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Expression and binding properties of two isoforms of the human growth hormone receptor

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Two isoforms of the human growth hormone receptor mRNA, one containing exon 3 (encoding an extracellular domain of the receptor), hGHR, and one excluding exon 3, hGHRd3, have been described. To study the cellular distribution of the two types of messengers we have analysed a panel of tissues. Both isoforms were expressed independently or simultaneously depending on the tissue studied. To investigate the binding properties of hGHRd3 we have cloned its cDNA in a eukaryotic expression vector; transient expression in COS-7 cells showed that the receptor without exon 3 was expressed on the plasma membrane and was able to bind human growth hormone (hGH) with the same high affinity as hGHR. Human lactogen (hCS) removed ^{125}I-hGH bound to the full-length and exon 3 – excluding receptors to the same extent. These results show that hGHR and hGHRd3 have tissue-specific expression and share identical binding properties for hGH and hCS and leave open the possibility that exon 3 might influence receptor signalling.

Human growth hormone receptor; Exon 3; Splicing; GH; CS; Binding

1. INTRODUCTION

Growth hormone (GH-N) is synthesized and secreted from the anterior pituitary lobe, and has multiple effects on both cells and the organism as a whole. They include promotion of body growth, stimulation of intermediary metabolism [1], and transcriptional regulation of specific genes (IGF-I and cytochrome P450 [2], c-*fos* and c-*jun* [3]). GH-N belongs to a family of proteins including the GH variant expressed in the placenta (GH-V) and chorionic somatomammotropin hormones (hCS-A and hCS-B). These forms of hGH are encoded by different genes (GH-N, GH-V, CS-A and CS-B) clustered on the short arm of chromosome 17. In addition, alternative splicing of the same transcript can generate different isoforms (GH-N 22 kDa, 20 kDa or 17.5 kDa) (for review, see [4]). These proteins have different metabolic properties; for example, the growth-promoting effect of hGH-V is similar to that of hGH-N whereas its lactogenic activity is lower [5]. It is now generally accepted that these hormones act on target cells through specific binding to the extracellular domain of transmembrane receptors. Although receptors for GH (GHR) are present in many cell types, an important question is whether a single GH receptor mediates the multiple effects of GH or whether different receptors exist. Various approaches, including direct radioligand studies using a panel of monoclonal antibodies [6], have provided evidence supporting the existence of membrane GH receptor subtypes. A cell-surface receptor was first cloned in rabbits and man [7] through its ability to bind to hGH-N 22 kDa with high affinity. Comparison of the primary sequences of GHR and related receptors isolated established that they were members of the new cytokine receptor family including receptors for prolactin, erythropoietin, granulocyte/macrophage colony-stimulating factor, interleukin-2, -3, -4, -5, -6, -7 and interferons [8] and, more recently, the receptors for IL-9 [9] and ciliary neurotrophic factor [10]. Structural and functional analyses of GHR should help to elucidate the different specific transducing pathways and the mechanisms by which GH binding initiates cellular responses.

The extracellular part of the receptor molecule is encoded by exons 2 to 7, which specify 244 of the 246 amino acids of the extracellular domain of the mature hGHR. Godowski et al. [11] have reported several GHR cDNA clones that diverge within the coding region. In one of these, the exon 3 sequence is missing. More recently, Urbanek et al. [12] showed that the expression of the human GH receptor isoform is predicted by tissue-specific alternative splicing of exon 3, and that the exon 3-excluding GHR is the single isoform expressed in the placental villi. The deletion of exon 3 gives rise to a 224-amino-acid exoplasmic domain, containing an aspartic acid in place of the alanine 24 at the exon 2–4 junction. Although the genomic organization has been only characterized in man, the striking conservation of this protein through evolution supports the hypothesis that exon junctions map to the equivalent

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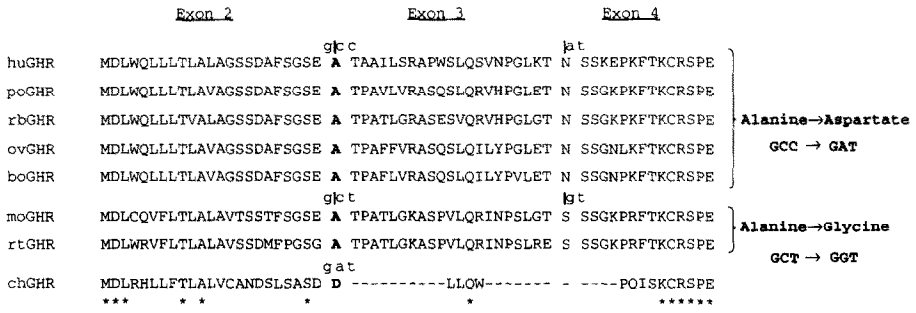

Fig. 1. Sequence alignment of the first 60 amino acids of the human (hu), porcine (po), rabbit (rb), ovine (ov), bovine (bo), mouse (mo), rat (rt) and chicken (ch) of the growth hormone receptor (GHR). This alignment was derived using the CLUSTAL program in CITI 2/BISANCE (Dessen, 1990). Exon junctions are based on the conservation of the human GH receptor sequence. At the exon junctions, nucleotide sequences are noted in small letters whereas amino acids are in bold. The change in amino acid resulting from exon 3 splicing is indicated on the right. (*), conservation of amino acid in all species.

locations in all species. The corresponding exon 3-excluding isoform in pigs [13], rabbits [7], cows [14] and sheep [15] apparently contain an aspartate at position 24, whereas an alanine is found at this position in the exon 3-containing isoform (Fig. 1). However, in rats [16] and mice [17], exon 3 splicing gives rise to a glycine in place of alanine. Interestingly, in chickens [18], in which the only cDNA cloned lacks exon 3, the residue present at the corresponding position is also an aspartate (Fig. 1). These observations raised the question as to whether this alternative inclusion/exclusion causes differences in hormone binding or signal transduction.

To elucidate the role of these isoforms, we studied the distribution of the two mRNAs in various tissues and analysed the abilities of the receptors, expressed in vitro, to bind hormones of the GH/CS family.

2. MATERIALS AND METHODS

2.1. *RNA preparation and cDNA synthesis*

Total cellular RNA was isolated from tissues, primary tissue culture cells and cell lines by means of guanidium thiocyanate phenol/chloroform extraction [19]. For PCR assays, 10 μg of total RNA was first reverse-transcribed into cDNA with hexanucleotides as primers (Pharmacia) and 40 U of AMV reverse transcriptase (Promega). One tenth of the reverse reaction product was used for amplification with primers PF (5′-CCCATGTCGACCTACAGGTATGGATCTCTGG-3′), located just upstream of exon 2, and PV (5′-CCACCATTGCTAGTTAGCTT-3′), located in the middle of exon 5, that provides a 420-bp fragment with exon 3 and a 354-bp fragment without exon 3. PCR was performed essentially according to the Perkin-Elmer/Cetus *Thermus aquaticus* polymerase protocol.

2.2. *Plasmid construction*

The full-length cDNA of the human growth hormone receptor has been previously cloned in the expression vector pECE [20]; the plasmid was named phGHRwt.

To obtain the exon 3-deleted form, recombinant PCR was performed, as described by Higuchi [21] on plasmid phGHRwt. As the *Sal*I and *Eco*RV cleavage sites are unique in the hGHR cDNA sequence and absent from the expression vector pECE, it was possible to remove the fragment with exon 3 from phGHRwt and to replace it with the amplified segment with exon 3 deleted. The plasmid was named phGHRd3.

2.3. *Transfection of expression vector into eukaryotic cells*

Sixty-percent-confluent COS-7 monkey kidney cells were transfected by the lipofectin method (Gibco/BRL, Life Technology, Grand Island, NY) with 2 μg of phGHRwt or phGHRd3 according to the manufacturer's instructions. After 48 h of expression the confluent monolayers were serum-privated for 12 h before being processed for binding studies.

2.4. *Binding assays*

COS-7 cells were scraped in 1 ml of phosphate-buffered saline and lysed in Eppendorf tubes by three freeze–thaw cycles. Total particulate membrane fraction was prepared by centrifugation for 30 min at 1,500 × *g*. The pellets were resuspended in 25 mM Tris-HCl, pH 7.4, 20 mM $MgCl_2$. One hundred micrograms of protein and 100,000 cpm of ^{125}I-labeled hGH (^{125}I-hGH) per tube were used for binding studies.

For Scatchard analyses, membranes were incubated with various amounts of unlabelled hGH. After incubation for 12 h at 4°C the mixtures were filtered through 0.22 μm GV membranes (Millipore), washed in 50 mM Tris, pH 7.4, 1 mg/ml bovine serum albumin. The amount of radioactivity was measured in a g counter. All determinations were done in triplicate. Human prolactin (hPRL) and human lactogen (hCS) (Sigma) were used as ligands for constructing competition curves. Two micrograms of hGH was labelled with 200 μCi of ^{125}I (1Ci = 37 GBq) (IMS 30, Amersham) as described elsewhere [22]. A specific activity of 30–60 μCi/μg was obtained.

3. RESULTS

3.1. *Expression pattern of the growth hormone receptor messenger RNA*

A wide variety of tissues were tested for the presence or absence of exon 3 in the human GH receptor mRNA. Total RNA was reverse transcribed and then amplified using primers PF and PV as described in Section 2. Fig. 2 shows the electrophoresis patterns of RT-PCR products. In each tissue, a semi-quantitative estimation of the relative proportion of both isoforms was made by inspecting the gels.

The three bands observed include a small fragment (354 bp) corresponding to the hGHRd3 species, a larger fragment (420 bp) corresponding to hGHR, and a slowly migrating segment. This latter band consists of heteroduplexes generated by the single-stranded reasso-

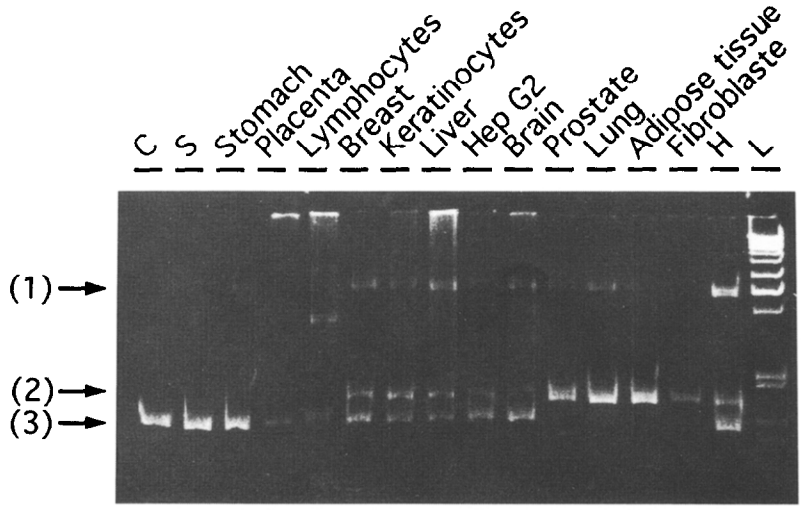

Fig. 2. Ethidium bromide stained gel of tissue distribution of hGHR and hGHRd3. RT/PCR analysis of the exon 3 splicing pattern of hGHR transcripts in primary cultures of human cytotrophoblast cells (C), primary culture of human syncitiotrophoblast cells (S), human stomach (stomach), total human placenta (placenta), B lymphocytes transformed with Epstein Barr virus (lymphocytes), human breast (breast), primary cultures of human keratinocytes (keratinocytes), adult human liver (liver), human hepatoma-derived cell line (HepG2), human brain (brain), human prostate (prostate), human lung (lung), human adipose tissue (adipose tissue), primary cultures of human fibroblasts (fibroblasts), (H) heteroduplex form amplified with PF and PV primers, (L) one-Kb ladder from Gibco/BRL Life Technology. (1)-heteroduplex formed with the two different single strands, (2)-420bp which corresponds to hGHR, (3)- 354bp which corresponds to hGHRd3.

ciation of the 354 and 420 bp fragments; indeed, when this DNA was removed from the gel and amplified with the same primers, the three species were generated in same proportions (Fig. 2, lane H). The use of primers able to generate heteroduplexes enhances the sensitivity of detection of both isoforms: the presence of heteroduplexes on a gel indicates the existence of both isoforms in the sample studied, even if one of the homoduplexes is not visible.

Five electrophoretic patterns were observed. (i) Only one isoform without exon 3 was expressed in the syncitiotrophoblast and cytotrophoblast. (ii) Both isoforms were found in the stomach, but the deleted isoform was predominant; the presence of the other undetected isoform is revealed by the heteroduplex. (iii) The two messenger RNAs showed the same level of expression in total placenta, EBV-transformed lymphocytes, breast, keratinocytes, liver, the HepG2 cell line and the brain. (iv) The isoform containing exon 3 was mainly expressed in the prostate and lung. (v) In adipose tissue and cultured fibroblasts, only the hGHR isoform was expressed.

3.2. *Determination of the relative association constant and binding specificity of hGHRd3 and hGHR*

GHR-cDNA lacking exon 3 was cloned as described in Section 2. The two constructs, phGHRwt and phGHRd3, were then transiently expressed in COS-7 cells. Binding studies were performed on cell membranes using ^{125}I-hGH. The association constant of hGHR and hGHRd3 were calculated from the Scatchard plot as 2.0 nM^{-1} and 2.8 nM^{-1}, respectively (Fig. 3).

Human prolactin (hPRL) and human lactogen (hCS) were assayed for their ability to displace hGH bound to the two receptor isoforms. The results confirmed that hPRL did not bind to hGHR. This hormone was also unable to displace hGH bound to hGHRd3. In contrast, hCS showed some affinity for hGHR, and similar results were obtained with cells transfected with phGHRd3 (Fig. 3).

4. DISCUSSION

Exons 2 to 7 of the human GH receptor (hGHR) gene have been shown to encode the exoplasmic domain of the protein. However, Godowski et al. [11], who studied hGHR cDNA clones derived from a human liver cDNA library, found that alternative splicing at the 5′ end of the hGHR transcripts could generate two receptors isoforms, one containing (hGHR) and the other excluding exon 3 (hGHRd3). This was recently confirmed by Urbanek et al. [12], who characterized the hGHRd3 species as the unique GH receptor isoform expressed in the placental villi. Given that the biological activities of GH depend on its target cells, an important question is whether or not these isoforms have the same metabolic functions. We have previously shown that the GHR gene is genetically linked to a hereditary GH-insensitivity condition (Laron syndrome, reviewed in [23]), thereby demonstrating that the receptor is indeed involved in growth [24]. However, since all the mutations discovered so far in affected families have been located outside exon 3 and within exons that are common to hGHR and hGHRd3 [11,20,25,26], they would result in sequence abnormalities in both isoforms, making it im-

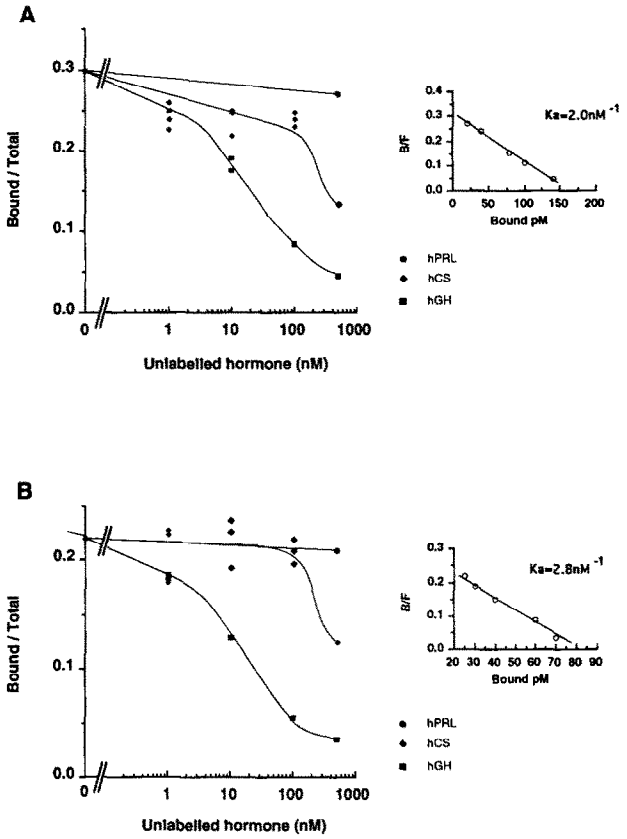

Fig. 3. Expression of hGHR and hGHRd3 cDNA clones in COS-7 cells. Binding of ^{125}-I-hGH to membranes from COS-7 cells transfected with phGHRwt (A) or phGHRd3 (B). Plasma membranes (100 μg) were incubated with ^{125}-I-hGH (100,000 cpm) and various concentrations of native hGH (n) and hCS (u). hPRL (l) was added in excess (2 μg). (*Inset*) Scatchard plot of the competition assay with unlabeled hGH. B/F, bound to free ratio.

possible to correlate one isoform with a particular function. In this study, to elucidate the respective roles of hGHR and hGHRd3, we first looked at their tissue distribution. One or both isoforms were detected, depending on the tissue analysed. The expression pattern was similar to that reported by Urbanek et al. [12], with some exceptions. In particular, we confirmed that total placenta contains both isoforms, whereas fetal placenta cells (syncitiotrophoblast and cytotrophoblast cells) express the hGHRd3 form only. However, in contrast to Urbanek et al. [12], who found only the hGHR form in liver, we clearly detected both isoforms in this tissue. No correlation was found between the expression of one or other isoform and the known metabolic functions of GH in the tissues analysed. For instance, GH does not promote cell proliferation in the brain [27], in which the two isoforms are equivalently expressed, but it does so in the liver [27] in which both isoforms are also expressed in the same ratio. In addition, GH promotes cell division in stomach where hGHRd3 is mainly expressed or in adipose tissue, in which only hGHR is expressed. Similarly, we found no correlation between the expression pattern and the embryonal origin of the different tissues.

However, studies aiming at deciphering the role of the hGHRd3 isoform have been based on mRNA analysis. Indeed, hGHRd3 protein has never been characterized, raising the question as to whether hGHRd3 mRNA is translated and the corresponding protein transported to the plasma membrane. The exon 4 spliced form of IL-2 receptor does not result in the expression of the receptors, even though specific mRNA is detected in transfected cells [28]. To address this question in the GHR system, we obtained transient expression of hGHRd3 in COS-7 cells. hGHRd3 was found to bind GH with a high affinity and to have a K_a value similar to that achieved in cells transfected with phGHRwt. These results are similar to those obtained in a prokaryotic expression system by Bass et al. [29]]. We therefore conclude that post-translational modifications of the GHR isoforms are not critical in terms of binding activity. This binding result is also consistent with data obtained by examination of crystal structure of the complex between GH and the exoplasmic domain of its receptor [30]. This later study confirmed the results of Cuningham et al. [31] who predicted that dimerization of GH would occur around one molecule of GH, and showed that the peptide encoded by exon 3 was located away from binding interfaces.

Given the similar binding properties of hGHR and hGHRd3, we speculate that different metabolic pathways are activated depending on the inclusion or exclusion of exon 3. It is striking that exclusion of exon 3 should result in the loss of four phosphorylation sites and one glycosylation site and in the replacement of alanine 24 by an aspartate at the end of exon 2. This latter modification involves a highly conserved amino acid and leads to a change in charge, size and hydrophobicity (Fig. 1). These modifications could possibly interfere with the ability of hGHRd3 to interact with a specific protein involved in signal transduction. Thus, although hGHR and hGHRd3 exhibit similar binding properties, hGHRd3 may transduce a specific signal. This hypothesis is strengthened by studies on signal transduction pathways in this superfamily of receptors which clearly suggest the involvement of multiple protein in receptor specifity and function [32].

No receptor for hCS has yet been identified. However, given the lactogenic activity of both this hormone and prolactin, this latter hormone acting through a specific and homologous receptor which lacks exon 3 [33], we tested CS and PRL binding to hGHRd3. Interestingly, hCS displaced hGH from both hGHRd3 and hGHR to a similar degree; however, PRL did not bind to either receptor. For the two types of receptor a high concentration of hCS (about 0.2 μM) is necessary to displace bound hGH; this result may be related to the very high level of hCS mRNA and protein observed in

total placenta at term of pregnancy (10–20% of the total placenta mRNA) [34].

Finally, since hGHRd3 is the only isoform expressed in fetal placenta cells and hGH-V is also produced exclusively by the placenta, it would be of interest to analyse the binding of hGH-V to hGHR and hGHRd3. Our understanding of these GHR isoforms should improve with the availability of appropriate functional assays to investigate signal transduction.

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