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Biochemistry

Alternatively spliced forms in the cytoplasmic domain of the human growth hormone (GH) receptor regulate its ability to generate a soluble GH-binding protein

(cytokine receptor/shedding)

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ABSTRACT **The mechanism underlying the generation of soluble growth hormone binding protein (GHBP) probably differs among species. In rats and mice, it involves an alternatively spliced mRNA, whereas in rabbits, it involves limited proteolysis of the membrane-bound growth hormone receptor (GHR). In humans, this latter mechanism is favored, as no transcript coding for a soluble GHR has been detected so far. To test this hypothesis, we analyzed COS-7 cells transiently expressing the full-length human (h) GHR and observed specific GH-binding activity in the cell supernatants. Concomitantly, an alternatively spliced form in the cytoplasmic domain of GHR, hGHR-tr, was isolated from several human tissues. hGHR-tr is identical in sequence to hGHR, except for a 26-bp deletion leading to a stop codon at position 280, thereby truncating 97.5% of the intracellular domain of the receptor protein. When compared with hGHR, hGHR-tr showed a significantly increased capacity to generate a soluble GHBP. Interestingly, this alternative transcript is also expressed in liver from rabbits, mice, and rats, suggesting that, in these four species, proteolysis of the corresponding truncated transmembrane GHR is a common mechanism leading to GHBP generation. These findings support the hypothesis that GHBP may at least partly result from alternative splicing of the region encoding the intracellular domain and that the absence of a cytoplasmic domain may be involved in increased release of GHBP.**

Growth hormone (GH), a multifunctional protein synthesized by the anterior pituitary, is involved in regulating postnatal body growth and several metabolic processes, including insulin- and antiinsulin-like effects on adipocytes, and stimulation of muscle cell and erythroid progenitor cell proliferation (1). This polypeptide hormone exerts its physiological effects through its interactions with a specific high-affinity receptor (the GH receptor, GHR) (2), an integral cell membrane protein (3) that is a member of the cytokine receptor superfamily (4). Many members of this receptor family, including GHR, exist in soluble forms (5, 6). The latter retain their ability to specifically bind their ligands and, therefore, to compete with membrane receptors, thus potentially acting as antagonists (6). In addition, soluble cytokine receptors have been described as carrier proteins that increase the half-life of the respective cytokine by potentially decreasing its metabolic clearance rate (6). Two different mechanisms leading to the generation of soluble receptors have been reported: (*i*) the major biosynthetic pathway, which involves an alternative pre-mRNA splicing of a common transcript, leading to the loss of transmembrane and cytoplasmic domains; this is the case of receptors for interleukin (IL) 4 (7), IL-5 (8), IL-7 (9), IL-9 (10), granulocyte–macrophage colony-stimulating factor (11), granulocyte colony-stimulating factor (12), leukemia inhibitory factor (13), erythropoietin (14), and c-mpl (15); (*ii*) in some cases [receptors for ciliary neurotrophic factor (16) and IL-6 (17)], the soluble variants are released from the cell surface by limited proteolytic cleavage of the membrane receptor, a process known as shedding.

The mechanism underlying GH-binding protein (GHBP) generation probably differs among species. Alternative splicing is responsible for soluble receptor generation in rats (18) and mice (19, 20). In these species, a short mRNA, in which sequences encoding the intracellular and transmembrane domains of GHR are replaced by a hydrophilic tail-encoding region, is easily detectable by Northern blot analysis. No analogue of murine alternatively spliced mRNA GHR has been described in humans or rabbits (3), raising the possibility that the soluble forms result from proteolytic cleavage of the full-length transmembrane proteins. Although the cleavage site and the protease responsible for generating GHBP are unknown, there is compelling evidence for this hypothesis: (*i*) release of GHBP has been elicited from IM-9 lymphocytes by sulfhydryl-reactive reagents like *N*-ethylmaleimide (21), suggesting that the loss of a sulfhydryl group, located on GHR or a neighboring protein, permits a membrane endopeptidase to release the GHBP; (*ii*) a soluble GHBP was detected in conditioned medium of human hepatoma Hep G2 cells briefly exposed to *N*-ethylmaleimide (22), as well as in the supernatant of Hep G2 cells stably expressing rabbit GHR cDNA (23); (*iii*) GHBP was shed from CHO cells transfected with rabbit (but not rat) GHR cDNA (24), a result consistent with the description of a specific mRNA encoding a soluble GHBP in rats only (18). Given the sequence similarity between rabbit and human GHR cDNAs (3) and the presence of a single mRNA in both species, it was postulated that GHBP generation in humans and rabbits involves a protease recognition site that is absent in rats (24).

With the aim of providing a molecular basis for GHBP generation in humans, we analyzed GHBP release from COS-7 cells transiently expressing the human or rabbit full-length GHR. We also looked for a human alternatively spliced GHR mRNA that could encode a soluble GHBP.

MATERIALS AND METHODS

Plasmid Construction and Site-Directed Mutagenesis. A plasmid vector for rabbit (rb) GHR expression in mammalian

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Abbreviations: GH, growth hormone; GHR, GH receptor; GHBP, GH-binding protein; ILR, interleukin receptor; h, human; rb, rabbit; IL, interleukin; EBV, Epstein–Barr virus; RT–PCR, reverse transcriptase-coupled PCR. Single mutants are designated by the wild-type residue (single-letter amino acid code) followed by its sequence position and the mutant residue.

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cells (designated prbGHR) was constructed by placing the full-length rbGHR cDNA coding region (provided by R. Barnard, University of Queensland, Australia) with synthetic adaptator sequences in the simian virus (SV40) promotor-based expression vector pECE (25). The cDNA coding for human (h) GHR-tr (see below) was prepared from pECE carrying the full-length hGHR cDNA sequence and designated phGHR(wt) (26); site-directed mutagenesis was performed by introducing the PCR fragment carrying the sequence unique to hGHR-tr and digested with *Eco*RV and *Eco*RI into the corresponding sites of phGHR(wt). The deletion construct, designated phGHR(tr), was checked by sequence analysis. The previously described plasmid phGHR(F239X), which was prepared from phGHR(wt) with a primer designed to insert a stop codon at position 239, results in a cDNA that encodes the extracellular domain only (27).

Transient Expression of hGHR, rbGHR, hGHR(F239X), and hGHR-tr in Eukaryotic Cells. Transient expression of each GHR construct [phGHR(wt), prbGHR, phGHR(F239X), or phGHR(tr)] was performed as described elsewhere (26). Briefly, transfections were performed at 60% confluence by the LipofectAmine method (Life Technologies, Gaithersburg, MD) in OptiMEM medium. Transfected and nontransfected COS-7 or CHO cells were maintained at 37°C in Iscove's medium supplemented with 10% fetal calf serum and processed for binding studies.

Binding Assays on Cell Membranes. hGH binding assays were performed on membrane receptors as described (28). Briefly, the total particulate membrane fraction was prepared by centrifugation for 30 min at 1500 × *g*; 80 μg of the resulting pellet was incubated with ^{125}I-labeled hGH (10^5 cpm). Two micrograms of hGH was labeled with 200 μCi of ^{125}I (1 Ci = 37 GBq) as described (29). A specific activity of 60 μCi/μg was obtained. Results are expressed as specific binding activity after subtraction of nonspecific binding in the presence of a large excess of unlabeled hGH (2 μg). The association constant (K_a) of hGHR and hGHR-tr was calculated by Scatchard analysis of the binding data obtained from three experiments, using the EBDA-LIGAND program (30). The slight difference in binding affinity observed between these two isoforms was taken into account to evaluate their membrane expression in each transfection experiment.

GHR Secretion Assay. Soluble receptor binding assays of COS-7 cell supernatants were performed essentially as described elsewhere (27). Briefly, 50 μl of supernatant from COS-7 cells transiently expressing each of the GHR constructs was incubated with ^{125}I-labeled hGH (10^5 cpm; 60 μCi/μg) in 0.1 M sodium phosphate buffer (pH 7.4). After 20 h at 4°C, the reaction products were analyzed by gel filtration on a Protein-Pak 300SW HPLC system (Waters). Elution was performed with buffer (0.1 M Na_2SO_4/0.1 M potassium phosphate, pH 7.0) at a flow rate of 0.5 ml/min. The molecular weight of the protein complexes was determined by size chromatography using standard molecular weight marker proteins. Fractions (0.250 ml) were collected and counted in a γ-counter (Minigamma, LKB). Specific binding activity was calculated after subtraction of nonspecific binding in the presence of a large excess of unlabeled hGH (2 μg), expressed as a percentage of the total radioactivity (cpm) recovered from the column.

The same assay was performed with (*i*) supernatants from CHO cells transfected with either phGHR(wt) or phGHR(tr) and (*ii*) a normal human serum sample that contains the naturally occurring GHBP, used as a control. To compare the ability of hGHR and hGHR-tr to generate a soluble GHBP, binding values were normalized to total membrane binding activity.

Reverse Transcriptase-Coupled PCR (RT–PCR) Analysis. Total RNA from several human tissues, including liver, stomach, mammary gland, kidney, muscle, placenta, and lung was obtained from CLONTECH. Total RNA from human adipose tissue, fibroblasts, and Epstein–Barr virus (EBV)-transformed lymphocytes, as well as rabbit, rat, and mouse liver, was extracted by means of the guanidium isothiocyanate method (31). RT–PCR analysis was performed using the Promega Access RT–PCR system (Promega). This system uses the avian myeloblastosis virus RT for cDNA synthesis and the thermostable *Thermus flavus* DNA polymerase for cDNA amplification. cDNA was prepared from 1 μg of total RNA according to the manufacturer's instructions. To detect a putative GHR transcript excluding exon 8 (which mainly encodes the transmembrane domain), we used primers h7 and h10, located in exons 7 and 10, respectively. Cycle parameters were 1 min of denaturation (94°C), 1 min of annealing (55°C), and 2 min of polymerization (68°C) for 40 cycles with a 7-min final elongation (68°C).

To test directly for the presence of hGHR-tr transcripts and to evaluate their abundance relative to hGHR transcripts in all human tissues tested, each isoform was amplified by means of RT–PCR using the same reverse primer (h10) and a specific forward primer, h9 or h9-tr, designed to amplify hGHR or hGHR-tr transcripts, respectively. These two primer sets were shown to be equally efficient in PCR assays performed on serial dilutions of phGHR(wt) and phGHR(tr) (data not shown). Similarly, primers rb10, rb9, and rb9-tr, and primers mu10, mu9, and mu9-tr were used in a PCR assay to detect both isoforms in rabbits and murine species (rats and mice), respectively. Primer sequences were as follows: h7, 5′-GCGAGTTCAGTGAGGTGCTC-3′; h10, 5′-CACTGTGGAATTCGGGTTTA-3′; h9 (identical to rb9), 5′-ATTTTCTAAACAGCAAAGGA-3′; h9-tr (identical to rb9-tr), 5′-ATTTTCTAAACAGCAAAGTT-3′; rb10, 5′-CATTGTAGAATTCAGGCTTA-3′; mu10, 5′-ATTGTAGAAGTCGGGTTTGT-3′; mu9, 5′-TATTTTCAAAGCAGCAAAGG-3′; mu9-tr, 5′-TATTTTCAAAGCAGCAAAGT-3′. To check the amount of RNA, total RNA (1 μg) from the same tissues was analyzed on 1% agarose gel stained with ethidium bromide. PCR products were analyzed by electrophoresis on either 6% acrylamide gel or 3% NuSieve GTG (FMC)/1% agarose gel stained with ethidium bromide.

RESULTS

Expression and Properties of hGHR and rbGHR in COS-7 Cells. To test whether the full-length hGHR behaves like the full-length rbGHR, the human and rabbit GHR cDNA clones were transiently expressed in COS-7 cells. Serum-free medium was collected from transfected cells and tested on HPLC columns for their capacity to bind ^{125}I-labeled hGH to examine whether these cells excreted GHBP. Spontaneous release of GHBP was observed in both situations (Fig. 1). As a positive control, COS-7 cells were transfected with an artificially terminated hGHR cDNA encoding the exoplasmic region only [hGHR(F239X)]. As expected, large amounts of GHBP were released in this cell supernatant.

These results suggest that proteolysis of the membrane-bound full-length receptor protein is a mechanism of GHBP generation in both humans and rabbits.

Isolation and Characterization of a Human Truncated GHR (hGHR-tr) Generated by Alternative Splicing. We also investigated whether in humans, by analogy with murine GHBP generation, alternative splicing leading to the loss of the transmembrane domain could generate GHBP. In humans, a single 4.4-kb mRNA species known to encode the transmembrane receptor is detected by Northern blot analysis (3), as opposed to the two mRNA species detected in mice and rats, encoding the membrane-anchored and soluble receptor proteins, respectively (18–20). This raised the possibility that a second mRNA species of similar size (4.4 kb) and, therefore, comigrating with the mRNA species encoding the membrane receptor could encode a soluble GHBP in humans.

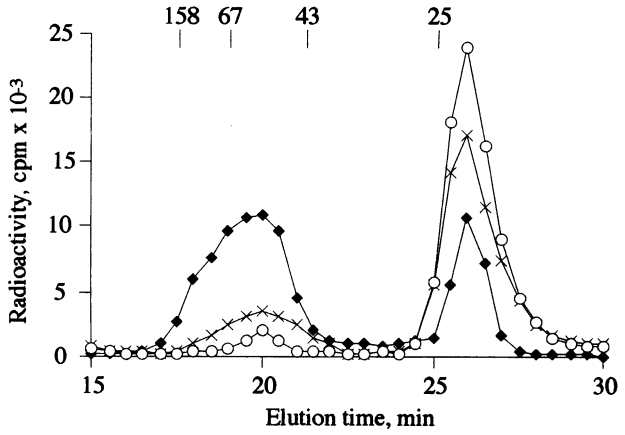

FIG. 1. HPLC gel filtration elution profile of ^{125}I-labeled hGH incubated with supernatant of COS-7 cells expressing the human GHR cDNA (○), the rabbit GHR cDNA (×), and an artificially terminated hGHR cDNA encoding the extracellular region only [hGHR(F239X)] (♦). The molecular weight of the markers used to calibrate the column is indicated above. The GHBP–^{125}I-labeled hGH complex eluted at 20 min (peak displaceable by unlabeled GH; data not shown), whereas free ^{125}I-labeled hGH eluted at 26 min. Results of one representative experiment are presented.

We thus applied PCR to reverse-transcribed human liver mRNAs to detect a putative transcript containing an exon encoding a hydrophilic tail unique to the binding protein in place of exon 8, a sequence that mainly encodes the transmembrane domain. A PCR product was generated with primers h7 and h10, flanking the candidate splicing sites encompassing exons 7 to 10.

To enhance sensitivity for a possible GHR isoform, PCR products were analyzed on polyacrylamide gel stained with ethidium bromide, a technique that has proven efficient for detecting GHR isoforms containing or excluding exon 3 (28). Three molecular species were visualized (Fig. 2, lane 1). One, the major species, was of the expected size (295 bp) and corresponded to the known hGHR cDNA, whereas the other two fragments, which were slowly migrating and faint, were heteroduplexes generated by single-stranded reassociation of hGHR cDNA and a new GHR species. This was confirmed as follows: when the slowly migrating DNA was removed from the gel and reamplified with primers h7 and h10, four species were generated in the same proportions; they consisted of the 295-bp fragment and a smaller PCR product (269 bp), as well as the two heteroduplexes resulting from the single-stranded reassociation of these two molecular species (Fig. 2, lane 2).

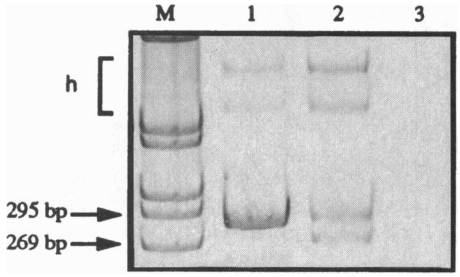

FIG. 2. Detection of hGHR and hGHR-tr splicing isoforms by RT–PCR analysis of human liver RNA on a polyacrylamide gel (6%) stained with ethidium bromide. RT–PCR products generated with a hGHR-specific primer pair (h7 and h10) (lane 1); PCR amplification of heteroduplexes (bands h) resulting from the single-stranded reassociation of hGHR and hGHR-tr transcripts and generating the 295-bp and 269-bp fragments that correspond to hGHR and hGHR-tr species, respectively, as well as the resulting heteroduplexes (lane 2); and control without RNA template (lane 3) are shown.

Sequencing of the smaller PCR product (269 bp) generated in this way confirmed that it corresponded to a new GHR mRNA. The relationship between this isoform and the previously described GHR mRNA species is depicted in Fig. 3. The nucleotide sequence of the shorter mRNA species, designated hGHR-tr, was identical to that of hGHR, except that it lacked 26 nt from positions 876 to 901. This deletion results in a novel translation reading frame that encodes 5 amino acids after the deletion point, leading to a stop codon at position 280 and thereby truncating 97.5% of the intracellular domain of the receptor protein. Genomic sequence examination confirmed that the two mRNA species, hGHR and hGHR-tr, are generated from a single gene by alternative splicing. Splicing for hGHR-tr mRNAs occurred at the sequence 5′-CTGATTCT-GCCCCCAG-3′, which is located within the ninth exon, instead of at 5′-TTTATATGTTTTCAAG-3′ in the case of hGHR mRNAs (Fig. 3*B*). The first 26 nt of the ninth exon of GHR are thus recognized as a part of the eighth intron in hGHR-tr.

To test directly for the hGHR-tr isoform and to evaluate its abundance relative to hGHR transcripts in human liver, two PCR forward primers, h9 and h9-tr, were designed to amplify hGHR and hGHR-tr transcripts, respectively, with the use of the same reverse primer (h10; Fig. 4*A*). This experiment confirmed the presence of hGHR-tr transcripts in human liver, but in small amounts relative to hGHR transcripts (Fig. 4*B*, lanes 1a and 1b).

Properties of hGHR-tr Expressed in Eukaryotic Cells and Comparison with hGHR and the Native hGHBP. We determined whether hGHR-tr cDNA encodes a GHR by transiently expressing phGHR(tr) in COS-7 cells. The total particulate membrane fraction from transfected cells was incubated with ^{125}I-labeled hGH and assayed for specific GH-binding activity. As shown in Fig. 5*A*, a high level of ^{125}I-labeled hGH binding was observed in 12 transfection experiments, as was also the case of COS-7 cells transiently expressing phGHR(wt). This contrasted with the absence of GH-binding activity of membranes prepared with COS-7 cells expressing hGHR(F239X) (data not shown) and strongly suggested that the protein encoded by hGHR-tr transcripts, which contains the hydrophobic segment corresponding to the transmembrane domain, is indeed a membrane-anchored receptor. In addition, Scatchard analysis revealed that hGHR-tr bound labeled GH with a K_a (1.82 ± 0.58 nM^{-1}) similar to that observed for the full-length human GHR (0.93 ± 0.27 nM^{-1}), indicating that a major deletion in the cytoplasmic domain has little effect on the GH-binding activity of the receptor. However, hGHR-tr showed a slight increase in binding affinity (approximately 2-fold). This difference in the affinity constant values was clear in three experiments.

To examine whether hGHR-tr protein could generate a soluble GHBP, the supernatant of COS-7 cells expressing hGHR-tr was assayed for GH-binding activity by means of HPLC gel filtration. As shown in Fig. 5*B*, large amounts of a protein encoded by hGHR-tr transcripts were secreted into the COS-7 cell medium in a form capable of binding efficiently to hGH. In each experiment, the ability of hGHR-tr to generate a soluble GHBP was compared with that of hGHR. Twelve transfection experiments, the results of which are normalized to total membrane binding activity and averaged in Fig. 5*B*, revealed that the hGHR-tr isoform exhibited a significant increase in GHBP generation (4.51 ± 1.34-fold) compared with the full-length receptor.

To test whether the GHBP generated by the cells transfected with phGHR(wt) or phGHR(tr) corresponded to the native serum hGHBP, a human serum sample incubated with ^{125}I-labeled hGH was analyzed in parallel (Fig. 6). When compared with the elution profile obtained with the transfected COS-7 cell supernatants, the human serum GHBP eluted in an earlier column fraction; however, supernatants of CHO cells tran-

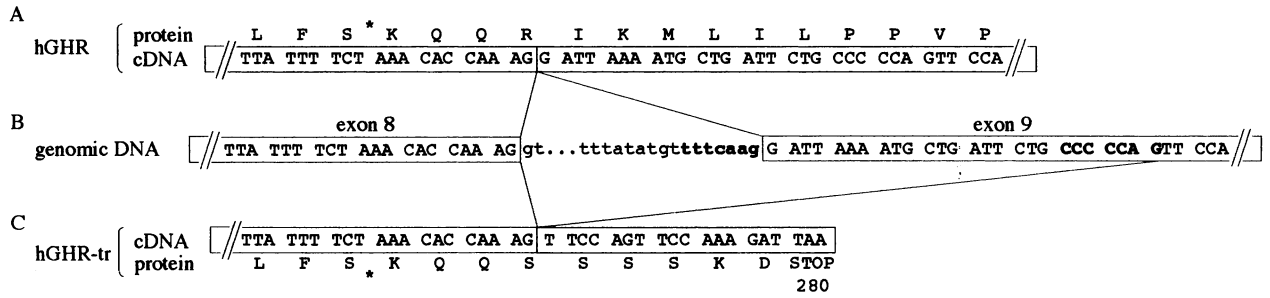

FIG. 3. Alternative splicing mechanism generating hGHR and hGHR-tr transcripts. (*B*) Diagrammatic representation of the genomic DNA fragment involved in the generation of hGHR (*A*) and hGHR-tr (*C*) transcripts. Intron and exon sequences are denoted by lowercase type and boxed uppercase type, respectively. Resulting amino acid sequences are shown above and below. Boldface type indicates the two 3′ splice consensus sites used in the GHR genomic sequence. The transmembrane–cytoplasmic junction is marked by an asterisk in the amino acid sequence.

siently expressing hGHR or hGHR-tr generated a GHBP eluting in column fractions identical to those corresponding to the native human serum GHBP, a result demonstrating physical similarity between the recombinant and native GHBPs.

Thus, these data indicate that both hGHR mRNA species generated by alternative splicing encode a transmembrane protein, but that one, designated hGHR-tr, that is translated into a receptor molecule that lacks 97.5% of its cytoplasmic domain, produces relatively large amounts of a soluble GHBP compared with the hGHR isoform.

Tissue Distribution of hGHR-tr mRNAs and Search for an Analogue in Rabbits, Rats, and Mice. The expression of hGHR-tr compared with that of hGHR was examined in various human tissues by means of a RT–PCR. Total RNA from adipose tissue, EBV-transformed lymphocytes, placenta, mammary gland, fibroblasts, lung, kidney, stomach, and muscle was subjected to RT–PCR as described above for liver RNA, using primers h10 in combination with either h9 or h9tr that correspond to hGHR and hGHR-tr mRNAs, respectively (Fig. 4*A*). The hGHR isoform was found to be expressed in all investigated tissues (Fig. 4*B*); the level of expression varied from tissue to tissue (Fig. 4 *B* and *C*), in keeping with previous studies (32). Interestingly, however, as deduced from this PCR assay, the ratio between hGHR and hGHR-tr mRNAs varied among tissues (Fig. 4*B*). These results suggest that, in liver, kidney, and fibroblasts, hGHR-tr mRNAs are minor splicing products when compared with the abundance of hGHR transcripts. By contrast, in mammary gland, EBV-transformed lymphocytes, and adipose tissue, in which hGHR-tr mRNAs were clearly detectable, the two isoforms showed a similar level of expression. In these experimental conditions, hGHR-tr mRNAs were only detectable in muscle, stomach, and lung when a second round of amplification was performed with nested primers (data not shown).

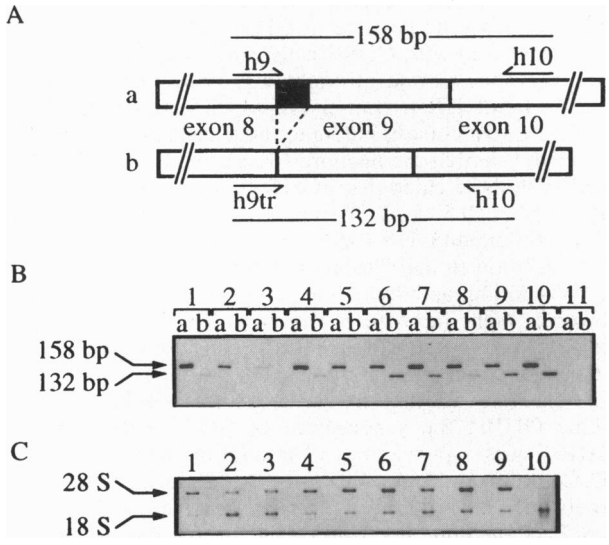

FIG. 4. RT–PCR analysis of hGHR and hGHR-tr isoforms in selected human tissues. (*A*) Schematic presentation of PCR primers (arrows), corresponding positions in each cDNA (a and b refer to hGHR and hGHR-tr transcripts, respectively), and expected sizes of PCR products. The solid box represents the alternatively spliced cDNA sequence. (*B*) RT–PCR products for hGHR (lanes a) and hGHR-tr (lanes b) detected on an agarose (1%)/NuSieve (3%) gel stained with ethidium bromide. Lanes were loaded with amplified cDNA derived from liver (lane 1), muscle (lane 2), stomach (lane 3), kidney (lane 4), lung (lane 5), mammary gland (lane 6), placenta (lane 7), fibroblasts (lane 8), EBV-transformed lymphocytes (lane 9), and adipose tissue (lane 10); control without RNA template (lane 11). (*C*) Visualization on agarose gel (1%) of 1 μg of total RNA collected from the same tissues (lanes 1–10) and used as a template in the RT–PCR experiment.

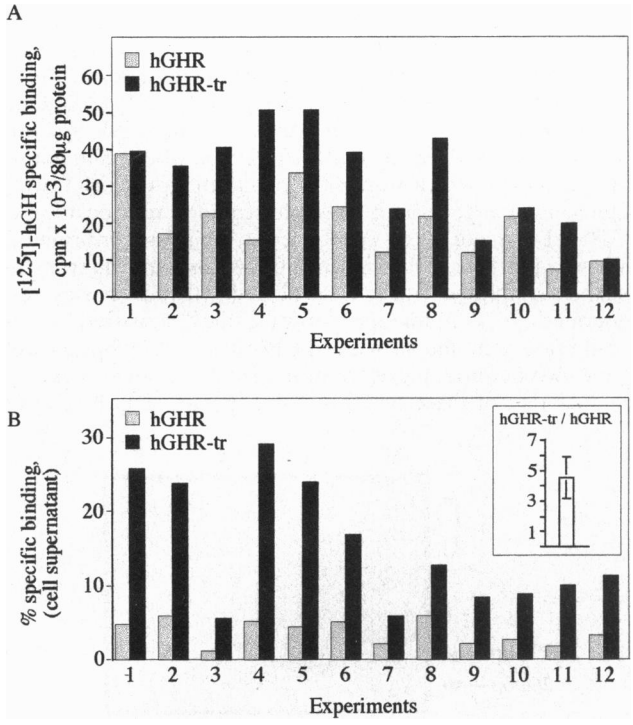

FIG. 5. Properties of hGHR-tr expressed in COS-7 cells and comparison with hGHR in 12 sets of transfection experiments (numbered 1 to 12). (*A*) Specific binding of ^{125}I-labeled hGH to total particulate membrane fractions of cells transfected with phGHR(wt) or phGHR(tr). (*B*) Amount of GHBP released in the supernatant of cells transfected with phGHR(wt) or phGHR(tr). Results are normalized to total membrane binding activity. (*Inset*) The difference observed in each experiment between hGHR-tr and hGHR, expressed in a ratio format (hGHR-tr to hGHR), is averaged.

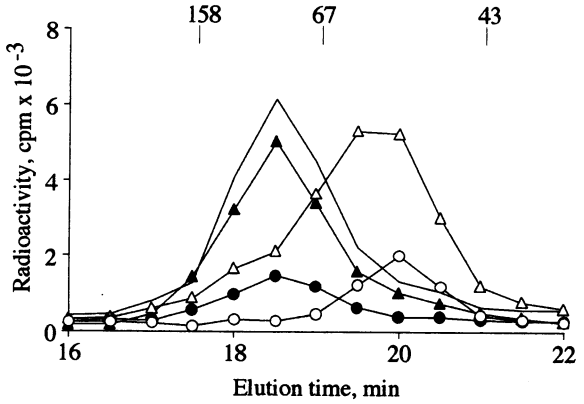

FIG. 6. HPLC gel filtration elution profile of ^{125}I-labeled hGH incubated with supernatant of CHO cells expressing hGHR (●) or hGHR-tr (♦), and COS-7 cells expressing hGHR (○) or hGHR-tr (◇). A human serum sample was analyzed in parallel (solid line). The molecular weight of the markers used to calibrate the column is indicated above. Results of one representative experiment are presented.

To search for an analogue to hGHR-tr in other species, similar RT–PCR experiments were performed on liver mRNA templates in rabbits, rats, and mice, using species-specific primers analogous to h10, h9, and h9-tr. Interestingly, an analogue of the human GHR-tr isoform was identified in these three species; as the GHR nucleotide sequence is somewhat different among the three species, it was possible to confirm this result by sequencing the RT–PCR products generated in this way (data not shown). These data, which are consistent with conservation of the alternative splice–acceptor site in exon 9 among these species, suggest that proteolysis of the corresponding truncated GHRs is a common mechanism leading to GHBP generation.

DISCUSSION

This study focuses on the mechanism by which the soluble human GHR is generated. Alternative splicing leading to the loss of the transmembrane domain and limited proteolysis of the membrane-bound receptor protein have been proposed as mechanisms underlying the synthesis/release of soluble variants of receptors for cytokines and growth factors. Interestingly, many members of the cytokine receptor superfamily have been shown to generate their soluble counterpart by alternative splicing of a common transcript, whereas several cytokine and growth factor receptors not belonging to this family generate a soluble receptor through proteolytic cleavage (6). The case of GHR seems to be more complex, as both mechanisms have been claimed to occur, depending on the species.

We assessed the ability of full-length human GHR cDNA, transiently expressed in COS-7 cells, to generate soluble GHBP. The corresponding GHR protein was correctly expressed, as shown by means of membrane GH binding. Little ^{125}I-labeled hGH binding was detected in the culture supernatant of the transfected cells, suggesting that spontaneous conversion of the membrane-anchored receptor into a soluble form is not a major mechanism of GHBP generation, at least in this system. This was also the case of COS-7 cells expressing the full-length rabbit receptor, as observed (23, 24). By contrast, in the same experimental conditions, GH-binding activity was readily detected in the supernatant of cells expressing an artificially terminated hGHR cDNA encoding the extracytoplasmic domain only.

These results, combined with the fact that soluble and membrane-anchored hGHRs arise from the same gene (3, 33), led us to consider whether GHR isoforms are one source of soluble GHBP. By means of RT–PCR, we identified a transcript that would encode a second transmembrane hGHR, designated hGHR-tr. Sequence comparison of GHR genomic DNA and hGHR-tr mRNA revealed that hGHR-tr is generated by alternative splicing through the use of a cryptic splice acceptor consensus sequence located in exon 9, 26 bp downstream of the splice acceptor used in the full-length hGHR. Indeed, the hGHR gene has the sequence 5′-CTGATTCT-GCCCCCAG-3′ in the ninth exon, which is similar to the consensus sequence (5′-(T/C)>10N(C/T)AG-3′) of the splice acceptor (34). Thus, part of the ninth exon of hGHR is recognized as the eighth intron and spliced out in hGHR-tr transcripts. This short deletion (also recently reported by Ross et al.[†]) that leads to a stop codon at position 280 is consistent with the apparent existence of only one GHR mRNA species in humans (3). Expression of hGHR-tr cDNA in COS-7 cells confirmed that this mRNA species encoded a membrane receptor. Interestingly, however, cells expressing this transcript generated a high level of soluble GHBP. When the results were normalized to GHR protein expression, hGHR-tr showed an approximately 4.5-fold increase in the ability to generate a soluble GHBP.

In theory, generation of soluble GHBP could result from a failure of the hydrophobic domain of hGHR-tr protein to stop translocation across the plasma membrane or from cleavage of the exoplasmic domain within a site presumably located close to the transmembrane domain (3). Several lines of evidence support the latter hypothesis: (*i*) binding assays performed on the total particulate membrane fraction of COS-7 cells expressing hGHR-tr revealed a high level of GH binding; (*ii*) the hydrophobicity of the GHR transmembrane domain, as assessed by using the HYDRAA program (35), is above the threshold required for it to function as a stop-transfer sequence (36); (*iii*) such truncated membrane-anchored proteins have been described in many other systems (5).

Our results strongly suggest that the absence of a cytoplasmic domain markedly stimulates cleavage of the hGHR exoplasmic domain, therefore, implying inside-out transfer of information across the membrane. This mechanism of conversion of membrane-bound proteins into soluble forms is reminiscent of that described for the transforming growth factor type α (TGFα) (37). However, in this latter case membrane TGFα (proTGFα) is able to generate the soluble growth factor through proteolytic cleavage, while deletion of the cytoplasmic tail prevents cleavage. It was shown that the cleavage step is dictated by the presence of a specific determinant (a valine residue) at the C-terminal position of the cytoplasmic domain of proTGFα. Interestingly, in the tumor necrosis factor receptor, cytoplasmic truncations (up to 96% of the cytoplasmic domain) do not prevent shedding (38). Thus, these observations further support the hypothesis that the structural determinants required for shedding differ among membrane proteins (39).

In the case of human GHR, we favor the hypothesis that the absence of a cytoplasmic domain may be involved in the release of GHBP by modifying the conformation of the region that includes the putative cleavage site, thereby exposing it to a proteolytic system. It is indeed unlikely that the C terminus of hGHR-tr contains a cleavage-specific determinant, as soluble GHBP was generated from two membrane-bound GHRs with different cytoplasmic domains, i.e., hGHR-tr and an artificially truncated receptor with a stop codon at residue 278 and with no modification of the translation reading frame upstream residue 278 (data not shown).

One striking observation was the very small amount of hGHR-tr transcripts found in liver, a tissue that expresses high

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levels of hGHR. As the weak expression of hGHR-tr was documented by *in vitro* amplification and after 40 cycles of PCR, it is not surprising that this isoform was not detected by means of RNase protection assays performed with liver RNA samples (40). However, as deduced from our PCR assays, the relative abundance of hGHR-tr is higher in other sites like mammary gland, placenta, adipose tissue, and EBV-transformed lymphocytes, thereby supporting an *in vivo* role for hGHR-tr. This hypothesis is further reinforced by the physical similarity between the soluble GHBP encoded by hGHR-tr expressed in CHO cells and the native serum hGHBP, as determined by gel filtration. It is however difficult to evaluate the contribution of hGHR-tr transcripts to overall expression of hGHBP since other cell type(s) not yet studied might be major sites of hGHR-tr production and other unidentified mechanisms could contribute to the accumulation of the circulating protein *in vivo*. Surprisingly, depending on the cell type used in the transfection experiments, the molecular mass of the released GHBP was different, with a lower value when expression was performed in COS-7 cells. One possible explanation is that post-translational modifications may be incomplete in this high-level expression system.

Another alternatively spliced GHR transcript has been described in humans; it consists of an mRNA species that lacks exon 3 (hGHRd3), which encodes a short peptide fragment of the exoplasmic domain (28, 32, 41–43) whose functional significance is unknown. By contrast, our study points to functionally distinct properties of hGHR and hGHR-tr isoforms. Possible linkage between these two splicing events remains to be investigated, bearing in mind that expression of the hGHRd3 isoform is probably regulated individually rather than tissue-specifically or developmentally (44). Finally, an analogue of hGHR-tr was detected not only in rabbits but also in rats and mice, whereas no analogue of murine GHR mRNA encoding a soluble receptor has been described in humans or rabbits. Interspecies differences in the expression of alternative forms have been found with other members of the cytokine receptor superfamily, including receptors for granulocyte colony-stimulating factor (12, 45), granulocyte–macrophage colony-stimulating factor (α chain) (11, 46), IL-5 (α chain) (8, 47) and c-mpl (15, 48), but their physiological role is unclear. In the case of GHR, such discrepancies may reflect species differences in the regulation of GHBP production. The study of potential modulators of the shedding reaction, and hybrid constructs, should provide insight into these regulatory phenomena.

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