. Examples of gold-labelled GHBP in nucleolar, nuclear and/or cytoplasmic compartments are indicated by arrowheads. Abbreviations; Nu, nucleolus; N, nucleus; C, cytoplasm

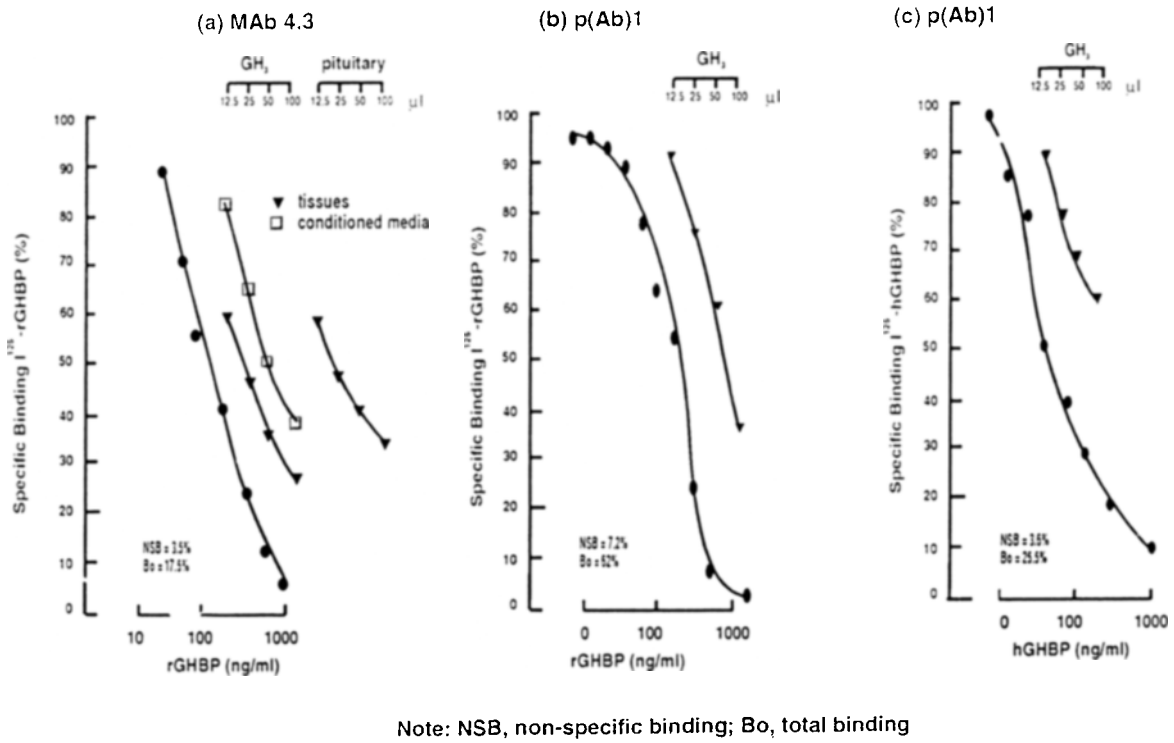

Figure 7 Cross-reactivity of GH_3 cells and GH_3-conditioned media with (**a**) MAb 4.3 and (**b**) p(Ab)1 in a radioimmunoassay using rGHBP as the standard and I^{125}-labelled tracer. (**c**) p(Ab)1 was also employed in a radioimmunoassay using hGHBP as the standard and I^{125}-labelled tracer. GH_3 cell-conditioned media did not, however, significantly displace I^{125}-labelled rGHBP binding to p(Ab)1. The cross-reactivity of rat pituitary extracts with MAb 4.3 is shown for comparison. Levels of total binding (Bo) and non-specific binding (NSB) are indicated for each assay

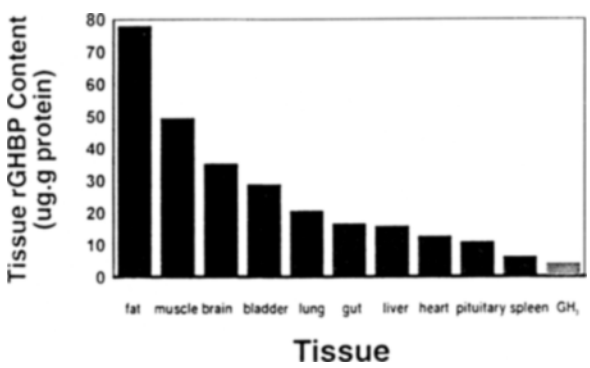

Figure 8 Immunoreactive GHBP concentrations (µg/g tissue protein), as determined by radioimmunoassay, in rat tissues and GH_3 cells

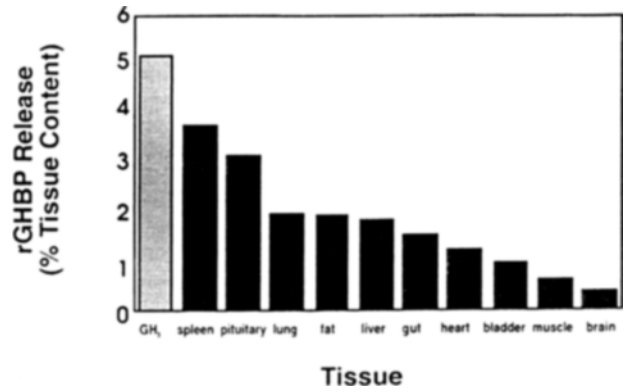

Figure 9 Release of immunoreactive GHBP from rat tissues and GH_3 cells during 5 h incubation period, expressed relative to tissue GHBP concentration

GH_3 cells was also much lower than in the rat pituitary gland and in other extrapituitary tissues, consistent with a reduced rate of GHR gene expression. This reduced GHBP concentration may thus facilitate receptor-mediated GH action and provide an explanation for the autoregulatory mechanism in tumorous somatotrophs that suppresses GH secretion. The presence of membrane binding sites for GH, linked to intracellular signal transduction systems, was not, however, directly assessed.

Although the intracellular GHBP content of GH_3 cells is far less than that in hepatic and extrahepatic rat tissues, the extracellular:intracellular GHBP ratio is far higher for incubated GH_3 cells than for incubated rat tissues (Figure 8). The augmented GHBP release from GH_3 cells may thus partly account for their relatively low intracellular GHBP concentration. This reduced tissue GHBP concentration may indicate that the competitive antagonism of GH receptors, by GHBP, would be reduced, thereby increasing the sensitivity of GH_3 cells to GH autoregulation. The constitutive release of GHBP from these cells may thus occur at a faster rate than in rat tissues that possess (e.g. pituitary and brain) or lack (e.g. liver and muscle) secretory granules. Thus, although the steady-state levels of GHR gene transcripts are lower than in normal rat tissues, their turnover rate may be accelerated in these tumorous cells. Moreover, since the intracellular GHBP content in GH_3 cells is less than in normal tissues, the rate of GHBP release may also be accelerated in these cells. This possibility is also supported by the demonstration that GH abundance is far lower in GH_3 cells than in rat somatotrophs but the constitutive rate of GH release is much greater from GH_3 cells, which have extracellular:intracellular GH ratios of 2:1 (Tixier-Vidal *et al.*, 1980).

GHBP has previously been shown to be constitutively released from hepatocytes (Amit *et al.*, 1994; Barnard *et al.*,

1994) but not from adipocytes (Frick *et al.*, 1994) or trophoblasts (Barnard *et al.*, 1994) that possess intracellular GHBP immunoreactivity. This therefore contrasts with our finding of GHBP immunoreactivity in the media of all the tissue explants incubated. The finding that media GHBP immunoreactivity was greater for several extrahepatic tissues than for liver explants was also unexpected, as this may indicate that numerous tissues contribute to circulating GHBP concentrations (Harvey & Hull, 1994). GHBPs were, for instance, abundant in fat-conditioned media. This observation may, in part, result from the extremely low protein content in fat in comparison to other tissues (i.e. liver), as GHBP abundance was quantified relative to tissue protein content. Nevertheless, because of its size, the liver would still probably be of primary importance in regulating plasma GHBP concentrations, even if other tissues contributed to an intravascular pool. It is, however, also possible that the presence of GHBP immunoreactivity in the media of all cultured tissues may merely reflect non-specific release of GHBP from damaged cells or the shedding of surface GHRs by tissue protease activity, especially as the amount of media GHBP was <5% of the tissue GHBP content. This possibility awaits investigation.

The subcellular distribution of GHR/GHBP immunoreactivity in GH_3 cells included both cytoplasmic and nuclear compartments. This is therefore similar to GHR/GHBP distribution in normal pituitary cells, hepatocytes, neurons and glia (Lobie *et al.*, 1991, 1992, 1993; Fraser & Harvey, 1992). Indeed, the ratio between nuclear and cytoplasmic staining by MAb 263 in GH_3 cells (1:2) was comparable to that in pituitary cells which contain secretory granules (1:1.7, calculated on figures presented by Harvey *et al.*, 1993). Most of the GHR/GHBP immunoreactivity in the nuclei of normal pituitary cells is, however, in the secretory granules, as the nuclear:cytoplasmic GHBP ratio falls to 1:0.85 in secretory granule-free cytoplasm. Harvey *et al.* (1993) found no MAb 4.3 GHBP immunoreactivity in the nuclei of normal pituitary cells. The occurrence of MAb 4.3 immunoreactivity in the nuclei of GH_3 cells therefore differs from normal pituitary cells and is more akin to the GHBP distribution in more traditional sites of GH action. Lobie *et al.* (1991), for instance, found significant MAb 4.3 GHBP immunoreactivity in hepatic nuclei. It is, however, unclear if nuclear GHBPs have direct or indirect effects on gene transcription within the nucleus.

In summary, these results demonstrate that GHR expression in GH_3 cells is comparable, but not identical, with that in normal pituitary cells. These tumorous somatolactotrophs could thus provide a potential model for studies on GHR/GHBP synthesis and release and for studying GH effects on pituitary function. The release of GHBP from pituitary cells may also indicate, for the first time, an extra-hepatic source of the GHBP in plasma.

Materials and methods

Tissue culture

GH_3 cells were obtained commercially (American Type Culture Collection, Bethesda, MD), and were cultured in Hams F-10 medium containing 12.5% horse serum and 2.5% fetal calf serum (Gibco BRL, Burlington, Ontario). Confluent cells (25 million) were washed in Hank's medium (containing 1% PMSF) (Gibco) to remove serum-containing culture medium prior to trypsinization and sedimentation. The cell pellets were then washed twice in serum-free medium prior to GHR analysis.

Northern blotting

Total RNA was extracted from the GH_3 cell pellet by resuspension in 5.5 M guanidinium thiocyanate, containing 25 mmol sodium citrate/l, 0.2% (w/v) β-mercaptoethanol and 0.5% (w/v) sodium lauryl sarcosine (pH 7.0). Total RNA was collected after centrifugation (125 000 *g*, at 21°C for 22 h) through a bed of cesium trifluoroacetic acid (Pharmacia Fine Chemicals, Uppsala, Sweden, density 2.01) containing 100 mmol EDTA/l, pH 7.0 (Okayama *et al.*, 1987). RNA was assessed for degradation by electrophoresis and quantified by fluorimetry. Total RNA (10 μg, liver; 20 μg, pituitary; 30–50 μg, GH_3 cells) was electrophoresed in 1% agarose-formaldehyde gels and transferred to a nylon membrane (Zetaprobe, Millipore). A rabbit GHR (rGHR) complementary RNA (cRNA) probe was constructed from a 638 base pair (bp) *BamHI/EcoRI* fragment (donated by Dr W.I. Wood, Genetech, San Francisco, California, USA) of the rabbit hepatic GHR cDNA sequence coding for portions of the extracellular and transmembrane domains (Leung *et al.*, 1987). The plasmid pGEM3Z (Promega Corporation, Madison, Wisconsin, USA) containing this sequence was linearized by *BamHI* digestion and transcribed by T7 polymerase using a riboprobe kit (Promega Corporation) in the presence of [^{32}P]aCTP (800 mCi/mmol; New England Nuclear, Mississauga, Ontario, Canada). The cRNA probe was then hybridized with the immobilized RNA in 60% (w/v) formamide (containing 0.75 mol NaCl/l, 25 mmol PIPES/l and 25 mmol EDTA/l, 0.2% (w/v) sodium dodecyl sulphate (SDS), 1 × Denhart's Reagent (0.1% (w/v) ficoll, 0.1% (w.v) BSA, 0.1% (w/v) polyvinylpyrrolidine), 100 mg salmon sperm DNA/l, pH 6.8) for 12 h at 55°C, following a 3 h incubation in the absence of the probe. Following a brief rinse in 2 × SSC, the nylon membranes were washed at room temperature in 0.1% (w/v) SDS containing 2 × SSC and subsequently at 75°C in 1% (w/v) SDS containing 0.1 × SSC (1 × SSC = 15 mmol sodium citrate/l and 150 mmol NaCl/l, pH 7.2). Membranes were then placed between intensifying screens and exposed to Kodak X-OMAT-AR film (Kodak, Rochester, New York, USA) for 2 days. The sizes of the RNA moieties hybridizing to the probe were determined by comparison with electrophoretically separable size markers (Boehringer Mannheim, Dorval, Quebec, Canada). As the transcripts in GH_3 cells and pituitary glands were of low abundance, hybridizing bands were also detected by laser densitometry of the autoradiographs, and the area under the absorbance curve for the 4.4 kb GHR mRNA band was determined by integration.

Polymerase chain reaction

Total GH_3 RNA was also reverse transcribed by Superscript (100 U, BRL) in the presence of 100 pmol of oligodeoxythymine primer (Boehringer Mannheim), excess nucleotides (10 mmoles/l each of dCTP, dTTP, dTKP and dGTP Boehringer Mannheim) and 5 × H-RT buffer (BRL). The reactions were diluted with double distilled water (500:1 vol/vol) and an aliquot of each (5% of total volume) was added to a polymerase chain reaction (PCR) cocktail containing 5'oligomer cGHR sense (CTGCGGCCGCAGGACCAGTTCCAAAGATTAA) and 3' antisense (AAGCGGCCGCGGCAGTAGTGGTAAGGCTTTC) oligonucleotides, deoxynucleotides (1.25 mmol/l of each), 1 × PCR reaction buffer (50 mmol KCl/l, 10 mmol Tris-Cl/l, pH 8.4, 1.5 mmol $MgCl_2$/l, and 20 μg/ml gelatin) and *Thermus aquaticus* (Taq) DNA polymerase (5 U, BRL) (Kawasaki, 1990). The primers were based on the known sequence of a portion of the rat GHR transcript coding for the intracellular domain (Baumbach *et al.*, 1989) (Figure 1). The reverse transcribed RNA was also amplified in the presence of 5' sense (CCTCCATTTGGATACCCTACTGCATTAAGC) and 3' antisense (GCATCACTGCTACTCCAAATATTCCAAAG) primers coding for a portion of the extracellular domain of the receptor. The mixture was overlayed with mineral oil (vol/vol), and heat denatured at 94°C for 3 min before 30 cycles of annealing (at 45°C for 1 min), extension (at 72°C for 3 min) and denaturing (at 94°C for 1 min) in a genetic thermal cycler (Minicycler,

Fisher Scientific, Edmonton, Alberta). The PCR products were also digested for 2 h with *Hinc*II for known cleavage sites in the regions coding for the extracellular and intracellular domains of the receptor. The PCR and digestion products were electrophoresed in 1.4% (w/v) agarose ethidium bromide-stained minigels and compared with *Hae*III digested pUC 18 or *Eco*RI/*Bam*HI digested lambda size markers.

The identity of the amplified PCR fragments was further elucidated by Southern blotting. Both intracellular and extracellular cDNA fragments were electrophoresed in 1.4% (w/v) agarose gels and transferred to Hybond N (Amersham, Oakville, Ontario) nylon membranes by capillary transfer (Turboblotter, Schleicher and Schuell, Keene, New Hampshire). Non-specific binding sites on the membrane were blocked by pre-hybridization in 30% (w/v) formamide (containing 0.75 mol NaCl/l, 25 mmol PIPES/l and 25 mmol EDTA/l, 0.2% (w/v) sodium dodecyl sulphate (SDS), 1 × Denhart's Reagent (0.1% (w/v) ficoll, 0.1% (w/v) BSA, 0.1% (w/v) polyvinylpyrrolidine), 100 μg salmon sperm DNA/l, pH 6.8) for 1 h. The immobilized cDNA was then hybridized with the pRatl-20 cDNA probe (Figure 1) in the same buffer for 12–18 h at 42°C. Following a brief rinse in 2 × SSC, the nylon membranes were washed at room temperature in 0.1% (w/v) SDS containing 2 × SSC. Additional washes were performed at 42°C in 1% (w/v) SDS containing 0.2 × SSC (15 min) and subsequently in 1% (w/v) SDS containing 0.1 × SSC (15 min) (1 × SSC = 15 mmol sodium citrate/l and 150 mmol NaCl/l, pH 7.2). Membranes were then exposed to Reflection autoradiography film (NEN) in the presence of a Reflection intensifying screen (NEN) for 2 h at −70°C.

Antibodies

A panel of monoclonal and polyclonal antibodies were employed in the immunological assays. Polyclonal antibodies 1 and 2 (p(Ab)1 and p(Ab)2 were raised against recombinant rat GHBP and recognize both GHR and GHBP (Sadeghi *et al.*, 1990; American Cyanamid). MAb263 (Agen Biomedicals Inc., Brisbane, Australia), raised against a common epitope in the extracellular domain of mammalian GHRs, also recognizes both GHR and GHBP, whereas MAb 4.3 (American Cyanamid) was raised against the hydrophilic tail of the rat GHBP and consequently recognizes GHBP but not GHR. Other polyclonal antibodies raised against the intracellular domain of the rat GHR (American Cyanamid) or monoclonal antibodies 1, 7 and 16 (Agen) were ineffective in Western blotting assays (data not shown).

Western analysis

GH_3 cells (approximately 25 million) were also homogenized in 1% (w.v) sodium dodecyl sulfate (SDS) containing 1 mM PMSF (Sigma, St. Louis, MO) and 10 mg/ml aprotinin (Sigma). Aliquots were subjected to electrophoresis in 15% acrylamide-SDS gels and subsequently electroblotted onto Immobilon P membranes (Millipore, Mississauga, Ont.) in 10 mM 3-[cyclohexylamino]-1-propane-sulfonic acid buffer (Sigma), pH 10, containing 10% methanol. The filter was incubated overnight with a p(Ab)2 (1:2000), in tris-buffered saline (TBS) (10 mM Tris (pH 7.5), 150 mM NaCl) containing 5% non-fat dried milk following a 1 h blocking step in the same buffer. Replicate blots were incubated in the absence of primary antibody or with antibody that had been preabsorbed with 100 mg recombinant rat GHBP for 1 h. The blot was subsequently washed in TBS (4 × 30 min) and incubated with horseradish peroxidase-labelled goat anti-rabbit IgG (Amersham, Lancashire, England) for 1 h at a dilution of 1:1500. Following four 30 min washes in TBS containing 1% bovine serum albumin (BSA, Sigma), the filter was developed using a ECL kit (Amersham) and autoradiographed.

Radioimmunoassay

A heterologous system was first developed using a polyclonal antibody raised against rat GHBP (p(Ab)1, Sadeghi *et al.*, 1990) as the primary antibody and human GHBP (hGHBP, kindly donated by Dr J.A. Wells, Genetech, San Francisco) as the standard and chloramine T-radioiodinated tracer. The tracer had a specific activity of approximately 40 μCi/ug and was >90.0% precipitable with excess primary antibody, which specifically bound 26.7% of the tracer when present at a final dilution of 1:3000. Non-specific binding was approximately 3%. Tubes containing GH_3 cell homogenates or incubation media (1:1–1:8) were incubated with approximately 10 000 c.p.m. of the tracer and p(Ab)1 for 24 h at 4°C prior to the addition (at a final concentration of 1:120) of the goat anti-rabbit secondary antibody (Sigma). After a further 24 h incubation at 4°C, bound and free radioactivity were separated by centrifugation and aspiration of the supernatants. The precipitation of bound radioactivity was assisted by the addition of 100 μl protein A (Tachisorb, Calbiochem Corporation, La Jolla, CA), at a final concentration of 1:120. The hGHBP standard (over the range 2500–2.4 μg/l) and GH_3 cell homogenates were diluted in 0.05 sodium phosphate buffer (contaianing 1% bovine serum albumin), pH 7.0, in a sample volume of 200 μl.

For comparative purposes the GH_3 samples were similarly assayed in homologous radioimmunoassays for rGHBP (American Cyanamid), using p(Ab)1 or MAb 4.3 as the primary antibodies (at final dilutions of 1:4000 and 1:6000, respectively).

GHBP release

The possibility that GHBP may be released from GH_3 cells and other rat tissues was also investigated by radioimmunoassay. Small fragments (approximately 3–5 mm^3) of fat, lung, brain, bladder, intestine, muscle, heart, spleen and liver and entire pituitary glands ($n = 5$) were quickly excised from Sprague Dawley rats (Health Sciences Laboratory Animal Services (HSLAS)) and incubated for 1 h in Medium 199 (M199). Harvested GH_3 cells were also isolated and resuspended (3 million/ml) in M199. The media were then aspirated and replaced with fresh M199. Following a 5 h incubation, the media was aspairated and the cells dissolved in phosphate-buffered saline by Polytron homogenization (tissues) or by sonication (GH_3 cells). As GHBP-like immunoreactivity was found to be of low abundance in tissues and media, the individual samples were pooled and concentrated in a Speedvac. The pooled media and cell homogenates were subsequently assayed for GHBP content by the RIA systems described above, and the data was expressed relative to protein content, measured by the Bradford method (Bradford, 1976). GHBP release into media was expressed relative to the amount of GHBP in the tissue, to permit analysis of the relative importance of retained and secreted GHBPs/GHRs.

Immunogold cytochemistry

GH_3 cells were pelleted in serum-free media, washed with PBS and fixed in 2% glutaraldehyde; 2% paraformaldehyde in 0.1 mM phosphate buffer, pH 7.4, for 30 min at room temperature. Samples were then prepared for cytochemistry as previously described (Harvey *et al.*, 1993). Briefly, the cell pellet was dehydrated in graded series of ethanols at −20°C prior to embedding in LR gold resin. This was polymerized in gelatin capsules under a u.v. lamp (360 nm) for 24 h. Ultrathin sections were cut and mounted on uncoated nickel grids.

For cytochemical staining, grids were floated on a drop of filtered 1% BSA for 10 min to block non-specific binding sites and transferred to a drop of primary antibody (MAb 263, raised against the rat GHR, Agen; MAb 4.3, raised against the rat hydrophilic GHBP tail, Sadeghi *et al.*, 1990)

for 2 h at room temperature. Control grids were floated on drops of PBS or of an unrelated monoclonal antibody raised against *Brucella* (Agen). Following washes with PBS, grids were floated on drops of a colloidal gold-labelled goat anti-mouse secondary antibody (1:10 in PBS) and washed in PBS and then water. Grids were stained with uranyl acetate and lead citrate to enable recognition of ultrastructural morphology.

The density of gold particle staining in photographs (magnification × 7400) was also compared in GH_3 cells and normal rat pituitary cells. The number of gold particles was counted in 12 separate 1 cm^2 boxes in nuclear and cytoplasmic compartments of GH_3 cells stained with MAb 4.3 ($n = 3$) and MAb 263 ($n = 2$). Nuclear/cytoplasmic ratios were also determined for pituitary cells stained with these antibodies (as described in Harvey *et al.*, 1993) for comparative purposes. As secretory granules are not present in GH_3 cells, the cytoplasm of pituitary sections was divided into secretory granule and non-secretory granule compartments.

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