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A single amino acid substitution in the exoplasmic domain of the human growth hormone (GH) receptor confers familial GH resistance (Laron syndrome) with positive GH-binding activity by abolishing receptor homodimerization

Philippe Duquesnoy, Marie-Laure Sobrier, Bénédicte Duriez, Florence Dastot, Charles R.Buchanan[1], Martin O.Savage[2], Michael A.Preece[1], Constantin T.Craescu[3], Yves Blouquit, Michel Goossens and Serge Amselem[4]

Laboratoire de Génétique Humaine, Institut National de la Santé et de la Recherche Médicale (INSERM) U.91, Hôpital Henri Mondor, 94010 Créteil, France, [1]Department of Growth and Development, Institute of Child Health, 30 Guildford Street, London WC1N 1EH, UK, [2]Department of Endocrinology, St Bartholomew's Hospital, London EC1A 7BE, UK and [3]INSERM U350, Institut Curie-Biologie, 92405 Orsay, France
[4]Corresponding author

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Growth hormone (GH) elicits a variety of biological activities mainly mediated by the GH receptor (GHR), a transmembrane protein that, based on *in vitro* studies, seemed to function as a homodimer. To test this hypothesis directly, we investigated patients displaying the classic features of Laron syndrome (familial GH resistance characterized by severe dwarfism and metabolic dysfunction), except for the presence of normal binding activity of the plasma GH-binding protein, a molecule that derives from the exoplasmic-coding domain of the GHR gene. In two unrelated families, the same GHR mutation was identified, resulting in the substitution of a highly conserved aspartate residue by histidine at position 152 (D152H) of the exoplasmic domain, within the postulated interface sequence involved in homodimerization. The recombinant mutated receptor protein was correctly expressed at the plasma membrane. It displayed subnormal GH-binding activity, a finding in agreement with the X-ray crystal structure data inferring this aspartate residue outside the GH-binding domain. However, mAb-based studies suggested the critical role of aspartate 152 in the proper folding of the interface area. We show that a recombinant soluble form of the mutant receptor is unable to dimerize, the D152H substitution also preventing the formation of hetero-dimers of wild-type and mutant molecules. These results provide *in vivo* evidence that monomeric receptors are inactive and that receptor dimerization is involved in the primary signalling of the GH-associated growth-promoting and metabolic actions.

Key words: dimerization/growth hormone receptor/Laron syndrome/signal transduction

Introduction

Growth hormone (GH), synthesized in and secreted from the anterior pituitary lobe, has multiple biological effects on growth, metabolism and the immune system (Isaksson *et al.*, 1985; Davidson, 1987; Edwards *et al.*, 1988). These effects are initiated by the binding of GH to specific high-affinity receptors (GHR) on the plasma membrane of target cells (Hughes and Friesen, 1985), followed by a series of post-receptor events including the generation of insulin-like growth factor-I (IGF-I). The isolation of a hepatic GHR cDNA clone in a number of species (Leung *et al.*, 1987; Baumbach *et al.*, 1989; Mathews *et al.*, 1989; Smith *et al.*, 1989; Adams *et al.*, 1990; Cioffi *et al.*, 1990; Hauser *et al.*, 1990; Burnside *et al.*, 1991) has made it possible to investigate its role in the biological effects of GH and to try to decipher the signal transduction process. This receptor is a transmembrane protein, mainly expressed in the liver, consisting of an exoplasmic domain (246 residues), a single transmembrane region (24 residues) and a large cytoplasmic domain (350 residues) (Leung *et al.*, 1987). The protein has been shown to belong to the cytokine receptor superfamily (Bazan, 1990), including receptors for prolactin, several interleukins (IL), erythropoietin, granulocyte- and granulocyte/macrophage-colony stimulating factors (GCSF and GMCSF), ciliary neurotrophic factor (Davis *et al.*, 1991), and leukemia inhibitory factor (Gearing *et al.*, 1991). Like many cytokine receptors in its family, GHR gives rise to a soluble high affinity GH-binding protein (GHBP).

Little is known about the factors that regulate differential expression of membrane versus soluble receptor forms and about the functional significance of the soluble receptor. However, the close structural relationship between the exoplasmic domain of the receptor and the plasma GHBP has led to the hypothesis that the binding properties of the plasma GHBP could reflect those associated with the extracellular domain of the membrane protein. Similarly, a recombinant soluble form of this domain may constitute a useful tool in the investigation of the nature of GH–GHR interactions (Leung *et al.*, 1987; Cunningham *et al.*, 1989; Fuh *et al.*, 1989; Cunningham and Wells, 1989, 1991). Such molecular interactions have been especially characterized by examination of the crystal structure of the complex between the human GH (hGH) and the extracellular domain of the human receptor (shGHR) produced in *Escherichia coli* (De Vos *et al.*, 1992). This study showed that (i) hGH binds to two identical subunits of the receptor, each recognizing different domains of the hormone, and (ii) dimerization of shGHR occurs around one molecule of hGH by a contact surface that includes the C-terminal domains of the two shGHR molecules. This ligand-induced receptor dimerization is probably relevant to receptor activation, as judged by the GH-dependent proliferative response of a mouse promyeloid cell line (FDC-P1) expressing hybrid GH/GCSF receptors (Fuh *et al.*, 1989) or the full-length GHR (Ishizaka-Ikeda *et al.*, 1993).

However, since no GHR-dependent functional assay specific for skeletal growth promotion has emerged, the critical role of the GHR in such biological activity was indirectly investigated by the molecular study of patients with

Laron syndrome, a rare GH-insensitive short-stature condition transmitted as an autosomal recessive trait (Laron *et al.*, 1966, 1971; Elders *et al.*, 1973). These patients are clinically (dwarfism and obesity), and in terms of metabolic responses (hypoglycaemia and high free fatty acids) indistinguishable from patients with isolated GH deficiency. However, in contrast to these latter patients, Laron patients have high levels of circulating GH and very low serum levels of IGF-I which do not respond to injections of GH. In addition, binding studies revealed the lack of plasma GH-binding activity in these patients (Baumann *et al.*, 1987; Daughaday and Trivedi, 1987), thereby suggesting involvement of the GHR in this disorder, a conclusion in agreement with other experimental data (Amselem *et al.*, 1991a). This disease was actually shown to be genetically linked to the GHR gene (Amselem *et al.*, 1989) in which a set of molecular defects was subsequently identified (Amselem *et al.*, 1989, 1991b, 1993; Godowski *et al.*, 1989; Berg *et al.*, 1992, 1993; Kou *et al.*, 1993). While some of the molecular defects (gene deletion, nonsense or frameshift mutations) so far described are consistent with a complete absence of binding of GH to its receptor, the other mutations require *in vitro* studies to evaluate their functional significance.

It was believed for some time that the absence of detectable GHBP was a constant biochemical feature of the Laron syndrome. However, this original hypothesis has been challenged since the recent description of patients displaying the classic features of Laron phenotype, except for the presence of high-affinity serum GHBP activity [similar in circulating levels and apparent affinity for GH to that of normal subjects (Aguirre *et al.*, 1990; Buchanan *et al.*, 1991)], raising the question: are the GHBP-positive and -negative phenotypes due to abnormalities within the same gene? Furthermore, if the GHR gene is shown to be involved in the GHBP-positive GH resistant phenotype, the subjacent molecular defect should provide an insight into GHR signal transduction.

In the present study we focus on the mutation underlying two unrelated families with Laron syndrome and positive GHBP. In both families we report the same mutation in the GHR gene, resulting in a single amino acid substitution leading to the replacement of aspartate 152 by a histidine (D152H) in the exoplasmic domain of the receptor close to the transmembrane-spanning region. The genetic analysis is consistent with a founder effect for this mutation. This natural GHR mutant expressed in eukaryotic cells retains GH-binding capability and is correctly expressed at the plasma membrane. Nevertheless, we present evidence for the involvement of the D152H mutation in the GH-resistant phenotype. We show that the mutant receptor has lost reactivity of one mAb epitope which is supposed to belong to the region where both receptor molecules contact each other, suggesting that the D152H substitution could interfere with the dimerization process. This mutation indeed abolishes completely homodimerization of the mutant receptor, such protein also being unable to form heterodimers with the wild-type molecules.

These *in vitro* data, along with the phenotype observed *in vivo*, provide further support for the 3-D model of the exoplasmic domain of GHR produced in *E.coli* and demonstrate the critical role of the GHR dimerization process in the growth-promoting action of GH, as well as in its metabolic effects.

Results

Characterization of the GHR gene frameworks associated with the GHBP-positive phenotype

To determine whether this new GH-resistance phenotype was the result of abnormalities within the GHR gene or another gene, we first examined the co-association of GHR polymorphic sites and phenotype in two unrelated affected families. The first patient is a child of healthy unrelated Indian parents (family A), whereas the second patient (family B) was born from unrelated Pakistani parents. Their clinical and biochemical characteristics have been described earlier (Buchanan *et al.*, 1991; Savage *et al.*, 1993). We have previously reported the presence of six polymorphic sites within an intervening sequence located in the part of the GHR gene (flanked by exons 9 and 10) encoding the intracellular domain of the receptor. Their association has been shown to define seven GHR gene frameworks (I–VII) that differ from each other by at least one nucleotide (Amselem *et al.*, 1989, 1993). The GHR gene frameworks were determined in both families. Unfortunately, family A was not informative for these DNA markers, both parents bearing the GHR framework I. In contrast, family B was found to be informative: both parents carried two different GHR frameworks (the father carried frameworks I and II and the mother carried frameworks I and V; data not shown), framework I being associated with the disease phenotype, a result consistent with the involvement of the GHR gene in this phenotype.

Identification of a missense mutation D152H in the exoplasmic domain of the GHR

In a first attempt to localize the genetic defect causing the Laron phenotype, we sought to sequence in both affected families the patients' GHR exons (2–10) encompassing the entire coding sequence. In patients from family A, there was agreement with the published normal nucleotide sequences and with those from a control subject, except in exon 6 at the nucleotide corresponding to base 508 of the GHR cDNA sequence, where a G residue was replaced by a C (Figure 1). The same substitution was identified in DNA from the family B patients. This substitution would result in the replacement of an aspartate (GAT) by a histidine (CAT) at position 152 of the mature GHR(D152H) within the extracellular domain close to the membrane-spanning region. The genomic nucleotide sequences for the parents from both families were also determined and they contained both G and C at position 738 of the GHR cDNA, in keeping with heterozygosity for the mutation. This nucleotide substitution abolishes a recognition site for the restriction endonuclease *Eco*RV, thereby allowing a distinction between both alleles by RFLP analysis. Amplification of a 263 bp fragment of normal GHR exon 6 followed by digestion with *Eco*RV yields 112 and 151 bp fragments (Figure 2A). The patient's amplified fragment will not be cleaved due to the loss of the *Eco*RV site (Figure 2B).

To test the hypothesis that the GHR gene is involved in the Laron phenotype, we examined the co-association of the D152H substitution and phenotype in both families. The segregation of the two mutant alleles could be followed in each family. As shown for family A in Figure 2B, restriction enzyme analysis of the parents gave a mixed digestion pattern which agreed with their being heterozygous for the same substitution, whereas affected individuals were homozygous

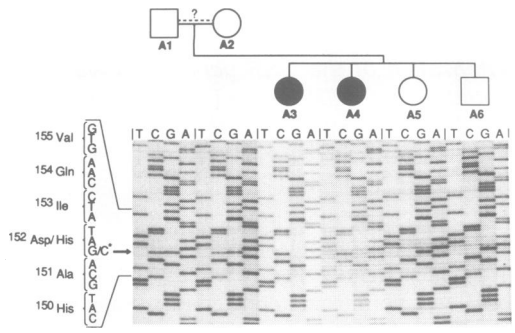

Fig. 1. Partial pedigree and corresponding GHR nucleotide sequence analysis of family A with autosomal recessive Laron syndrome and apparent normal high-affinity serum GHBP. Patients (A3 and A4) are homozygous for a G→C transversion within codon 152, generating a CAT codon (His) in place of a GAT codon (Asp). The phenotypically normal parents (A1 and A2) bear the wild-type and the mutant (asterisk) alleles, whereas the siblings bear only the wild-type allele.

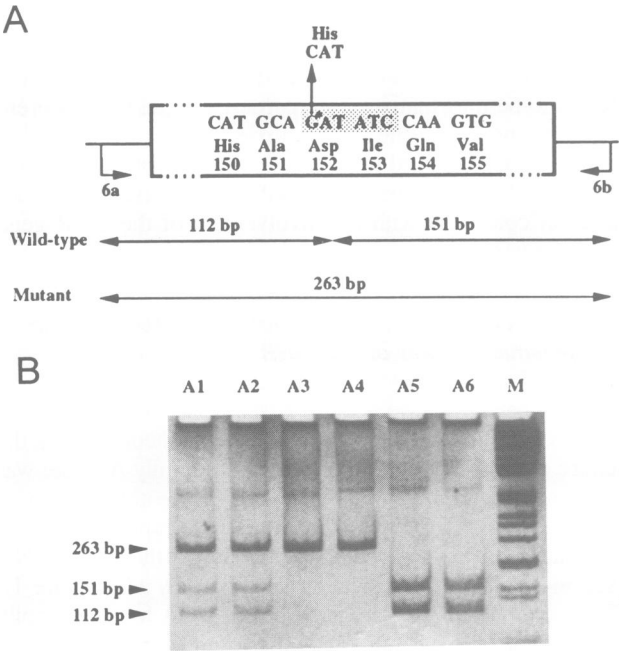

Fig. 2. Restriction enzyme mapping of PCR-amplified DNA from family A members. (**A**) A 263 bp genomic DNA containing exon 6 was amplified from each member of the affected family using oligonucleotide primers 6a and 6b (arrows). (*), site of the mutation in exon 6 obliterating an *Eco*RV site (shaded) and the predicted fragment sizes after *Eco*RV digestion of a wild-type (upper) and a mutant (lower) alleles. (**B**) PAGE of PCR-generated DNA after *Eco*RV digestion. Heterozygous parents (A1 and A2) yield a banding pattern consistent with the presence of both wild-type and mutant alleles, whereas both patients A3 and A4 bear only the mutant allele and the siblings (A5 and A6) display both the wild-type alleles only. The size marker is a 1 kb ladder from Gibco/BRL.

for the mutation, as indicated by the presence of a single 263 bp fragment, and siblings were homozygous for the wild-type allele.

The mutant GHR(D152H) retains GH-binding capability

To evaluate the functional importance of the aspartate→histidine substitution in the binding activity of the receptor, membranes of COS-7 cells expressing either the mutant or the wild-type receptor were incubated with [^{125}I]hGH. The

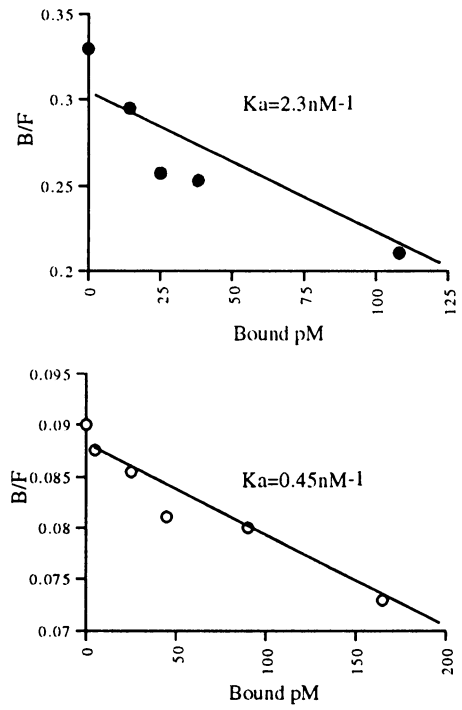

Fig. 3. Scatchard analysis of radiolabelled hGH binding to membranes of COS-7 cells expressing the wild-type hGHR (●) or the mutant D152H hGHR (○). B/F, bound:free ratio.

study was performed with COS-7 cells transfected with the expression plasmid pECE carrying the hGHR cDNA that contains the G→C transversion resulting in the substitution of histidine for aspartate at amino acid 152 [phGHR(D152H)] or the wild-type GHR [phGHR(wt)]. The association constants of the wild-type and mutant receptors were calculated from Scatchard plots as 2.3 and 0.45 nM^{-1}, respectively (Figure 3a and b). These results showed that the GHR(D152H) retains GH-binding capability. However, although the mutant and wild-type recombinant receptors bound hGH with closed affinity values, the D152H mutant showed a slight reduction in binding affinity to hGH of ~4-fold. This difference in the affinity constant values was clearly detectable in three independent experiments. Control untransfected COS-7 cells showed no detectable binding of [^{125}I]hGH.

The mutant GHR(D152H) retains a normal subcellular distribution despite a structural change

To determine the cellular distribution of the mutant GHR compared with the wild-type GHR, we used indirect immunofluorescence microscopy (Figure 4). Two anti-GHR mAbs (mAb263 and mAb5) were compared with respect to their reactivity towards wild-type and mutant receptors. In cells transfected with phGHRwt and examined with mAb263, GHR fluorescence revealed multiple bright peripheral spots at the periphery of cells (Figure 4a). After plasma membrane permeabilization, these cells exhibited intense cytoplasmic staining (Figure 4c). The mutant protein was unchanged in reactivity towards this mAb (Figure 4b and d).

However, different results were obtained using the second anti-GHR mAb (mAb5). Whereas cells transfected with phGHR(wt) and examined with mAb5 after plasma membrane permeabilization exhibited a staining pattern similar to that obtained with mAb263 (Figure 4e), no

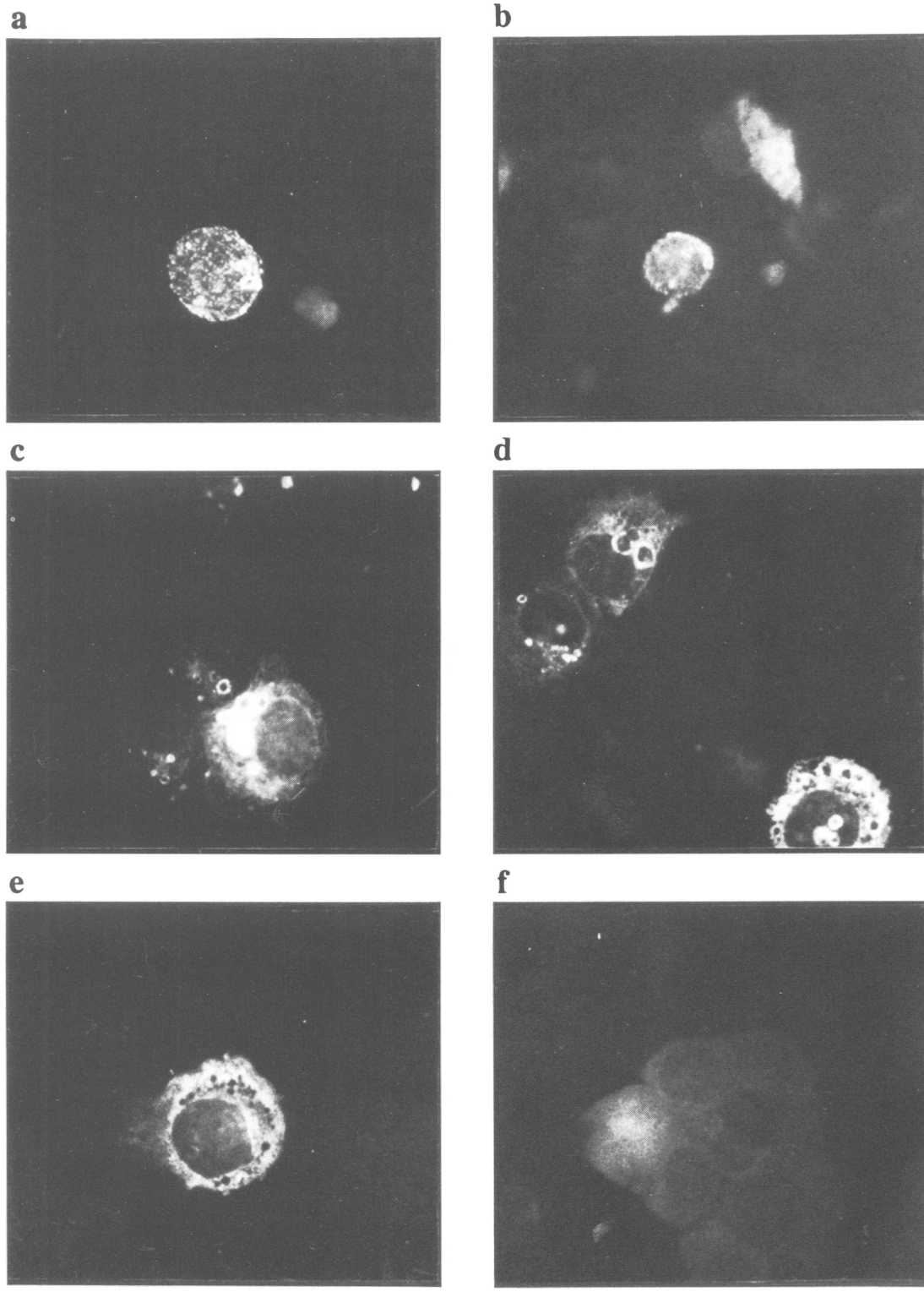

Fig. 4. Localization of wild-type and mutant hGHR proteins by immunofluorescence and their reactivity towards two different anti-GHR mAbs. COS-7 cells were transfected with phGHR(wt) (left panels) or phGHR(D152H) (right panels), labelled with mAb263 (**a**–**d**) or mAb5 (**e** and **f**), and examined before (a and b) and after (c–f) cell permeabilization. Magnification is ×1400.

labelling was detected in cells transfected with the mutant clone and examined in the same conditions (Figure 4f). These results indicate that mAb5 recognized only intact wild-type GHR.

Taken together, these results suggest that the mutant protein is expressed and that its subcellular distribution is similar to that of the wild-type receptor, but its structure is somewhat different from the wild-type protein; in particular, epitope for mAb5 may be disturbed. In view of the observation that mAb5 possibly binds to the region where both receptors contact each other (Cunningham *et al.*, 1991; Fuh *et al.*, 1992), and given the location of the D152H

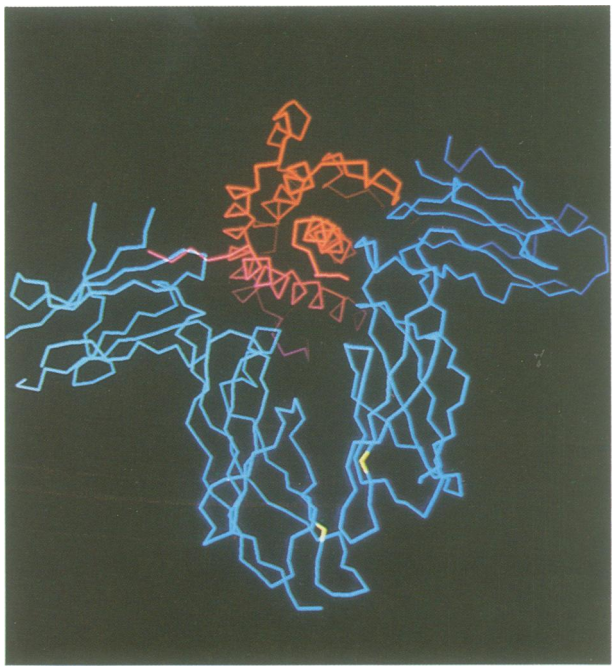

Fig. 5. 3-D carbon α trace of the hGH−shGHR complex showing the position of aspartate 152. The hormone (red) is bound to two molecules of the extracellular domain of its receptor (blue). The position of the aspartate 152 residue, which is mutated in the two families with GH-resistant syndrome, is depicted in yellow in both shGHR molecules. The graphics were produced using the deposited crystallographic coordinates (De Vos *et al.*, 1992).

mutation within the postulated interface sequence of GHR (Figure 5) (De Vos *et al.*, 1992), these results prompted us to analyse the potential of the mutant GHR to dimerize.

Dimerization of the wild-type GHR extracellular domain occurs in solution

To study the dimerization process, a sensitive solution assay based on gel filtration chromatography of the complexes between hGH and the wild-type GHR extracellular domain was adapted from that developed by Cunningham *et al.* (1991). A soluble hGHR protein [shGHR(wt)] lacking transmembrane and cytoplasmic regions and expressed in COS-7 cells was prepared from phGHR(wt) with a termination codon at position 239, since it was previously demonstrated that this genetically engineered receptor expressed in *E.coli* retained GH-binding capability (Fuh *et al.*, 1989).

Recombinant shGHR secreted in the medium of transfected COS-7 cells was concentrated, incubated with [^{125}I]hGH and fractionated by HPLC gel filtration. The resulted profiles are depicted in Figure 6a. Four radioactive peaks are typically seen with this separation method. The first peak consists of a non-specific fraction which is not displaceable by GH; peak III corresponds to free GH. Peaks IIa and IIb correspond to fractions displaying specific GH-binding activity. Indeed, increasing amounts of unlabelled hGH resulted in progressive diminution of these fractions (data not shown), leading to their disappearance with large excess (2 μg) of hGH (Figure 6a). A molecular mass of ~110 kDa was observed for the receptor−ligand complex eluted in peak IIa, while peak IIb contained a 65 kDa receptor−ligand complex. These data suggest that peak IIb contains a complex of 1 mol shGHR (~43 kDa) per mol

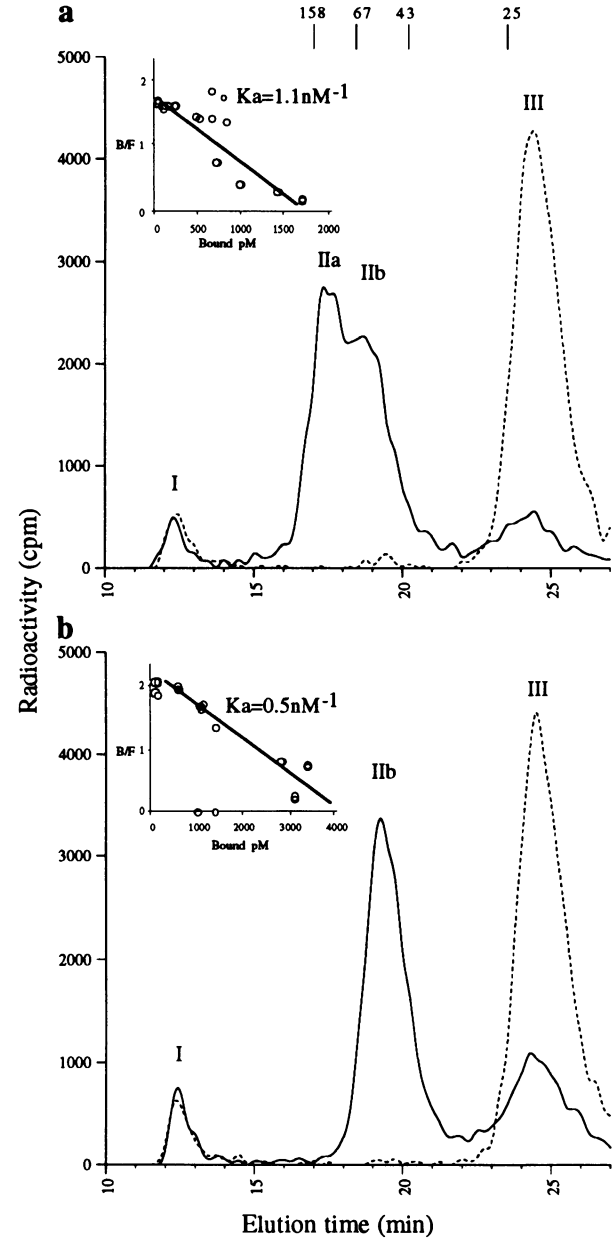

Fig. 6. Elution profile from the HPLC gel filtration column of [^{125}I]hGH incubated with supernatant of COS-7 cells expressing the wild-type shGHR (**a**) or the mutant shGHR (**b**). The resulting mixture was incubated without (—) or with (---) increasing amounts of unlabelled hGH. The molecular weight of the markers used to calibrate the column is indicated at the top of the figure. Inset: Scatchard plots of the competition assays with unlabelled hGH. B/F, bound:free ratio.

hGH (22 kDa), and that peak IIa corresponds to a hGH−shGHR(wt) complex in a ratio of 1:2 [hGH:shGHR(wt)]. The affinity constant calculated from Scatchard analysis was 1.1 nM^{-1}. No binding activity could be detected in the media of non-transfected COS-7 cells or cells transfected with the native plasmid only (data not shown).

To characterize further peaks IIa and IIb and to confirm the stoichiometry of both complexes, mixtures of shGHR(wt) and radiolabelled hGH in different ratios were separated by HPLC gel filtration (Figure 7). Depending on this ratio, different elution patterns were observed: at high shGHR(wt) concentrations (Figure 7a), peak IIa was clearly detectable; in contrast, when the ratio shGHR(wt):hGH was progres-

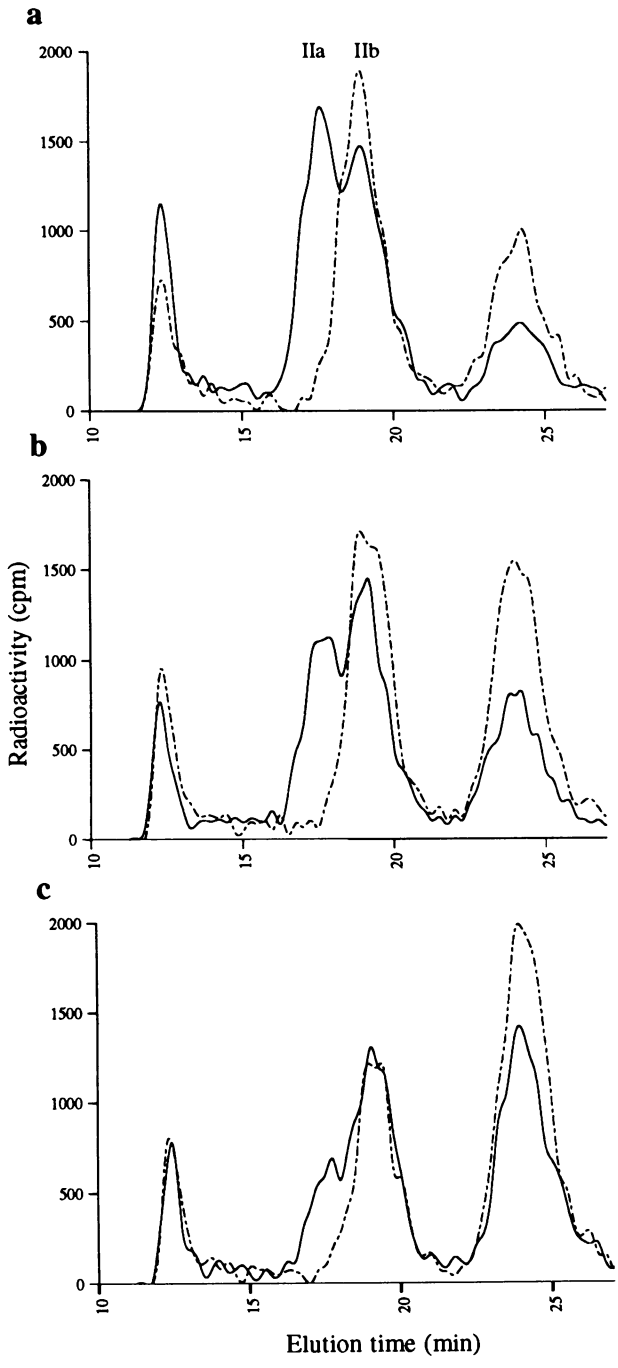

Fig. 7. HPLC gel filtration chromatography of various ratios of shGHR:hGH. A constant amount of [^{125}I]hGH was incubated with decreasing amounts of shGHR (**a**–**c**). The specific complexes generated with the wild-type shGHR (—) consist of two peaks (peaks IIa and IIb). As the shGHR:hGH ratio decreases, peak IIa (dimer) progressively diminishes, whereas peak III (free hGH) progressively increases. In all ratios tested, the mutant shGHR (---) was only able to generate peak IIb.

sively reduced (Figure 7b and c), this fraction diminished, whereas free radiolabelled hGH accumulated in the peak III region.

As a control, we also studied the dimerization status of the soluble receptor in the absence of ligand. The medium of transfected cells secreting recombinant shGHR(wt) molecules was first fractionated by HPLC gel filtration without addition of any hGH. Several fractions that eluted at different times were subsequently assayed for their ability to bind hGH as follows: after incubation with [^{125}I]hGH, each fraction was reapplied to the gel filtration column. Depending on the fraction analysed, different elution profiles were observed: late column fractions eluting with protein of molecular mass ~45 kDa (corresponding to monomeric receptors) generated elution patterns comprising not only peaks I and III (corresponding to a non-specific fraction and free hGH, respectively), but also peaks IIa and IIb [corresponding to specific hGH–shGHR(wt) complexes in ratios of 1:2 and 1:1, respectively, and, as expected, which were displaceable by cold hGH]. By contrast, early column fractions (eluting with protein of molecular mass ~90 kDa) did not generate peaks IIa and IIb, but only peaks I, III and a non-specific fraction which was not displaceable by hGH (data not shown).

Evidence gathered by these methods therefore indicated that (i) in the presence of hGH only, shGHR(wt) dimerized in solution, and (ii) this assay system could be used to evaluate the potential of the shGHR(D152H) mutant to dimerize.

The D152H mutation abolishes receptor homodimerization

It was then examined whether the shGHR(D152H) mutant retains the capability to dimerize in the presence of hGH. [^{125}I]hGH was added to different amounts of COS-7 supernatant containing shGHR(D152H) and the resulting mixtures were fractionated by gel filtration. At all hGH:shGHR(D152H) ratios tested (Figure 7a–c), we detected only three peaks, peak IIa [which corresponds to a hGH–shGHR(D152H) complex in the ratio of 1:2] being absent. From competition Scatchard analysis, the binding affinity for the peak IIb fraction was identical to that of the membrane mutant receptor (Figures 3b and 6b). These results show that the D152H mutation abolishes the dimerization process.

The D152H mutation prevents the formation of heterodimers of wild-type and mutant receptors

To define the extent to which 'hybrid' heterodimers of wild-type and mutant receptors are assembled, we examined whether shGHR(D152H) was able to compete for dimerization of shGHR(wt) in the presence of [^{125}I]hGH by HPLC gel filtration (Figure 8). The specific complexes generated with only the wild-type shGHR molecules (Figure 8a) consisted of two peaks (peaks IIa and IIb corresponding to dimers and monomers, respectively), whereas the mutant shGHR molecules gave rise to peak IIb only (Figure 8c). Mixing shGHR(wt) with shGHR(D152H) resulted in a marked reduction of peak IIa, together with an increase of peak IIb (Figure 8e). By contrast, as expected, in a control experiment in which the amount of shGHR(wt) was twice as much as in the first assay, opposite results were obtained (Figure 8b), whereas in another control experiment, doubling the amount of the mutant molecules resulted in the increase of peak IIb (Figure 8d). These competition data are consistent with the lack of any tendency of shGHR(D152H) molecules to dimerize with the wild-type molecules.

Taken together, these results show that the D152H mutation does not affect the ability of hGHR to bind hGH, but rather impairs its dimerization.

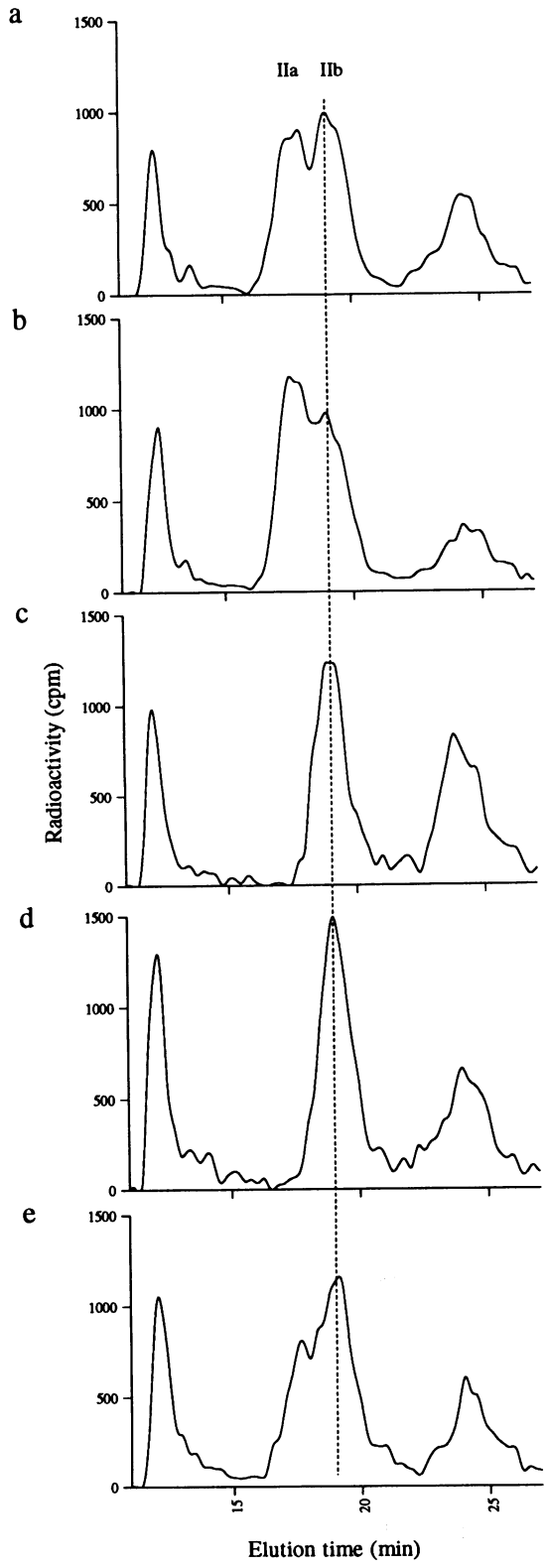

Fig. 8. Effect of shGHR(D152H) molecules upon dimerization of the wild-type receptor molecules, as shown by HPLC gel filtration. To study the interactions of shGHR(D152H) with shGHR(wt), a constant amount of [^{125}I]hGH was incubated with the supernatant of COS-7 cells expressing the wild-type shGHR and/or that of cells expressing the mutant shGHR(D152H), and the resulting mixtures were analysed by HPLC gel filtration. (**a**) shGHR(wt) alone; (**b**) shGHR(wt) at a higher concentration (2-fold increase); (**c**), shGHR(D152H) alone; (**d**), shGHR(D152H) at a higher concentration (2-fold increase); (**e**), mixture of shGHR(wt) and shGHR(D152H) (molar ratio 1:1).

Discussion

Cosegregation of a missense mutation involving a conserved residue with the disease phenotype

Our result that a mutant GHR gene allele cosegregates with the disease phenotype in both families strongly argues for a decisive role of this gene in the aetiology of familial GHBP-positive Laron syndrome. The same mutation consisting of a G→C transversion at position 508 of the human cDNA, which results in the replacement of aspartate by a histidine at residue 152, was found in the two affected families. In both families, patients who are homozygous for the codon 152 mutation are also homozygous for the same GHR framework (I), suggesting that there is a common origin (i.e. a founder effect) for this mutation. A number of lines of evidence indicate that the D152H mutation underlines the genetic basis for the disease phenotype. No other amino acid substitution or deletion was found in the entire coding region of the patients' GHR. The defect is located in a highly conserved region of the GHR and involves an invariant residue in all species studied so far, including human, rabbit, mouse, rat, pig, cow, sheep and chicken. In addition, the residue is also conserved in the receptor for IL-7 (Bazan, 1990), another member of the cytokine receptor superfamily. This high degree of conservation of aspartate 152 suggests that amino acid substitutions at this position would be detrimental to receptor function. Interestingly, this residue does not belong to the intracellular domain of the receptor, but to a region of the exoplasmic domain close to the transmembrane region.

Observed GHR structural change

Examination of the GH-binding properties of the plasma membranes of COS-7 cells expressing the mutant membrane receptor revealed an affinity constant identical to that found *in vivo* for the plasma GHBP of these patients. However, since the plasma GH-binding activity was normal and the affinity constant for the corresponding recombinant membrane receptor was slightly decreased, we conclude that the D152H mutation does not interfere with the binding activity of the soluble GHBP, but rather with that of the membrane receptor.

To understand how this natural mutation impairs the gene product and at what subcellular level, we analysed transfected COS-7 cells by indirect immunofluorescence. This study showed that expression of the mutant receptor protein was normal, as judged by the labelling pattern obtained with one anti-GHR mAb (mAb263), but that the mutation disrupted the binding site for another mAb (mAb5). The recently determined 3-D structure of the shGHR–hGH complex provides a structural context in which to explore these data (De Vos *et al.*, 1992). The crystallographic study confirmed the data of Bazan who predicted that the exoplasmic part of the GHR and related members of the cytokine receptor superfamily consist of two duplicated domains (N- and C-terminal domains), each containing seven β-strands divided into two sheets (Bazan, 1990). It also confirmed the results of Cunningham *et al.* (1991), who showed that dimerization of the GHR should occur around one molecule of GH. Examination of the crystal structure showed that the same largely nonpolar faces of the receptors bound to the two distinct regions on opposite sites of the hormone. Combination with the hormone promotes association between

the two receptor molecules (shGHRI and shGHRII) through the three-stranded sheets of the C-terminal receptor domains. The differences in GHR staining with mAb263 and mAb5 found in the present study can be explained by the different ways they bind to the shGHR. Indeed, it has been shown that mAb5 prevents binding of a second shGHR to the hGH–shGHR complex (Cunningham *et al.*, 1991), possibly by binding to the region where the two receptor molecules contact each other (Fuh *et al.*, 1992). In contrast, mAb263 binds at a site located away from the receptor–receptor interface (Fuh *et al.*, 1992). Our immunofluorescence data together with the binding results are therefore consistent with a localized effect of the D152H substitution, which perturbs the epitope for mAb5 without altering the global tertiary structure of the receptor.

Effect of the D152H substitution upon the dimerization step: implications for signal transduction

It is noteworthy that aspartate 152, which lies within the C-terminal domain, is part of the three-stranded sheet that creates the contact surface between the two receptor molecules (Figure 5) (De Vos *et al.*, 1992). Interestingly, according to the crystallographic structure, in each monomer this residue is involved in specific electrostatic interactions: aspartate 152 of shGHRII should interact with serine 145 and threonine 147 of shGHRI, whereas that of shGHRI is close to tyrosine 200 of shGHRII. These interactions may be critical for the stabilization of the molecular complex. Introduction of a histidine at position 152 should result in the loss or decrease of these intermolecular interactions. In addition, we cannot rule out a local effect of an aspartate→histidine substitution which causes a change in size and charge. Taken together, these data strongly suggest that the D152H substitution interferes with normal GHR dimerization.

Using *in vitro* dimer reconstitution we were able to confirm this hypothesis. Gel filtration studies of the complexes formed between radiolabelled hGH and wild-type shGHR showed that two molecular species could be discerned. Determination of the molecular mass of the hGH–shGHR(wt) complexes by sizing column chromatography, showed that one complex consisted of one hGH molecule per two shGHR molecules, whereas the other complex contained a monomeric shGHR. This result is consistent with a previous gel filtration analysis (Cunningham *et al.*, 1991). As expected, the relative proportion of each complex depended on the hGH:shGHR ratio, the hGH–shGHR 1:2 complex being favoured by increasing the amount of shGHR; conversely, the amount of hGH–shGHR 1:1 complex increased upon addition of hGH. In contrast, in a similar study performed with shGHR(D152H), whichever hGH and shGHR concentrations we used, only the hGH–shGHR 1:1 complex was present, thereby demonstrating the inability of shGHR(D152H) to dimerize.

In theory, the failure to dimerize could be due to either a mutation-induced abnormality in one of the two ligand-binding domains on hGH, so that the ligand can bind to the receptor only in a monomeric manner, or the destruction of a necessary contact area between the two receptors. However, according to the 3-D model of the hGH–shGHR complex, both receptors donate essentially the same residues to interact with the hormone; it is therefore very unlikely that the mutant receptor, which retains hGH-binding activity, could not dimerize because of abnormalities in one of the two ligand-binding domains. In addition, if the mutation would impair one of the two ligand-binding domains, addition of wild-type molecules should allow the dimerization process to occur, which is not the case. For these reasons, the inability of shGHR(D152H) to dimerize most probably results from an impaired contact surface between the C-terminal domains of the receptors.

The mechanism for signal transduction is just beginning to be elucidated. Among the different molecular events that are relevant for GHR activation the dimerization step is required, as shown by *in vitro* studies on the basis of a cell proliferation assay of a myeloid leukaemia cell line (FDC-P1) expressing the GHR (Fuh *et al.*, 1992; Ishizaka-Ikeda *et al.*, 1993). Our *in vitro* results, along with the disease phenotype observed *in vivo*, now demonstrate that the growth-promoting action of GH requires GHR dimerization. They also strongly suggest that the monomeric receptor is inactive because both clinical and metabolic features of patients with the D152H substitution were identical to those of patients with typical Laron syndrome in whom nonsense mutations have been identified (Amselem *et al.*, 1991b, 1993; Berg *et al.*, 1993). GHR dimerization should therefore also be relevant to the metabolic actions of GH (i.e. on carbohydrate and lipid metabolism). In addition, this study demonstrates that generation of the plasma GHBP is not dependent on activation of GHR-associated intracellular signals because patients with the D152H substitution display GHBP similar in circulating level to that of normal subjects.

The structural requirements for dimerization are interesting to compare with data obtained in related receptors. It is striking to notice that ligand-induced receptor homodimerization has also been proposed for the IL-7 receptor (Goodwin *et al.*, 1990), a member of the cytokine receptor superfamily, in which an aspartate residue is also conserved at the same position (Bazan, 1990). Similarly, compelling evidence supports the hypothesis that erythropoietin binding induces erythropoietin receptor dimerization, which is in turn critical for signal transduction. Interestingly, in this latter case such hypothesis has emerged after *in vitro* examination of a mutant receptor obtained by a retroviral selection strategy and that conferred a constitutive receptor activation (Yoshimura *et al.*, 1990). This mutation, which consists of the replacement of arginine 129 by cysteine, is part of a peptide domain sharing structural homologies with the GHR domain involved in receptor homodimerization. This substitution occurs at a position occupied by the threonine 140 residue in the GHR, this amino acid together with the aspartate 152 residue belonging to the same three-stranded sheet of the interface area. The constitutively active mutant, which was shown to form disulfide-linked homodimers, requires the presence of cysteine at residue 129 (Watowitch *et al.*, 1992). Conversely, the D152H substitution described here represents a naturally occurring mutation that results in a constitutive absence of dimerization.

Recessive nature of the GHBP-positive disease phenotype

It is noteworthy that the phenotype linked to the D152H mutation is clinically recessive. This observation suggests that compensatory mechanisms may be acting *in vivo* to mitigate the effects of the heterozygous state. Strikingly, our competition data between wild-type and mutant GHR showed

that the D152H mutation prevents the formation of hybrid complexes consisting of heterodimers of wild-type and mutant receptors. Is a similar mechanism operating *in vivo*? To answer this question, additional experiments are needed to determine how signal transduction at the cell surface is regulated under such circumstances. Nevertheless, we have to keep in mind that given the large amount of hGH available in heterozygous individuals, ~50% of the GHR dimers consist of functional wild-type molecules.

In conclusion, the aspartate→histidine mutation of the hGHR found in two affected families with GH resistance points to an important domain of GHR, distinct from the GH-binding region, involved in the homodimerization of the receptor. This defect provides the first *in vivo* and biochemical confirmations of the X-ray structural model that infers this aspartate residue in the dimerization process. It would be of interest to determine whether mutations of the other residues contained within the postulated interface sequence of GHR and related receptors would cause similar defects in receptor assembly. This study shows that the molecular characterization of natural mutants is a very useful approach to identifying functional domains in hGH–hGHR complexes and should be of further aid in mapping other sites involved in signal transduction.

Materials and methods

Genetic analyses

Both clinical and biochemical phenotypes from the patients investigated here have been reported: family A is of Indian descent (Buchanan *et al.*, 1991), whereas family B is of Pakistani origin (patient number 23 from Savage *et al.*, 1993). In the two families, there is no known consanguinity and the patients displayed the classic features of the Laron syndrome, except for the presence of high-affinity serum GHBP activity (Buchanan *et al.*, 1991; Savage *et al.*, 1993).

Genomic DNA was isolated from peripheral blood leukocytes. All nine GHR coding exons and the surrounding intronic sequences, as well as intron 9 that has been shown to contain polymorphic sites (Amselem *et al.*, 1989), were analysed by PCR. The primer sequences were deduced from the published sequence of the hGHR gene (Godowski *et al.*, 1989). Direct sequencing of the PCR products was performed according to Gyllensten and Erlich (1988).

Rapid diagnostic identification of the single-point mutation in GHR exon 6 was accomplished by *Eco*RV digestion of the PCR-amplified products. Digestion products were analysed on a 6% polyacrylamide gel stained with ethidium bromide. 1 kb ladder (Gibco/BRL) was used as a molecular size marker.

Site-directed mutagenesis

A hGHR cDNA containing the G→C transversion (resulting in the substitution of aspartate for histidine at amino acid 152) was prepared from the expression vector pECE carrying the full-length hGHR cDNA coding sequence and designated phGHR(wt) (Duquesnoy *et al.*, 1991). Site-directed mutagenesis was performed by a previously described PCR procedure (Higuchi *et al.*, 1988) on plasmid phGHR(wt) using synthetic oligonucleotides designed to replace aspartate 152 (GAT) by a histidine (CAT). The resulting plasmid was designated phGHR(D152H).

Site-directed mutagenesis of the phGHR(wt) and phGHR(D152H) templates was performed to produce pshGHR(wt) and pshGHR(D152H), encoding the corresponding soluble secreted forms of the receptor using the oligonucleotide 5′-CCCATGAGCTCAAGAAAGGCTATTGGCTCA-TCT-3′. This introduced a translation stop codon at codon 239 followed by an *Sst*I restriction site allowing insertion of this 238 residue-coding sequence into pECE.

DNA transfection

Eighty per cent confluent COS-7 cells were transfected by the Lipofectin method (Gibco/BRL, Life Technology, Grand Island, NY) either with the wild-type or the mutant constructs encoding the full-length or the soluble GHR, according to the manufacturer's instructions. The confluent monolayers obtained were processed for binding studies and immunofluorescence. The cell culture supernatants were collected, 10-fold concentrated by ultrafiltration with a Centricon 30 (Amicon) and used as such for gel filtration.

Binding assays on cell membranes

hGH-binding assays were performed on membrane receptors as previously described (Duquesnoy *et al.*, 1991). Briefly, total particulate membrane fraction was prepared by centrifugation for 30 min at 1500 *g*; the resulting pellet was incubated with ^{125}I-labelled hGH with various amounts of unlabelled hGH. After incubation for 16 h at 4°C, bound and free hGH were separated by filtration through 0.22 μm GV membranes (Millipore) and specifically bound [^{125}I]hGH was determined using a γ counter.

Indirect immunofluorescence staining

The recombinant hGHRs were detected on the cell surface and in the cytoplasm of COS-7 cells using an anti-GHR mAb (mAb263 or mAb5) on intact cells and on cells permeabilized with pure methanol as previously described (Duquesnoy *et al.*, 1991). The anti-GHR mAbs 263 and 5, which recognize different epitopes on GHR, were obtained from Agen Biomedical Limited (Australia).

HPLC gel filtration of shGHR–hGH complexes

10 μl of the 10-fold concentrated COS-7 cell supernatant containing either the wild-type or the mutant shGHR were incubated with ^{125}I-labelled hGH (10^5 c.p.m.) in 0.1 M phosphate buffer (pH 7.4). After 20 h at 4°C, the reaction products were analysed by gel filtration on an HPLC Protein-Pak 300SW (Waters). Elution was performed by using a buffer (0.1 M Na_2SO_4, 0.1 M potassium phosphate, pH 7.0) pumped at a rate of 0.5 ml/min. The molecular weights of the protein complexes were determined by size chromatography using standard molecular weight marker proteins. 0.125 ml fractions were collected and counted in a γ counter (Minigamma, LKB).

Structure analysis and molecular graphics

Analysis of the 3-D structure of shGHR was performed on a Silicon Graphics 4D310GTX system using the BIOSYM package. The coordinates of the shGHR structure (De Vos *et al.*, 1992) used are those deposited in the Brookhaven Protein Data Bank (Bernstein *et al.*, 1977).

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References

Adams,T.E., Baker,L., Fiddes,R.J. and Brandon,M.R. (1990) *Mol. Cell. Endocrinol.*, **73**, 135–145.

Aguirre,A., Donnadieu,M., Job,J.-C. and Chaussain,J.-L. (1990) *C. R. Acad. Sci. Paris*, **311**, 315–319.

Amselem,S., Duquesnoy,P., Attree,O., Novelli,G., Bousnina,S., Postel-Vinay,M.-C. and Goossens,M. (1989) *New Engl. J. Med.*, **321**, 989–995.

Amselem,S., Duquesnoy,P. and Goossens,M. (1991a) *Trends Endocrinol. Metab.*, **2**, 35–40.

Amselem,S., Sobrier,M.-L., Duquesnoy,P., Rappaport,R., Postel-Vinay,M.-C., Gourmelen,M., Dallapiccola,B. and Goossens,M. (1991b) *J. Clin. Invest.*, **87**, 1098–1102.

Amselem,S., Duquesnoy,P., Duriez,B., Dastot,F., Sobrier,M.-L., Valleix,S. and Goossens,M. (1993) *Hum. Mol. Genet.*, **2**, 355–359.

Baumann,G., Shaw,M.A. and Winter,R.J. (1987) *J. Clin. Endocrinol. Metab.*, **65**, 814–816.

Baumbach,W.R., Horner,D.L. and Logan,J.S. (1989) *Genes Dev.*, **3**, 1199–1205.

Bazan,J.F. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 6934–6938.

Berg,M.A., Guevara-Aguirre,J., Rosenbloom,A.L., Rosenfeld,R.G. and Franke,U. (1992) *Hum. Mutat.*, **1**, 24–34.

Berg,M. *et al.* (1993) *Am. J. Hum. Genet.*, **52**, 998–1005.

Bernstein,F.C. *et al.* (1977) *J. Mol. Biol.*, **112**, 535–542.

Buchanan,C.R., Maheshwari,H.G., Norman,M.R., Morrell,D.J. and Preece,M.A. (1991) *Clin. Endocrinol.*, **35**, 179–185.

Burnside,J., Liou,S.S. and Cogburn,L.A. (1991) *Endocrinology*, **128**, 3183–3192.

Cioffi,J.A., Wang,X. and Kopchick,J.J. (1990) *Nucleic Acids Res.*, **18**, 6451.
Cunningham,B.C. and Wells,J. (1989) *Science*, **244**, 1081–1085.
Cunningham,B.C. and Wells,J. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 3407–3411.
Cunningham,B.C., Jhurani,P., Ng,P. and Wells,J. (1989) *Science*, **243**, 1330–1336.
Cunningham,B.C., Ultsch,M., De Vos,A.M., Mulkerrin,M.G., Clauser,K.R. and Wells,J.A. (1991) *Science*, **254**, 821–825.
Daughaday,W.H. and Trivedi,B. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 4636–4640.
Davidson,M. (1987) *Endocrine Rev.*, **8**, 115–131.
Davis,S., Aldrich,T.H., Valenzuela,D.M., Wong,V., Furth,M.E., Squinto,S.P. and Yancopoulos,G.D. (1991) *Science*, **253**, 59–63.
De Vos,A.M., Ultsch,M. and Kossiakoff,A. (1992) *Science*, **255**, 306–312.
Duquesnoy,P., Sobrier,M.-L., Amselem,S. and Goossens,M. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 10272–10276.
Edwards,C.K., Ghiasuddin,S.M., Schepper,J.M., Yunger,L.M. and Kelley,K.W. (1988) *Science*, **239**, 769–771.
Elders,M., Garland,J., Daughaday,W., Fisher,D., Whitney,J. and Hughes,E. (1973) *J. Pediat.*, **83**, 253–263.
Fuh,G., Mulkerrin,M., Bass,S., McFarland,N., Brochier,M., Bourell,J., Light,D. and Wells,J. (1989) *J. Biol. Chem.*, **265**, 3111–3115.
Fuh,G., Cunningham,B.C., Fukunaga,R., Nagata,S., Goeddel,D. and Wells,J. (1992) *Science*, **256**, 1677–1680.
Gearing,D., Thut,C., VandenBos,T., Gimpel,S., Delaney,P. and Beckmann,M. (1991) *EMBO J.*, **10**, 2839–2848.
Godowski,P.J. *et al.* (1989) *Proc. Natl Acad. Sci. USA*, **86**, 8083–8087.
Goodwin,R. *et al.* (1990) *Cell*, **60**, 941–951.
Gyllensten,U.B. and Erlich,H.A. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 7652–7656.
Hauser,S.D., McGrath,M.F., Collier,R.J. and Krivi,G.G. (1990) *Mol. Cell. Endocrinol.*, **72**, 187–200.
Higuchi,R., Krummel,B. and Saiki,R. (1988) *Nucleic Acids Res.*, **16**, 7351–7367.
Hughes,J.P. and Friesen,H.G. (1985) *Annu. Rev. Physiol.*, **47**, 469–482.
Isaksson,O.G.P., Eden,S. and Jansson,J.-O. (1985) *Annu. Rev. Physiol.*, **47**, 483–499.
Ishizaka-Ikeda,E., Fukunaga,R., Wood,W., Goeddel,D. and Nagata,S. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 123–127.
Kou,K., Lajara,R. and Rotwein,P. (1993) *J. Clin. Endocrinol. Metab.*, **76**, 54–59.
Laron,Z., Pertzelan,A. and Mannheimer,S. (1966) *Isr. J. Med. Sci.*, **2**, 152–155.
Laron,Z., Pertzelan,A., Karp,M., Kowadlo-Silbergeld,A. and Daughaday, W.H. (1971) *J. Clin. Endocrinol. Metab.*, **33**, 332–342.
Leung,D.W. *et al.* (1987) *Nature*, **330**, 537–543.
Mathews,L.S., Enberg,B. and Norstedt,G. (1989) *J. Biol. Chem.*, **264**, 9905–9910.
Savage,M.O. *et al.* (1993) *J. Clin. Endocrinol. Metab.*, **77**, 1465–1471.
Smith,W.C., Kuniyoshi,J. and Talamantes,F. (1989) *Mol. Endocrinol.*, **3**, 984–990.
Watowitch,S., Yoshimura,A., Longmore,G., Hilton,D., Yoshimura,Y. and Lodish,H. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 2140–2144.
Yoshimura,A., Longmore,G. and Lodish,H.F. (1990) *Nature*, **348**, 647–649.

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