Multimeric Growth Hormone Receptor Complexes Serve as Signaling Platforms

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Background: The current concept is that preformed homodimeric cytokine receptors initiate signaling.

Results: We utilized blue native PAGE to identify novel functional GHR-GH complexes.

Conclusion: GH induces higher order ~900-kDa receptor complexes that are phosphorylated and polyubiquitylated and that act as signaling platforms.

Significance: Insight in cytokine receptor complex formation is important for engineering cytokines that control cell responses.

Growth hormone (GH) signaling is required for promoting longitudinal body growth, stem cell activation, differentiation, and survival and for regulation of metabolism. Failure to adequately regulate GH signaling leads to disease: excessive GH signaling has been connected to cancer, and GH insensitivity has been reported in cachexia patients. Since its discovery in 1989, the receptor has served a pivotal role as the prototype cytokine receptor both structurally and functionally. Phosphorylation and ubiquitylation regulate the GH receptor (GHR) at the cell surface: two ubiquitin ligases (SCFTrCP2 and CHIP) determine the GH responsiveness of cells by controlling its endocytosis, whereas JAK2 initiates the JAK/STAT pathway. We used blue native electrophoresis to identify phosphorylated and ubiquitylated receptor intermediates. We show that GHRs occur as ~500-kDa complexes that dimerize into active ~900-kDa complexes upon GH binding. The dimerized complexes act as platforms for transient interaction with JAK2 and ubiquitin ligases. If GH and receptors are made in the same cell (autocrine mode), only limited numbers of ~900-kDa complexes are formed. The experiments reveal the dynamic changes in post-translational modifications during GH-induced signaling events and show that relatively simple cytokine receptors like GHRs are able to form higher order protein complexes. Insight in the complex formation of cytokine receptors is crucially important for engineering cytokines that control ligand-induced cell responses and for generating a new class of therapeutic agents for a wide range of diseases.

The growth hormone (GH) receptor (GHR) is a key regulator of postnatal growth and, together with insulin, is an important regulator of lipid and carbohydrate metabolism. It belongs to the class I cytokine receptor family, which, among others, includes prolactin and erythropoietin receptors (1). Members of this family lack intrinsic kinase activity and rely on JAK family member non-receptor tyrosine kinases to initiate signaling (2). Binding of GH to the GHR initiates conformational changes that lead to full activation of JAK2 and initiation of signaling mainly via the STAT5/MAPK pathways.

The GHR is synthesized as a precursor, provided with 5-high mannose oligosaccharides, and dimerizes in the endoplasmic reticulum (ER) (3). Next, the receptor travels to the Golgi complex, where it becomes complex-glycosylated and appears on the cell surface. The GHR is constitutively endocytosed (4). Previously, we identified two ubiquitin ligases, SCFTrCP2 and CHIP, as necessary factors for GHR endocytosis (5, 6). SCFTrCP2 ubiquitylates the GHR, which is subsequently internalized via clathrin-coated pits and degraded in lysosomes (4, 7). GHR endocytosis depends on a functional ubiquitin system, but Lys-48 ubiquitylation of the receptor itself is not necessary for this event (8). Recently, we showed that also Lys-63-linked ubiquitylation via CHIP and Ubc13 is required for GHR endocytosis (6). Upon binding to the GHR, GH stimulates its internalization (7). This leads to the following scenario: JAK2 becomes active, phosphorylates both itself and the receptor, and dissociates from the GHR (7). Next, the two ubiquitin ligases, SCFTrCP2 and CHIP/Ubc13, assemble on the receptor, and finally, ubiquitylation of yet unidentified substrates through either Lys-48 or Lys-63 linkages initiates clathrin-mediated endocytosis. The relative abundance of the GHR at the cell surface is a key determinant of cellular GH sensitivity. Thus, insight in the dynamics of the protein complexes involved in receptor signaling is needed.

Multiprotein complexes play crucial roles in nearly all cell biological processes. Blue native electrophoresis (BN-PAGE) is a powerful tool to analyze these protein complexes in native conditions. Combining BN-PAGE with second dimension SDS-PAGE (second dimension BN/SDS-PAGE) allows separation and analysis of proteins within the complexes according to their size, subunit composition, and relative abundance (9, 10). We utilized BN-PAGE to study the GHR at early stages of synthesis and endocytosis. We took advantage of the relative low affinity of GH-(His-TEV-STREP3-His) (referred to as Strep-
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GH) that allowed isolation and purification of the GHR-containing protein complexes. We show that there are mainly two complexes present at the cell surface. Additionally, we provide evidence that only receptors within the bigger complex are subjected to phosphorylation and ubiquitination. If Strep-GH and the GHR are expressed in the same cell (autocrine mode), our data reveal that, although the assembly process starts in the ER, the signaling output differs from endocrine signaling. Using wild-type and mutant GHRs, we show the dynamics of the JAK2 signalosome by analyzing the GHR together with two main binding partners, JAK2 and βTrCP2 (β-transducin repeat-containing protein 2).

EXPERIMENTAL PROCEDURES

Cells, Plasmids, Antibodies, and Reagents—HEK293 cells were used as described previously (7). FLAG-tagged wild-type mouse JAK2 constructs were a generous gift of Prof. Carter-Su (University of Michigan, Ann Arbor, MI). Plasmid cDNAs expressing ubiquitin variants and FLAG-tagged βTrCP2 were generous gifts of Tomoki Chiba (Tokyo Metropolitan Institute of Medical Science). pCAGGS3.1-HA-K1Ub-K48 and pCAGGS3.1-HA-K1Ub-K63 were kindly provided by Dr. Kazuhiro Iwai (Osaka City University, Osaka Prefecture, Japan). The wild-type and truncated rabbit GHR cDNAs in pcDNA3 have been described (11). GH-(His-TEV-STREP3-His) (referred to as Strep-GH) was kindly provided by Dr. R. A. P. Romijn (U-Protein Express BV, Utrecht, The Netherlands). Mouse anti-phosphotyrosine antibody was from Millipore (clone 4G10), anti-HA tag antibody (clone 12CA5) was from BabCO (Richmond, CA), anti-ubiquitin antibody (clone FK2) was from Enzo, and anti-FLAG tag M2 antibody was from Sigma. Rabbit anti-GHR B antibody was described previously (4, 11). Monoclonal antibody against phosphorylated Tyr-1007 and Tyr-1008 of JAK2 was from Abcam (ab32101). Alexa Fluor 680- and IRDye 800-conjugated goat anti-mouse and anti-rabbit IgGs were obtained from Molecular Probes. GHR-expressing HEK293 cells grown as described (7) were transfected with FuGENE 6 (Roche Applied Science) according to standard conditions or with the calcium phosphate method.

Strep-GH Pulldown and Elution for BN-PAGE—Cells were stimulated with 180 ng/ml Strep-GH for 5–15 min in a CO2 incubator at 37 °C, washed four times with ice-cold PBS, and lysed for 30 min in 1% Triton X-100 in lysis buffer (100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, and inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 15 mM NaF, 1 mM Na3VO4, and 15 mM N-ethylmaleimide)). Cells were scraped, and the lysates were clarified by centrifugation for 5 min in 4 °C. At this point, samples were taken to analyze total cell lysates. The supernatants were filtered in Microcon centrifugal filter devices (Millipore) and washed once with BN base buffer (20 mM Bis-Tris (pH 7.0), 0.5 M 6-aminocaproic acid, 20 mM NaCl, 2 mM EDTA, 10% glycerol, and inhibitors). The samples were loaded on BN-polyacrylamide gels. For the Strep-GH pulldown procedure, supernatants were incubated with Strep-Tactin beads for 2 h at 4 °C. Complexes were eluted three times for 30 min with lysis buffer containing 2.5 mM desthiobiotin. Eluates were combined, filtered, and washed once with BN base buffer to decrease the salt concentration.

RESULTS

GHR Complexes at Steady State—We analyzed GHR complexes isolated via Strep-GH from either unstimulated or stimulated GHR-expressing HEK293 cells by second dimension BN/SDS-PAGE (Fig. 1A). In this method, Coomassie G-250 is used to bind to proteins, which enables separation of protein complexes according to their masses. At steady state, the cells contained several distinct GHR complexes with both mature and immature GHR polypeptides (Fig. 1A, panel a). The smallest GHR complex with immature chains (precursor GHR) probably corresponds to monomeric receptor molecules (spot 1). Based on our previous studies that show that dimerization occurs within 1–2 min after termination of translation (3), it is likely that spot 2 contains dimeric folding intermediates and that the majority of immature complexes that range from ~200 kDa up to a few thousands represent multimeric high mannose GHRs, ready to leave the ER. This is in agreement with a study of van den Eijnden et al. (12), who showed that GHR folding is extremely efficient, enabling GH to bind to the GHR within 2 min after being synthesized. The discrete spot 5 represents the mature GHR with an estimated size of three or four monomers. At steady state, without GH, the majority of the mature GHR is in complexes ranging between ~500 and 2000 kDa (indicated at spot 6). Previously, we have shown that, at steady state, the majority of the mature GHR resides at the cell surface (13). In Fig. 1A (panels b), the cells were stimulated with Strep-GH for 10 min, and the GHRs were isolated on streptavidin beads and analyzed. Under these conditions, the majority of the GH-bound receptor had entered the cell via clathrin-mediated endocytosis (5). Two main GHR complexes could be distinguished, an ~500-kDa (spot 5) and an ~900-kDa (spot 6) complex. Minor complexes migrated in between.
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To confirm that the mature complexes were indeed present on the cell surface at steady state, we treated wild-type GHR and endocytosis-defective GHR F327A mutant cells with proteinase K on ice. The GHR F327A mutant lacks a crucial phenylalanine residue within the ubiquitin-dependent endocytosis motif required for endocytosis (5). Proteinase K removes the extracellular part of the GHR, thereby leaving the transmembrane and cytosolic domains intact (14). Although the ER species were unchanged, in both cell lines, the mature receptor complexes almost completely shifted to lower molecular masses (Fig. 1B, panels b and d, arrows). Remarkably, the shift in the first dimension reflected roughly the loss of the extracellular domains, indicating that the composition of the complexes remained the same. We conclude that, at steady state, most of the GHR complexes reside at the cell surface and that the extracellular domain has no part in the composition of the complexes.

Covalent Post-translational Modifications of the GHR Complexes—Our previous study has shown that GH stimulation accelerates GHR endocytosis (7). Upon GH binding, the GHR is phosphorylated by JAK2 and ubiquitylated by the SCF<sup>TrCP2</sup> ubiquitin ligase. Therefore, we asked what happens to the complexes isolated via Strep-GH as depicted in Fig. 1A. Analyses with antibodies recognizing phosphorylated tyrosines or ubiquitin showed that only proteins present or derived from spot 6 were either phosphorylated or ubiquitylated (Fig. 1A, panels b). Because the molecular mass of JAK2 is close to that of GHR, we cannot exclude that the phosphotyrosine signal partially originated from JAK2, although our previous finding that phosphorylated JAK2 detaches from its receptor renders this unlikely (7). The smear originating at the GHR position upwards (Fig. 1A, upper panel b, spot 6) very likely represents ubiquitylated GHR, as both staining patterns overlap. The nature of diagonal modification (originating from spot 6 in Fig. 1A, panel b) is unknown. However, as its molecular mass increases equally in both dimensions, it may represent multimeric covalently linked GHRs that are insensitive to reducing conditions (15). As the signals for phosphorylation and ubiquitylation only partially overlap in the lower part of the blot, few if any ubiquitylated receptors seem to be phosphorylated.

GHR Is Subjected to Dynamic Changes upon GH Binding—As we concluded from the distribution of mature GHR complexes in the lysate at steady state (Fig. 1A), the GHR occurs in multiple complexes ranging from 500 to >2000 kDa. To analyze the effect of GH binding and receptor activation, we compared GHR complexes at the cell surface with the situation after stimulation for different periods of time. In Fig. 2A, Strep-GH was allowed to bind for 2 h on ice, and the complexes were isolated and analyzed for GHR, ubiquitin, and phosphotyrosine. The differences from the steady-state situation in the total cell lysate are striking. Although spot 5 kept its appearance upon GH binding, the heterogeneity of the complexes of spot 6 (Fig. 1A, panel a) disappeared and changed into a single GHR complex of ~900 kDa. This was not caused by a selective binding of GH to a subpopulation of complexes, as pulldown experiments with a GH antagonist showed that the GHR binds equally well (14). Thus, GH binding to GHRs at 0 °C seems to induce complexes of uniform size. Obviously, this complex showed neither phosphorylation nor ubiquitylation. Next, we incubated the cells with Strep-GH for 5, 10, and 15 min at 37 °C (Fig. 2, B–D). GH treatment caused increased ubiquitylation that was maximal after 10 min while phosphotyrosine labeling was induced, and the signal remained steady over the incubation period. Remarkably, the 500-kDa complex (white-boxed spot 5 in Fig. 2, B–D) gradually disappeared, and the 900-kDa complex (black-boxed) gradually increased in size (quantification is depicted in the graph in Fig. 2E). Thus, in the presence of GH, the GHRs are organized mainly in discrete complexes of ~900 kDa in size that constitute the signaling platforms.

Autocrine GH is a potential regulator of tumor neovascularization in mammary neoplastic progression (16). An important question is thus how the complexes signal if GH and the GHR are made in the same cell (autocrine function). Previously, we...
have shown that, in these cells, the hormone-receptor complexes arrive at the cell surface, and exogenously added GH is unable to activate these receptors (17). Autocrine GH activates the GHRs to a limited extent, but signal transduction occurs only after the GHRs have exited the ER (17). To address this further, we transfected our GHR-expressing cells with Strep-GH and analyzed the complexes (Fig. 3A). The majority of the GHRs in the ER (110 kDa) were in the 500-kDa configuration; only a small amount migrated as ~900-kDa species. The same applied for the 130-kDa mature GHRs, present mainly at the cell surface. This is in striking contrast to GHRs isolated from the cell surface in control cells, in which the 900-kDa complexes were the dominant species (Fig. 3B). Without GH in the ER (Fig. 1A, panel a, and Fig. 3B), the precursor GHRs occurred in heterogeneous complexes from ~200 kDa up to a few thousands kDa. In the presence of GH, these complexes assembled in the 500-kDa configuration, presumably containing one or two GH molecules (as they were isolated via Strep-Tactin beads). Strikingly, GH in the ER induced the conversion to 900-kDa complexes only to a limited extent, and little of this species was present in the isolated fraction (pulldown on Strep-Tactin beads). As the latter complexes act as signaling platforms (Fig. 2), this probably explains why we detected little or no phosphotyrosine signal on the GHR (Fig. 3A) and only a limited STAT5b signaling capacity in autocrine GHR-GH cells (17). An important difference between the endocrine and autocrine situations is that endocrine signaling induces rapid signal down-regulation via SOCS, whereas in the autocrine situation, steady-state conditions prevail in which SOCS, the GHR, JAK2, and STAT proteins generate a continuous (low) signal.

Analysis of GHR-βTrCP2 Complexes—The SCTβTrCP2 ubiquitin ligase complex is responsible for ubiquitylation and subsequent endocytosis of the GHR (5). The ligase acts in a complex of βTrCP2, Skp1, Cul1 (modified with Nedd8), Rbx1, and UbcH7 as E2, with a total molecular mass of ~200,000 Da. Based on structural studies, it might act as a dimer (18–20). The
we overexpressed only one factor of the SCF ligase, it is unlikely that the βTrCP2 label is in SCF complexes. However, as a presumed dimer, it might associate with many different substrates both in the cytosol and in the nucleus, explaining the heterogeneity. Strikingly, in isolated GHR complexes (Fig. 4B), βTrCP2 did not co-migrate with the 900-kDa complex. Although weakly, it co-purified with receptors in complexes with molecular masses above 10⁶ Da, suggesting that the GHR transiently associates with the SCF complex. As only 900-kDa complexes are polyubiquitylated, ubiquitylation might induce the dissociation of the ligase from the receptor.

To further analyze the mechanism of GHR ubiquitylation, we used our truncated GHR mutant lacking lysine residues (GHR399K-less) (21). GHR complexes were isolated from transfected HEK293 cells after 10 min of incubation with Strep-GH. Both GHR399K-less- and GHR399-containing complexes showed similar patterns as the control GHR (compare Fig. 4C with Fig. 1A, panel b). Previously, we showed that endocytosis and degradation of full-length GHR, GHR399, and GHR399K-less depend equally on TrCP activity and on active proteasomes (5, 11, 22), indicating that the three receptors are specific substrates for TrCP via the ubiquitin-dependent endocytosis motif. Interestingly, the size of the complexes in the first dimension was approximately half the size of full-length GHR: 250 and 500 kDa, respectively. Although the 500-kDa GHR399 complexes were clearly ubiquitylated, the ubiquitylated state of the 250-kDa complex was less clear. GHR399K-less was not ubiquitylated. The only difference was the increase in GHR complexes above 500 kDa (boxed in Fig. 4C and quantified in Fig. 4D) in the case of GHR399K-less. That the mutant cannot be ubiquitylated but can still associate with βTrCP2 via the ubiquitin-dependent endocytosis and DSG motifs (23) strengthens the notion that the GHR can interact with the ligase until ubiquitylation is completed. Again, both GHR399 and GHR399K-less displayed diagonal GHR-positive signals starting from the 550-kDa complexes (Fig. 4C, arrows).

**GHR Accepts Both Lys-48- and Lys-63-linked Ubiquitin Chains**—Although the significance is unknown, it has been shown that the GHR is subjected to Lys-48-linked ubiquitylation (7). To explore the effect of ubiquitylation further, we overexpressed two different ubiquitin mutants: HA-ubiquitin K48R, which precludes the formation of Lys-48 polyubiquitin chains, and HA-ubiquitin K63R, which prevents Lys-63-linked ubiquitylation. Cells were stimulated for 10 min with Strep-GH, and the isolated complexes were analyzed. Staining with anti-GHR antibody showed that the GHR can be modified by both types of chains, as overexpression of ubiquitin K48R caused a clear reduction in polyubiquitylation of a complex slightly bigger than 900 kDa, whereas overexpression of ubiquitin K63R diminished ubiquitylation of the 900-kDa complex itself (Fig. 5). Due to the low intensity of the anti-HA staining in control cells, the complexity of the ubiquitin pattern is difficult to discern. However, comparison of the patterns of the mutants (ubiquitin K48R and ubiquitin K63R) with the pulldown in Fig. 2C clearly illustrates the effects. Although the experiments are not full-proof, they suggest that both Lys-48- and Lys-63-linked polyubiquitin chains can be attached to the GHR. This is in line with our recent findings that two ligases, CHIP and SCFβTrCP2,
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FIGURE 5. Lys-48- and Lys-63-linked ubiquitylation is involved in GHR endocytosis. GHR-expressing HEK293 cells were transfected with HA-ubiquitin (HA-ubi; upper panels), K48R mutant (middle panels), or K63R mutant (lower panels) DNA. Cells were stimulated for 10 min with Strep-GH. GHR complexes were pulled down (PD) on Strep-Tactin beads, and analyzed by Western blotting (WB) as indicated. All data in this figure are representative of three independent experiments.

Analysis of GHR-JAK2 Complexes—GHR conducts signaling via association with the non-receptor tyrosine kinase JAK2. To analyze the involvement of JAK2 in the GHR complexes, GHR-expressing HEK293 cells were transfected with FLAG-JAK2, and total cell lysates and GHR complexes were analyzed. As shown in Fig. 6A, JAK2 overexpression resulted in accumulation of high numbers of the mature GHR at the cell surface (7). JAK2 occurred mostly in high molecular mass complexes partly overlapping with the 900-kDa GHR complexes (Fig. 6A, box). However, in GHR complexes isolated with Strep-GH, JAK2 was not detectable (Fig. 6B). Even if the complexes were analyzed in one dimension in the presence of SDS, visualization of GHR-associated JAK2 after incubation with Strep-GH at both 0 and 37 °C was difficult (data not shown). Previously, we showed that, upon GH stimulation, JAK2 detaches from the GHR due to phosphorylation at Tyr-119 in JAK2 (7). This predicts that only a small amount of JAK2 is bound to the GHR at any given time, confirming that the interaction between the GHR and JAK2 is transient. We conclude that, given the size of JAK2, it is unlikely that JAK2 is part of the 900-kDa GHR complex.

DISCUSSION

In this study, we analyzed GHR complexes in the ER and at early stages of endocytosis with second dimension BN/SDS-PAGE. We identified two main GHR complexes of ~500 and 900 kDa. Upon GH stimulation, there is a transition from 500- to 900-kDa complexes. We have also shown that both JAK2 and βTrCP2 are present only in very high molecular mass complexes, confirming that the GHR interaction with these proteins is transient. Only the 900-kDa complex is both ubiquitylated and phosphorylated. In addition, we showed that trimeric complexes can bind GH already in the ER.

In unstimulated cells, mature GHRs are present in discrete complexes of 500 kDa as well as in heterogeneous complexes ranging from 800 to >2000 kDa (mature GHR) (Fig. 1A, panel a). Mature GHR complexes represent receptors trafficking from the cell surface to lysosomes. Immature GHR complexes represent receptors trafficking from the ER to the Golgi complex. A molecular mass of 500 kDa indicates that it can comprise three or four GHR molecules.

Previous studies on GHR suggest that receptor dimers are preformed in the ER (3, 24), but they do not exclude higher order complexes. Our present studies show that GHRs indeed act as higher order complexes. Crystal structure studies of 2:1 complexes of the GHR with GH were performed only with the extracellular part of the receptor. This and other studies show that the transmembrane and intracellular parts (up to amino acid 399) (Fig. 4C) are responsible for higher order complex formation.

Other studies on cytokine receptors show that they can form complex structures consisting of two, three, and more...
receptor molecules. On the basis of our current study, we propose a new model for the GHR (Fig. 7). The model explains why the 500-kDa complex is not phosphorylated despite its capacity to accommodate one or two GH molecules. Either a trimer does not allow the conformational change needed for JAK2 activation, or the trimer does not offer the correct binding conditions for JAK2. In the case of a GH-JAK2 complex, one GH might be able to induce the proper conformational change that activates JAK2. Therefore, we propose that the 500-kDa complex represents GHR trimers, whereas the 900-kDa complex contains a hexameric GHR with no ancillary proteins attached. As blue native electrophoresis does not provide sufficient accuracy to precisely determine the molecular mass of the complexes, other methods will be required to prove our model.

Although the heterogeneous complex (spot 6 in Fig. 1A, panel a) is very likely composed of GHRs associated with ancillary proteins (enzymes and chaperones), GH-bound complexes isolated from cells at either 0°C or after incubation at 37°C show two well defined species (Fig. 2A, spots 5 and 6). At this moment, we cannot exclude that GHR complexes in spots 5 and 6 contain ancillary proteins. However, the results shown in Fig. 4C render this possibility unlikely. The GHR399 truncation with 120 amino residues less in the intracellular tail is still able to form complexes that correspond in size to spots 5 and 6 of full-length GHR. This hypothesis is further strengthened by the finding that both the proteinase K-digested GHR lacking the GH-binding domain and the C-terminally truncated GHR yield well defined complexes of relatively the same masses (Figs. 1B and 4C, respectively).

At present, the cellular location of the complexes remains uncertain. Fig. 2A, the results in Fig. 1B (in which endocytosis-deficient GHRs were used), and the coexpression study depicted in Fig. 3 suggest that 900-kDa complex formation occurs at the cell surface. If cells are incubated with Cy3-GH or 125I-GH, 50% of the label is internalized within 15 min (11, 25). Therefore, the majority of the GHR complexes isolated after incubation with Strep-GH for 5–15 min are most likely inside the cells at the moment of cell lysis. In the absence of GH, the GHR is constitutively endocytosed and uses identical ubiquitination factors and routes as the GH-bound GHR (7). The experiments with proteinase K clearly show that most of the GHRs present in the various GHR complexes reside at the cell surface. It remains to be determined in which configuration the complexes that are devoid of GH are endocytosed.

The mode of JAK2 action on cytokine receptors is still enigmatic. Our data show that JAK2 occurs in large complexes ranging from 700 Da up to several thousand kDa. However, even at high expression levels of both the GHR and FLAG-JAK2, we were unable to visualize JAK2-containing wild-type GHR complexes in our second dimension gels. As both the GHR and JAK2 were abundantly phosphorylated, this finding that both the proteinase K-digested GHR lacking the GH-binding domain and the C-terminally truncated GHR yield well defined complexes of relatively the same masses (Figs. 1B and 4C, respectively).

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![Model for the GHR](image)

**FIGURE 7. Model for the GHR.** Complex 1 is the trimer, both at the cell surface and in the ER, representing spots 5 and 3, respectively, in Fig. 1A (panel a). The trimer contains one asymmetric GHR (light pink). Complexes 2a and 2b are the double spots 5 in Figs. 1A (panel b) and 2A, containing either one or two GH molecules. Complex 3 is composed of 1:1 complex 2a and complex 2b, representing spot 6. Unexplained is the observation that complexes 2a and 2b seem to occur in equimolar amounts. We assume that the GH molecules in complex 2 are unable to initiate signaling because they showed virtually no phosphotyrosine labeling. Complex 3 is the business molecule that can accommodate both JAK2 and the ubiquitin ligases, probably via the asymmetric GHR molecules. As the monovalent GH antagonist B2036 endocytoses the GHR399 truncation with 120 amino residues less in the intracellular tail is still able to form complexes that correspond in size to spots 5 and 6 of full-length GHR. This hypothesis is further strengthened by the finding that both the proteinase K-digested GHR lacking the GH-binding domain and the C-terminally truncated GHR yield well defined complexes of relatively the same masses (Figs. 1B and 4C, respectively).

Two trimeric GHR complexes together into a GH$_3$-GHR$_6$ complex. The model also explains why the 500-kDa complex is not phosphorylated despite its capacity to accommodate one or two GH molecules. Either a trimer does not allow the conformational change needed for JAK2 activation, or the trimer does not offer the correct binding conditions for JAK2. In the case of a GH-JAK2 complex, one GH might be able to induce the proper conformational change that activates JAK2. Therefore, we propose that the 500-kDa complex represents GHR trimers, whereas the 900-kDa complex contains a hexameric GHR with no ancillary proteins attached. As blue native electrophoresis does not provide sufficient accuracy to precisely determine the molecular mass of the complexes, other methods will be required to prove our model.

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At present, the cellular location of the complexes remains uncertain. Fig. 2A, the results in Fig. 1B (in which endocytosis-deficient GHRs were used), and the coexpression study depicted in Fig. 3 suggest that 900-kDa complex formation occurs at the cell surface. If cells are incubated with Cy3-GH or 125I-GH, 50% of the label is internalized within 15 min (11, 25). Therefore, the majority of the GHR complexes isolated after incubation with Strep-GH for 5–15 min are most likely inside the cells at the moment of cell lysis. In the absence of GH, the GHR is constitutively endocytosed and uses identical ubiquitination factors and routes as the GH-bound GHR (7). The experiments with proteinase K clearly show that most of the GHRs present in the various GHR complexes reside at the cell surface. It remains to be determined in which configuration the complexes that are devoid of GH are endocytosed.

The mode of JAK2 action on cytokine receptors is still enigmatic. Our data show that JAK2 occurs in large complexes ranging from 700 Da up to several thousand kDa. However, even at high expression levels of both the GHR and FLAG-JAK2, we were unable to visualize JAK2-containing wild-type GHR complexes in our second dimension gels. As both the GHR and JAK2 were abundantly phosphorylated, this finding that both the proteinase K-digested GHR lacking the GH-binding domain and the C-terminally truncated GHR yield well defined complexes of relatively the same masses (Figs. 1B and 4C, respectively).
The action of ubiquitylating enzymes in GHR function and degradation has been a major subject in our group for many years. Lys-48-specific ubiquitylation by SCF^{βTrCP} is required for GHR endocytosis (5). Recently, we discovered that also Lys-63 ubiquitylation occurs, presumably by the activity of Ubc13 in collaboration with the ubiquitin ligase CHIP (6). The experiment of Fig. 5 reveals that the two types of ubiquitin chains occur in different 900-kDa GHR complexes, confirming that the two ligases (SCF^{βTrCP} and CHIP) act in different stages of endocytosis (6). Assuming that the exogenously expressed ubiquitin mutants dominated endogenous ubiquitin, overexpression of the ubiquitin K48R mutant caused a clear reduction in the length of polyubiquitin chains, confirming previous results that the GHR is modified with Lys-48-linked ubiquitin chains (7). The Lys-63-linked complexes were slightly bigger than the Lys-48-containing complexes. As the latter bear shorter ubiquitin chains, the ubiquitin load of the GHRs may contribute to their complex sizes. The experiments with mutant ubiquitin clearly demonstrate the potential of a method that not only shows dynamic changes in post-translational modifications during complex signaling events but also enables stoichiometric analysis of the factors involved in these processes.

In this study, we have shown that the GHR resides at the cell surface in higher order complexes. Upon GH binding, there is a transition from a smaller to a larger complex, the latter being subjected to phosphorylation and ubiquitylation. However, neither JAK2 nor βTrCP2 is present in these complexes, confirming that interaction between the GHR and the enzymes is transient. Additionally, the data show that the GHR and GH are sufficient to cause multimerization of the GHR. The study shows for the first time that, in addition to the “complex” cytokine homodimerizing receptors (GH and erythropoietin receptors) may form high order complexes.

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