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A Membrane-Fixed, Truncated Isoform of the Human Growth Hormone Receptor*

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ABSTRACT

Previously, we reported the identification of a new human GH receptor (hGHR) messenger RNA species that encodes a smaller hGHR isoform, termed hGHRtr. Its messenger RNA is expressed in several human tissues and predicts a severely truncated GHR protein that lacks 97.5% of the intracellular domain. Because these two hGHR isoforms, which display similar binding affinity, are coexpressed in several tissues, they may reside side by side and, therefore, interrelate. To further characterize the biological properties of hGHRtr in comparison with hGHR, we generated Chinese hamster ovary (CHO) cell lines stably expressing each of these hGHR isoforms. Cross-linking of [^{125}I]hGH to CHO/hGHRtr cells revealed a majored specific complex with apparent Mr of ~100 kDa, which would indicate the hGHRtr to be in molecular mass form of about 80 kDa. When compared with CHO/hGHR, CHO/hGHRtr cells secreted higher amounts of soluble GH-binding protein (GHBP). In contrast to CHO/hGHR cells, CHO/hGHRtr cells did not exhibit any GH-induced receptor down-regulation, and internalization was markedly reduced. Analysis of the constitutive turnover of cellular hGHR and soluble GHBP showed that incubation of CHO/hGHR cells with cycloheximide caused parallel disappearance of hGHR and GHBP. This contrasted with the stability of GHRtr, which showed no decline after cycloheximide treatment for up to 4 h, suggesting that the bulk GHRtr and GHBP may be derived from preformed proteins. Thus, in contrast to hGHR, hGHRtr is fixed at the cell membrane; it undergoes minimal internalization, no down-regulation by hGH, no constitutive turnover for as long as 4 h, but increased capacity to generate a soluble GHBP. Because hGHRtr failed to undergo ligand-induced internalization, the source of the continuous, undisturbed GHBP released into the medium may be from an intracellular storage pool. The relative abundance of these two hGHR isoforms, through regulation of splicing, could be of critical importance in modulating the biological effects of GH. (*J Clin Endocrinol Metab* **82:** 3813–3817, 1997)

THE GH receptor (GHR) is a single transmembrane polypeptide chain of 620 amino acid residues with a 350-amino acid cytoplasmic portion, a 24-amino acid transmembrane domain, and 246 residues in the exoplasmic domain containing 5 N-linked oligosaccharides (1).

Upon GH binding to cell surface GHR, a single molecule of GH is bound by a dimer of GHR (2), leading to receptor internalization and down-regulation and, finally, processing by one of two pathways: a small rapid, nondegradative pathway, which results in recycling or exocytosis, and a slower, lysosomal degradative pathway (3). Cell-surface GHRs turn over rapidly and constitutively and are not replaced in the absence of protein synthesis (3, 4). In addition, like many cytokine receptors in its family, GHR exists in a soluble form of a GH-binding protein (GHBP) that corresponds to the extracellular domain of GHR (5, 6).

In humans, 2 isoforms of the human GHR (hGHR) messenger RNA (mRNA) have been identified: exon 3 can be either retained or deleted, leading to an in-frame deletion of 22 amino acids within the extracellular domain of the receptor, with no apparent impact on binding isotherms or on signal transduction. (7–9). An alternatively spliced form in the cytoplasmic domain of hGHR (hGHRtr) has recently been described in several human tissues. hGHRtr mRNA is identical in sequence to hGHR, except for a 26-bp deletion, leading to the creation of a stop codon at position 280, thereby truncating 97.5% of the intracellular domain of hGHR (10). As shown by transient expression studies, when compared with hGHR, hGHRtr showed a significantly increased capacity to generate a soluble GHBP (10, 11).

The present study was undertaken to further characterize this new, naturally occurring hGHRtr isoform. To this aim, Chinese hamster ovary (CHO) cells were stably transfected with expression vectors encoding hGHR or hGHRtr and tested for GH-binding properties, GH-mediated internalization, and down-regulation and GHR turnover, in relation to GHBP generation.

Materials and Methods

Construction of CHO cell lines overexpressing hGHR and hGHRtr

To obtain cell lines overexpressing the two GHR constructs, CHO cells were cotransfected with a neomycin resistance plasmid and phGHR or phGHRtr corresponding to the previously described SV40 promoter-based expression vector pECE (12) carrying the full-length hGHR complementary DNA sequence or hGHRtr, respectively (10). Transfections were performed at 60% confluence by the LipofectAmine method (Life Technologies, Grand Island, NY) in Iscove's medium, and selection in

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500 μg/mL G418 was carried out for 2 weeks before subcloning survivors. Stably transfected cells (designated CHO/hGHR and CHO/hGH-Rtr) and nontransfected CHO cells were maintained at 37 C in Ham's F-12 medium, supplemented with 10% FCS (Kibbutz Beth-Haemek, Israel) and processed for mRNA analysis, binding, and cross-linking studies. Nontransfected CHO cells exhibit no GH binding, as previously reported (4).

RNA Isolation and Northern blot analysis

Total cellular RNA was isolated in parallel from nontransfected and GHR-overexpressing CHO cells and transferred by capillary blotting onto Hybond nylon membranes (Amersham, Aylesburg, UK). Filters were hybridized with the hGHR complementary DNA probe labeled by random hexanucleotide priming (Life Technologies), as previously described (13). The relative abundance of RNA in each lane was judged to be similar by comparing ethidium bromide-staining intensity of the ribosomal bands. For further confirmation, the blots were hybridized with a probe for an endogenous housekeeping gene, β-actin (data not shown).

GH-binding assays

Recombinant authentic hGH (a kind gift from Bio-Technology General, Rehovot, Israel) was radiolabeled with [^{125}I]Na (Nuclear Research Center-Negev, Beersheva, Israel) by the Chloramine-T method. The specific activity of [^{125}I]hGH ranged from 70–80 μCi/μg.

Cellular GH binding

Confluent cells were incubated with [^{125}I]hGH (1 ng) in the absence (total binding) or presence (nonspecific binding) of 1 μg hGH in a final vol of 200 μL binding buffer containing 10 mmol/L PO_4 buffer, 1% BSA, and 30 mmol/L $MgCl_2$ (pH 7.4) for 90 min at 30 C or for 20 h at 4 C. After removal of the binding buffer, cell monolayers were washed three times with ice-cold 10 mmol/L PBS, pH 7.4. Cell-bound activity was measured in a multiwell γ-counter. All determinations were carried out in triplicate. Specific binding was expressed as a percentage of the total radioactivity added, and data were normalized to 200 μg cellular protein, determined by the Lowry method. The affinity constant was calculated from incubation with increasing concentrations of unlabeled hGH, according to Scatchard, using the Ligand program (14).

Determination of secreted GHBP

Conditioned media of confluent cells were centrifuged at 3,000 × *g* (20 min, 4 C) to remove cell debris, and the cleared supernatants were concentrated 10-fold by lyophilization. To ascertain removal of all cell debris, medium that was ultracentrifuged at 100,000 × *g* (60 min, 4 C) yielded similar binding results. GHBP released into the medium during incubation was measured by specific binding of [^{125}I]hGH, as previously described (4). Briefly, binding of [^{125}I]hGH (1 ng) was performed in a final vol of 270 μL binding buffer, with or without 1 μg hGH for 20 h at 4 C. Free and bound [^{125}I]hGH were separated by adding 1 mL dextran-coated charcoal (0.2% Dextran T-70 and 2% Norit-A charcoal) in 10 mmol/L PO_4 buffer, pH 7.4. After incubation on ice and centrifugation, the radioactivity was measured in the supernatant. Specific binding was expressed as a percentage of the total radioactivity incubated, and data were normalized to 200 μg cellular protein, so that cellular GHR and medium GHBP for each well were jointly normalized.

GH internalization and down-regulation

Surface-bound radiolabeled ligand was differentiated from internalized ligand using an acid extraction procedure (15). Briefly, after washing with PBS, cell surface-bound radioactivity was removed by incubation of cells with 500 μL 10 mmol/L PO_4 containing 50 mmol/L HCl, pH 3, for 1 min at 4 C. The fraction containing the internalized, acid-resistant ligand was lysed with 0.1% SDS and counted in a multiwell γ-counter. Internalized GHR was calculated as a percentage of the total cell-associated radioactivity. Cell surface receptor was estimated by incubation of cells at 4 C for 20 h with [^{125}I]hGH. Down-regulation was measured by incubating cells with different concentrations of hGH in serum-free medium for 90 min at 30 C. The cells were placed on ice and associated hGH was removed by acid wash, as described (15). Residuals GHRs at the cell surface were measured by [^{125}I]hGH binding for 20 h at 4 C.

Affinity cross-linking

Confluent cells were incubated with [^{125}I]hGH (10 ng), in the absence or presence of 10 μg hGH (nonspecific), at 30 C for 90 min. Covalent cross-linking was achieved by the addition of 1 mmol/L disuccinimidyl suberate (Pierce Chemical Co., Rockford, IL), freshly dissolved in dimethylsulfoxide for 1 h at 4 C. Cells were homogenized in ice-cold 10 mmol/L Tris containing 300 mmol/L sucrose and protease inhibitors, pH 7.4 (homogenization buffer) and centrifuged at 15,000 × *g* for 5 min. The protease inhibitors used were 1 mmol/L ethylenediamine tetraacetate, 3.2 μmol/L aprotinin, 2 mmol/L phenylmethylsulfonyfluoride, 10 μg/mL leupeptin, and 10 mmol/L benzamidine (Sigma Chemical Co. St. Louis, MO). Samples were dissolved in an equal volume of 2-fold concentrated Laemmli sample buffer, boiled for 3 min, and equal amounts of protein were subjected to 10% SDS-PAGE. After drying, autoradiography was performed using Kodak X-omat AR film (Sigma Chemical Co).

Statistical analysis

Experiments were repeated at least three times, and the data were analyzed by ANOVA, followed by Student's *t* test.

Results

Affinity cross-linking

Cross-linking of [^{125}I]hGH to CHO/hGHR and CHO/hGHRtr cells is shown in Fig. 1. In both cases, specific complexes were displaced in the presence of an excess unlabeled hGH. For the full-length hGHR, we observed two cross-linked specific bands with apparent Mr of 150 kDa and 120 kDa. Subtraction of the molecular mass of hGH yields Mr values of ~130 kDa and ~100 kDa, that may represent the native hGHR and a degradation fragment or a GHBP dimer, respectively. CHO cells expressing hGHRtr revealed a majored specific complex with an apparent Mr value of ~100 kDa, which would indicate the hGHRtr to be, in molecular mass form, about 80 kDa, after accounting for the hormone component. The larger band of about 170 kDa could represent a dimeric form of the receptor.

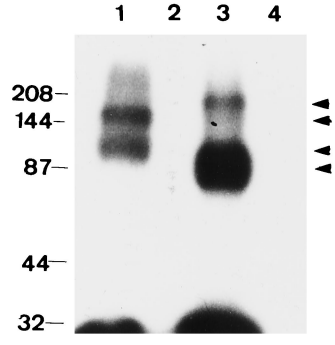

FIG. 1. Affinity cross-linking demonstration of cellular GHR. Confluent CHO/hGHR (lanes 1 and 2) and CHO/hGHRtr (lanes 3 and 4) cells were incubated with [^{125}I]-hGH (10 ng) for 20 h at 4 C without (lanes 1 and 3) or with (lanes 2 and 4) excess of hGH (10 μg). The cross-linked receptors were analyzed by 10% SDS-PAGE and autoradiographed. The positions of the molecular mass markers (× 10^3) are shown on the *left*, and the specific bands are indicated by *arrows* on the *right*.

GHR and GHBP levels

Fig. 2 shows the levels of cellular GHR and secreted soluble GHBP in CHO cells transfected with hGHR or hGHRtr. The amount of secreted GHBP by CHO/hGHRtr cells was significantly higher than that generated by CHO/hGHR (2.8 ± 0.1-fold and 4.2 ± 0.7-fold for 1 h and 4 h, respectively). Scatchard analysis revealed a single-component, high-affinity constant of 8.3×10^9 mol/L^{-1} and 4.3×10^9 mol/L^{-1} for hGHR and hGHRtr, respectively.

GH internalization

We examined the ability of CHO/hGHRtr cells to internalize [^{125}I]hGH, compared with the full-length hGHR. CHO/hGHR showed rapid internalization of [^{125}I]hGH, which reached an apparent equilibrium within 30 min, when around 60% of the total specific binding was localized intracellularly (Fig. 3). In contrast, in CHO/hGHRtr cells, hGH internalization was markedly reduced with only ~10% of the total specifically bound hGH internalized by 1 h.

GH-induced receptor down-regulation

In addition to internalization studies, we further examined GH-induced down-regulation of hGHRtr, compared with hGHR. As shown in Fig. 4, CHO/hGHR cells showed a dose-dependent down-regulation of GHR. After 90 min incubation with 100 and 5,000 ng/mL hGH, followed by an acid wash to expose occupied binding sites, ~50% and ~70%, respectively, of specific GHRs were down regulated. In contrast, the apparent up-regulation of hGHRtr, observed with increasing hGH concentrations up to 1,000–5,000 ng/mL ($P < 0.05$ *vs.* control) and declined with 50,000 ng/mL. The control experiment, in the absence of an acid wash, verifies an initial binding to cell surface receptors of both cell lines.

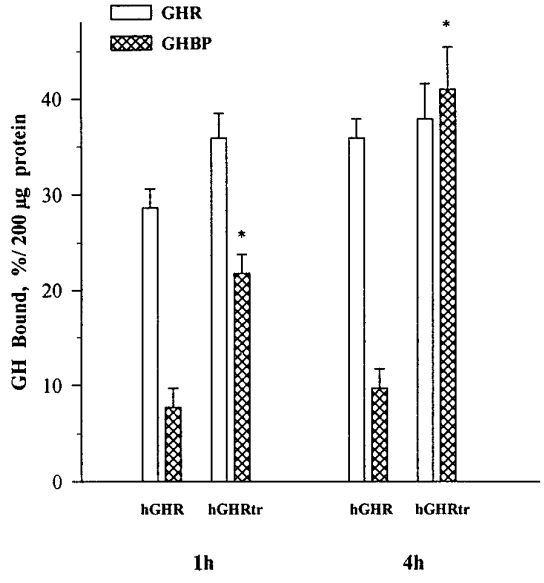

FIG. 2. [^{125}I]hGH binding to cellular GHR and soluble GHBP. GHR and GHBP levels were determined in CHO/hGHR and CHO/hGHRtr confluent cells and media, after incubation with Ham's F-12 medium containing 10% FCS, for 1 or 4 h. Results are expressed as a percentage of specific binding per 200 μg cellular protein. Data are shown as the mean ± SE of triplicate determination. *, $P < 0.05$ *vs.* hGHR.

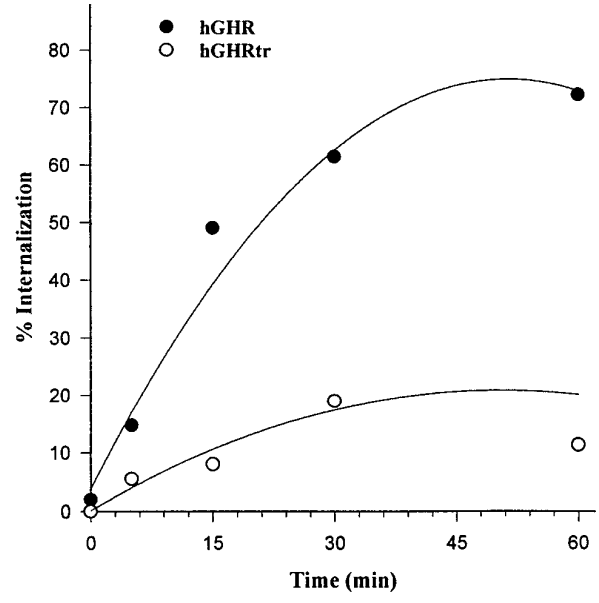

FIG. 3. GH internalization. Confluent CHO/hGHR and CHO/hGHRtr cells were incubated for 90 min at 30 C, and [^{125}I]hGH binding was determined. Data are expressed as percentage of the total specific cell-associated binding from a representative experiment that was repeated 2 more times.

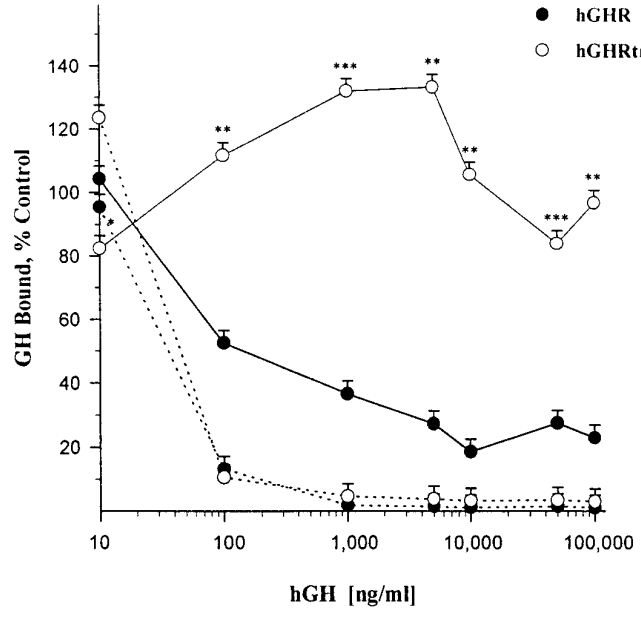

FIG. 4. Down-regulation of hGHR. Confluent CHO/hGHR and CHO/hGHRtr cells were incubated with the indicated concentrations of hGH for 90 min at 30 C, and GHR down-regulation was measured with (---) or without (—) acid wash. Results are expressed as the mean ± SE of triplicate determination from a representative experiment that was repeated 2 more times. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$ *vs.* hGHR.

Turnover of GHR and GHBP

Analysis of the turnover of cellular GHR and soluble GHBP in CHO/hGHR and CHO/hGHRtr cells was per-

formed after inhibition of protein synthesis by cycloheximide. Confluent cells were incubated with cycloheximide (20 μg/mL) for 1–4 h before measuring [^{125}I]hGH binding to cells (Fig. 5A) and medium (Fig. 5B). In CHO/hGHR cells, cycloheximide caused a time-dependent decrease in GHR and GHBP, with a half-time of about 2 h and 3 h, respectively. In contrast, in CHO/hGHRtr cells, cycloheximide had no effect on GHR and GHBP levels.

Discussion

GH binding to its cell surface receptor is followed by a sequence of events including dimerization, internalization, and receptor down-regulation and, subsequently, transportation into a degradative or nondegradative recycling pathway (2, 3). Alongside, it is assumed that GHBP is generated by proteolytic cleavage of GHR. This model of receptor processing is now revisited in the light of the present study, which focuses on the turnover of a newly described hGHR isoform (hGHRtr). hGHRtr mRNA is expressed in several human tissues and is identical in sequence to the full-length hGHR mRNA, except for a 26-bp deletion, leading to a stop codon at position 280, thereby truncating 97.5% of the intracellular domain of hGHR (Fig. 6).

Results obtained in this study, using CHO cells stably expressing the full- length hGHR or with hGHRtr isoform, indicate that in contrast to hGHR, hGHRtr is relatively fixed at the cell membrane. First, hGHRtr undergoes minimal internalization. This feature of the naturally occurring GHR isoform is consistent with several data generated by *in vitro* mutagenesis of rat GHR that demonstrated the structural importance of the cytoplasmic domain for internalization (16–18). Second, hGHRtr shows no down-regulation, in spite of GH concentration rising up to 1,000-fold of that active for hGHR. Third, this truncated receptor undergoes no constitutive turnover, for as long as 4 h after inhibition of protein synthesis. However, when compared with hGHR, hGHRtr showed a significantly increased capacity to generate a soluble GHBP, as described recently in COS-7 cells (10) and in 293 cells (11). Thus, we show, for the first time in the same model system of CHO/GHRtr cells, that failure to internalize the receptor is associated with increased GHBP generation.

The present study shows that hGHRtr misses completely both constitutive and ligand-regulated turnover. Cell-surface GHRs turn over rapidly, under both ligand-regulation and constitutively in various cell types, and are not replaced after blocking of protein synthesis (3, 4). Consistent with our earlier observations (4) and with the present results, incubation of CHO/hGHR cells with cycloheximide caused a parallel disappearance of GHR and GHBP, with t1/2 values that are comparable with those reported earlier (3, 4). This turnover time is in marked contrast to the relative stability of hGHRtr, which shows no measurable decline after cycloheximide treatment up to a 4-h test period, suggesting that the bulk GHR and GHBP may be derived from preformed proteins. For the rat GHR, it has been shown that the domain(s) involved in GH internalization is located in the cytoplasmic domain region between positions 294 and 454 (16). Furthermore, a region of the rat GHR between amino acids 319 and 380 has been reported to be essential for GH-induced internalization, with a single phenylalanine (Phe 346) being critical (17). It was recently shown that ubiquination of the cytoplasmic domain is essential for endocytosis and degradation of the GHR (19), which may also fit with the markedly reduced ability of GH internalization of hGHRtr.

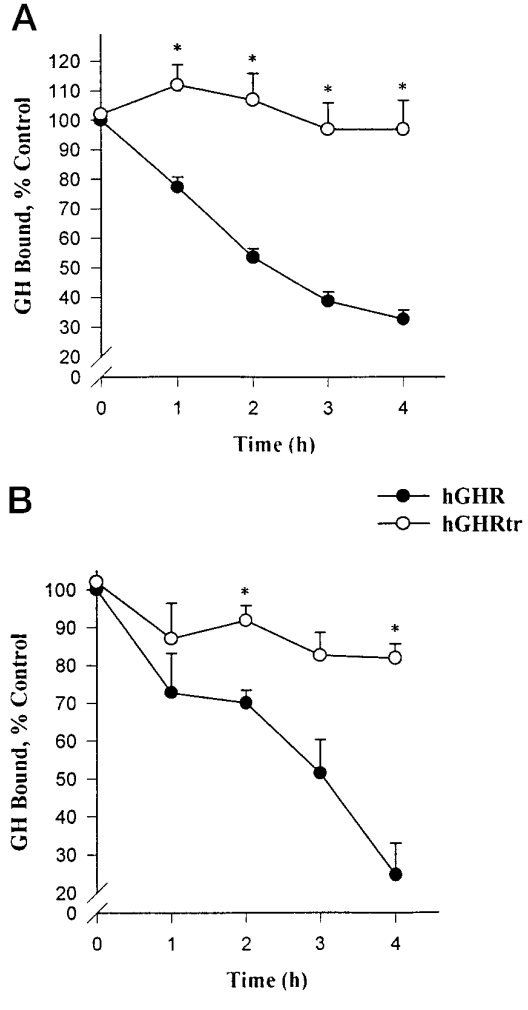

FIG. 5. Turnover of cellular GHR and soluble GHBP. Confluent CHO/hGHR and CHO/hGHRtr cells were incubated in the absence (control) or presence of 20 μg/mL cycloheximide for 1–4 h, and specific binding of [^{125}I]hGH to cells (A) and media (B) was determined. Results are expressed as a percentage of the value in control untreated cells, and data represent the mean ± SE from a representative experiment that was repeated two more times. *, $P < 0.05$ *vs.* hGHR.

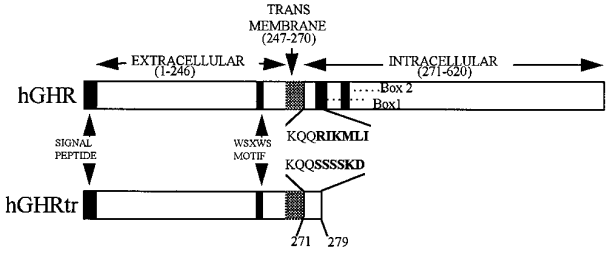

FIG. 6. Schematic representation of hGHR and the alternatively spliced truncated hGHRtr. Frame-shifting by 26-bp deletion results in a novel translation, 6 intracellular amino acids, and an early termination, truncating 97.5% of the intracellular sequence. Box 1 is interrupted and Box 2 is deleted.

We have previously reported on the synchronization of GHR turnover with GH pulsatility (20). hGHRtr adds a new dimension to this concept. Whereas hGHR (in response to a hGH pulse) internalizes, degrades, or recycles and restores itself at the cell surface (to receive the next GH pulse), hGHRt would remain fixed at the cell membrane, either occupied with a preload of hGH, guarding leftover ligand for a next hGHR emergence, or receiving a ligand during the refractory period.

Thus, the intracellular processing of hGHRtr is significantly different from the usual model suggested for cellular processing of GH and GHR, in which, upon GH binding, the [GH-GHR] complexes are rapidly internalized, the majority of the internalized GH and GHR are degraded, and rapid receptor synthesis is required to maintain GH binding capacity of the cell (21). Moreover, because the hGHRtr failed to undergo ligand-induced internalization, the source of the continuous, undisturbed, soluble GHBP released into the medium may be from an intracellular storage pool. This is consistent with the observation that in CHO/hGHRtr cells, cycloheximide did not affect GHR and GHBP levels, and with earlier findings suggesting that cycloheximide does not interfere with externalization of receptors from the intracellular compartment (22). The receptor expressed on the cell surface may not be the source of the GHBP released through the action of an exoprotease, but rather, GHBP may originate from within the cell of a receptor protein that perhaps is generated at an early posttranslational state.

The present results suggest that internalization/down-regulation are not required for GHBP generation and that these may be inversely related processes. It is speculated that because internalization is a path for GHR degradation, it may result in rapid receptor clearance, leading to cellular desensitization (23). The absence of this attenuation mechanism in CHO/hGHRtr cells could be compensated for by the enhancement of GHBP generation. Indeed, GHBP competes with GHR for the ligand, which results in an inhibition of receptor binding and consequent GH action *in vitro* (24, 25).

The existence of hGHRtr (a naturally occurring isoform of hGHR that fails to internalize and to undergo GH-induced down-regulation, but fully retains the capacity to generate GHBP) may reflect the different functions associated with the relative abundance of hGHR and hGHRtr in different cell types. The relative abundance of hGHR and hGHRtr differ among tissues (10), implying a regulated process. Under such conditions, alternative splicing may be regulated to favor one of these species over the other. GHBP generation may be favored in conditions when alternative splicing deviates towards hGHRtr. Interestingly, hGHRtr mRNA is favorably expressed in placenta and mammary gland (10), where estrogen activity is obvious, and indeed, estrogens have been suggested to increase serum GHBP (26). This principle may apply to other regulators of GHR, such as GH itself. *In vitro* studies performed in 293 cells have suggested that heterodimerization of the two isoforms could result in eventual negative regulation of GHR by GHRtr (11). The relative abundance of the two hGHR isoforms, which, as documented in the present study, is regulated at the plasma membrane, could be of critical importance in modulating the biological effects of GH.

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