Conformational Changes Required in the Human Growth Hormone Receptor for Growth Hormone Signaling*

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Growth hormone (GH) plays a significant role in normal growth and development. Signaling to the cell is believed to require growth hormone receptor (GHR) dimerization, which occurs following binding of a single growth hormone molecule to each of two receptors. We have developed human growth hormone receptor-specific monoclonal antibodies, one of which was used here to characterize hormone/receptor interactions. This antibody, GHR05, is directed against the hinge spanning subdomains I and II of the receptor’s extracellular region. Antibody binding to the cell surface receptor increases upon receptor binding to growth hormone, but not when it binds a mutant form, hGHG120R, which does not trigger receptor activation. Growth hormone binding thus appears to lead to a conformational change in the receptor epitope recognized by GHR05, giving rise to the active dimer configuration, necessary for signal transduction. Using a chimeric receptor-expressing, growth hormone-dependent murine cell line, we find that GHR05 binds to the receptor in the absence of human GH and delivers a signal leading to cell proliferation. Finally, GHR05 treatment of IM-9 cells, a human cell line expressing a functional human GHR, leads to cell proliferation mediated by the generation of GHI-specific signals, including phosphorylation of the JAK2 tyrosine kinase and activation of STAT5.

The 22-kDa polypeptide human growth hormone (hGH),1 essential for normal growth and development, induces a variety of biological effects including linear growth, lactation, nitrogen retention, diabetogenic and insulin-like effects, and macrophage activation (1–4). Each of these effects is initiated by hGH interaction with specific cell receptors. The hGH receptor (hGHR) belongs to a large cytokine receptor family (5), comprising a heavily N-glycosylated extracellular ligand-binding domain, a single transmembrane segment and an intracellular domain, the last of which shares little sequence identity within the family (11). The hGHR extracellular region has two subdomains, one implicated mainly in hGH binding (subdomain I) and the other in hGHR dimerization (subdomain II) (7, 8).

The extracellular hGHR domain is found in serum in the form of a hormone binding protein (hGHBP), which binds hGH with approximately the same affinity and specificity as the intact receptor (12). Nonglycosylated recombinant bacterial hGHBP (13) has the same binding affinity (Kd = 0.4 nM) (14) and specificity for hGH as the mammalian binding protein. Crystallization studies of hGH and hGHBP show that a single hGH molecule binds two hGHBP molecules (15). Each hGH molecule is bivalent, containing two separate hGHBP binding sites; site I is a high affinity site and site II, a low affinity site. In contrast, the hGHBP is univalent, as it uses the same amino acids to bind either hGH site I or site II. An hGH excess dissociates the hGH-(hGHBP), complex to form a monomeric complex. Biological response triggering requires sequential binding of the first receptor subunit to site I on a GH molecule, followed by binding of the second receptor to GH site II, forming the GH-(hGHR)2 complex (16). In addition to the hormone-receptor interactions, there is also substantial surface contact between the subdomain II subunits of the extracellular regions of the two receptor molecules. This sequential dimerization model has been confirmed using several approaches including crystallization, size exclusion chromatography, calorimetry, and fluorescence quenching assays (14).

Although little is known of the hGHR signal transduction mechanism, several lines of evidence indicate that hGHR activation triggers tyrosine phosphorylation of JAK2 kinase (17) and some STAT transcription factors, mainly STAT2, STAT3, and STAT5 (18–21).

We have developed a panel of anti-hGHR monoclonal antibodies (mAb), used for the characterization of signals triggered by hGH binding. One antibody, GHR05, directed against the hinge region between subdomains I and II of the receptor’s extracellular region, recognizes uncomplexed hGHR on the cell surface. Binding assays indicate a higher affinity of GHR05 for the hGH-hGHR complex than for hGHR alone, indicating that the mAb better recognizes a receptor conformation induced following ligand binding.

To analyze the consequences of hGHR/hGH interaction, we tested the effect of GHR05 on hGH-expressing IM-9 cell line and on the Ba/F3(8/6) cell line. These latter cells were generated from the IL-3-dependent murine pro-B cell line Ba/F3 (22).
Conformational Changes in hGHR upon Activation

TRANSGENIC ACTIVATION

Once activated, hGHR binds hGHBP, which has a high affinity for hGH at physiological concentrations.

EXPERIMENTAL PROCEDURES

Proteins, Antibodies, and Cell Lines

Recombinant human GH 22K (rGH-22K, Genetropin) and recombinant human GHBP were obtained from Pharmacia and Upjohn (Stockholm, Sweden). hGHG120R was kindly donated by Dr. Gunnar Norstedt (Center for Biotechnology, Karolinska Institute, Huddinge, Sweden). IM-9 cells were from the ATCC (Rockville, MD). BA/F3(8/6) cells were generated by transfection of BA/F3 cells with the chimeric construct hGHR/h-CSFIR and cultured in RPMI 1640 medium supplemented with IL-3 (10 units/ml) and 10% fetal calf serum (FCS) at 37 °C in 5% CO2. Anti-human JAK2 mAb was from Upstate Biotechnology, Inc. (Lake Placid, NY), anti-human STAT5, anti-murine c-myc and anti-murine bax mAb were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), anti-murine bcl-2 mAb was from OncoGene Science, Inc. (Cambridge, MA), and anti-murine p53 mAb was from Transduction Laboratories (Lexington, KY).

Preparation of Monoclonal Antibodies BALB/c mice were immunized subcutaneously with 10 μg of GHBP in 0.1 ml sterile phosphate-buffered saline (PBS) and Freund’s complete adjuvant (Difco, Detroit, MI). They were boosted subcutaneously on days 30 and 60 with 10 μg of protein in Freund’s incomplete adjuvant and intraperitoneally in PBS on day 90. Mice were boosted intravenously with 10 μg of protein in PBS on days 3–2 before cell fusion. Mouse spleen and/or lymph node cells were fused with the P3X63-Ag8.653 myeloma cell line (CRL 1580, American Type Culture Collection, Rockville, MD) using polyethylene glycol 4000 (Merck, Darmstadt Germany) following standard protocols (23, 24). Supernatants were tested for antibodies using an enzyme-linked immunosoraya (EIA), and positive hybridomas were selected. Hybridomas were cultured in RPMI 1640, 10% FCS at 37 °C in 5% CO2. Monoclonal antibodies were produced in tissue culture supernatants for 30 min at 4 °C. Cells were washed twice with PBS, incubated with 100 μl of undiluted supernatants for 30 min at 4 °C. Samples were analyzed in an EPICS XL flow cytometer (Coulter Electronics Inc., Hialeah, FL).

Enzyme-linked Immunoassays

Four different EIA were performed that varied in their mechanism of antigen presentation to the antibody.

Antibody Capture Assay—Biotin-labeled GHBP (0.5 μg/ml in PBS, 100 μl) were adsorbed to avidin-coated microtiter plates (3 μg/ml) (Maxi-sorb, Nunc, Copenhagen, Denmark) for 90 min at 37 °C. Remaining protein-binding sites were blocked with 0.5% BSA in PBS. Plates were washed with distilled water and mAb incubated for 60 min at 37 °C, followed by a PO-labeled goat anti-mouse immunoglobulin antibody (Ago). Antibody was added and incubated for 90 min at 37 °C, followed by PO-streptavidin and OPD. The reaction was terminated as above.

Antigen Capture Assay—Monoclonal antibodies were adsorbed to the solid phase, directly (3 μg/ml in PBS) or via an affinity-purified GAM antibody. After blocking, biotin-labeled GHBP (1/1000) in PBS-0.5% BSA was incubated for 60 min at 37 °C, followed by PO-labeled streptavidin (Sigma) for 30 min at 37 °C, and OPD. The reaction was terminated as above.

Sandwich Capture Assay—Purified mAb (3 μg/ml in PBS) was adsorbed to microtiter plates. After overnight incubation at 4 °C and blocking with 0.5% BSA, GHBP dilutions in PBS-0.5% BSA were added and incubated for 60 min at 37 °C. After washing, a biotin-labeled second mAb was added, followed by PO-streptavidin and OPD. The reaction was terminated as before.

 competitive assay—GHBP (1 μg/ml in PBS) was adsorbed to microtiter plates overnight at 4 °C, then blocked with PBS-0.5% BSA for 60 min at 37 °C. Plates were washed, and biotinylated mAb, alone or mixed with competitor antibody, was added and incubated for 90 min at 37 °C, followed by PO-streptavidin. The reaction was terminated as above. Competition for GHBP binding was assumed when a signal decrease of more than 40% was observed in the presence of the added antibody compared with that of GHRO5 alone; that is, the two mAb recognized similar or proximal epitopes on the GHBP molecule.

Determination of Kinetic Parameters Using Real-time Interaction Analysis

BIACore real-time interaction analysis (Pharmacia, Uppsala, Sweden) was used to determine the kinetic parameters of mAb binding to hGHBP and the hGH-hGHBP complex. The carboxylated dextran CM-5 matrix of a sensor chip was activated with an N-ethy-l-N’-(3-dimethylaminopropyl)carbodiimidehydrochloride/N-hydroxysuccinimide mixture (Pharmacia). Rabbit anti-mouse immunoglobulin light chain (35 μl at 50 μg/ml, Pharmacia) solubilized in 10 mM acetic buffer, pH 4.5, was then immobilized on the matrix via primary amine groups in 10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.05% surfactant P20, pH 7.4 (HBS) at a flow rate of 5 μl/min. Unreacted groups were blocked with 35 μl of ethanalamine-HCl, pH 8.5. Determinations were performed at 25°C using eight GHBP concentrations (4 to 500 nM), alone or complexed with a 20 x molar excess of hGH. GHBP or GH/GHBP were injected in PBS at a constant 5 μl/min flow rate over the GHRO5 mAb, previously captured by dextran-immobilized rabbit anti-mouse light chains. After each determination, the surface was regenerated for 3 min with 30 mM HCl. Kinetic rate constants (k_on, k_off) and the apparent equilibrium affinity constants (K_D = k_off/k_on) were determined using BIAalogue Kinetics Evaluation Software (Pharmacia).

Cytofluorimetric Analysis

Cells (2 x 10^6/100 μl) were plated in V-bottom 96-well plates and incubated with 100 μl of 70% ethanol for 10 min at 4 °C for permeabilization, or in PBS with 2% BSA and 2% FCS (PBSa). After washing with PBSa, cells were incubated with 1 μg/ml HGH or PBSa for 30 min at 4 °C, washed twice, and incubated with 100 μl of undiluted supernatants for 30 min at 4 °C. Cells were washed twice with PBSa, fluorescein isothiocyanate labeled-GAM (Southern Biotechnologies, Birmingham, AL) was added and incubated for 30 min at 4 °C. Samples were analyzed in an EPICS XL flow cytometer (Coulter Electronics Inc., Hialeah, FL).

Immunoprecipitation, SDS-Polyacrylamide Gel Electrophoresis, and Western Blot Analysis

Hormone- or antibody-treated cells (2 x 10^6) were lysed in 50 mM Tris-HCl, pH 7.6, 1% Nonidet P40, 250 mM NaCl, 0.5 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate with protease inhibitors for 20 min at 4 °C and centrifuged at 15,000 x g. Protein extracts were separated in 12.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. For Western blot analysis, membranes were blocked with 5% non-fat dry milk in PBS for 1 h at room temperature. Free biotin was removed by dialysis against PBS. Biotin-labeled protein was diluted 1:2 with glycerol and stored at -20 °C.

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Cell Proliferation Assay

Ba/F3(8/6) cells (20 \times 10^5/ml) were washed in basal RPMI 1640 medium without IL-3. Then, 25 \mu l of the cell suspension were added to 96-well plates and treated with hGH (0.001–10 \text{nM}) or mAb in a final volume of 100 \mu l for 18 h at 37 °C. Confluent IM-9 cells (1 \times 10^6/ml) were washed in serum-free RPMI 1640, and 100 \mu l of the cell suspension were incubated in 96-well plates in the presence of hGH or mAb for 8 h at 37 °C. To measure DNA synthesis, 1 \mu Ci/well [\text{^3}H]thymidine (Amersham) was added (5 \muCi/\text{mmol}), incubated 6 h at 37 °C in 5\% CO_2. Cells were harvested and washed on glass fiber filters, and [\text{^3}H]thymidine uptake was quantified by liquid scintillation counting.

Cell Cycle Analysis

Cell cycle stage was assessed by cell DNA content using propidium iodide staining and flow cytometry analysis. Briefly, subconfluent cells were washed three times in RPMI-10\% FCS without IL-3 (basal medium), plated at 3 \times 10^5 cells/well in 24-well plates (Nunc), and treated with hGH (10 \mu g/ml) or mAb (1 \mu g/ml), to a final volume of 1 ml. After 24 h of incubation at 37 °C in 5\% CO_2, cells were centrifuged 10 min at 200 \times g and resuspended in 100 \mu l of PBS. Cell were permeabilized and DNA-stained by adding 100 \mu l of detergent followed by 1.2 ml of propidium iodide solution (Coulter DNA-Prep® reagents). After vortexing, samples were incubated at 37 °C for 30 min and analyzed in an EPICS-XL cytometer (Coulter).

RESULTS

Characterization of Anti-growth Hormone Receptor Antibody—Using Escherichia coli-derived recombinant hGHBP as immunogen, we generated a series of mAbs, one of which (GHR05) was further characterized. GHR05 is an IgM, \kappa anti-body specific for the hGH receptor, with a $K_a$ of 2.5 or 6 \times 10^6 \text{M}^{-1} as measured in BIAcore using GH-GHBP complex or GHBP, respectively. The BIAcore measures real-time interac-
tions and thus determines the association of GHR05 to GHBP or GH/GHBP with no appreciable differences (1.7 $\times 10^3$ and 3.2 $\times 10^3$ M$^{-1}$ s$^{-1}$, respectively) (Fig. 1, A and B). However, GHR05 dissociates less rapidly from GHBP (2.6 $\times 10^{-4}$ s$^{-1}$) than it does from the GH-GHB complex (1.2 $\times 10^{-3}$ s$^{-1}$). Thus, this antibody binds with similar $K_{on}$ to both GHBP and the complex, although the dissociation data indicate that the GHR05-GHBP complex is more stable than that of GHR05-GH-GHB.

As one characteristic of cytokine receptors is their heavy glycosylation, and the ultimate use of GHR05 was the study of the GHR, we tested its recognition of the CHO-derived GHBP. In antigen capture EIA, GHR05 recognizes GHBP, whether glycosylated or not (Fig. 1C). Recognition was also apparent in antibody capture EIA, Western blot, immunoprecipitation and real-time specific interaction using GHR05 coupled to a dextran matrix or in liquid phase (not shown).

To determine the hGHR region recognized by GHR05, three different assays were designed, based on the ability of this mAb to bind simultaneously with or to compete for GHBP binding with other anti-GHR mAb that recognize sequential or conformational epitopes. These assays include a sandwich EIA, a competitive EIA and an additive assay in BIAcore. The data provide information on those mAbs that bind simultaneously to a single GHBP molecule, those that bind to overlapping or identical epitopes and, using hGH, those with interfering or non-interfering interactions with the ligand binding site. With these assays, the epitope recognized by GHR05 was determined to lie in the hinge between subdomains I and II of the extracellular receptor domain. This epitope does not form part of the hGH binding site.

We assessed GHR05 reactivity with the hGHR expressed on the surface of two different cell lines. We tested IM-9, a human...
B cell line which expresses a functional hGHR (28) as shown by its ability to respond to hGH by enhancing cell proliferation (29), IgG production (30) and \([\mathrm{Ca}^{2+}]_{\text{i}}\), influx (31). We also used the IL-3-dependent murine pro-B cell line Ba/F3 (22), transfected with a chimeric gene containing the hGHR extracytoplasmic domain and the intracytoplasmic domain of the human granulocyte colony-stimulating factor receptor (Ba/F3(8/6)). Although both wild-type and transfected cells grow in the presence of exogenous IL-3, only transfected cells proliferate in the presence of hGH (32). GHR05 recognizes hGHR in both IM-9 and Ba/F3(8/6) cell lines, as a significant fluorescence shift (15%) was observed in flow cytometry, while no binding was seen in non-transfected Ba/F3 cells (Fig. 2).

The GHR05 Antibody Recognizes an Activating Epitope on the Growth Hormone Receptor—Dimerization of the hGHR appears essential for correct signal delivery. To evaluate the capacity of GHR05 to recognize the hGH(hGHR)\(_2\) dimer complex, binding of several mAbs to Ba/F3(8/6) cells pretreated with saturating hGH concentrations (0.45 \(\times\) 10^{-8} M) was studied. GHR05 binding to the complexed receptor increases, while the binding of other mAbs is unaffected (mAb 263) or diminished (GHR25) (Fig. 3, A–C). These phenomena appear not to be due to stabilization of mAbs binding to the hGH:hGHR, at least in terms of affinity, since BIAcore measurements show that GHR05 has similar apparent \(K_a\) for both hGHBP and the hGH:hGHBP complex (Fig. 1, A and B).

GHR05 binding to hGH increases dramatically when cells are previously permeabilized (Fig. 2E). This phenomenon is GHR05-specific, as this mAb binds to 100% of cells following permeabilization, which is not the case for mAb specific for other hGHR domains (Fig. 3F). This increase cannot be ascribed to hGH:hGHR complex internalization, since the experiment was performed in the absence of hGH, nor to the receptor glycosylation pattern, as glycosylation did not alter GHR05 recognition (Fig. 1C). Thus, the binding increase may be due to improved exposure of the GHR05 binding epitope.

To analyze the possible relationship between the GHR05 binding increase and hGH-induced receptor conformational change, an hGH mutant (hGHG120R) was employed that is able to bind the hGH but not to form the active hGH:hGHR\(_2\) complex. hGHG120R has a Gly\(^{120} \rightarrow\) Arg mutation affecting the second GH binding site, which impedes correct receptor dimerization and subsequent proliferation induction (33). This molecule has been shown to act as a GH antagonist both in vitro (13, 16, 34) and in vivo (34). In contrast to hGH, the presence of hGHG120R does not modify GHR05 receptor binding (Fig. 3D), permitting us to conclude that GHR05 better recognizes an epitope which becomes exposed in the active, but not in the inactive, receptor complex.

GHR05 Signals through the hGHR and Promotes Cell Proliferation—As shown earlier, GHR05 binds to hGHR-expressing cells in the absence of ligand. To study the biological significance of this binding, we studied cell cycle status in hGH-dependent Ba/F3(8/6) cells treated with hGH, GHR05 or GHR19, an isotype-matched hGHR-specific mAb. After 24 h, cell cycle distribution was analyzed by flow cytofluorometry. The hGH-treated cells were in subconfluent growth, while the GHR19-treated cells showed a marked increase in the sub-G\(_1\) population, indicative of apoptosis. When cells were GHR05-treated, however, the apoptosis ratio was significantly lower than that of the GHR19-treated cells, while the ratio of proliferating cells increased (Fig. 4). This indicates that GHR05 binding induces a GH-like signal.

The GHR05-triggered cell survival effect was further characterized by studying the expression of the cell survival control proteins bcl-2 (35), bax, p53, as well as of c-myc, whose deregulated expression drives certain cells into apoptosis following growth factor deprivation. hGH or GHR05 treatment of cells causes bcl-2 expression while preventing induction of bax, p53, and c-myc, which are expressed upon treatment with other anti-hGHR mAb (Fig. 5).

To further assess the proliferative effect induced by GHR05...
binding, we measured hGH- and GHR05-promoted [3H]thymidine incorporation into Ba/F3(8/6) cells. GHR05 induces significantly greater cell proliferation (25%) than does a control mAb (8%, equivalent to background) (Fig. 6A). The maximum GHR05-induced [3H]thymidine incorporation level is equivalent to that promoted by 4 pM hGH. GHR05, or GHR25 as control. [3H]Thymidine was added for 6 h, and cells harvested on glass-fiber filters. Lower panel, Proliferation of IM-9 cells in the presence of hGH (500 nM) or GHR05 (100 ng/ml) was determined after 8 h at 37 °C by [3H]thymidine incorporation as above. Results of three experiments performed in quadruplicate are presented and S.D. are indicated. Histograms show cell treatment (x axis) versus [3H]thymidine incorporation in counts/min (y axis).

As Ba/F3(8/6) are artificially generated GH-responsive cells, the results were validated using the human IM-9 cell line, which expresses the full-length hGHR. Both GHR05 and hGH promote IM-9 cell proliferation (Fig. 6B). To further verify GHR05 signaling specificity, IM-9 cells were treated with hGH and GHR05, and the tyrosine phosphorylation status of JAK2 and STAT5 determined. The same JAK2 and STAT5 expression pattern is observed with both treatments, indicating the agonistic behavior of GHR05 (Fig. 7). We therefore conclude that GHR05 triggers GH-like cell activation and proliferation following hGHR binding.

**DISCUSSION**

Using *E. coli*-derived rhGHBP as immunogen, we obtained the hGHR-specific mAb GHR05. Fine characterization of its reactivity using the soluble extracellular region of the receptor (GHBP) and competitive binding with other anti-GHR mAb allow mapping of the epitope recognized by this mAb to the hinge region between GHR subdomains I (binding domain) and II (dimerization domain). This epitope is unrelated to sequences involved in GH binding to the receptor (15). These data correlate well with the fact that this mAb recognizes both GHBP and GHR complexed with GH. However, some interesting differences are apparent when the behavior of the mAb with GH/GHBP or with GH-GHR complexes is compared. GHR05 binding to cell membrane hGHR increases following hGH activation, as determined by flow cytometry analysis using cells expressing either the native hGHR or a chimeric hGHR/hG-CSFR form. Its affinity for the soluble receptor form, GHBP, is similar to that for the GH-GHBP complex, indicating that the epitope recognized by GHR05 is not altered by GH binding in saturating conditions. When the association and dissociation constants are further analyzed, GHR05 binding to...
the GH-GHBP complex appears less stable than its binding to GHBP alone. Characteristics differ in each of these assays, as the continuous flow in BIAcore allows measurement of association and dissociation to a single GHBP molecule, while the measurement of binding to intact cells in flow cytometry permits equilibrium with multiple GHR molecules. Nonetheless, the data suggest that the conformation of the GHR extracellular domain varies considerably, depending on whether it is in liquid phase (GHBP) or membrane-bound (native hGHR or chimeric hGHR/hG-CSFR). Models which use GHBP to characterize GH/GHR interactions should thus be interpreted with caution.

To ascertain whether these GH binding-induced conformational changes in the receptor are related to signaling, we examined whether or not hGH120R induces similar changes. As this mutant lacks hGH binding site 2, it cannot induce functional receptor dimerization and thus acts as an hGH antagonist (13, 16, 34). As also described for FDC-P1 cells transfected with a hybrid of the hGHR extracellular domain linked to the murine granulocyte/macrophage-CSF receptor transmembrane and intracellular domains (16), hGH120R cannot induce Ba/F3(8/6) or IM9 cell proliferation (34, 36). This is linked to the murine granulocyte/macrophage-CSF receptor antagonist (13, 16, 34). As also described for FDC-P1 cells, functional changes in the receptor are related to signaling, we caution. Characterization of GH/GHR interactions should thus be interpreted with caution.

Conformational Changes in hGHR upon Activation

Our results are compatible with a model in which the hGH is transported to the membrane as a single chain that, upon expression on the cell surface, either aggregates or associates with other proteins. In such a complex, GHR05 recognition of the hinge region amino acid sequence between domains I and II is impeded. Upon binding to hGH, the GHR05 undergoes transition to an active stage; this stage is distinguished by a modified conformation recognized by the antibody. This active conformation triggers a transduction pathway involving the phosphorylation of specific tyrosine motifs in the hGHR cytoplasmic domain, as well as the induction of specific transcriptional factors that lead to cell proliferation. Either a fraction of cells or a fraction of the hGHR in each cell is in the active conformation and thus subject to antibody recognition. These differential conformational or domain accessibility changes may be relevant in the physical recognition and association of the hGHR intracytoplasmic domain with JAK tyrosine kinases or in its interaction with the appropriate STATs. The characterization of this “active” state might therefore be germane for a clearer understanding of growth hormone-triggered signaling pathways.

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