Disulfide bonds determine growth hormone receptor folding, dimerisation and ligand binding

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Summary
The growth hormone receptor contains seven cysteine residues in its extracellular domain. The six in the growth hormone binding domain form disulfide bonds, and help the receptor to gain its correct three-dimensional structure. In this study we replaced the cysteine for serine and alanine residues and investigated their role in growth hormone receptor folding, dimerisation and signal transduction. Folding and growth hormone binding capacity of the wild-type growth hormone receptor require less than two minutes for completion. Although less efficient, all mutant receptors arrive at the cell surface as pre-formed dimers.

Disulfide bond C38-C48 is important for efficient maturation. The middle disulfide-bond, C83-C94, is important for ligand binding. Removing disulfide bond C108-C122 has little effect without affecting signalling. When two or all disulfide bonds are changed, ligand binding and activation are blocked. Dimerisation is delayed when all disulfide bonds are destroyed.

Key words: Growth hormone, Cytokine receptor, Folding, Dimerisation, ER, Disulfide bonds

Introduction
The growth hormone receptor (GHR) is a type I transmembrane protein of 620 amino acids, belonging to the superfamily of cytokine receptors. Other members of this family are the prolanctin receptor, the erythropoietin receptor and several interleukin receptors (Bazan, 1990; Grotzinger, 2002). The crystal structure of the GHR in complex with growth hormone (GH) was solved in 1992 (de Vos et al., 1992). Recently, the unliganded GHR was crystallised (Brown et al., 2005). The GHR has an intracellular domain of 350 amino acids, containing the Box1 and the UbE motifs (Frank et al., 1999; Sotiropoulos et al., 1994; Govers et al., 1999). The transmembrane domain of the GHR has 24 amino acids and the extracellular domain 246. The extracellular domain consists of two subdomains, connected through a hinge region. The N-terminal part of the extracellular domain is involved in ligand binding, whereas the membrane-proximal part has a structural (supportive) function (de Vos et al., 1992; Chen et al., 1997). The extracellular domain of the GHR contains five putative N-glycosylation sites and seven cysteines (Leung et al., 1987; Fuh et al., 1990; Harding et al., 1994). Six cysteines form intramolecular disulfide bonds (see Fig. 1), and the seventh, at position 241, may form an intermolecular disulfide bond (Zhang et al., 1999). After protein synthesis, high-mannose glycosylation and dimerisation in the ER, complex glycosylation occurs in the Golgi system (Gent et al., 2002). As the GHR has no intrinsic kinase activity, two Jak2 kinases are recruited to the dimerised GHR. Upon ligand binding at the cell surface, a conformational change occurs, bringing the Jak2 kinases in close proximity (Gent et al., 2003; Brown et al., 2005). The kinases phosphorylate the receptor, crossphosphorylate themselves, and start signal transduction (Argetsinger et al., 1993; Argentsinger and Carter-Su, 1996). The fate of the GHR can be twofold: (1) the extracellular domain can be proteolytically cleaved by TACE, a metalloproteinase, generating the GH binding protein (GHBP) (Zhang et al., 2000; Conte et al., 2002; Schantl et al., 2003). Presumably, GHBP serves as a buffer capable of binding free GH in the blood (Baumann, 2001). The remaining part of the GHR is a target for presenilin-dependent γ-secretase activity (Cowan et al., 2005). (2) The major portion of the liganded or unliganded GHR internalises ligand-independently via clathrin-coated pits (Sachse et al., 2001; van Kerkhof et al., 2001b; van Kerkhof et al., 2002). Internalisation depends on the ubiquitin system; the UbE motif in the intracellular tail is required for this process (Strous et al., 1996; Govers et al., 1999). After internalisation, the GHR is degraded via the endosomal and lysosomal system (van Kerkhof et al., 2001a; van Kerkhof and Strous, 2001; Sachse et al., 2002).

Until now most research on the GHR was performed on the mature GHR: its signalling, shedding and internalisation. Not much is known about the synthesis and folding of the GHR. In this study, we mutated the three disulfide bonds of the extracellular domain of the GHR to investigate their role in folding, dimerisation and activation of the GHR.

Results
The disulfide bonds of the GHR
The organisation of the N-terminal part of the extracellular domain of the GHR is schematically depicted in Fig. 1. This figure is based on the work of Bazan (Bazan, 1990), Bass et al. (Bass et al., 1991) and de Vos et al. (de Vos et al., 1992). The extracellular domain of the GHR consists of two subdomains, each built of two antiparallel β sheets, one of four
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(D,C,F,G) and the other of three strands (A,B,E). Both domains are separated by a short linker sequence. Only the N-terminal domain, which is shown in Fig. 1, contains cysteine residues. They form disulfide bonds as indicated: C38-C48 connects neighbouring strands A and B of the three-stranded sheet (bond 1). C83-C94 links strand D to strand E, thereby crosslinking the two β sheets (bond 2). The third disulfide bond, C108-C122, links strands F and G of the four-stranded sheet (bond 3). The role of the disulfide bonds in achieving the tertiary structure of the GHR was examined in this study.

Folding of the GHR

First, we investigated folding of the wild-type GHR (wtGHR) in a pulse-chase assay. The receptor was transiently transfected in ts20 cells. To ascertain that no folding intermediates of the GHR were missed, immunoprecipitations were performed with a polyclonal antibody raised against the membrane-proximal cystosolic tail (Fig. 2A, left panel, anti-GHR-T). After a 10-minute pulse-label period (0-minute chase), the precursor (ER) form of the GHR is seen as a double band just above 100 kDa. During longer chase times, the double band slightly diminished and shifted downwards in the gel. There are two additional bands visible around 90 kDa which disappear after 60 minutes. The complex glycosylated, mature 130-kDa GHR (present at the cell surface) appeared after 30 minutes of chase. After 240 minutes of chase, no degradation can be seen.

To investigate how folding relates to the capacity of the newly formed protein to bind ligand, we combined pulse-chase labelling with a pull-down procedure using biotinylated GH (btGH) (Fig. 2A, right panel). In the case of the btGH pull-down, only GHR that bound GH will be isolated. In Fig. 2A equal aliquots for both immunoprecipitation and btGH pull-down were used. After a pulse-label period of 10 minutes (0-minute chase) GH already bound GHR equally as well as the antibody. If folding into a GH-binding-competent molecule takes more than 2 minutes, the first lane (0-minute chase, right panel) would be significantly less intense compared with the second lane (5-minute chase). However, both lanes are of equal intensity. Assuming that the anti-GHR-T antibody recognised GHR immediately after completion, and that it takes approximately 30 seconds to synthesise a GHR molecule, we conclude that folding of GHR into a GH-binding-competent molecule occurs within 2 minutes. When the left and right panels are compared, at each time point both bands have the same intensity (verified through image quantification, results not shown) indicating that the two isolation methods are equally efficient. The only difference between the two panels is that after immunoprecipitation, two additional bands run around 90 kDa, which cannot be seen after btGH pull-down.

Fig. 1. Schematic representation of the β-sheet and disulfide bond organisation of the N-terminal portion of the extracellular domain of the GHR. The three-stranded β-sheet, with strands A,B,E, is indicated in dark grey. The four-stranded β sheet, with strands D,C,F,G, is indicated in light grey. Disulfide bonds are connected with lines. Bond 1 is C38-C48, bond 2 is C83-C94 and bond 3 is C108-C122.

Fig. 2. Folding of the wild-type GHR. Transiently transfected ts20 cells were pulse-labelled for 10 minutes and chased for the indicated times. (A) Postnuclear lysates were split in equal amounts and either immunoprecipitated with anti-GHR-T (left panel), or isolated with btGH and streptavidin beads (right panel). Samples were subjected to reducing SDS-PAGE. p, 110-kDa precursor form; m, 130-kDa mature form. (B) After immunoprecipitation, samples were treated with EndoH (+) or mock treated (–). All samples were immunoprecipitated with anti-GHR-T and subjected to reducing SDS-PAGE. (C) Cells were pre-incubated with MG132 for 1 hour and subsequently treated with MG132 during pulse and chase periods (+), or mock treated (–). Samples were immunoprecipitated with anti-GHR-T and subjected to reducing SDS-PAGE. (D) After immunoprecipitation, samples were subjected to reducing (+) and non-reducing (–) SDS-PAGE. Relative molecular mass standards are indicated.
To investigate the nature of the 90-kDa bands, we treated the samples with endoglycosidase H (Endo H) (Fig. 2B). Endo H specifically cleaves high-mannose N-linked glycans in their pre-Golgi state. Upon treatment with Endo H, the two 110-kDa bands shifted towards the 90-kDa bands, indicating that the 110-kDa bands are Endo-H-sensitive forms of the GHR. As the lower bands did not shift, we conclude that they represent non-glycosylated forms of the GHR. The 130-kDa band was not sensitive to Endo H treatment, indicating that this GHR is complex glycosylated and is in or passed the Golgi complex. The untreated 110-kDa bands shifted downwards with time (Fig. 2A). Most likely, this is due to mannose trimming of the molecule, because after Endo H treatment both the 10-minute and the 120-minute chase bands run at exactly the same height (Fig. 2B). Note that the 90-kDa bands do not bind GH (Fig. 2A).

To explain the disappearance of the 90-kDa bands with time, we incubated the cells with MG132, a proteasome inhibitor (Fig. 2C). After 10 minutes of chase, no differences were observed. After 100 minutes of chase, the non-glycosylated bands started to disappear in the non-treated cells. After 180 minutes of chase the lowest bands almost completely disappeared in the non-treated cells, whereas in the MG132-treated cells, the 90-kDa bands were still present. This indicates that the non-glycosylated, 90-kDa bands are subjected to the ER-associated protein degradation (ERAD) system (Sommer and Wolf, 1997; Brodsky and McCracken, 1999; Meusser et al., 2005) and that they do not convert to mature GHR. After 180 minutes of chase, no differences were observed between MG132-treated or non-treated cells in the 110-kDa and 130-kDa forms, indicating that the glycosylated GHR species are not sensitive to ERAD.

When reduced samples are compared with non-reduced samples (Fig. 2D), the non-reduced (–DTT) samples run slightly lower. No folding intermediates were observed. This means that the disulfide bonds, which are destroyed after DTT treatment, create a more compact GHR and are already fully formed after a 10-minute pulse period. The mannose-trimming pattern is observed in both the reduced and non-reduced lanes.

In conclusion, the newly formed GHR polypeptide folds quickly, without any discernable intermediates, into its correct precursor form. GH recognizes this form but not the non-glycosylated 90-kDa GHR species.

Disulfide bond mutations and folding
The GHR contains seven cysteine residues in the extracellular domain. Six of them form intramolecular disulfide bonds, as shown in Fig. 1. We mutated these cysteine residues into serine or alanine residues. The cysteine-to-alanine mutations are disruptions of entire disulfide bonds. The cysteine-to-serine mutations are single point mutations, to investigate the difference between removing an entire disulfide bond and destroying one side of a bond. To determine the effect of the various mutations on the kinetics of folding of the GHR, we transiently transfected the wild-type and mutant receptors into ts20 cells, pulse-labelled the cells for 10 minutes, and analysed the GHRs by immunoprecipitation after a short (15-minute) and a long (180-minute) chase period (Fig. 3A). In most cases the precursor forms are slightly smaller at 180 minutes of chase compared with a 15-minute chase time. As explained before, this is probably due to mannose trimming and occurs with all mutants. No folding intermediates were observed. All mutant GHRs matured less efficiently than the wtGHR. Notably, with the exception of the C018S mutant, all of the mature, mutant receptors migrated as more compact species.

In Fig. 3B, the maturation efficiencies are shown, quantified as the ratio of mature to total at 180 and 240 minutes of chase, and presented relative to the wtGHR maturation efficiency. The mature receptors have maturation efficiencies between 26% and 68% when compared with the wtGHR. The mutants that mature least efficiently lack the first disulfide bond (Fig. 3B, mutants A, AB, AC, C485). The mutants that mature best lack the third disulfide bond (Fig. 3B, mutants C, C108S). We conclude that the first disulfide bond, C38-C48, is important for efficient maturation of the GHR.

![Fig. 3. Maturation efficiency of wild-type and mutant GHRs.](image-url)

(A) Folding and maturation of wild type (WT) and mutated GHRs transiently transfected in ts20 cells. Cells were pulse labelled for 10 minutes and chased for 15 or 180 minutes (as indicated). Samples were immunoprecipitated with anti-GHR-T and subjected to reducing SDS-PAGE. p, 110-kDa precursor form; m, 130-kDa mature form; A, GHR(C38A-C48A); AB, GHR(C83A-C94A); C, GHR(C108A-C122A); AB, AC, BC and ABC are combinations of mutations A,B and/or C. Relative molecular mass standards are shown on the right. (B) Quantification of the maturation efficiencies of the wild-type and mutant receptors. The amount of radioactivity was determined using ImageQuant (Molecular Dynamics). The amount of mature GHR species (130 kDa) was divided by the amount of precursor (110 kDa) and mature GHR species at 180 and 240 minutes chase and represented as a percentage relative to the maturation rate of wtGHR. The values represent the mean ± s.d. of at least two different experiments.
Disulfide bonds and functional GHR folding

In order to determine the capability of the mutant receptors to bind ligand, we used btGH to pull down the mutant GHRs after a 90-minute and 240-minute chase (Fig. 4A). The two time periods allow analysis of both rapid- and slow-folding GHRs. In all cases the amount of radioactivity was compared with and expressed as the radioactive GHR species after immunoprecipitation. We observed no differences between the 90- and 240-minute chases. Fig. 4B shows the quantified data. In the wild-type situation, the receptor immunoprecipitation signal and the btGH signal were equal and confirmed the data in Fig. 2A. btGH bound mutant A at 19% of the wild-type level, mutant C at 32% and mutant B was not recognised (Fig. 4B). The C48S mutant was recognised only by a very small amount of ligand (7%). btGH bound C108S at 37% of the wild-type level, but the C83S mutant was not recognised. Mutating two or more disulfide bonds destroyed the possibility of binding ligand. Apparently, the middle disulfide bond, C83-C94, is important for ligand recognition.

Disulfide bond mutations and dimerisation

To examine whether the disulfide bond mutations can interfere with dimerisation of the receptors, we performed co-immunoprecipitation assays. Ts20 cells were transiently cotransfected with wild-type or mutant GHRs, either in a full-length (FL) form or as a truncated, epitope-tagged GHR(1-369; HA-His6-Myc) (369-HA). Fig. 5A contains lysates of the samples and Fig. 5B immunoprecipitations. Immunoprecipitations were performed with anti-GHR-C, recognising only full-length GHRs. Detection of the western blots was performed with an anti-HA antibody, showing the epitope-tagged truncated receptors. These receptors can only be seen in Fig. 5B when heterodimer formation occurs between full-length and truncated receptors. To exclude the possibility that the interaction occurred after lysis, lysates from ts20 cells expressing full-length wild-type and mutant receptors were mixed with lysates from ts20 cells expressing 369-HA-tagged wild-type and mutant receptors. Under those conditions, no interaction was observed (Fig. 5B, M lanes). Reprobing the same blot with the GHR antibody Mab5 revealed similar amounts of precipitated full-length GHR species (Fig. 5C). Apparently, all disulfide bond mutations are able to form dimers. Comparison of the ratios of mature to precursor before and after the pull-down procedure reveals whether the different mutants dimerise with equal efficiencies. When the ratios were compared between direct lysates (Fig. 5A) and co-immunoprecipitations (Fig. 5B), they were all approximately the same, except for the ABC mutant. Here, the ratio doubled after immunoprecipitation. Because the amount of mature receptors does not change, there is relatively less precursor form of the ABC mutant immunoprecipitated in the dimers. This indicates that dimerisation is delayed for this mutant, whereas for the wtGHR and the other mutant GHRs, all precursor forms have dimerised with equal efficiencies.

Fig. 4. Ligand-binding capacities of the mutant receptors. (A) Transiently transfected ts20 cells were pulse-labelled for 10 minutes and chased for 90 or 240 minutes as indicated. Samples were split into equal amounts and immunoprecipitated (IP) with anti-GHR-T or isolated with btGH and streptavidin beads (btGH) and subjected to reducing SDS-PAGE. Receptor species are indicated as in Fig. 3, p. 110-kDa precursor form; m, 130-kDa mature form. Relative molecular mass standards are shown on the right.

(B) Quantification of the ligand binding capacity of wild-type and mutated receptors. The amount of radioactivity was determined using ImageQuant (Molecular Dynamics). Precursor form intensities of the wild-type and mutant receptors after btGH pull-down were divided by precursor form intensities of the wild-type and mutant receptors after immunoprecipitation and represented as a percentage. The values represent the mean ± s.d. of at least two different experiments.
Cell surface appearance of the mutant receptors

The expression of wild-type and mutant GHRs at the cell surface was measured by flow cytometry analysis. As a negative control, empty-vector-transfected cells were used, visible in each panel of Fig. 6 as a solid histogram. The monoclonal antibody Mab5 is able to detect cell surface expression of the wtGHR (Fig. 6, upper left panel, open histogram). The mutant receptors (open histograms) are named in each panel. Compared with the empty vector control, all the mutants show a shift to the right, indicating expression at the cell surface. For mutants A, B, AB, AC and C48S the shift is very small, but this is in agreement with Fig. 3, which showed that these mutants mature least well. As the flow cytometry method is dependent on a conformationally sensitive antibody, the actual amount of mutant receptors at the cell surface may be higher. To control this, we performed proteinase K digestion and analysed the disappearance of the mature form of the GHRs on western blot (results not shown). For all mutant receptors, the extracellular domain disappeared upon proteinase K treatment. We conclude that for all mutants, the 130 kDa mature GHR arrives at the cell surface.

Signalling capacities of the mutant receptors

Finally, we examined whether the mutant receptors that are ligand-binding competent, GHR(C48S), GHR(C108S), mutant A and mutant C, are also able to initiate signal transduction. This should result in tyrosine phosphorylation of the receptors after btGH and not after btGH antagonist (btGHA) treatment and pull-down. The mutant receptors C48S and A have such a low amount of mature receptors, that it was not possible to obtain sufficient mature receptor to detect tyrosine phosphorylation. Fig. 7A shows the lysates of the wtGHR, mutant C108S and mutant C, transiently transfected in ts20 cells. Equal amounts of receptors were used for the pull-downs. In Fig. 7B the isolated proteins were detected with the anti-GHR-C antibody, showing efficient pull-down of only the mature species with both btGH and btGHA. After 15 minutes of incubation with btGH, a phosphorylated tyrosine (PY) signal was visible for all three receptor species at the same height as the mature GHR (Fig. 7C). No PY signal was visible in the cells incubated with btGHA. In conclusion, even though disulfide bond C108-C122 is partly or completely changed, the receptors that mature and arrive at the cell surface are still capable of initiating signal transduction.

Discussion

The functional relevance of the three disulfide bonds of the GHR was investigated in this study. The results can be clarified by the crystal structure of the receptor (see Fig. 1). C83-C94 connects both sheets of domain 1, whereas the other two bonds connect strands within one sheet. Therefore, this disulfide bond is probably most important and, indeed, when disrupting C83-C94, the mutated GHR is no longer capable of binding its ligand. Bond C38-C48 is more conserved than C108-C122 and probably needs to fold first, to allow the rest of the extracellular domain to form. This could explain its role in maturation efficiency. The minor effects of mutating C108-C122 are explained by the fact that this disulfide bond is not conserved among the cytokine receptor family (Bazan, 1990). In the salmon GHR it is also absent (Fukada et al., 2004). When the cysteine-to-alanine mutations are compared with the cysteine-to-serine mutations, no major differences are observed. The trend in maturation efficiency is the same, with the first disulfide bond, C38-C48 having the lowest and the third having the highest maturation efficiency. The middle disulfide bond loses GH recognition in both situations, whereas C108-C122 can still be activated in both types of mutation. In this study, mutating one or two cysteines of a disulfide bond does not cause different effects. The membrane-proximal domain of the extracellular part of the GHR contains one unpaired cysteine at position 241. This cysteine may form a disulfide bond with a dimersing GHR but was not investigated in this study (Zhang et al., 1999).

To our knowledge, only one patient has been described with
a missense mutation at a cysteine position (C38S) (Sobrier et al., 1997). This person had Laron syndrome, with detectable plasma GH-binding activity (Laron et al., 1971). According to the authors the mutation affected a highly conserved residue, which is a key element of secondary structure, involved in the edification of the GH-binding site. Our data confirm these results. In addition, even though we mutated the opposite site of the disulfide bond and removed the entire bond, our results predict a reduced amount of GHRs at the plasma membrane. The mutant GHR can bind GH, it may even be able to signal, but it matures with such a low efficiency that its signal transduction capacities will probably be insufficient.

In this study, transient transfections were used. We found some differences between the stable and the transient system regarding GHR turnover, being prolonged in the transient system. Van Kerkhof et al. found the stably transfected GHR to have a half-life of about 75 minutes (van Kerkhof et al., 2000; van Kerkhof et al., 2002). In the experiments described here, the half-life of the GHR is over 4 hours. In the pulse-chase assay (Fig. 2A), the intensity of the ER form of the GHR only slightly reduces in 4 hours (verified by image quantification), whereas in the stable system, almost all precursor forms become mature. Transient transfection probably overloads the ER and the glycosylation machinery to such an extent that a large proportion of the precursor forms of the GHR never matures. Our MG132 experiments have shown that in the first 180 minutes, the 110- and 130-kDa species of the GHR are not being degraded (Fig. 2C). But we do see mannose trimming, especially after 60 minutes of chase, which signals the onset of ERAD (Sommer and Wolf, 1997; Brodsky and McCracken, 1999). If chase times are prolonged, we observe the disappearance of the wild-type precursor (results not shown).

The 90-kDa non-glycosylated species are also typical for transient transfections. They seem to appear under strong ER overload and are removed by ERAD relatively quickly. On the extent to which non-glycosylated GHRs are recognised by GH, Fuh et al. concluded that glycosylation of the GHR, produced in bacteria, is not important for ligand recognition (Fuh et al., 1990). The crystal structure of the GHR in complex with GH was determined with non-glycosylated GHR, produced in bacteria (de Vos et al., 1992). Harding et al. and Szecowka et al. state that mutating glycosylation sites or interfering with glycosylation strongly reduce GH-binding capacities (Szecowka et al., 1990; Harding et al., 1994). The non-glycosylated GHR molecules in this study cannot be recognised by GH and run at exactly the same height as normal precursor forms with the glycans removed (Fig. 2B,D). This
lysates and co-immunoprecipitations are compared. This implies that the non-glycosylated species generated in the ER, although correctly folded, cannot bind GH.

Mutating disulfide bonds does not prevent dimerisation of the GHR. However, for the ABC mutant, a different mature to precursor ratio was observed, implicating delayed dimerisation. Probable, dimerisation occurs co-translationally or very early after translocation into the ER lumen.

In conclusion, this study provides novel information on the role which disulfide bonds have in achieving a correctly folded, dimerised and functional GHR. Understanding how folding and dimerisation govern GHR biogenesis will help in understanding how cells regulate GHR availability at the cell surface. Since the two most conserved disulfide bonds are present in all cytokine receptors these results might explain structure-function relationships in other family members.

Materials and Methods

Materials and antibodies

Anti-GHR rabbit antiserum generated against amino acid residues 271-320 (anti-GHR-T), 327-493 (anti-GHR-B) and 493-620 (anti-GHR-C) were raised as previously described (Strous et al., 1996; van Kerkhof et al., 2000). Monoclonal anti-HA antibody 16B12 was purchased from Eurogentec (Belgium, CA). Antibody Mab5, recognising the extracellular domain of the GHR was obtained from AGEN (Parsippany, NJ). Monoclonal antibody 4G10, recognising phosphorytorysine residues, was obtained from Upstate Biotechnologies (Lake Placid, NY). Human GH was kindly provided by Eli Lilly & Co. Research Labs (Indianapolis, IN). Human GH antagonist B2036 (GHA), containing a G120K mutation in binding site 2 and eight additional mutations that enhance the binding affinity of site 1 (Cunningham et al., 1991; Ross et al., 2001), was a generous gift from William F. Bennet of Sensus Drug Development Cooperation (Austin, TX). Biotinylated GH (btGH) or GHA (btGHA) were created according to the manufacturer’s instructions. (Pierce, Rockford, IL) (Bentham et al., 1994). MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucyl-L-leucinal) was from Calbiochem (San Diego, CA).

GHR mutants and cell lines

Disulfide bond mutations of the GHR extracellular domain were created by QuikChange Site-directed Mutagenesis (Stratagene). Briefly, pcDNA3 plasmids (Invitrogen) encoding wild type rabbit GHR (wtGHR) and GHR(1-369; HA-His6-Myc) (369-HA) were used as a template in a polymerase chain reaction with 3’ and 5’ oligonucleotides encoding for the C48S, C83S, C108S, C38A-C48A (A), C83A-C94A (B), C108A-C122A (C) mutants or combinations of mutations: A and B (AB), A and C (AC), B and C (BC), A, B and C (ABC). The oligonucleotides also introduced a silent mutation that either created or disrupted a restriction site. All constructs were verified by restriction analysis and sequencing. The construction of a triple-epitope-tagged truncation mutant GHR(1-369; HA-His6-Myc) was described before (Gent et al., 2002).

Chinese hamster ts20 cells, bearing a thermolabile ubiquitin-activating (E1) enzyme, were used for experiments. Transient transfections were performed with FuGENE6 (Roche) according to the manufacturer’s description. 48 hours after transfection, cells were used for experiments. Cells were cultured at 30°C in MEMα (Gibco) supplemented with 10% fetal bovine serum (Sigma), 4.5 g/l glucose and 100 U/ml penicillin/streptomycin (Gibco).

Pulse-chase assay

Subconfluent ts20 cells, grown in 6 cm dishes, were used 48 hours post-transfection for pulse-chase analysis as described (Jansens and Braakman, 2003). The cells were washed in phosphate-buffered saline (PBS) and pre-incubated in starvation medium lacking methionine and cysteine for 15 minutes at 30°C. Cells were pulse-labelled for 10 minutes with 125 μCi/ml RepliV×PRO-MIX™ L-[35S] in vitro cell labelling mix (Amersham Biosciences) and chased with excess cold methionine and cysteine and 1 mM cycloheximide in MEMα medium (with HEPES) at 30°C for the indicated times. Incubations were stopped by transferring the cells to ice, aspirating the medium and adding ice-cold PBS, containing 0.5 mM MgCl2, 1 mM CaCl2 and 20 mM N-ethylmaleimide (MEM α) (Sigma). The cells were lysed in ice-cold lysis buffer containing 1% Triton X-100, 1 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride (PMSF) and 20 mM NEM in PBS. Cell lysates were centrifuged to pellet the nuclei and post-nuclear supernatants were used for immunoprecipitation or btGH pull-down. GHR molecules were immunoprecipitated with anti-GHR antibodies in 1% Triton X-100, 1% SDS, 0.5% sodium deoxycholate, 1% BSA, 1 mM EDTA, 1 mM PMSF, 50 mM NaF, 1 mM Na3VO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 20 mM NEM. Immunocomplexes were isolated with protein-A-conjugated agarose beads (Repligen, Waltham, MA). Alternatively, cell extracts were incubated with 100 ng btGH. btGH-GHR complexes were isolated with Immunopure immobilised streptavidin (Pierce). Immunoprecipitates or btGH-GHR complexes were subjected to SDS-PAGE. Gels were stained in a Coomassie Brilliant Blue solution, destained in 10% methanol, dried, and visualised using a Molecular Dynamics Phosphorimager.
The amounts of radioactivity were determined using ImageQuant (Molecular Dynamics). Endoglycosidase H (Endo H) treatment (Boehringer): After immunoprecipitation, 15 μl of 100 mM sodium acetate pH 5.5 and 0.2% SDS was added, the beads were resuspended and boiled for 5 minutes. Next, 15 μl of 100 mM sodium acetate pH 5.5, 1 mM PMSF, 1 mM NaF, 1 mM NaVO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2% Triton X-100 and 0.0025 U Endo H were added and the samples were incubated for 90 minutes at 37°C. Control samples were treated in the same way, except for the addition of Endo H. After incubation, samples were prepared for reducing SDS-PAGE.

Co-immunoprecipitation

Cells were grown in 6 cm dishes and 48 hours post-transfection. Dishes were put on ice and washed three times with ice-cold PBS. The cells were lysed in ice-cold co-immunoprecipitation (co-ip) buffer containing 0.5% Triton X-100, 1 mM EDTA, 50 mM NaF, 1 mM NaVO4, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM PMSE in PBS. Cell lysates were centrifuged to pellet the nuclei and post-nuclear supernatants were mixed as indicated and used for immunoprecipitation with anti-GHR-C. Immune complexes were isolated with protein-A-conjugated goat-anti mouse beads (Repligens). The immunoprecipitates were washed twice with co-ip buffer and twice with PBS. The proteins were subjected to reducing SDS-PAGE and transferred to Immobilon-FL polyvinylidenedifluoride (PVDF) membrane (Millipore). Blots were immunostained with the indicated antibodies followed by Alexa Fluor 680- (Molecular Probes) or IRDye800 (Rockland, Gilbertsville, PA)-conjugated goat-anti-mouse antibodies. Detection and quantification were performed with an Odyssey System (LI-COR Biosciences, Lincoln, NE). When indicated, blots were reprobed after stripping twice for 15 minutes with stripping buffer from Escherichia coli and disulide bonding pattern of the extracellular domain. J. Biol. Chem. 265, 3111-3115.

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