The D152H mutation found in growth hormone insensitivity syndrome impairs expression and function of human growth hormone receptor but is silent in rat receptor

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ABSTRACT

In two patients with growth hormone (GH) insensitivity syndrome (Laron syndrome), in whom the GH receptor is able to bind the hormone, the D152H mutation was identified, and lack of dimerization was proposed to explain GH resistance in these patients. To examine further the consequences of the substitution of conserved aspartate 152 on the function of the GH receptor (GHR), we reproduced the mutation *in vitro* on the full length GH receptor cDNA from man and rat. Effects of the mutation on expression and activity of the GHR were analyzed in 293 cells transfected with wild-type and mutant GHR cDNAs. Mutant human receptor protein was expressed at a lower level than wild-type receptor and its activity was reduced: GH-dependent signal transducer and activator of transcription 5 (Stat5)-mediated transactivation of a reporter gene was lower in 293 cells transfected with mutant GHR cDNA than in transfected cells expressing a comparable level of wild-type GHR. The membrane-bound form of the mutant and of the wild-type human GHR were able

to homodimerize, as suggested by the size of the complexes detected in cross-linking experiments with ¹²⁵I-human (h) GH, and also by the activity in the functional test. With the soluble GHR resulting from proteolysis of the wild-type membrane form, no dimeric complexes could be detected. However, when a soluble receptor lacking the transmembrane and cytoplasmic domains of the receptor was expressed, wild-type and not mutant GH binding protein (GHBP) was able to form dimers in the presence of hGH. The amino acid substitution has no effect on either expression or function of the rat receptor. Structural modeling of D152H soluble human and rat GHR (GHBP) supports the species-specific functional consequences of the mutation. Evaluation of the functional importance of the mutation strongly suggests that impairment in expression and activity of the mutant receptor, rather than complete lack of dimerization, explains the GH resistance of the patients.

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INTRODUCTION

The growth hormone (GH) receptor (GHR) belongs to the superfamily of cytokine receptors (Bazan 1990). In addition to the membrane-bound receptor, a GH binding protein (GHBP) has been characterized in the serum of man (Baumann *et al.* 1986) and of many other species, including rabbit (Ymer & Herington 1985), rat (Baumbach *et al.*

1989), and mouse (Peeters & Friesen 1977). The amino acid sequence of the GHBP is identical to that of the extracellular domain of the membrane-bound GH receptor (Leung *et al.* 1987). In rat and mouse, GHR and GHBP are encoded by two distinct mRNAs generated by alternative splicing of a single primary transcript (Baumbach *et al.* 1989, Smith *et al.* 1989). In tissues of other mammals, including man, only one mRNA has been identified

by Northern blot analyses (Delehay-Zervas *et al.* 1994) and it has been suggested that, in these species, GHBP is mainly produced by proteolysis of the membrane GHR (Spencer *et al.* 1988, Sotiropoulos *et al.* 1993).

Structural, mutational, and functional studies have provided evidence that ligand-induced dimerization of receptors of this family is a crucial step in receptor activation leading to signal transduction (Heldin 1995). High resolution mutational analysis of the human GHR and X-ray crystallographic studies have shown that the GH-GHR complex is a homodimer consisting of one molecule of hormone interacting with two receptors (Cunningham *et al.* 1991, De Vos *et al.* 1991, Fuh *et al.* 1992).

Laron syndrome is a rare genetic disease with complete GH resistance due to molecular defects in the GHR gene (Laron *et al.* 1966, Berg *et al.* 1993). In most cases, GH binding activity of binding protein (BP)/receptor is undetectable and this is consistent with a defect in the extracellular domain of the GHR, which results in impaired hormone binding (Daughaday & Trivedi 1987). However, recent reports have described patients with positive GHBP (Buchanan *et al.* 1991, Savage *et al.* 1993). In two such patients, the substitution of aspartate by histidine at position 152 in the extracellular domain of the GHR was demonstrated (Duquesnoy *et al.* 1994). This group reproduced the mutation in human GHR cDNA which was transiently transfected in COS cells, and they also engineered a soluble GHR protein. While they reported normal binding characteristics for the membrane-bound mutant receptor, their results, based on chromatographic analyses, suggested that the recombinant soluble mutant receptor was unable to dimerize.

Our goal was to characterize better the functional consequences induced by substitution of the conserved aspartate residue by histidine at position 152 (D152H), and to do the study using human (h) and rat (r) GHRs. We compared the expression of both the membrane-bound form and the soluble receptor in cells transfected with wild-type or mutant GHR cDNAs. A functional test to measure activity of the receptor forms using the activation of signal transducer and activator of transcription 5 (Stat5) by GHR has been reported (Wood *et al.* 1995). Wild-type or the mutant GHR cDNA, and a reporter gene containing DNA binding element for Stat5 were used to cotransfect 293 cells. Our results show that the amino acid substitution in human GHR results in partial defects of expression, dimerization, and function of the receptor, the combination of which could explain the GH resistance in the patients. In addition, although the

aspartate residue is conserved among species, the functional consequences of its substitution vary, since expression and activity of D152H rat GHR are not altered.

Moreover, three dimensional models have been constructed for wild-type and mutant forms of human and rat receptors. To understand further the effects of point mutations, trajectories of molecular dynamics have been computed and compared between mutant and wild-type forms of the receptors.

MATERIALS AND METHODS

Construction of expression plasmids and mutagenesis

The full length hGHR cDNA coding sequence was obtained from W Wood (Genentech) (Leung *et al.* 1987). The cDNA was subcloned into BamHI-SnaBI sites of pcDNA1-derived mammalian expression vector that contains a cytomegalovirus promoter, and the bacteriophage f1 intergenic region. Rat GHR cDNA (a gift from G Norstedt) was subcloned into pcDNA3 vector. Single-stranded DNA from pcDNA1 and pcDNA3 was isolated. Oligonucleotides containing the G→C transversion, resulting in the substitution of aspartate in histidine at amino acid 152, were used to mutagenize the receptor cDNA. The resulting mutation was confirmed by DNA sequencing.

Site-directed mutagenesis was performed to produce soluble wild-type hGHR (wt sGHBP) and soluble D152H hGHR (D152H sGHBP) encoding the corresponding soluble secreted forms of the receptor using oligonucleotide 5-CTTCTTCAC ATGTTTATTGGCTCATC-3 which introduced a translation stop codon at the amino acid 239 of the hGHR cDNA sequence.

Cell culture and transfection

Dulbecco's modified Eagle's medium (DMEM nut F12) containing 10% fetal calf serum (FCS), glutamine, and antibiotics ('growth medium') was used to grow 293 cells to confluence at 37 °C in a humidified atmosphere of 5% CO₂. Three hours prior to transfection, the cells were plated in 'rich medium' (2/3 DMEM nut F12, 1/3 DMEM with 4.5 g/l glucose, 10% FCS, glutamine, and antibiotics) in six-well plates. Transfections were performed using the calcium phosphate method: the DNA solution (plasmid, 0.25 M CaCl₂) was mixed with BES (N,N-bis [2-hydroxyethyl]-2-aminoethanesulfonic acid)-buffered saline (50 mM BES, 280 mM NaCl, 1.5 mM Na₂HPO₄), pH 6.9.

The mix was added to each well and then the cells were incubated for 15–20 h at 37 °C in 3% CO₂, and replaced in 5% CO₂ 24 h prior to assay.

Binding assays

¹²⁵I-hGH was prepared using Chloramine-T; its specific activity ranged from 80 to 120 µCi/µg. Twenty-four hours after transfection, the cells were serum-starved for 12 h. The culture medium was removed and concentrated (20-fold) to be analyzed for the presence of GHBP. Cells were washed with PBS containing 1% BSA and incubated with ¹²⁵I-hGH (10⁵ c.p.m./well) for 3 h at room temperature, in the absence or presence of various concentrations of unlabeled hGH. The cells were then washed with the same buffer and solubilized in 1 ml NaOH (1 M) for counting.

GHBP was assayed in culture media using HPLC gel filtration (Tar *et al.* 1990). Twentyfold concentrated supernatants containing either the wild-type or the D152H mutant soluble GHR were incubated with ¹²⁵I-labeled hGH (1.5 × 10⁵ c.p.m.) in 0.1 M phosphate buffer (pH 7.4). After 20 h at 4 °C, the reaction products were analyzed by gel filtration on an HPLC Protein Pak 300 SW column (Waters, Milford, MA, USA). Elution was performed with degassed buffer (0.1 M Na₂SO₄, 0.1 M K₂HPO₄, pH 7.0) pumped at a rate of 0.5 ml/min. Radioactivity was recorded on line using a Berthold LB2040 detector (Berthold, Elancourt, France) connected to a Compaq computer. Scatchard analyses were performed with the program Ligand (Munson & Rodbard 1980).

Membrane fractions of cells transfected with 5 µg plasmid were prepared by differential centrifugation. Membrane protein (300 µg) was incubated with ¹²⁵I-hGH (1.5 × 10⁵ c.p.m.) as described in Dinerstein *et al.* (1995). Bound and free hormones were separated by centrifugation at 17 000 g for 15 min, and the radioactivity in the pellet was counted.

Cross-linking studies

Cells were grown in six-well plates and transfected with 5 µg plasmid containing the wild-type or the mutant cDNA. ¹²⁵I-hGH (5 × 10⁵ c.p.m./well) was added to the dishes in the absence or presence of 5 µg/ml unlabeled hGH, and incubated for 30 min at 37 °C. Cells were then washed with PBS, and 2 ml PBS were added to each well. Di-succinimidylsuberate (0.5 mM) was added to the cells and incubated for 20 min at room temperature. The reaction was stopped with 2 ml 50 mM Tris, 150 mM NaCl. Electrophoresis was

performed on an 8% SDS polyacrylamide gel. The gel was dried and exposed to X-ray film (Kodak, France).

Transcription assays

We prepared the fusion gene consisting of lactogenic hormone response element (LHRE) linked to the luciferase reporter gene. It was constructed using a pUC18 vector containing the thymidine kinase (TK) minimal promoter linked to luciferase reporter gene (TK-luc) subcloned into the BamHI site. Double stranded oligonucleotides, 5-CTG CAGTGTGGACTTCTTGGGAATTAAGGGAC TTTTGCTGCAG-3 for LHRE (six copies) were inserted into the PstI site of the vector. 293 cells were plated in 6-well plates at 0.4 × 10⁶ cells per well before being transfected with 0.001 to 0.1 µg expression vector containing the different mutant forms of GHR, 1.5 µg LHRE/TK-luciferase reporter gene and 3 µg pCH110 (β-galactosidase expression vector; Pharmacia, Bromma, Sweden). Cells were incubated for 24 h with serum-free medium containing 20 nM hGH and 250 mM dexamethasone or 250 mM dexamethasone alone. Cells were lysed as described previously (Dinerstein *et al.* 1995) and whole cell extracts were used for determination of luciferase and β-galactosidase activities. Luciferase activity was normalized to β-galactosidase activity. Fold induction of luciferase activity (in the presence of hormone) was calculated from the basal level (in the absence of hormone).

Molecular modeling

Four GH-(GHBP)₂ molecular models were built in order to study structural effects of the D152H mutation in human and rat receptors. The common pattern used for molecular modeling was the human wild-type complex hGH-(hGHBP)₂ as described by De Vos *et al.* in 1991 and revised in the Protein Data Bank (Bernstein *et al.* 1977) in 1994 (PDB:3HHR). The human D152H mutant complex, in which each receptor bears the D152H mutation, was constructed using InsightII (Biosym, San Diego, CA, USA) to perform point mutations. As extracellular sequences of hGHR and rGHR are 70% identical, the ExpASy (Peitsch 1995) automatic modeling server was used to build a hGH-(rGHBP)₂ complex based on the human complex. Then a D152H mutant rat complex was built. All these complexes were protonated and energy minimized (1000 steps) *in vacuo* using the CHARMM (Brooks *et al.* 1996) force field (X-Plor program, Brünger *et al.* 1989).

Extracellular C-terminal domains of receptors were solvated in a box containing 2200 water molecules at 300 K, corresponding to two layers. As the hormone binding activity of these receptors is normal, the hormone and the N-terminal domains are kept as a referential: these regions were not solvated and were constrained (fixed coordinates) during the molecular dynamics. Then structures were energy minimized (2000 steps) using the CHARMM force field and first 100 ps, then 400 ps molecular dynamics were performed. A temperature of 300 K is stable after 20 ps. Final solvated complexes were once again energy minimized (500 steps).

Surface accessibility (S) calculations were performed using the Access program (Richmond & Richards 1978). Interface surface between two A and B proteins was calculated by computing the difference between the sum of the accessibility surface of each protein and the accessibility surface of the complex: $S(\text{interface}) = S(A) + S(B) - S(A+B)$.

The axes of the extracellular C-terminal domains of GHR have been approximated by defining 'slices' and calculating the center of gravity for each slice. Choice of these slices was determined according to the general orientation of domains in the human GH-(GHBP)₂ complex. Four slices were defined for each domain, and for each one, a peripheric atom was chosen to detect rotations: it corresponds to α carbons of residues 145, 161, 191 and 199.

RESULTS

Expression of GH receptors in 293 cells

Expression of the wild-type (wt) GH receptors (wt GHRs) and of the mutant D152H GHRs was first examined by measuring hGH binding in transiently transfected 293 cells. Receptor expression as a function of the quantity of cDNA transfected in 293 cells is shown in Fig. 1. At all amounts of plasmid transfected, specific binding of ¹²⁵I-hGH to cells expressing mutant human GHR (hGHR) was much lower than binding to cells expressing wt hGHR, the defect being less severe when more than 1 µg plasmid was used in transfections. Scatchard analyses from competition experiments were carried out with cells transfected with 5 µg plasmid encoding the hGHR: the number of sites per cell transfected with the vector coding for wt hGHR was twofold higher than in cells transfected with the vector coding for the mutant hGHR. The dissociation constant (K_d) ranged from 0.92 to 1.90 nM, with a small 1.7-fold difference in affinity between the wt hGHR and D152H hGHR (Table 1).

FIGURE 1. Specific binding of ¹²⁵I-hGH to wt and D152H hGHRs as a function of increasing amount of cDNA transfected. Transiently transfected 293 cells were incubated with ¹²⁵I-hGH (10⁵ c.p.m.) in the absence or presence of unlabeled hGH (2 µg). The specific binding is expressed as the percentage of total radioactivity. Each value represents the mean ± s.e.m. of triplicate measurements from one of three independent experiments. No specific hGH binding was detected in cells transfected with 0.05 µg plasmid of D152H hGHR cDNA. * $P < 0.05$ compared with wt hGHR.

Comparable results were obtained in ten independent experiments and with different plasmid preparations. Specific binding of ¹²⁵I-hGH to membranes of transiently transfected (with 5 µg plasmid) 293 cells confirmed the lower binding in membranes of cells expressing the mutant human receptor and suggested absence of abnormal receptor cellular trafficking for the mutant receptor (not shown).

There was no significant difference in GH binding affinity in 293 cells transfected with plasmid encoding wt or mutant rat GHR (Table 1). The number of binding sites was lower in cells expressing the D152H rat GHR.

Soluble forms of GHR in the culture medium of transfected 293 cells

GHBP of non murine species are thought to be produced by proteolysis of the membrane-bound GH receptor. We analyzed culture media from 293 cells transfected with full length cDNA encoding either wt (thick line) or D152H mutant form of hGHR (thin line) (Fig. 2A). Soluble GH receptors, generated by proteolysis were produced in the two culture media and superimposable elution profiles were observed. Two peaks were detected, the second representing free ¹²⁵I-hGH (Fig. 2A). The

TABLE 1. Characteristics of hGH binding to 293 cells transfected with different forms of the GH receptor. 293 cells were transfected with 5 µg cDNA of the different receptor forms. Binding affinities (K_d) and number of sites per cell have been calculated from Scatchard plots obtained from competition experiments. Mean $\pm$ S.E.M. of the results obtained in three experiments are presented

	K_d (nM)	Sites/cell
wt hGHR	1.01 $\pm$ 0.09	333 500 $\pm$ 39 500
D152H hGHR	1.70 $\pm$ 0.20	186 000 $\pm$ 9000
wt ratGHR	1.29 $\pm$ 0.15	363 000 $\pm$ 5000
D152H rat GHR	1.11 $\pm$ 0.05	277 500 $\pm$ 24 500

elution times of the peak corresponding to wt GHBP and to mutant GHBP are very close: 17 min 12 s and 17 min 16 s respectively. We never observed high molecular weight radioactive peaks representing potential receptor dimers in elution profiles obtained with media containing wt or mutant GHBP whatever the amount of concentrated media used in the experiments (data not shown). Binding characteristics of wt and mutant GHBP were also analyzed by Scatchard plots obtained from competition experiments. Comparable dissociation constants (4.5 nM for the wt GHBP and 5.0 nM for the mutant soluble receptor), similar to values reported for human plasma GHBP (Tar *et al.* 1990) were found. However, the maximum binding capacity was twofold lower for the mutant GHBP (2.6 nM) compared with the wt GHBP (5.5 nM). No GH binding activity was detected in the media of untransfected 293 cells.

Different results were obtained when media containing an artificially secreted soluble GHBP were studied. A stop codon was introduced at position 239 to generate a GHR protein with no transmembrane domain, as previously performed by Duquesnoy *et al.* (1994). Elution profiles are shown in Fig. 2B: under such conditions, a difference in the elution times of the complexes of 125 I-hGH with the wt and the mutant extracellular domain of GHR is observed. With medium containing the extracellular domain of GHR, the radioactive complex is shifted towards a higher molecular weight, suggesting the presence of receptor dimers (Fig. 2C); there is a difference of 60 s between the elution times of the peaks corresponding to the highest (50 µl) and the lowest (0.5 µl) volume of medium. Elution times obtained with various amounts of media containing the secreted mutant GHBP remain identical whatever the concentration of GHBP used; it corresponds to the elution time expected for a complex consisting of one receptor molecule and one hormone molecule (not shown). Thus, under

these particular experimental conditions, the soluble, secreted mutant GHBP lacks the ability to dimerize.

Cross-linking studies

Cells were transiently transfected with wt or mutant forms of human or rat GHR, and cross-linking of 125 I-hGH to the different GHR forms was performed. The complexes were analyzed by SDS/PAGE and detected by autoradiography (Fig. 3). Two complexes, of approximately 280 and 150 kDa, were revealed for wt and D152H GHR; both are specific since their formation was blocked by an excess of unlabeled hGH. The size of the two complexes suggests that the 150 kDa form represents one hormone molecule bound to one receptor, while the 280 kDa complex could consist of a receptor dimer and one hGH molecule. As a control, cells were also transfected with a cDNA encoding a truncated form of human GHR (GHR₁₋₃₁₇) missing 303 residues in the carboxyterminal region of the cytoplasmic domain; the two complexes obtained were of smaller size compared with those formed with the full length receptor (Fig. 3). The cross-linking studies suggest that both wild-type and mutant GHRs are able to dimerize.

Functional studies of GHR forms

The activation of the reporter gene in cells transfected with wt or D152H GHR cDNA from human (A) and rat (B) is shown in Fig. 4. hGH stimulation of luciferase activity was measured in cells transfected with various quantities of GHR cDNAs. In cells transfected with human GHR cDNAs (wt or mutant), the relative luciferase units measured in stimulated cells and the fold induction increased with the amount of cDNA transfected. In cells transfected with the mutant GHR, the GH effect, as also expressed by the fold induction, was much lower than in cells transfected with wt hGHR cDNA (Fig. 4A).

In contrast to that which we observed for human GHR, the mutation D152H did not alter the activity of rat GHR in the functional test. However, it can be observed in Fig. 4B that the fold induction obtained at 0.1 µg cDNA transfected for both the wt and the mutant rat GHR is lower than the fold induction reached at 0.01 µg cDNA, and that it is due to an increased basal level of luciferase activity; the high basal level could be related, at least in part, to high receptor expression.

The dose-response of hGH on Stat5 transcriptional activity in 293 cells expressing wt or D152H hGHR is shown in Fig. 5. Cells were transfected

with 0.1 µg plasmid of either wt or D152H hGHR or with 1 µg plasmid of D152H hGHR to avoid a low expression of mutant receptors; under these conditions, the specific binding of ¹²⁵I-hGH to the transfected cells was 1.6%, 0.1%, and 3.3% of the radioactivity respectively. GH stimulation of luciferase activity was measured in the three cell cultures. A GH effect was detected with 10 ng/ml GH and reached a maximum at 1 µg/ml (Fig. 5). At higher hormone concentrations, transcriptional activity decreased. A bell-shaped curve was observed under the three transfection conditions, suggesting that dimerization of GH receptors also

FIGURE 3. Autoradiography of cross-linked ¹²⁵I-hGH to GH receptor forms. 293 cells expressing wt or D152H human or rat GHR, and human GHR₁₋₃₁₇ as a control were incubated with ¹²⁵I-hGH (5×10^5 c.p.m.) without or with an excess of unlabeled hGH. Solubilized proteins were analyzed on an 8% SDS-polyacrylamide gel. Numbers on the left indicate molecular mass (kDa) of protein markers.

occurs in cells expressing the mutant receptor. The median effective concentration of stimulation (EC_{50}) of ~30 ng/ml was identical for cells expressing wt or D152H hGHR (Fig. 5). However, luciferase activity measured in cells expressing the mutant hGHR was always lower than in cells expressing the wt hGHR, even though the former cells were transfected with ten times more cDNA and had a twofold higher level of GH binding. For the same amount of GHR cDNA transfected (0.1 µg plasmid/plate), the D152H hGHR was eight times less active on gene transcription than the wt hGHR, as shown in Fig. 5.

FIGURE 2. Elution profiles of ¹²⁵I-hGH incubated with concentrated culture medium of transfected 293 cells. The panels show the elution profiles from HPLC gel-filtration column of ¹²⁵I-hGH incubated with concentrated supernatants of 293 cells expressing (A) the wt proteolyzed form of the GHR (wt GHBP) (thick line) or the D152H GHBP (thin line), and (B) the wt secreted form of the GHR (wt sGHBP) (thick line) or D152H sGHBP (thin line). The elution times of the complexes of wt GHBP, D152H GHBP, wt sGHBP, and D152H sGHBP are estimated at 17 min 16 s, 17 min 12 s, 16 min 42 s, and 17 min 26 s respectively. (C) The same concentration of ¹²⁵I-hGH ($\sim 10^5$ c.p.m.) was incubated with 50, 5, 1, or 0.5 µl medium expressing the wt sGHBP. The complexes elute at different times: the peak is seen at 16 min 15 s for 50 µl medium, at 16 min 41 s for 5 µl medium, at 16 min 51 s for 1 µl medium, and at 17 min 15 s for 0.5 µl medium.

FIGURE 4. Transcriptional activity of GHR forms in the LHRE/TK-luciferase system. 293 cells were transiently co-transfected with various amounts of plasmids encoding either the wild-type or the indicated mutated GHR cDNA, together with the fusion gene LHRE/TK-luciferase. After transfection, cells were incubated in the presence (+) or absence (-) of 20 nM hGH for 24 h. Results are expressed in luciferase units, and also, above the bars, in fold induction of luciferase activity due to GH (see Materials and Methods). Results are shown for cells expressing different forms of human (A) and rat (B) receptors. Each value represents the mean $\pm$ S.E.M. of triplicate measurements.

FIGURE 5. Transcriptional activity induced by hGH in 293 cells transfected with 0.1 µg plasmid/plate of wt hGHR (●), and 0.1 (▲) or 1 (△) µg plasmid/plate of D152H hGHR cDNA. Cells were stimulated in serum-free medium with various concentrations of hGH for 12 h and luciferase units were measured. Values represent the mean of triplicate measurements. One representative experiment out of three is shown.

Effects of the mutation on the GHR dimer structure

Structural analysis based on distance and angular measurements shows five possible hydrogen bonds between the two human wt receptors (Table 2). The two Asp152 residues are involved in three of the five stabilizing interactions between receptors and seem to be crucial for dimerization. These potential hydrogen bonds differ from those found by De Vos *et al.* in 1991, presumably because we used the latest structure available in the Protein Data Bank (1994). Three possible hydrogen bonds were still found after molecular dynamics in the D152H mutant human complex (Table 2). Distance and angular measurements suggest that the D152H mutation in hGHR does not break the dimerization (Fig. 6), although the interface surface is reduced from 480 Å² in the wt to 390 Å² in the mutant receptor.

In the case of the rat receptor, the interface stability does not seem to be affected by the D152H mutation, as can be observed from surface calculations (about 220 Å² for both wt and mutant rat complexes). However, the number of hydrogen bonds between receptors decreased from three in the wt to two in the mutant receptor (Table 2).

Schematic representation of the axes was used to visualize changes induced by the mutation on the relative orientation between C-terminal domains of GHBP in human and rat complexes (Fig. 7). In the human complex, the two mutant GHBP C-terminal

TABLE 2. Hydrogen bonds in intermolecular interface of GH receptor dimers. The possible intermolecular hydrogen bonds between two GHBP molecules and the distance between atoms of residues involved are presented

	GHBP-I atom	GHBP-II atom	Distance (Å)
Human wildtype	O _{δ1} ASP-152	HH TYR-200	1·98
	H _{ε2} HIS-150	O _{δ1} ASN-143	1·99
	HN LEU-146	O _γ SER-201	1·95
	H _{γ1} THR-147	O _{δ2} ASP-152	2·00
	H _γ SER-145	O _{δ1} ASP-152	1·96
Human D152H	O ALA-151	HH TYR-200	1·90
	O _{γ1} THR-147	H _{ε2} HIS-152	1·96
	H _γ SER-145	O _γ SER-201	1·91
	H _γ SER-201	OH TYR-200	1·98
Rat wild type	HN LEU-146	O _γ SER-201	1·98
	H _γ SER-145	O _{δ2} ASP-152	2·03
	O TYR-200	HH TYR-200	1·86
Rat D152H	HN LEU-146	O _γ SER-201	1·96

FIGURE 6. Residues involved in the stabilization of the interface between (A) wt and (B) D152H human receptor complexes. Results of 400 ps molecular dynamics are presented. Only residues of interest are shown in bold lines on the first (GHBP-I) and second (GHBP-II) receptor. Dotted lines represent potential hydrogen bonds between receptors. A, B and E β -strands of the GHBP involved in the dimerization are shown (thin lines).

axes are displaced 1 Å from those of the wt and are closer than in wt by about 1 Å. The most affected regions are those where the Asp152 residues are located, as shown by shaded circles in Fig. 7. To study the effects of these displacements on the interface between receptors, distances between Asp152 on one receptor and the nearest residue on the other were measured; they remain the same in wt and mutant receptors (about 6·5 Å). Indeed the change in relative distance between C-terminal domains of hGHBP induced by the mutation, as measured by the displacement of centers of gravity of slices, is associated to a rotation (Fig. 7) which presumably keeps these two domains at the same distance.

In contrast, in the rat complex both axis displacement and rotation around the axis are absent. Thus the D152H mutation does not affect the mean relative orientation of the extracellular C-terminal domain of each receptor molecule.

DISCUSSION

The first defect of the GH receptor identified in patients with Laron syndrome and normal binding activity of GH receptor/GHBP was the substitution of aspartate 152 by histidine (Duquesnoy *et al.* 1994). In these patients, the molecular defect of the GH receptor results in failure to transduce

FIGURE 7. Schematic representation of the axis of extracellular C-terminal domains of the GHR involved in (A) human and (B) rat complexes. Results of 400 ps molecular dynamics are presented. Centers of gravity of slices chosen (see Materials and Methods) to visualize the axis of GHBP C-terminal domains are shown: G1, G2, G3, G4 for GHBP-I and G'1, G'2, G'3, G'4 for GHBP-II. Bold lines represent axes of wild-type receptors and dotted lines those of D152H mutant receptors. $C\alpha$ points represent α carbons of residues chosen to detect rotations around the axis. Shaded circles represent the position on the axis of Asp152. The D152H mutation makes the two extracellular hGHBP domains closer by 1 Å in the rat receptor, and induces a rotation in the human receptor.

the signal, whereas GH binding affinity is not altered.

GH binding to its receptor induces dimerization of the receptor, and activation of the tyrosine kinase JAK2 (Argetsinger *et al.* 1993) which results in phosphorylation of the kinase, of the receptor, and of a cascade of other cellular proteins including members of the Stat (signal transducers and activators of transcription) protein family (Heldin 1995). The development of functional systems has made possible the evaluation of GH receptor mutants for their ability to transduce hormonal signals (Goujon *et al.* 1994, Sotiropoulos *et al.* 1994). Our goal has been to compare the binding characteristics, expression and function of the D152H and of the wild-type GHR.

Specificity and affinity of the human wild-type GHR expressed in 293 cells were those expected for a human receptor (Hocquette *et al.* 1989). The binding affinity of the D152H receptor was similar to the wild-type GHR, whereas the number of receptors expressed in 293 cells, whatever the amount of cDNA transfected, was lower for the mutant than for the wild-type GHR. The processing of the mutant receptor to the plasma membrane did not seem to be altered since the number of total receptors was decreased.

A soluble form of mutant human receptors, as well as wild-type receptors, was secreted into the culture medium of transfected cells, suggesting that proteolysis of membrane receptors occurred for both forms. The lower concentration of the mutant GHBP in cell medium was probably related to the lower number of mutant membrane receptors. The GH binding affinity of the wild-type and mutant GHBPs was similar to the affinity reported for human plasma GHBP (Tar *et al.* 1990). This result was expected, as the binding activity of the plasma GHBP of the patients was found to be normal (Duquesnoy *et al.* 1994). A decrease in the serum GHBP concentration reflecting a low number of membrane GHRs would be difficult to demonstrate in these patients, considering the wide range of GHBP concentrations found in normal subjects. Moreover, measurement of GH receptor number in accessible cells obtained from the patients is not possible.

Chromatographic profiles of GHBPs generated through proteolysis of mutant and wild-type membrane receptors were similar. We did not observe the presence of two radioactive peaks or a peak eluting at an earlier time, which could have suggested the existence of monomers and dimers of wild-type soluble GHRs, even when high

concentrations of soluble receptors were incubated with ^{125}I -hGH. Duquesnoy *et al.* (1994), who identified this mutation in two patients, proposed that the inactivity of the D152H GHR was due to failure to dimerize. Their conclusion was based on chromatographic studies of soluble proteins engineered by introducing a stop codon at position 239. We also observed that the secreted extracellular domain of the wild-type GHR can bind GH as a monomer or as a dimer. In contrast, the complex of hGH with secreted mutant extracellular domain appears to consist of receptor monomers only whatever the ratio of soluble receptor versus hormone. Soluble GHRs generated by two different mechanisms must behave differently to explain the discrepancy between the results; dimers of the extracellular domain can probably form more easily in secretory vesicles. Taken together, these results indicate that it is only under particular conditions of secretion that a failure to form dimers can be demonstrated for the mutant GHBP.

Ligand-induced receptor dimerization has been reported for many receptors of the superfamily, and appears to be a critical step for signal transduction (Cunningham *et al.* 1991, Ishizaka-Ikeda *et al.* 1993, Heldin 1995). Receptor dimerization has been shown to be necessary for GH effects such as proliferation of promyeloid FDC-P1 cells (Fuh *et al.* 1992), stimulation of lipogenesis in rat adipocytes and of receptor down regulation in IM-9 lymphocytes (Ilondo *et al.* 1994). From cross-linking experiments, we obtained evidence that membrane-bound D152H, as well as wild-type GHR, could form dimers, and that similar sized complexes were observed with wild-type and mutant forms of receptors of the different species.

To evaluate the activity of the various forms of the GHR, we studied GH-induced activation of transcriptional activity mediated by Stat5. GH can activate Stat5 phosphorylation and DNA binding to promoter regions of GH-responsive genes, such as the serine protease inhibitor (Spi 2.1) or the β casein (Sotiropoulos *et al.* 1994, Wood *et al.* 1995). Stat5-mediated transcription was evaluated with multimerized LHRE fused to the luciferase gene; LHRE is present in the promoter of the β casein gene. The fact that the mutant human receptor is able to activate Stat5, as shown in the functional test, implies that the mutant receptor is able to dimerize. It has been shown that, at very high concentrations, hGH is able to antagonize signaling by preventing dimerization (Fuh *et al.* 1992); accordingly, bell-shaped curves were obtained for the dose-responses of GH in the functional test, suggesting that receptor dimerization occurs with the mutant as well as with the wild-type receptor.

The wild-type and the mutant human GHRs were able to activate Stat5; but, under all conditions of cDNA transfected, the relative luciferase activity remained lower in cells transfected with the D152H GHR cDNA than in cells transfected with the wt GHR cDNA. Low expression is not the only explanation for the impaired activity of the mutant receptor: ten times more cDNA was transfected in 293 cells, so that the GH binding level was higher in cells expressing the mutant GHR; however, under these conditions, maximum luciferase activity, attained at 0.1 to 1 $\mu\text{g}/\text{ml}$ GH, remained lower in cells expressing the D152H GHR. The defects of the D152H GHR, including low expression and reduced activity, are more pronounced in cells transfected with small amounts of cDNA, under conditions which are closer to the *in vivo* situation.

In contrast to the results obtained with the mutant human receptor, D152H rat GHR had normal activity in the functional test. Even if the mutant rat receptor was expressed at a somewhat lower level than the wild-type receptor, it did not alter its functional activity. Thus, despite the fact that the mutation is located in a conserved region of the GHR and involves an invariant residue in the protein of the different species, the consequences of the mutation vary.

These observations can be correlated with results of molecular modeling performed on GH-(GHBP)₂ complexes. The D152H mutation affects dimerization by reducing the number of hydrogen bonds between receptors for both human and rat complexes (from 5 to 3 for the human and from 3 to 2 for the rat receptor). It also decreases the interface surface in the case of the human receptor. The fact that the interface surface in wild-type and mutant rat receptors is about half that found in human complexes could be a consequence of the presence in rat receptor of a proline at position 147 instead of a threonine in the human receptor: thus fewer residues are involved in dimerization of the rat receptor.

The substitution of Asp152 by His at the interface between receptors induces local changes: for instance the role of Asp152 seems to be replaced by Ser201 in the mutant human receptor with respect to hydrogen bond formations, but presumably the mutation does not break the dimerization. In the case of the human receptor, the mutation induces long-distance conformational effects, shown by the displacements of the axis of C-terminal GHBP domains and rotations around these axes, which are not evidenced in the rat complex. However, the calculations were only performed on GHBP, without taking into account the anchorage of receptors into the membrane.

The calculations are in agreement with our experimental results. The possible formation of dimers with the mutant human GHR is confirmed; however, decreased stability of the homodimer with the mutant receptor is not excluded, and represents a possible explanation for the lower activity of D152H human receptor. Other hypothetical explanations can be proposed: conformational changes induced by the mutation in the human GHR could affect the interaction of the cytoplasmic domains of the two receptors molecules and/or of JAK2 with the receptor.

In conclusion, the substitution of aspartate 152 by histidine is associated with defects in expression and function of the human GHR. Dimeric complexes with the mutant human receptor can form, but may not be equivalent to wild-type GHR dimers. The extreme GH resistance presented by the patients could result from several combined abnormalities, including low receptor expression and partial activity in signal transduction, rather than complete lack of receptor dimerization. Although the aspartate residue at position 152 is conserved in GHR of different species, the mutation in rat GHR does not affect expression and function of the receptor.

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