A Short Isoform of the Human Growth Hormone Receptor Functions as a Dominant Negative Inhibitor of the Full-Length Receptor and Generates Large Amounts of Binding Protein

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The GH receptor (GHR) is a member of the cytokine receptor family. Short isoforms resulting from alternative splicing have been reported for a number of proteins in this family. RT-PCR experiments, in human liver and cultured IM-9 cells, using primers in exon 7 and 10 of the GHR, revealed three bands reflecting alternative splicing of GHR mRNA: the predicted product at 453 bp and two other products at 427 and 383 bp. The 427-bp product (GHR1-279) utilized an alternative 3′-acceptor splice site 26 bp downstream in exon 9; the predicted C-terminal residues are six frameshifted exon 9 codons ending in an inframe stop codon. The 383-bp product (GHR1-277) resulted from skipping of exon 9; the predicted C-terminal residues are three frameshifted exon 10 codons ending in an in-frame stop codon. RNase protection experiments confirmed the presence of the GHR1-279 variant in IM-9 cells and human liver. The proportion of alternative splice to full length was 1–10% for GHR1-279 and less than 1% for GHR1-277. The function of GHR1-279 was examined after subcloning in an expression vector and transient transfection in 293 cells. Scatchard analysis of competition curves for [^{125}I]-hGH bound to cells transfected either with GHR full length (GHRfl) or GHR1-279 revealed a 2-fold reduced affinity and 6-fold increased number of binding sites for GHR1-279. The increased expression of GHR1-279 was confirmed by cross-linking studies. The media of cells transfected with GHR1-279 contained 20-fold more GH-binding protein (GHBP) than that found in the media of cells transfected with the full-length receptor. Immunoprecipitation and Western blotting experiments, using a combination of antibodies directed against extracellular and intracellular GHR epitopes, demonstrated that GHRfl and GHR1-279 can form heterodimers and that the two forms also generate a 60-kDa GHBP similar in size to the GHBP in human serum. Functional tests using a reporter gene, containing Stat5-binding elements, confirmed that while the variant form was inactive by itself, it could inhibit the function of the full-length receptor. We have demonstrated the presence of a splice variant of the GHR in human liver encoding a short form of the receptor similar in size to a protein previously identified in human liver and choroid plexus. Expression studies in 293 cells support the hypothesis that while the expression of the splice variant accounts for only a small proportion of the total GHR transcript, it produces a short isoform that modulates the function of the full-length receptor, inhibits signaling, and generates large amounts of GHBP. The differential expression of GHR receptor short forms may regulate the production of GHBP, and truncated receptors may act as transport proteins or negative regulators of GHR signaling. (Molecular Endocrinology 11: 265–273, 1997)

0888-8809/97/$03.00/0
Molecular Endocrinology

INTRODUCTION

The GH receptor (GHR) is a member of the cytokine family of receptors that possess a single transmembrane domain, are devoid of intrinsic enzyme activity, and associate with cytoplasmic tyrosine kinases to form multisubunit receptor complexes. Binding of a single molecule of GH results in receptor dimerization and activation. A short isoform of the receptor identical to the extracellular domain of the receptor circulates as a binding protein. In rodents there is alternative splicing of mRNA just proximal to the transmembrane domain with the full-length message (4.5 kb) coding for the GHR and a truncated message (1.2 kb) coding for the GH-binding protein (GHBP). In the rat the sequence for the GHBP is identical to that of the GHR up to three amino acids before the putative transmembrane domain where an additional 17 amino acids are encoded before a stop codon is encountered (1). In contrast, in the human no alternative splice is seen on Northern analysis, and it has been proposed that the GHBP is derived by proteolytic cleavage of the extracellular domain of the GHR. Support for this concept comes from transfection studies with the GHR (2, 3) and studies of GHBP in the media from cultured IM-9 cells (4).

The human GHR and GHBP are products of a single gene (5). Exon 2 codes for the signal peptide, the extracellular domain is coded by exons 3 to 7, the transmembrane domain is coded by exon 8, while exons 9 and 10 encode the cytoplasmic domain and the 3′-untranslated region (6). Different isoforms for various members of the cytokine receptor superfamily have been reported. For the GHR an exon 3 skipped isoform (exon 3-) is expressed in placenta, liver, and various cultured cells (7). For the rat PRL receptor, short isoforms with a limited or absent cytoplasmic domain have been identified (8, 9). For PRL the short and long isoforms are expressed in a tissue-specific manner and regulated by estrus (10). Five different human granulocyte-colony stimulating factor (G-CSF) isoforms, arising from alternative splicing, have been isolated and are identical in the extracellular domain but differ in their downstream sequences (11). Three different isoforms of the α-subunit of the granulocyte macrophage (GM)-CSF receptor have been reported, one of which encodes a soluble receptor (12).

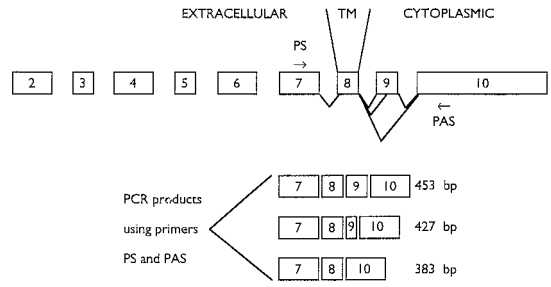
We postulated that there is alternative splicing of the human GHR around the transmembrane domain but that this may not have been identified on Northern blotting if all transcripts were of approximately the same size or if transcripts were of low abundance. To test this hypothesis we designed primers in exons 7 and 10 and, using a RT-PCR-based technique, tested for alternative splicing in mRNA from human liver and cultured cells. In this paper, we show that two variants could be identified, and functional studies indicate that, in spite of their low abundance, they could have a physiological role generating large amounts of GHBP and interacting with the full-length receptor.

RESULTS

Identification of Splice Variants in Human Liver and IM-9 Cells

Using primers PS and PAS in exons 7 and 10 of the human GHR (Fig. 1) RT-PCR of human liver revealed three products at 453, 427, and 383 bp (Fig. 2). The individual bands were extracted from polyacrylamide, cloned into pCRII, and sequenced. The 453-bp product had an identical sequence to that previously published for the GHR (GHRfl) (5). The 427-bp (GHR1-279) product utilized an alternative 3′-acceptor splice site in exon 9. Exon 8 was as expected but spliced to a 3′-splice acceptor site within exon 9, thus omitting the upstream 26 bp of exon 9. The nucleotide sequence at

a)



b)

Nucleotide Sequence

	902 Exon 8	/ Exon 9		
GHRFL	TTTTCTAAA	CAGCAAAG/GA	TTAAAATGCT	GATTCTGCCC
GHR1-279	TTTTCTAAA	CAGCAAAG/. .	. . . . .	. . . . .
GHR1-277	TTTTCTAAA	CAGCAAAG/. .	. . . . .	. . . . .
	941			
GHRFL	CCAGTTCCAG	TTCCAAAGAT	TAAAGGAATC	GATCCAGATC
GHR1-279	. . . . TTCCAG	TTCCAAAGAT	ta a	
GHR1-277	. . . . .	. . . . .	. . . . .	. . . . .
	981	/ EXON 10		
GHRFL	TCCTCAAG/GA	AGGAAAATTA	GAGGAGGTGA	ACACAATCTT
GHR1-279				
GHR1-277	. . . . . /GA	AGGAAAAT t a	g	

Translated sequence

	TM	Cytoplasmic domain
GHRFL	F S	KQQRIKMLILPPVPVPKI
GHR1-279	F S	KQQ'ssssksd
GHR1-277	F S	KQQRken

Fig. 1. Schematic Representation of the GHR Gene and Alternative Splice Products

a, Human GHR gene alternative splicing and position of the primers used for PCR. Exons are shown as *boxes*, primers as *arrows*, and TM indicates transmembrane domain. b, Nucleotide and translated sequence of the full-length GHR and alternative splices GHR1-279 and GHR1-277. Exon boundaries are marked by *slashes*, deleted sections by *periods*, in-frame stop codons *underlined*, and the end of the putative transmembrane domain by |. Nucleotide and amino acid residue homologies are in *uppercase*.

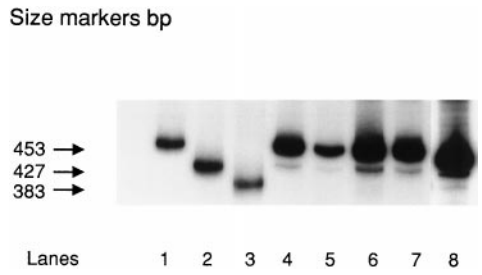


Fig. 2. Detection of GHR Isoforms in Human Livers

RT-PCR products from five different livers using primers PS and PAS were analyzed by Southern blot with labeled gel-purified products from pGHR453. Lanes 1–3 are markers at 453, 427, and 383 bp generated in a PCR reaction with clones pGHR453, pGHR1-279, and pGHR1-277 as templates. Five liver samples (lanes 4–8) demonstrated products at 453 and 427 bp. Overexposure (lane 8) revealed three bands at 453, 427, and 383 bp although the 427 is partly obscured.

this position in exon 9 matches the mammalian consensus for a 3′-acceptor CAG/TT at position 944 in the Genbank sequence (X06562). The 383-bp (GHR1-277) product skipped exon 9 with exon 8 splicing to exon 10. The predicted peptides have been labeled by their amino acid number according to Leung *et al*. (5).

The predicted C-terminal residues of the GHR1-279 peptide are six frame-shifted exon 9 codons ending in an in-frame stop codon and for the GHR1-277 peptide are three frame-shifted exon 10 codons ending in an in-frame stop codon (Fig. 1). The translated sequence is the same as the full-length receptor for the first three to four amino acids of the cytoplasmic domain of GHR1-279 and GHR1-277, respectively, but the predicted peptides deviate from each other before the proline-rich box I region of the receptor that is required for signal transduction (13, 14).

The alternative splice variants of the GHR were also identified in cultured IM-9 cells. Since all three splice variants compete for the same primers and PCR reagents in a common reaction, the relative optical densities of the three bands were assumed to reflect the proportion of the variants in the template cDNA. The 453 bp and 427 bp products are clearly seen in Fig. 3a. The 383 product was seen only on overexposed autoradiographs (data not shown). Quantification on appropriately exposed autoradiographs by an image densitometer revealed that the proportions of the alternative splice products were similar in all experiments. Thus, GHR1-279 was 1–10% of GHRfl, and GHR1-277 was consistently less than 1%. RNase protection (Fig. 3b) confirmed the presence of the GHR1-279 alternative splice product in human liver and IM-9 cells in similar proportions to the full-length receptor as seen by RT-PCR. The GHR1-277 splice variant was not identified by RNase protection.

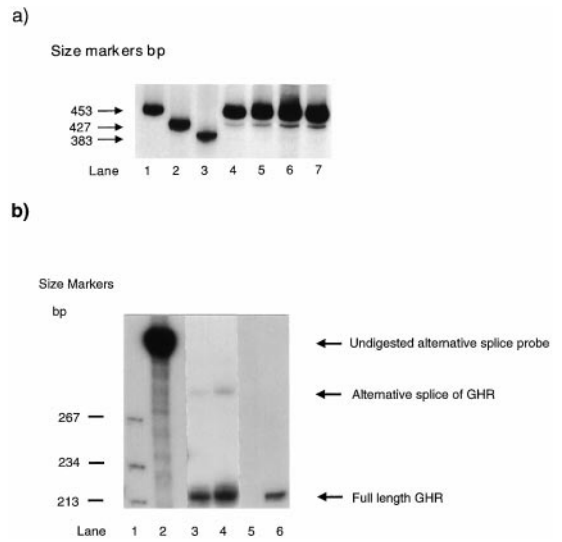


Fig. 3. Alternative Splicing for GHR in IM-9 Cells

a, GHR gene expression detected in four separate cultures of IM-9 cells (lanes 4–7) by RT-PCR and Southern blotting. Lanes 1–3 are markers at 453, 427, and 383 bp. b, RNase protection for the GHR using a GHR1-279 probe. Lane 1 markers: 2, undigested probe; 3, IM-9 cells; 4, human liver; 5, untransfected HepG2 cells; 6, HepG2 cells transfected with GHRfl. The alternative splice for GHR1-279 is seen at 296 bp in human liver (lane 4) and IM-9 cells (lane 3). The full-length receptor is detected at 217 bp in IM-9 cells, human liver, and transfected HepG2 cells (lane 6).

Expression of GHR1-279 in 293 Cells

293 cells were transiently transfected with GHRfl or GHR1-279 cDNAs. Expression of the two receptor forms was analyzed in binding experiments of [^{125}I]-hGH to intact cells. Scatchard analysis of the competition experiments indicated a 2-fold reduced affinity for GHR1-279 compared to GHRfl; association constant (K_a) for GHRfl = 1.2×10^9 M^{-1} and GHR1-279 = 0.6×10^9 M^{-1}. The level of expression was high, as expected in 293 cells. The calculated number of binding sites per cell was about 1.2×10^5 when 1 μg GHRfl cDNA was transfected into 3×10^6 cells, and 7×10^5 sites per cell for 1 μg GHR1-279 cDNA transfected.

Cross-linking studies with [^{125}I]-hGH on cells transiently transfected with GHRfl or GHR1-279 cDNAs are shown in Fig. 4. In both cases specific complexes, displaced in the presence of an excess of native hormone, were observed. In cells transfected with GHRfl, the apparent sizes of the radioactive bands ($\sim$140 and $\sim$260 kDa) were those expected for complexes of one or two molecules of receptor and one hormone molecule, respectively. In cells transfected with GHR1-279 cDNA, the amount of radioactivity in both complexes is much higher than that observed with the full-length receptor. This is consistent with the data from binding experiments indicating that the number of receptors in the cell membrane is greater after transfection with the GHR1-279 cDNA. The apparent

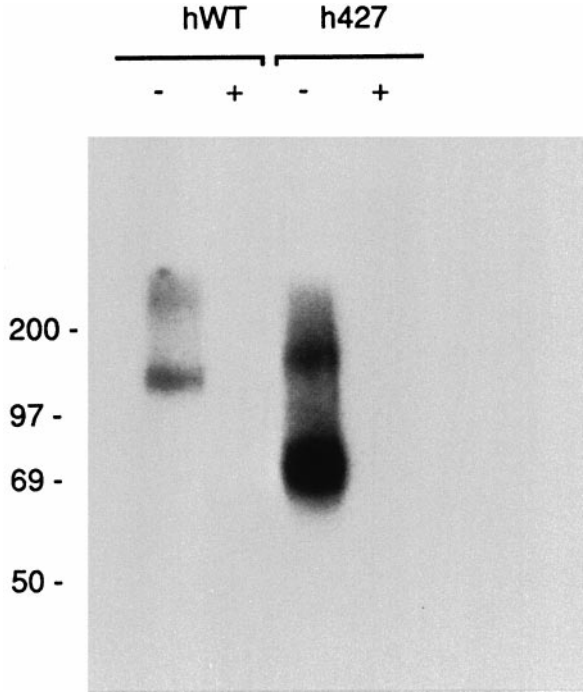


Fig. 4. [^{125}I]-hGH Cross-Linking to the GHRfl and GHR1-279 Receptor Forms in Transfected 293 Cells

The cross-linked receptors were analyzed by SDS gel electrophoresis as described in *Materials and Methods*. Cross-linking was performed without (−) or with (+) 3 μg unlabeled hGH. *Numbers* indicate the size of protein standards in kilodaltons.

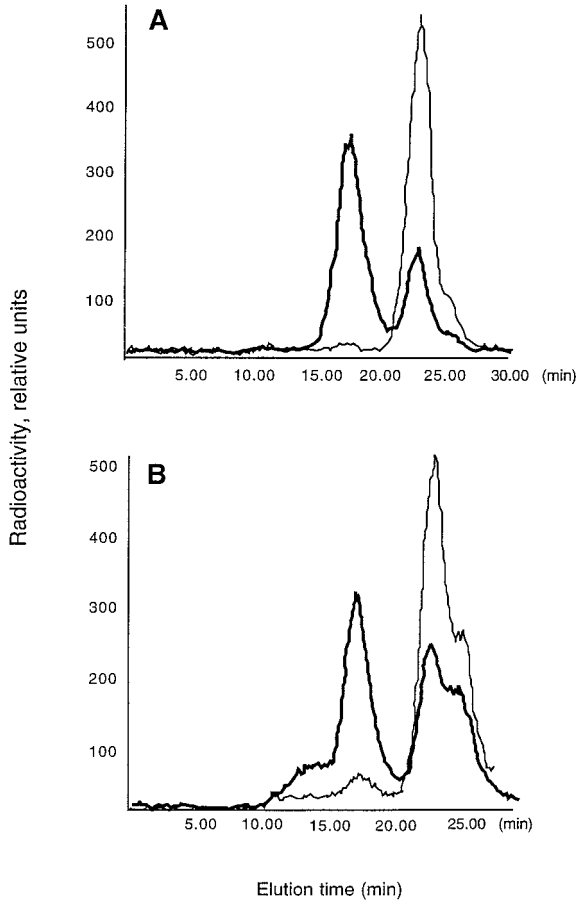


Fig. 5. Elution Profiles of [^{125}I]-hGH Incubated with Medium from Transfected 293 Cells

Cells were transfected with 5 μg human GHRfl or GHR1-279. Medium was recovered and analyzed as described in *Materials and Methods*. Incubation was performed in the absence (total binding, *solid line*) and presence (nonspecific binding, *thin line*) of 2 μg native hGH; 100 μl of media were analyzed for GHRfl (A) and 5 μl GHR1-279 (B).

sizes of the two complexes are approximately 75 kDa and 150 kDa and correspond to the sizes expected for one or two molecules of GHR1-279 bound to one molecule of hormone.

Soluble forms of the receptor generated by GHRfl or GHR1-279 in the media of transfected cells were studied by HPLC gel filtration. The elution profiles of [^{125}I]-hGH incubated with concentrated media (20-fold) of cells transfected with GHRfl and GHR1-279 revealed two peaks (Fig. 5). The first peak was completely displaced by an excess of native hGH, and its elution time corresponded to that previously described for GHBP in human serum (15) or media of human hepatoma cells (16). The second peak corresponds to the free [^{125}I]-hGH. Sixty percent of radioactivity was in the first peak when 100 μl of culture media from cells transfected with GHRfl were analyzed. A similar binding activity, corresponding to GHBP, was found when only 5 μl GHR1-279 media were used, demonstrating a 20-fold increase of GHBP in media from cells transfected with GHR1-279.

Immunoprecipitation and Western blot analysis were used to assess the sizes of the membrane-bound and soluble forms of GHR in cells transfected with GHRfl and GHR1-279 (Fig. 6). The expression of GHRfl was detected by immunoprecipitation using a polyclonal antibody (αGHR-intra) raised against an epitope in the intracellular domain of human GHR (not present in GHR1-279) (2). To purify the receptor in cells transfected with GHR1-279 alone, we incubated the cells with biotinylated hGH and purified the complexes with streptavidin beads after lysis. The soluble forms in media were also purified with biotinylated hGH and streptavidin beads. In order to detect a possible heterodimerization between GHRfl and GHR1-279, cells were cotransfected with 5 μg of each cDNA and stimulated with hGH (in order to induce dimerization) before lysis. The complexes formed were then immunoprecipitated with the αGHR-intra antibody. Western blots were performed with mAb263, which recognizes all membrane and soluble forms of the GHR. The results are shown in Fig. 6. GHRfl appeared as a single band around 120 kDa (lane 1). GHR1-279 consisted in two bands; the size of the major band was around 55-60 kDa; the minor band, around 46 kDa, was likely a degradation product (lane 3). Soluble receptors in the supernatant of cells transfected with either GHRfl

(lane 5) or GHR1-279 (lane 6) were single bands of apparently 60 kDa, similar in size to the upper band identified in normal serum (lane 7). In cells cotransfected with GHRfl and GHR1-279, three bands were detected with the mAb263 (lane 2). The upper band migrated as the GHRfl (lane 1), and the two other bands migrated as GHR1-279 (lane 3). No band could be detected when lysates of cells transfected with GHR1-279 alone were immunoprecipitated with the αGHR-intra antibody (lane 4). These results demonstrate that GHRfl and GHR1-279 can heterodimerize on GH stimulation.

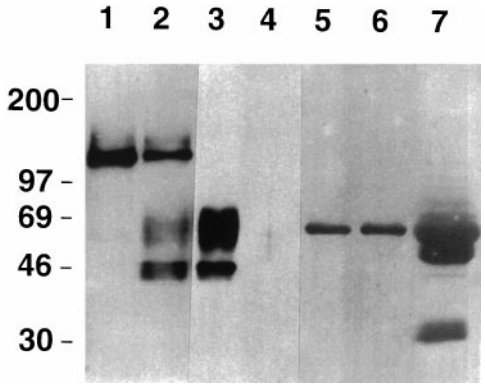


Fig. 6. Western Blot Analysis of Membrane-Bound and Soluble Forms of Receptors Expressed in 293 Cells

Cells were transfected with GHRfl or GHR1-279 or cotransfected with both cDNAs. Forty eight hours after transfection, cells were incubated with hGH or biotinylated hGH. Medium from cells transfected with GHRfl or GHR1-279 and human serum were also incubated with biotinylated hGH. Cell lysates were immunoprecipitated with αGHR-intra or streptavidin-agarose. Soluble receptors in medium and serum were purified with streptavidin-agarose. Purified proteins were separated on a 10% SDS/PAGE and analyzed by Western blot with mAb263. Lane 1, Cells transfected with GHRfl and immunoprecipitated with αGHR-intra; lane 2, cells cotransfected with GHRfl and GHR1-279 and immunoprecipitated with αGHR-intra; lane 3, cells transfected with GHR1-279 purified with biotinylated hGH and streptavidine agarose; lane 4, cells transfected with GHR1-279 immunoprecipitation with αGHR-intra; lane 5, medium of cells transfected with GHRfl; lane 6, medium of cells transfected with GHR1-279; lane 7, human serum.

Functional Studies of GHR1-279

The function of the wild type and variant form of the GHR was examined in 293 cells transfected with a reporter gene containing a Stat5-binding element (LHRE) fused to a minimal TK promoter and luciferase. Such a test allows analysis of GH-dependent Stat5 transactivation (17). As box 1 is not present in the short cytoplasmic domain of the variant receptor, it would not be expected to activate Stat5, but we could speculate that when coexpressed with wild type receptor it could competitively inhibit receptor signaling. To test this hypothesis, functional tests were performed. Cells were transfected with 0.1 μg GHRfl cDNA per six-well plates (to give $\sim 2 \times 10^3$ receptor sites per cell) and increasing amounts of GHR1-279 cDNA. In conditions providing maximal stimulation of the reporter gene (20 nM hGH), we observed a 12-fold stimulation of luciferase activity in cells transfected with GHRfl alone. No stimulation of luciferase was observed, as expected in cells transfected with GHR1-279 alone. When increasing amounts of GHR1-279 cDNA (from 0.01 to 1.0 μg) were cotransfected with 0.1 μg of GHRfl cDNA, a dose-dependent inhibition was observed (Fig. 7). For a ratio similar to that observed *in vivo* (GHR1-279 *vs*. GHRfl = 10%), the inhibition of luciferase activity was 5% when 20 nM hGH was used in the functional test but 30% when a more physiological level, 1 nM hGH, was used. This physiological hormone concentration induced a 3-fold stimulation of the reporter gene in cells transfected with GHRfl. As the degree of inhibition appears dependent on the concentration of hGH in the medium, this suggests that the inhibition of GHRfl signaling by the short form could be also due to a competition between GHRfl and GHR1-279 for binding of GH.

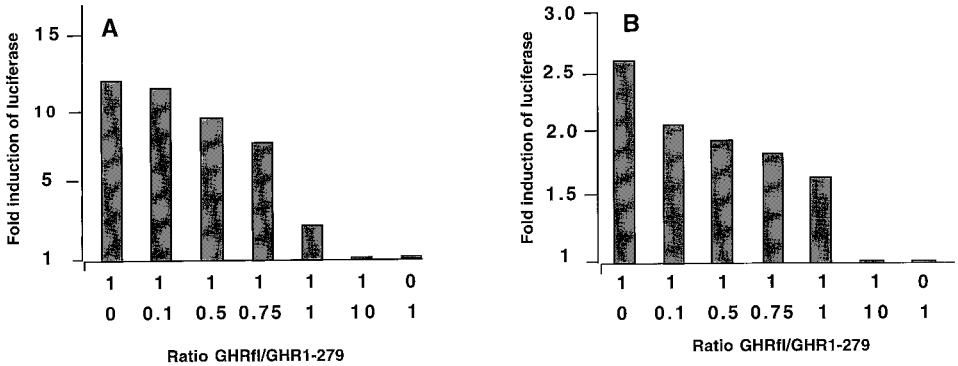


Fig. 7. Inhibition of STAT5-Dependent Transcriptional Activity Mediated by GHR in the Presence of the Short Form GHR1-279

293 cells were transiently cotransfected with plasmids containing cDNAs encoding GHRfl and increasing amounts of the GHR1-279 cDNA together with the reporter gene LHRE/TK/luciferase. After transfection the cells were incubated in the presence or the absence of 20 nM (A) or 1 nM (B) hGH for 16 h. One representative of several experiments is shown: the fold induction represents the ratio of luciferase activity in the presence and in absence of hGH.

DISCUSSION

The studies presented here demonstrate alternative splicing in the cytoplasmic domain of the human GHR. We have sequenced two clones derived from human liver that code for short isoforms of the GHR with partial or complete skipping of exon 9. Based on RT-PCR and RNase protection experiments, these alternative splices represent a small proportion of the total GHR transcript. We detected transcripts with an alternative 3′-acceptor site in exon 9 (GHR1-279) and transcripts with complete deletion of exon 9 (GHR1-277) in human liver and IM-9 cells. The GHR1-279 represented 1–10% and the GHR1-277 less than 1% of the total transcript. GHR1-279 and GHR1-277 encode short isoforms of the GHR with very short cytoplasmic domains of nine and seven residues, respectively, which differ from the full-length receptor three to four residues after the transmembrane domain and before the proline-rich box region that is required for signal transduction (13, 14).

Following transfection of the short form, GHR1-279, in mammalian cells we identified a ~75-kDa protein in cell membranes by cross-linking with GH. A similar size protein had been previously identified in human liver (18). At that time, two molecular forms of the hormone-receptor complex were observed in human liver with estimated sizes of 124 and 75 kDa (18). It was not clear whether the smaller form was a degradation product of the expected 124-kDa receptor complex. Taking into account our current results it is possible it could be generated from the splice variant that we detected in human liver. In human choroid plexus, cross-linking studies identified only one hormone-bound complex again at ~75 kDa (19). In the rat there is evidence for a short form of the receptor bound in the membrane of adipocytes (20), and it has been speculated that similar forms will be found in the human (21).

This paper investigated the functional significance of the most abundant alternative splice variant reported, GHR1-279. This short isoform of the receptor, which retains the transmembrane domain but is divergent in the cytoplasmic domain, was subcloned into an expression vector and transfected into 293 cells. Binding assays with entire cells indicated that in spite of its short cytoplasmic domain, GHR1-279 is held in the cell membrane. However, the GHR1-279 had a reduced affinity and increased binding capacity compared to the full-length receptor. The binding affinity (K_a) for GHRfl was 1.2×10^9 M^{-1} similar to that previously published for the human liver GHR (18). GHR1-279 had a 2-fold lower affinity of 0.6×10^9 M^{-1} similar to the GHBP in human serum (22), which has a 5-fold lower affinity compared to the human liver GHR (23). A possible explanation for these results is that the length of the cytoplasmic domain could affect the general structure of the receptor and its ligand affinity. The differences in the binding capacity for expressed GHR1-279 *vs*. GHRfl is consistent with that previously observed for truncated GHRs. In CHO clones stably expressing a truncated form of the rabbit GHR (which retains only 46 amino acids in its cytoplasmic domain) the number of binding sites for this mutant was 8 times higher than that for CHO clones stably expressing GHRfl (14). We have observed that the increase in binding sites correlates with an impaired internalization of the receptor (L. S. Moutoussamy *et al.*, manuscript in preparation). A similar finding has been reported with short isoforms of the rat GHR (24). Critical residues for internalization of the receptor have been mapped for the rat GHR (24), which are located in a cytoplasmic domain that is absent in the truncated rabbit GHR or in GHR1-279. Recently the involvement of the ubiquitin system has been demonstrated in GHR internalization and the putative amino acid sequence for ubiquitination present in the GHR sequence is absent in GHR1-279 as well as in the short forms of rat and rabbit receptor mentioned above (25). However, when coexpressed in cells with the GHRfl, a proportion of GHR1-279 could be internalized through heterodimerization with GHRfl. The extent of this phenomenon remains to be established.

GHBP in the media of cells was studied by HPLC gel filtration and Western blotting after affinity purification. By HPLC, a GHBP with similar characteristics to human serum GHBP was identified in the media of cells transfected with GHR1-279. There was an increased amount of GHBP in the medium of cells transfected with GHR1-279 as compared with that measured in the media of cells transfected with GHRfl, when similar amounts of cDNA were transfected. This could be related to increased levels of the short isoform at the cell surface and/or reduced internalization allowing more receptor to be available for proteolysis. On Western blotting human serum revealed two bands of the expected sizes for the GHBP (60 and 55 kDa) (22, 23, 26). Media from cells transfected with GHRfl and GHR1-279 demonstrated a protein of 60 kDa similar to the predominant protein in human serum. We can postulate that, in spite of its low level of mRNA expression, the spliced variant could generate a proportion of the circulating GHBP.

Immunoprecipitation experiments demonstrated that heterodimers could be formed between GHRfl and GHR1-279. Western blot analysis of the complexes immunoprecipitated with a cytoplasmic domain antibody, αGHR-intra, and probed with an extracellular domain antibody, mAb263, indicated that GHR1-279 could only be immunoprecipitated when complexed with GHRfl. The identity of GHR1-279 in the heterodimeric complex was assessed by its comigration with the very large band detected when GHR1-279 was precipitated with streptavidin after binding to biotinylated hGH. The finding of heterodimerization suggests that GHR1-279 may act in a dominant negative fashion to inhibit receptor signaling in addition to competitively binding GH.

GHR signaling involves GH-dependent receptor dimerization, activation of the tyrosine kinase JAK2, and the subsequent recruitment and tyrosine phosphorylation of various Stat proteins including Stat1, Stat3, and Stat5 (27). JAK2 activation is dependent on its association with the receptor that is mediated by the juxtamembranous region of the cytoplasmic domain, including the proline-rich region box1 (14, 28). Functional tests using a reporter gene containing the Stat5-binding element were performed to test the possibility that GHR1-279 could act as a negative regulator of the full-length receptor. We observed such an effect even when the ratio of the cDNAs transfected was 1:10 for GHR1-279 to GHRfl. As the degree of inhibition was dependent on the concentration of hGH, it suggests that inhibition was also related to a decreased availability of the ligand for the active GHRfl dimer when the short form receptor is expressed. Preliminary data have been reported suggesting a dominant negative effect for other GHR mutants (29), and a dominant negative effect was seen with the PRL receptor when similar proportions of cDNA encoding a short form were transfected (30). In addition, there is a report of a patient heterozygous for a GHR mutation resulting in the expression of a protein identical to GHR1-277 (31). This patient presented with short stature, and the authors suggest the short form of the receptor may act in a dominant negative fashion. Our data support this hypothesis. Studies with the G-CSF receptor have shown that expression of the various isoforms was tissue specific; aberrations in the expression of these various isoforms have been reported and postulated to play a role in the pathogenesis of disorders of granulopoiesis (11). Thus, it is possible that differential expression of GHR isoforms could play a role in the physiological regulation of receptor function in some genetic or acquired GH disorders.

The truncated receptor GHR1-279 has recently been identified in rabbit as well as in human tissues (32). Using PCR analysis there was differential expression of the truncated receptor with low abundance of the alternative splice in liver, kidney, and fibroblasts but similar expression to the full-length receptor in mammary gland and adipose tissue. The differential tissue expression of alternative splicing may regulate the production of GHBP. In addition, the truncated receptor may act to inhibit GH signaling in some tissues or act as a transport protein, as has been suggested for the truncated form of leptin receptor expressed in the choroid plexus (33).

Our results indicate that while only a single mRNA species is detected by Northern blotting, alternative forms of mRNA for the GHR are transcribed. These splice variants encode short forms of the receptor that could play a role in the generation of a GHBP in the human and can modulate the function of the full-length receptor.

MATERIALS AND METHODS

Tissues

All samples were human tissues removed at operations, frozen immediately in liquid nitrogen, and stored at −80 C. Local Ethical Committee approval and informed consent or assent from patients or relatives were obtained. Normal liver samples (n = 5 subjects) were from brain-dead liver transplant donors or patients undergoing resection of a single lesion, metastasis, or hydatid cyst.

RNA Extraction and RT-PCR

This was performed as described (34). Total RNA was extracted using an acid phenol/guanidinium isothiocyanate method (RNAzol B, Biogenesis, Poole, U.K.). RNA quantification was performed spectrophotometrically and quality assessed by agarose-formaldehyde gel electrophoresis. Complementary DNA was made using 5 μg total RNA with 200 U MMLV reverse transcriptase (BRL, Gaithersburg, MD), 5 μg random hexamer primers (Boehringer Mannheim, Indianapolis, IN), and 200 μM final concentration of deoxynucleosidetriphosphate in a 50-μl reaction. The reaction was performed at 24 C for 10 min, 37 C for 60 min, and 92 C for 10 min. PCR amplification of GHR transcripts was performed using 5 μl cDNA (equivalent to 0.5 μg total RNA) in a 50-μl volume with: 200 μM final concentration of deoxynucleosidetriphosphate; 2.5 mM final concentration $MgCl_2$, 2 μM final concentration of each primer, and 1 U Taq DNA polymerase. After heating to 94 C for 3 min, 30 cycles were performed at 94 C for 30 sec, 56 C for 1 min, and 72 C for 2 min, before a final step of 72 C for 10 min. All PCR reactions included negative controls with water.

Primers (Fig. 1)

Primers were manufactured by Genosys (Cambridge, U.K.). For the GHR, primer PS is in exon 7 starting at nucleotide 709 (5′-GGATAAGGAATATGAAGTGC-3′) and was used with primer PAS in exon 10 starting at nucleotide 1161 (5′-GATTTCTCATGGTCACTGC-3′) predicting a product of 453 bp.

Cloning, Sequencing, and Southern Blotting

PCR products were cloned into the pCRII vector (Invitrogen, San Diego, CA) according to the manufacturer's protocol, and dideoxy sequencing was with the Sequenase 2.0 kit according to the manufacturer's instructions (USB, Cleveland, OH). RT-PCR products were transferred from polyacrylamide gels (6–12%) onto a nylon membrane (Hybond N+, Amersham, Little Chalfont, U.K.) by electroblot (Bio-Rad, Hemel Hempstead, U.K.). GHR cDNA probes were prepared by PCR using 0.1 μg of the GHR453 plasmid as a template; products were run on 1% agarose, excised, purified (Geneclean, Bio 101) and random primer labeled with 30 μCi [^{32}P]dCTP (Oligolabeling kit, Pharmacia, Piscataway, NJ). The probes were separated by a Sephadex G50 column. Prehybridization (4 h) and hybridization (18 h) were performed at 42 C in 50% formamide. Posthybridization washes were twice in 2× sodium citrate chloride/0.2% SDS for 30 min at room temperature, then twice in 0.1 × sodium citrate chloride/0.1% SDS at 55 C for 30 min each. The membranes were exposed to Kodak XAR film.

Quantification

Autoradiographs were scanned by an image densitometer (GS 670, Bio-Rad) and optical densities analyzed by Molecular Analyst software (Bio-Rad). A standard PCR reaction was

performed on normal liver cDNA with product being sampled after every tenth cycle for 40 cycles to assess the kinetics of the reaction.

IM-9 Cell Cultures

Human IM-9 lymphocytes were grown in RPMI 1640 medium (all reagents, from Sigma, St. Louis, MO), containing 10% (vol/vol) FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin, 2 nM L-glutamine at 37 C in 5% CO_2. The cells were cultured to stationary phase, counted with a hemocytometer, centrifuged at 200 $\times$ *g*, washed three times in RPMI 1640, and resuspended in RPMI 1640 and 0.1% wt/vol BSA. After 24 h cells were harvested and total RNA extracted as described above. HepG2 and 293 cells were cultured and transfected by the $CaPO_4$ method as previously described (17, 34).

RNase Protection

The GHR riboprobe was constructed using the GHR1-279 plasmid in pCRII (Invitrogen). The plasmid was linearized by digestion with *Ncol*, and *in vitro* transcription by T7 RNA polymerase produced an antisense probe of 385 bp, which was gel purified. RNase protection was performed on 25 μg total RNA from yeast (negative control), IM-9 cells, human liver, HepG2 cells, and HepG2 cells stably transfected with the GHRfl (this stably transfected cell line was used as a positive control for the full-length GHR message). Total RNA was hybridized with labeled GHR antisense probe (GHR1-279) overnight at 45 C in 80% (vol/vol) formamide, 40 mM piperazine N,N′-bis (2-ethanesulfonic acid, pH 6.4), 400 mM sodium chloride, and 1 mM EDTA. After hybridization, 8 μg/ml RNase A and 0.4 μg/ml RNase T1 (Sigma, Dorset, UK) were added and incubated for 1 h at 30 C to digest nonhybridized RNA. Protected hybrids were isolated by ethanol precipitation and separated on a 6% polyacrylamide/7 M urea denaturing sequencing gel. The dried gel was exposed to x-ray film. The expected protected fragments were 296 bp for the GHR1-279 alternate splice and 217 bp for the GHRfl (the smaller hybrid for the full length as GHR1-279 was used as the probe).

Construction of GHRfl and GHR1-279 Expression Vectors

The full-length human GHR has proved difficult to assemble and propagate in *E. coli*. This problem has been overcome by changing 24 nucleotides largely in the transmembrane domain while maintaining the native amino acid sequence (35). This construct, kindly provided by Genetech, was subcloned into the expression vector pcDNAI/Amp (Invitrogen) using the *Bam*HI and *Sna*BI restriction sites to produce the GHRfl. This construct contains a unique *Bst*BI site engineered at the end of exon 8, which is not found in the native sequence. The expression vector GHR1-279 was constructed by introducing a *Bst*BI restriction site by PCR amplification of GHR1-279, subcloning back into the pCRII vector (Invitrogen), then digesting with *Bst*BI and *Not*I, and ligating into the GHRfl at the same sites. The construct was then sequenced to confirm the modified sequence.

Binding Assays

Twenty four hours after transfection, the cells were serum starved for 12 h. The culture media was removed and concentrated (20×) to be analyzed for the presence of GHBP using gel filtration and HPLC (15). Cells were then washed with PBS containing 1% BSA and incubated with [^{125}I]-hGH (5×10^5 cpm/well) for 3 h at room temperature in the absence or presence of various concentrations of unlabeled hGH. The cells were then washed in the same buffer and solubilized in 1 ml NaOH 1 N for counting radiation.

Cross-Linking

Cells were grown in six-well plates and transfected with 5 μg cDNA; 5×10^5 cpm/well of [^{125}I]-hGH were added to the dishes in the absence or presence of 5 μg/ml of cold hGH, and incubated for 30 min at 37 C. The cells were then washed with PBS, and 2 ml PBS were added to each well. Dissuccinimidylsuberate (0.5 mM in dimethyl sulfoxide, Pierce, Rockford, IL) was added to the cells, mixed, and incubated for 20 min at room temperature. The reaction was stopped with 2 ml Tris, 50 mM, NaCl, 150 mM, and cells were lysed in 50 μl SDS-sample buffer. Electrophoresis was performed on a 8% SDS-polyacrylamide gel. The gel was dried and exposed to x-ray film.

Immunoprecipitations and Western Blotting

Human GH (Genotropin, Pharmacia) was biotinylated using the Boeringher kit at a ratio of 1:5 molar. For immunoprecipitations, 5×10^6 cells were transfected with 10 μg of GHRfl or GHR1-279 cDNA alone or with a combination of 5 μg of each cDNA. Twenty four hours after transfection, cells were incubated in starvation media for 16 h and then stimulated with either 2 μg/ml of hGH or biotinylated hGH for 5 min at 37 C. One milliliter of normal human serum was also incubated with 2 μg biotinylated hGH. Cell lysates were then incubated overnight at 4 C with 4 μg/ml affinity-purified αGHR-intra antibody (2) and protein A Sepharose or with 30 μl of streptavidin beads. Supernatants (10 ml) and serum were incubated with 30 μl of streptavidin beads. Purified proteins were applied to a 10% polyacrylamide-SDS gel, transferred to a nitrocellulose membrane, and probed with 5 μg/ml of the GHR antibody mAb 263 (Biogenesis). Detection was performed with the chemiluminescent detection system (Amersham).

Transcription Assays

293 cells were plated in six-well plates at 0.4×10^6 cells per well before being transfected with 0.1 μg of the pcDNA1 expression vector containing the GHRfl and/or 0 to 1 μg GHR1-279, 1.5 μg LHRE/TK-luciferase reporter gene (17), and 3 μg pCH110 (fl-galactosidase expression vector, Pharmacia). Cells were then incubated for 24 h in serum-free medium with or without 20 or 1 nM hGH. Cells were lyzed and luciferase and galactosidase activities measured. Luciferase activity was normalized to the galactosidase activity. All experiments were performed in triplicate.

Acknowledgments

We are grateful for the support and advice of Professor GM Besser and Professor PA Kelly.

Received April 11, 1996. Re-revision received December 19, 1996. Accepted December 20, 1996.

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S.L.C. is supported by a Wellcome Trust Clinician Scientist Fellowship, and S.V.L. by a grant from the YCRC. The work was supported by grants from the Clinical Endocrinology Trust, Royal Society, Northern General Hospital Research Committee, and Serono Laboratories, UK.

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