A 36 residues insertion in the dimerization domain of the growth hormone receptor results in defective trafficking rather than impaired signaling

M Maamra¹, A Milward¹, H Zarkesh Esfahani³, L P Abbott¹,
L A Metherell², M O Savage², A J L Clark² and R J M Ross¹

¹Division of Clinical Sciences (North), University of Sheffield, Clinical Sciences Centre, Northern General Hospital, Sheffield S5 7AU, UK.

²Department of Endocrinology, Barts and the London, Queen Mary University of London, West Smithfield, London EC1A 7BE, UK.

³Department of Biology, Faculty of Sciences, Isfahan University, Isfahan 81746–73441, Iran

(Requests for offprints should be addressed to R J Ross; Email: r.j.ross@sheffield.ac.uk)

Abstract

Growth hormone insensitivity syndrome (GHIS) has been reported in a family homozygous for a point mutation in the GH receptor (GHR) that activates an intronic pseudoexon. The resultant GHR (GHR1–656) includes a 36 amino-acids insertion after residue 207, in the region known to be important for homodimerization of GHR. We have examined the functional consequences of such an insertion in mammalian cells transfected with the wild type (GHRwt) and mutated GHR (GHR1–656). Radioligand binding and flow cytometry analysis showed that GHR1–656 is poorly expressed at the cell surface compared with GHRwt. Total membrane binding and Western blot analysis showed no such difference in the level of total cellular GHR expressed for GHR1–656

vs GHRwt. Immunofluorescence showed GHR1–656 to have different cellular distribution to the wild type receptor (GHRwt), with the mutated GHR being mainly perinuclear and less vesicular than GHRwt. Western blot analysis showed GH-induced phosphorylation of Jak2 and Stat5 for both GHR1–656 and GHRwt, although reduced Stat5 activity was detected with GHR1–656, consistent with lower levels of expression of GHR1–656 than GHRwt at the cell surface. In conclusion, we report that GHIS, due to a 36 amino-acids insertion in the extracellular domain of GHR, is likely to be explained by a trafficking defect rather than by a signalling defect of GHR.

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Introduction

Laron syndrome or GH insensitivity syndrome (GHIS) is a rare autosomal recessive condition associated with post-natal growth failure leading to extreme short stature, mid-facial hypoplasia, truncal obesity, and hypoglycaemia in infancy. Patients have raised GH levels, associated with deficiency of insulin-like growth factor (IGF-I), and IGF binding protein-3 (IGFBP-3) (Rosenfeld *et al.* 1994, Woods *et al.* 1997). These features result from GHR dysfunction and consequent failure of GH action.

The mature wild type human GHR (GHRwt) is 620 amino acids long, possessing 246 extracellular amino acids, a 24 amino acid transmembrane spanning region, and 350 cytoplasmic amino acids (Leung *et al.* 1987). GH interacts with its receptor in a 1:2 stoichiometry, and this is essential to trigger signalling (Maamra *et al.* 1999). The GHR extracellular domain constitutes two subdomains, joined by a short hinge region. Subdomain 1 spans from residue 1 to 123 and subdomain 2 from residue 128 to 246.

Subdomain 1 is mainly involved in the GH–GHR interaction (Behncken & Waters 1999). The two molecules of GHR also interact, and the dimerization interface of the two receptors is confined to subdomain 2 of the receptors. In this subdomain, the unpaired cysteine 241 is thought to play a role in the conformational change of GHR induced by GH, rather than in the dimerization of the receptor (Zhang *et al.* 1999).

The GHR lacks intrinsic kinase activity and relies on the regulation and activation of cytosolic Janus kinase 2 (Jak2). Jak2 is constitutively associated with the receptor at the proline rich Box1 site (amino acids 276–287) and it is thought that ligand binding may stabilise the preformed receptor–Jak2 complex (Argetsinger *et al.* 1993, Colosi *et al.* 1993). Box2 (amino acids 325–338) of the GHR is thought to be required for full activation of Jak2 by GH (Colosi *et al.* 1993, Wang & Wood 1995). GH binding results in a conformational change in the receptor dimer, and transphosphorylation of associated Jak2 (Argetsinger *et al.* 1993, Ross *et al.* 2001). Following GH stimulation,

phosphorylated Jak2 has been reported to activate Stat1, Stat3, Stat5 (Meyer *et al.* 1994, Campbell *et al.* 1995, Wood *et al.* 1995), and the MAP kinase pathway including Erk1/2, p38, and Jnk (Winston & Bertics 1992, Zhu *et al.* 1998, Zhu & Lobie 2000).

The majority of patients with GHIS have mutations in exons encoding the extracellular domain of the GHR (Rosenfeld *et al.* 1994, Woods *et al.* 1997). These mutations usually result in low or absent GH binding protein (GHBP) in serum and failure of GH to bind and signal at GH responsive tissues (Woods *et al.* 1997, Wojcik *et al.* 1998). The relatively few mutations described in DNA encoding the transmembrane or intracellular domain of the GHR result in truncated GHRs lacking most of the intracellular domain of the receptor (Ayling *et al.* 1997, 1999, Woods *et al.* 1996, Kaji *et al.* 1997, Iida *et al.* 1998, Gastier *et al.* 2000). Rarely, GHIS has been reported to be due to post-receptor defects (Iida *et al.* 1999, Rosenfeld *et al.* 2004).

Four patients from one family, with an atypical form of GHIS, have been previously identified having a homozygous point mutation in intron 6 of the GHR. The point mutation lies within the pseudoexon (A₋₁ to G₋₁ at the 5'pseudoexon splice site) and may improve the interaction of the pre-mRNA with U1 snRNP resulting in spliceosome action. This results in the activation of a pseudoexon encoding a 36 residue insertion between exons 6 and 7 which lies in the dimerization region of the extracellular domain of the GHR (Metherell *et al.* 2001). Failure of dimerization was thus thought to be the most likely cause of GHIS. To clarify, the effect of this mutation on GH function, we have expressed the mutant GHR in mammalian cells and investigated its functional properties.

Materials and Methods

Phenotype and genotype

The index cases were four male cousins from a highly consanguineous Pakistani family. The patients presented with marked short stature, and low IGF1 levels (20–29 ng/ml, levels >50 ng/ml), however they had normal levels of GHBP and a normal facial appearance. Genotyping revealed a 108 bp nucleotide insertion between exons 6 and 7, resulting from the inclusion of a pseudoexon, giving a receptor of 656 amino-acids (GHR1–656) (Metherell *et al.* 2001).

Plasmids

The full-length wild type human GHR (GHRwt) subcloned into pcDNA1/amp (Invitrogen) was previously described (Maamra *et al.* 1999). To amplify the sequence of insertion of GHR1–656, patient cDNA was subjected to PCR using primers within exons 6 and 7 (sense primer

5'-CAT GCA GAT ATC CAA GTG AGA TGG-3') and reverse primer 5'-GAT CTC ACG CGT ACT TCA TAT TCC TTA TCC ACT TTC AAT GAG -3'). Two fragments were amplified from the patient cDNA. The 302 bp fragment contains the inserted pseudoexon, whereas the lower 194 bp band corresponds to the low levels of GHRwt expressed in the patient. The 302 bp fragment was subcloned in PcDNA1 GHRwt following digestion with EcoRV and MluI. The reporter plasmids, Stat5/Stat3 responsive firefly luciferase reporter vector containing the lactogenic hormone response element LHRE-TK-luc has been described previously (Ross *et al.* 1997, Maamra *et al.* 1999).

Cell culture and transfection

Human embryonic kidney (HEK293) cells were routinely grown respectively in DMEM Nut F12 medium supplemented with 10% foetal calf serum (FCS), 100 IU penicillin and 100 µg /ml streptomycin, 2 mg/ml fungizone, 2 mM L-glutamine, and buffered with HEPES (all GIBCO). Cells were routinely grown at 37 °C in a 95% humidified atmosphere of 5% CO₂. Transfection of HEK293 cells was performed using a calcium phosphate transfection kit (GIBCO) according to the manufacturer's instructions.

Reporter assays

HEK293 cells were used to investigate the effect on the pseudoexon insertion on signaling. This cellular system was preferentially chosen against Chinese ovary cell line and human hepatoma cell line HuH7 as it responds more strongly to GH-induced signalling (unpublished observations, personal). Cells were incubated in 60% DMEM F12/30% DMEM 4.5 g/l glucose (GIBCO BRL) and 10% FCS for 6 h. Cells were transfected with 5 µg GHR expression vector or empty vector, 1.5 µg LHRE/TK-Luc and 30 ng of a renilla luciferase expression vector per plate. Sixteen h later, the cells were stimulated with GH in the presence or absence of 0.5 µM dexamethasone. The cells were harvested and assayed for firefly (LHRE) and Renilla (internal control) luciferase activities using the Promega dual luciferase kit (Promega). The activities were measured in the same aliquot sample using an Automat plus LB953 luminometer (Berthold, Redbourn, UK). Firefly Luciferase activity reflecting GH-dependent Stat activation was normalised using renilla luciferase activity to adjust for transfection efficiency between samples. Samples were measured in duplicate and experiments were repeated three times. The data are presented as fold induction (mean ± S.E.M.).

GH binding by monolayer cells

HEK293 cells were transfected with 3 µg of GHR expression plasmids and cells incubated in serum free medium

12 h prior to binding. Cells were then washed with PBS containing 1% BSA (Sigma) and incubated with I^{125} -labelled GH (10^5 c.p.m./well) (Novo Nordisk Bagsvaerd, Denmark) for 3 h at room temperature in the absence (total binding) or presence (non-specific binding) of excess unlabelled hGH (2 µg) (Genotropin, Pharmacia and Upjohn, Milton Keynes, UK). The cells were washed in the same buffer and solubilized in 1 M NaOH for counting on a gamma-counter. Specific binding of the radioligand was calculated as the difference of total and non-specific binding and expressed as percentage of the total count. Samples were measured in duplicate and experiments were repeated three times. The data are presented as percentage specific binding (mean $\pm$ S.E.M.).

Total GHR binding to GH

To estimate the total cellular content of functional GHR (cell surface and intracellular), GH binding activity of surface and intracellular membranes derived from cell sonicates was measured as follows. Cell monolayers were set up in 10-cm dishes. The cells were washed in PBS 0.2% BSA and detached by scraping in 1 ml of ice-cold PBS containing a mixture of protease inhibitors (Roche Diagnostics). After centrifugation at 800 r.p.m., the packed cells were resuspended in 2 ml of ice-cold inhibitor mixture and disrupted by sonication. The cell sonicates were centrifuged at 40 000g for 60 min at 4 °C to yield pellets of total cellular membranes. To measure GH binding, the membrane pellets were resuspended in 6.5 ml of ice-cold PBS 0.2% BSA.

The membrane suspensions were incubated in triplicate with I^{125} -labelled GH (2×10^5 c.p.m./ml) at room temperature for 2 h. Non-specific binding was defined as the binding in the presence of 10 µg/ml unlabelled GH. At the end of the assay, the membrane suspensions were centrifuged, washed twice with ice-cold PBS/0.2% BSA and counted on a gamma-counter. The data are presented as percentage specific binding (mean $\pm$ S.E.M.). Samples were measured in triplicate and experiments were repeated three times. To confirm equivalence of transfection efficiency, binding experiments were performed under identical conditions with simultaneous transcription assays with renilla luciferase as an internal control. In addition, for total membrane binding experiments. BCA was performed to evaluate the total amount of proteins in the membranes preparation. Membranes were pelleted by centrifugation at 14 000 r.p.m. for 15 mins at 4 °C. The pellet was dissolved in 1 M NaOH/0.2% Triton X-100 and BCA using a the Bio-Rad DC Protein Assay Kit II according to the manufacturer's instructions (Hemel Hempstead, Hertfordshire, UK). Cell surface binding and total membrane binding experiments were performed on the same transfection samples.

Flow cytometry

HEK293 cells (4×10^5 cells) were transfected with 3 µg of GHR expression plasmid. After preincubation in FCS free medium for 2 h, cells were dislodged from the culture dish using cell dissociation solution (Sigma Genosys). Cells were suspended in 600 µl of PBS 1%BSA (washing buffer). Cells (100 µl) were incubated with 5 µg of an anti-GH receptor antibody (2C8 antibody was a kind gift from Prof. CJ Strasburger, Berlin, Germany) or isotype-matched negative control antibody (R&D Systems, Abingdon, UK) for 30 min on ice. The binding epitope of the 2C8 antibody is on the centre of the hGH binding domain but does not overlap the epitopes of Mab263 (hinge region of GHR) and Mab10B8 (dimerisation domain) (Rowlinson *et al.* 1998, Z. Wu, unpublished observations). Primary antibody binding was detected by incubation with biotinylated goat anti-mouse IgG polyclonal antibody (1 µg; Calbiochem, Nottingham, UK), followed by incubation with Streptavidin-R.Phycoerythrin (SAV-R-PE) conjugate (10 µl; Serotec, Oxford, UK) for 30 min on ice. Flow cytometry was performed using a FACScan flow cytometer (BD Biosciences, Oxford, UK) and CellQuest data acquisition and analysis software (Beckton Dickinson, San Jose, CA, USA).

Immunofluorescence

Immunofluorescence was performed on fixed cells to determine the cellular distribution of GHR. Labelling was carried out on cells transfected with GHRwt or the mutated receptor GHR1–656. Cells were fixed in 2% paraformaldehyde (PFA) for 15 min and permeabilized or not with Triton X-100 (0.1% in 2% PFA) for 2 min. All washings were performed in PBS with 0.1% saponin. For blocking, cells were incubated in PBS 5% goat serum plus 0.1% saponin for 1 h. Biotin and streptavidin sites were blocked using the avidin–biotin blocking kit (Vector Laboratories UK, Peterborough, England). Cells were rinsed and subsequently incubated with the anti-GHR extracellular domain 2C8 monoclonal antibody (10 µg/ml) in antibody buffer (PBS 1% goat serum, 0.1% BSA, 0.1% saponin). After washing, cells were incubated with biotinylated Fab fragment of goat anti-mouse antibody and streptavidin Alexa 488 (Molecular probes, Eugene, OR, USA). When immunofluorescence was performed with a polyclonal rabbit antibody against the intracellular domain of GHR (Antibody 12, kindly provided by J Finidori, Paris, France) detection was performed with a secondary antibody directly coupled to Alexa 594. Slides were mounted in Vectashield mounting medium (Vector Laboratories UK). Fluorescence was detected using a LEICA TCS confocal fluorescent microscope (LEICA, Wetzlar, Germany). Cells were excited at 488 nm and detection was through a 530 nm band pass filter for Alexa 488 detection. For Alexa 594 detection, excitation was at

594 nm and detection was through a 610 nm band pass filter.

Jak–Stat Western blotting

Cells (2×10^6 cells per 10 cm dish) transfected with GHR expression plasmids were incubated overnight in serum-free medium and stimulated at 37 °C with GH. Cells were washed in 5 ml of PBS supplemented with 1 mM sodium orthovanadate and lysed in 1 ml of PBS-TDS buffer (PBS, 0.1% Triton X-100, 12 mM sodium deoxycholate, 3.5 mM SDS, 1 mM sodium orthovanadate). Lysates were immunoprecipitated with 10 µl anti-Jak2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-Stat5 antibody clone sc-835 (Santa Cruz) overnight at 4 °C. Lysates were briefly spun and incubated with 40 µl of protein A/G beads suspension (Pierce, Rockford, IL, USA). Western blot analysis was carried out in duplicate with equivalent amounts of protein loaded on gel confirmed by measuring total protein expression. Proteins were separated on a 7.5% SDS–PAGE gel for Jak2 and 10% for Stat5, and blotted onto PVDF membrane. Phosphorylation of Jak2 and Stat5 was detected using an anti-phosphotyrosine antibody (4 G10; 1:650; UBI, Lake Placid, NY, USA). The secondary antibodies were coupled to horseradish peroxidase (HRP; Amersham Pharmacia Biotech.) and the enzyme was detected using an enhanced chemiluminescence (ECL) kit according to the manufacturer's instructions (Amersham Pharmacia Biotech). All blots were reprobed for total Jak2 and Stat5.

GHR Western blotting

To estimate the amount of total GHR present and its size in the cells, cells transfected with GHR were incubated overnight in serum free medium and lysed in 250 µl of PBS-TDS lysis buffer. Crude lysates were separated on a 7.5% PAGE and blotted onto PVDF membrane. A rabbit polyclonal antibody against the intracellular domain of GHR (Antibody 12, kindly provided by J Finidori, Paris, France) was used for detection. ECL detection was performed as described for Jak–Stat.

Statistical analysis

The paired *t*-test was used with Bonferroni correction for multiple comparisons. For analysis of dose–response and time–course studies repeated measures ANOVA was used with Bonferroni correction as appropriate. Data are expressed as the mean $\pm$ S.E.M. and a $P < 0.05$ was considered to indicate statistically significant differences. For the detection of GH-induced gene transcription in GHR expressing cells, the data was analysed using a 2-way factorial ANOVA with blocking structure. Comparisons were made between grand means using appropriate

Figure 1 Cell monolayer binding by GHRs. Radioligand binding was carried out on HEK293 cells expressing either GHR1–656 or GHRwt, using I¹²⁵-hGH in the absence (total binding) or presence (non-specific binding) of excess cold GH. Non-transfected cells were used as a negative control. Results of 3 experiments are expressed as percent specific binding of labelled GH (mean $\pm$ S.E.M.).

contrasts, and *P*-values were calculated by reference to *t*-distributions. Again, $P < 0.05$ was considered to be statistically significant.

Results

Binding of GH to cell surface GHR

GHR1–656 and GHRwt were transfected into HEK293 cells and binding at the cell surface detected using iodinated human GH (I¹²⁵-GH) (Fig. 1). GH binding to GHR1–656 was consistently markedly reduced compared with GHRwt ($P = 0.01$). The same results were obtained in Chinese ovary cell line and human hepatoma cell line HuH7 cells (data not shown).

Flow cytometry detection of GHR expressed at the cell surface

GHR1–656 binds less GH at the cell surface than GHRwt. To further clarify the protein expression at the cell surface, flow cytometry detection was carried out using the 2C8 antibody against the extracellular domain of GHR (Fig. 2). This antibody recognises a site distant from the pseudoexon insertion. Both GHRwt and GHR1–656 exhibited higher fluorescence than untransfected cells. Mean fluorescence levels for expressed GHR1–656 were much lower than for GHRwt.

Binding of GH to total cellular GHR

GHR1–656 and GHRwt were transfected into HEK293 cells and total cell membranes prepared. Binding was performed using iodinated human GH (I¹²⁵-GH) (Fig. 3).

Figure 2 Flow cytometry analysis on GHR1-656 and GHRwt expressing cells. Untransfected HEK293 cells and HEK293 cells transfected with either GHR1-656 or GHRwt were incubated with 2C8 (mouse anti-human GHR). Biotinylated goat anti-mouse and phycoerythrin linked to streptavidin were used for detection. 2C8 recognises an epitope in the extracellular domain of GHR. Flow cytometry analysis results are expressed as mean fluorescence of cells with 2C8.

No significant binding was detected in cells transfected with control plasmids. Transfection of GHR gave a significant increase in GH binding to membranes (ANOVA, $P<0.002$). In cells transfected with GHR1-656, consistently higher levels of GH binding to membranes was detected compared with GHRwt ($P<0.03$). The same results were obtained in Chinese ovary cell line and human hepatoma cell line HuH7 cells (data not shown).

Subcellular localisation of GHR by immunofluorescence

To visualize the receptors independently of their ability to bind GH, we performed immunofluorescence studies with the monoclonal antibody 2C8 recognizing an epitope in the extracellular domain of the receptor. Cells were fixed and incubated directly or after permeabilization with Triton to visualize receptor present at the cell surface (Fig. 4a) and in intracellular compartments

Figure 3 Total cell membranes binding by GHRs. Radioligand binding was carried out on total membranes preparations from HEK293 cells expressing either GHR1-656 or GHRwt, using ^{125}I -hGH in the absence (total binding) or presence (non-specific binding) of excess cold GH. Non-transfected cells were used as a negative control. Results of 3 experiments are expressed as percent specific binding of labelled GH (mean $\pm$ S.E.M.).

Figure 4 Immunofluorescence cellular localization of GHRs. Cells transfected with control plasmid pcDNA3, GHRwt, or mutated GHR1–656 were grown on coverslips. Cells were fixed with 2% PFA and then labelled with 10ug/ml anti-GHR antibody for which the epitope is in the extracellular domain of the receptor and biotinylated anti-mouse and streptavidin labelled Alexa 488 (a). Cells were also permeabilized with 0.1% Triton X-100 prior to staining (b). Alternatively, permeabilized cells were then labelled with a rabbit polyclonal antibody against the intracellular domain of the receptor (c) and anti-rabbit antibody labelled with Alexa 594.

(Fig. 4b). In non-permeabilized cells, no fluorescence was detected in cells not transfected with GHR. A bright cell surface fluorescent signal was detected in cells expressing the GHRwt. In contrast, only a faint signal was detected on cells expressing the mutant receptor GHR 1–656. When intracellular staining was performed, comparable levels of immunofluorescence were observed in intracellular compartments of cells expressing GHRwt and GHR1–656. However, the pattern of fluorescence localisation was different. GHRwt was localized in cytoplasmic vesicles and in a region adjacent to the nucleus consistent with the Golgi apparatus. In contrast, the mutant GHR was mainly circumferential to the nucleus and only a few vesicles were detected in the cytoplasm consistent with the endoplasmic reticulum (ER) and compatible with the

Golgi apparatus in 293 cells as described previously (Maamra *et al.* 1999, Wojcik *et al.* 1998) (Fig. 4b). The same results of intracellular pattern were obtained with the antibody 12 directed against the intracellular region of GHR (Fig. 4c).

Detection of total GHR by Western blot

To further determine the level of protein expression of the different GHRs, Western blotting was performed (Fig. 5). The antibody used was directed against the intracellular domain of GHR. The GHRwt exhibited several bands, consistent with different glycosylation states and degradation by GHR turnover. The two lower bands of GHRwt were around 60 and 65 kDa and the same bands were

Figure 5 Detection of GHR cell expression by Western blot. Cells transfected with control plasmid pcDNA3, GHRwt, or mutated GHR1-656 were lysed and crude lysates run on SDS-PAGE gel and blotted onto PVDF membranes. Membranes were probed with a rabbit polyclonal antibody against the intracellular domain of the receptor and antibody binding detected by ECL.

detected for GHR1-656. Two bands migrated at around 110 kDa and 130 kDa consistent with previous reports for GHRwt. In GHR 1-656, the two corresponding bands were slightly higher consistent with the insertion of 36 amino acids in the mutant.

GH-induced Jak2 phosphorylation

293 cells were transfected with plasmids expressing GHR1-656 or GHRwt and stimulated with 100 ng/ml GH

for the times indicated. No phosphorylation of Jak2 was detected in cells transfected with mock control plasmid (data not shown). Phosphorylated Jak2 was detected in both cells expressing GHRwt and GHR1-656 when stimulated with GH (Fig. 6). However, the level of activation was lower in GHR1-656 than in GHRwt expressing cells.

GH-induced Stat5 phosphorylation

Cells were stimulated with 'low' (25 ng/ml) and 'high' (100 ng/ml) dose GH (Fig. 7a) for 15 min. No activation of Stat5 was detected in cells transfected with empty vector. In cells expressing GHRwt, phosphorylation of Stat5 was detected after stimulation with 25 ng/ml of GH for 15 min. At the higher concentration of GH (100 ng/ml), the level of phosphorylated Stat5 was increased. At both concentrations, GHR 1-656 expressing cells showed phosphorylation of Stat5, but to a lesser extent than on GHRwt expressing cells. A time response was performed. In GHRwt expressing cells, Stat5 phosphorylation was detected at 15 min after GH stimulation and was sustained for 60 min (Fig. 7b). The same pattern was observed in GHR1-656 expressing cells, although GH seemed to induce Stat5 phosphorylation to a lesser extent.

GH-induced gene expression through LHRE

To further quantify the difference in signaling between GHRwt and the mutant GHR, cells were transfected with 1.5 µg per 12 well plate of receptor expression plasmid and with the Stat5 responsive reporter LHRE-TK-Luc (Fig. 8). Cells were stimulated with varying doses of GH in the presence (b) or absence (a) of dexamethasone. Dexamethasone has been shown to exert a synergetic effect on GH-induced gene expression through LHRE. In cells transfected with control plasmid, no activation of transcription through LHRE was detected (Fig. 8a). When GHRwt was transfected, a six hours exposure to GH induces a dose-dependent increase in luciferase

Figure 6 Detection of Jak2 phosphorylation in GHR expressing cells by Western blot. Cells transfected with control plasmid pcDNA3, GHRwt, or mutated GHR1-656 were stimulated with 100 ng/ml GH for the time indicated. Cells were lysed and lysates immunoprecipitated with an anti-Jak2 antibody. Samples were run on SDS-PAGE gel and blotted onto PVDF membranes. Membranes were probed with the anti-phosphotyrosine antibody 4 G10 and antibody binding detected by ECL. Blots were reprobed for total Jak2.

Figure 7 Detection of Stat5 phosphorylation in GHR expressing cells by Western blot. (a) Cells transfected with control plasmid pcDNA3, GHRwt, or mutated GHR1-656 were stimulated for 15 min with GH at the concentration indicated. Cells were lysed and lysates immunoprecipitated with an anti-Stat5 antibody. Samples were run on SDS-PAGE gel and blotted onto PVDF membranes. Membranes were probed with the anti-phosphotyrosine antibody 4 G10 and antibody binding detected by ECL. Blots were reprobed for total Stat5. (b) Cells transfected with control plasmid pcDNA3, GHRwt, or mutated GHR1-656 were stimulated with 100 ng/ml GH for the time indicated. Samples were analysed as in (a).

activity compared with unstimulated cells ($P < 0.001$; GHRwt vs control), with activation visible at doses as low as 6.25 ng/ml GH. In cells expressing GHR1-656, GH also induces a dose-dependent LHRE activation, although at a lower level than in GHRwt expressing cells ($P < 0.001$; GHR1-656 vs GHRwt). Similar results were obtained when cells were transfected with lower levels of GHR (data not shown). When the experiments were repeated in the presence of dexamethasone, similar results were obtained, with lower activation of LHRE by GHR1-656 compared with GHRwt ($P = 0.022$ GHR1-656 vs control; $P < 0.001$ GHRwt vs control; $P < 0.001$ GHR1-656 vs GHRwt; Fig. 8b). All P values quoted are for differences between grand means of each GHR type.

Discussion

This manuscript reports *in vitro* studies of a GHR mutant identified in siblings with GHIS. The patients presented with atypical GHIS and our goal was to investigate the functional capacity of the mutant GHR.

An important first-step in GH signaling is the formation of a trimer complex with one molecule of ligand and two molecules of receptors. Recent studies suggest that GHR pre-exists as a dimer when synthesised (Ross *et al.* 2001, Gent *et al.* 2002), and that GH binding results in a

conformational change (Ross *et al.* 2001) and the formation of a cysteine bond (Zhang *et al.* 1999). As the pseudo-exon insertion in the GHR mutant lies between exons 6 and 7 (Metherell *et al.* 2001), a region previously described as critically involved in receptor dimerization (Cunningham *et al.* 1991), it had been suggested that the mutation resulted in a dimerization defect resulting in GHI. However, our studies indicate that the primary cause underlying the GHIS was related to a trafficking defect in the mutant receptor.

Binding studies on cell monolayers indicated that GHR1-656 binds less GH than GHRwt at the cell surface. We considered that GHR1-656 could have a reduced affinity for GH, however, our flow cytometry analysis confirmed that the levels of cell surface GHR1-656 protein were lower than for GHRwt. Some mutations in the GHR result in a defect in expression (Esposito *et al.* 1998), but this is not the case with GHR1-656 as RT-PCR from the patient's fibroblasts to construct the expression vector confirmed the previous studies that the mutant GHR mRNA was expressed at nominal levels (Metherell *et al.* 2001). Although cell surface binding was reduced for the mutant the overall binding for the mutant from total membranes was if anything increased. As the total cellular GHR binding of GH was not reduced in GHR mutant compared with GHRwt, this suggests that the affinity for GH is not a major defect for this mutation.

Figure 8 Detection of GH-induced gene transcription in GHR expressing cells. Cells were transfected with control plasmid pcDNA3, GHRwt, or mutated GHR1-656 plus 1.5 μg LHRE/TK-luc and an internal transfection control CMV-renilla luciferase expression vector. Cells were incubated without (a) or with (b) dexamethasone (0.5 μM) in the presence of increasing amounts of GH and cell lysates were used for determination of luciferase activity. Values of 3 experiments are expressed as fold induction (mean ± S.E.M.).

The signaling capacity of GHR 1-656 was evaluated by Western blotting and transcription assays. Both GHRwt and GHR1-656 were able to activate the Jak-Stat pathway, but activation was lower in cells transfected with the GHR1-656. GH-induced Stat5 and Jak2 phosphorylation seemed to be lower in cells transfected with the mutant receptor. Although at 60 min, Stat5 phosphorylation at 60 min appears to be comparable for GHRwt and mutant, this is more likely due to antibody maximal binding capacity and detection sensitivity. This was confirmed quantitatively in the LHRE transcription assay. In this assay, GHR1-656 showed at least 2-fold reduction in signaling capacities compared with GHRwt. At low concentrations of GH, the mutant receptor appeared to induce a similar level of transcription to the wt receptor, however, we suspect this is an artifact of the assay. It maybe that at low levels of GH, the assay is unable to differentiate different levels of signaling. Low total cellular

expression of the mutant is not a likely explanation for this defect, as by Western blot, the levels of GHRwt and GHR1-656 were similar. The explanation for reduced signaling probably relates to lower cell surface expression of the mutant GHR.

Glycosylation occurs with the GHR and is thought to play a role in protection of the protein. According to previous reports on GHR, glycosylation and ubiquitination are likely to account for an important part of the GHR size in mammalian cells (Harding *et al.* 1994, Alves dos Santos *et al.* 2001). Given the sizes detected in the Western blots, it is unlikely that glycosylation is affected in the GHR mutant.

Stat5 has been shown to be a major player in GH action. Generation of Stat5a/b knockout mice has been shown to result in mice significantly reduced in size in both sexes and size correlated with reduced levels of insulin-like growth factor (IGF-1) (Udy *et al.* 1997, Teglund *et al.* 1998). Before Stat5 can be phosphorylated by GH, it must first bind via its SH2 domain to specific phospho-tyrosyl residues in the cytoplasmic domain of the GHR. The GHR intracellular domain undergoes constitutive and GH-induced degradation, leading to the detection of remnant GHR fragments in the cell (Zhang *et al.* 2001). One could potentially hypothesise that the mutation in GHR 1-656 could influence GHR degradation and thereby alter Stat5 binding. However, the Western blotting confirmed the presence of a full size GHR, and the remnant GHR at lower size (50–75 kDa) in both GHR 1-656 and GHRwt, making abnormal internal degradation of the mutant GHR unlikely. Both WT and mutant appear to have similar relative abundances of mature and precursor GHR which, is reflected by the two higher forms of the full-length receptor (Fig. 5). This would suggest that the mature (fully glycosylated) and therefore post-Golgi) mutant receptor is less likely to be on the surface than the mature WT receptor. The lower cell surface expression of the mutant at the cell surface is translated into the lower signalling capacities. To determine exactly if the trafficking differs during biogenesis (processing ER to Golgi to surface) and/or after GH-independent endocytosis (lower cell surface half-life) and will require further investigation.

Our immunofluorescence data on fixed cells confirmed the low level of mutant receptor at the cell surface. More interestingly, it revealed a different fluorescent pattern for the mutant GHR when compared with GHRwt. Whereas the GHRwt is present in the Golgi Apparatus and in numerous small vesicles in the cytoplasm and at the plasma membrane, GHR 1-656 seemed to be trapped around the nucleus in a circumferential pattern of fluorescence. The pattern of fluorescence is at compatible with the Golgi apparatus in 293 cells as described previously (Wojcik *et al.* 1998, Maamra *et al.* 1999). This is consistent with the hypothesis that the mutant GHR is retained inside the cells, because of a failure of proper protein

folding. This is the most likely cause of the disease observed in the patients. These patients have an atypical GHIS phenotype. This appears to be explained by the fact that (1) low levels of GHRwt are expressed from a minor wild type GHR transcript and (2) because the mutant GHR retains the capacity for apparently normal signaling, despite significantly reduced expression at the cell surface. Both the wt and mutant GHRs may give rise to circulating GHBP explaining the normal serum levels of this marker found in these patients.

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