Growth Hormone (GH)-induced Dimerization Inhibits Phorbol Ester-stimulated GH Receptor Proteolysis*

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Growth hormone (GH) initiates its cellular action by properly dimerizing GH receptor (GHR). A substantial fraction of circulating GH is complexed with a high-affinity GH-binding protein (GHBP) that in many species can be generated by GHR proteolysis and shedding of the receptor’s ligand-binding extracellular domain. We previously showed that this proteolysis 1) can be acutely promoted by the phorbol ester phorbol 12-myristate 13-acetate (PMA), 2) requires a metalloprotease activity, 3) generates both shed GHBP and a membrane-associated GHR transmembrane/cyttoplasmic domain remnant, and 4) results in down-regulation of GHR abundance and GH signaling. Using cell culture model systems, we now explore the effects of GH treatment on inducible GHR proteolysis and GHBP shedding. In human IM-9 lymphocytes, which endogenously express GHRs, and in Chinese hamster ovary cells heterologously expressing wild-type or cytoplasmic domain internal deletion mutant rabbit GHRs, brief exposure to GH inhibited PMA-induced GHR proteolysis (receptor loss and remnant accumulation) by 60–93%. PMA-induced shedding of GHBP from Chinese hamster ovary transfectants was also inhibited by 70% in the presence of GH. The capacity of GH to inhibit inducible GHR cleavage did not rely on JAK2-dependent GH signaling, as evidenced by its continued protection in JAK2-deficient γ2A rabbit GHR cells. The GH concentration dependence for inhibition of PMA-induced GH receptor proteolysis paralleled that for its promotion of receptor dimerization (as monitored by formation of GHR disulfide linkage). Unlike GH, the GH antagonist, G120K, which binds to but fails to properly dimerize GHRs, alone did not protect against PMA-induced GHR proteolysis; G120K did, however, antagonize the protective effect of GH. Our data suggest that GH inhibits PMA-induced GHR proteolysis and GHBP shedding by inducing GHR dimerization and that this effect does not appear to be related to GH site 1 binding, GHR internalization, or GHR signaling. The implications of these findings with regard to GH signaling and GHR down-regulation are discussed.

Growth hormone (GH) acts on its target tissues by interacting with the transmembrane GH receptor (GHR) (1, 2). In multiple species, a substantial fraction of circulating GH is complexed with a high-affinity GH-binding protein (GHBP) that corresponds to the ligand-binding extracellular domain of the GHR (3–5). By virtue of its interaction with GH, GHBP may function as either a potentiator (by delaying GH clearance) or an inhibitor (by GH sequestration) of GH bioavailability (3, 6–9). Whereas in some species GHBP arises as a secreted alternatively spliced form of the GHR that lacks the transmembrane and cytoplasmic domains (10–12), GHBP in humans, rabbits, and several other species is formed posttranslationally by shedding of the proteolytically cleaved GHR extracellular domain (14). As a GHBP-generating mechanism, GHR proteolysis can also lead to down-regulation of receptor abundance and GH signaling, thus further contributing to regulation of GH bioavailability (13–15).

We and others have shown that GHR proteolysis and GHBP shedding can be observed in cell culture model systems (13–24). Our work indicated that such processing can be induced by the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) and by growth factors such as platelet-derived growth factor and serum (13, 15). In addition, the alkyllating reagent N-ethylmaleimide can also cause GHR proteolysis (13, 15–17, 19, 22–24). Each of these stimuli promotes rapid GHR proteolysis that, in addition to causing GHBP shedding, results in formation of a cell-associated receptor cytoplasmic domain remnant (13, 15). Although the exact site of cleavage (presumably in the proximal extracellular domain) is not yet known, * This work was supported in part by VA Merit Review awards (to S. J. F. and G. B.), grants from the National Science Foundation and the Northwestern Memorial Foundation (to G. B.), National Institutes of Health Grant DK46395 (to S. J. F.), and the State of Ohio Eminent Scholar Program, which includes a grant from Milton and Lawrence Goll, and by Sensus Corp. (to J. J. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡‡ The abbreviations used are: GH, growth hormone; GHBP, GH-binding protein; GHR, GH receptor; PMA, phorbol 12-myristate 13-acetate; CHO, Chinese hamster ovary; rb, rabbit; TACE, tumor necrosis factor α-converting enzyme; GH, human GH; BB, binding buffer (25 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 1.2 mM MgCl2, 0.1% (w/v) bovine serum albumin, and 1 mM dextrose); FLB, fusion lysis buffer (1% (v/v) Triton X-100, 150 mM NaCl, 10% (v/v) glycerol, 50 mM Tris-HCl, pH 8.0, 100 mM NaF, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM benzamidine, and 10 μg/ml aprotinin); SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TNF, tumor necrosis factor.
GHR proteolysis and GHBP shedding in each instance are prevented by the metalloprotease inhibitor, Immunex Compound 3 (13–15). Our genetic reconstitution experiments strongly suggest that the transmembrane metzincon metallo-protease, tumor necrosis factor α-converting enzyme (TACE or ADAM-17) (25–27), can function as a GHR sheddase (14).

An additional important step in understanding the molecular mechanism(s), physiological role(s), and regulation of GHR proteolysis is to determine the impact, if any, of GH on this process. GH initiates intracellular signals by interacting with the cell surface transmembrane GHR to form a complex of 1:2 GH:GHR stoichiometry (28, 29). GH-induced formation of a GHR dimer is believed to be essential for normal GH signaling; GH antagonists with mutations at GH site 2 that do not allow proper formation of receptor dimers cannot themselves promote signaling but rather antagonize signaling by wild-type GH (30–32). Whereas it is known that the long-standing excess GH levels found in acromegalic patients are often associated with decreased GHBP levels (33–35), it is unclear how this effect is exerted. Furthermore, it may be that steady-state GHBP levels reflect tissue GHR levels (4, 36–38); thus, effects of chronic GH excess or deficiency on GHBP levels may not solely reflect the effect of GH on the proteolytic process itself.

Using human HepG2 cells stably transfected with the rabbit GHR, Harrison et al. (22) noted a significant 30% decline in GHBP spontaneously released over a 24-h interval when the cells were incubated with 100 ng/ml human or bovine GH. Amit et al. (39) also addressed the issue of the effects of GH on GHBP production and postulated an inverse relationship between GHR internalization and proteolytic GHBP generation in a CHO-GHR reconstitution system similar to ours. These authors suggested that the ability of GH to cause GHR internalization and degradation may account for its ability to prevent GHBP shedding induced in that study by N-ethylmaleimide treatment.

In this study, we examine in model tissue culture systems the acute effects of GH on PMA-induced GHR proteolysis and GHBP shedding. Our results present interesting mechanistic and physiological implications concerning metalloprotease-mediated GHR processing.

**EXPERIMENTAL PROCEDURES**

**Materials**—PMA, hygromycin B, and routine reagents were purchased from Sigma Chemical Co. unless otherwise noted. Restriction endonucleases were obtained from New England Biolabs (Beverly, MA). Recombinant hGH was kindly provided by Eli Lilly Co. Recombinant hGH-G120K was kindly provided by Sensus Corp. (Austin, TX).

**Cells, Cell Culture, and Transfections**—COS-7, IM-9, and CHO (a gift of J. Kudlow, University of Alabama at Birmingham, Birmingham, AL) cells were maintained as described previously (13, 15, 32, 40). COS-7 cells were transiently transfected with pSX rGHR, pSX rGHRdel 297–406, pSX rGHRdel 297–406, or pSX rGHRdel 297–406 (rabbit GHR mutants with deletion of residues 297–406, 294–498, or 391–620, respectively) as described previously (41). Stable transfection of CHO cells was achieved by introducing either pSX rGHR, pSX rGHR C241S (a point mutant rabbit GHR in which cysteine 241 is changed to serine (32)), or pSX rGHRdel 297–406 (20 μg each in 3 ml of Dulbecco's modified Eagle's medium in 60 × 15-mm dishes), along with 1 μg of pSP65-SRα.2-HAtag-Hygro using Lipofectin according to the manufacturer's protocol. Transfected cells were grown in complete Dulbecco's modified Eagle's growth medium for 48 h. After dilution, clones were negatively selected in medium supplemented with 500 μg/ml hygromycin B and screened for GHR expression by anti-GHR immunoblotting (untransfected y2A cells express no detectable GHR (Fig. 4)).

**Plasmid Construction**—The pSX plasmid (a gift of Dr. J. Bonifacino (National Institutes of Health, Bethesda, MD) and Dr. K. Araki (DNAx)), which drives eukaryotic protein expression from the SV40 promoter (43), has been described previously (32). Preparation of the rGHR cDNA (kind gift of W. Wood; Genentech, Inc., South San Francisco, CA) and pSX cDNAs encoding rGHR C241S, rGHRdel 297–406, rGHRdel 294–498, and rGHRdel 391–606, and their ligation into pSX have been described previously (32, 41, 44).

**Antibodies**—The 4G10 monoclonal anti-phosphotyrosine antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The rabbit polyclonal anti-GHR CTD-ALT, which was directed against residues 271–620 of the human GHR (the entire cytoplasmic domain (45)), and anti-GHR cyt, which was directed against residues 317–620 of the human GHR, have been described previously (46, 47). Anti-GHR CTD-ALT is a rabbit serum raised against a bacterially expressed N-terminally His-tagged fusion protein incorporating human GHR residues 271–620. The cDNA encoding this fusion was created by polymerase chain reaction in the pET vector system (Novagen) (polymerase chain reaction primers are available upon request). Molecular biology techniques, cDNA sequencing for verification of fidelity, bacterial fusion protein expression, and preparation of Ni2+-agarose-purified fusion protein were as described previously (41, 46–49) and followed the manufacturer’s suggestions. For the purposes of the experiments in this study, the three anti-GHR cytoplasmic domain antibodies each behaved similarly for immunoblotting of GHRs and remnants.

**Cell Stimulation, Protein Extraction, Electrophoresis, and Immunoblotting**—Serum starvation of COS-7 transfectants, IM-9, CHO transfectants, y2A, and y2A transfectants was accomplished by substitution of 0.5% (w/v) bovine serum albumin (fraction V; Roche Molecular Biochemicals) for serum in their respective culture media for 16–20 h before experiments. Unless otherwise noted, stimulations were performed at 37 °C. Details of the hGH (at indicated concentrations) and PMA (at 1 μg/ml) treatment protocols have been described previously (14, 15, 32, 40). Briefly, for IM-9 cells, cells were stimulated in suspension at 10 million cells/ml in binding buffer (BB). Stimulations were terminated, and cells were collected by centrifugation (800 × g for 1 min at 4 °C). For the stimulation of the BR COS-7 transfectants, CHO transfectants, y2A cells, and y2A transfectants were stimulated in confluent 150 × 50-mm dishes (Falcon) in BB. Stimulations were terminated by washing the cells once and harvesting by scraping in ice-cold phosphate-buffered saline in the presence of 0.4 mM sodium orthovanadate. Pelleted cells were collected by brief centrifugation. For each cell type, pelleted cells were solubilized for 15 min at 4 °C in fusion lysis buffer (BL) (45). Immunoblotting with antibodies 4G10 (1:2000), anti-GHR CTD-ALT (1:1000), anti-GHR cyt (1:1000), or anti-GHR AL47 (1:1000) with horse-radish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:2000) and ECL detection reagents (all from Amersham Pharmacia Biotech) and stripping and reprobing of blots were accomplished according to the manufacturer's suggestions.

**GHBP Assay**—GHBP activity was measured in conditioned media by a standard GH binding assay, as reported previously (13, 50). Conditioned medium (1 ml) from cells treated as indicated was incubated with freshly labeled 125I-hGH (∼0.5 ng) for 45 min at 37 °C. Bound GH was then immediately separated from free GH by gel chromatography on a Sephadex G-100 column at 4 °C. The fraction of GH bound was determined by peak integration. Statistical analysis was performed by unpaired Student's t-test.

**Densitometric Analysis**—Densitometric quantitation of ECL immunoblots was performed using a video camera and the Image 1.49 program (developed by W.S. Rasband; Research Services Branch, National Institute of Mental Health, Bethesda, MD). The fractional loss of full-length GHRs in extracts from PMA-treated cells was estimated by measuring the intensity of the GHR signal relative to that present in non-PMA-treated cell extracts from the same experiment. The PMA-
induced loss of receptors determined thus in samples not pretreated with GH was considered the maximum (100%) within that particular experiment. PMA-induced GH loss in the presence of GH was expressed relative to this maximum. PMA-induced GH remnant accumulation was estimated by subtracting the remnant signal present in non-PMA-treated cell extracts from that present in the PMA-treated cells. Maximum (100%) remnant accumulation within each experiment was that arising from PMA treatment without prior GH stimulation. PMA-induced GHR remnant accumulation in the presence of GH was expressed relative to this maximum. As indicated when graphically shown, pooled data from several experiments are displayed as the mean ± S.E. The significance of differences of pooled results are estimated by unpaired t tests.

RESULTS

GH Inhibits PMA-induced GHR Proteolysis and GHBP Shedding—Proteolytic generation of the GHBP can be detected in both cells that endogenously express GHRs and cells that heterologously express GHRs (13–24). In our previous studies with human IM-9 lymphoblasts and CHO cells stably expressing the rbGHR, we have shown that metalloprotease-mediated GHBP shedding in response to activation of protein kinase C with the phorbol ester PMA is associated with loss of the full-length GHR and accumulation of cell-associated 60–68-kDa cytoplasmic domain-containing remnant proteins (13, 15). As we observed previously, expression of CHO-rbGHR cells to PMA for as little as 10 min allowed detection of receptor loss and remnant accumulation by immunoblotting with an antiseraum against the GHR cytoplasmic domain (Fig. 1A, lane 2 versus lane 1).

Because GHBP shedding has been observed in cells that express GHR isoforms that lack a large portion of the cytoplasmic domain (51, 52), we tested whether this biochemical signature of inducible shedding (receptor loss and remnant accumulation) could be detected in the absence of the full receptor cytoplasmic domain. We previously characterized a rbGHR mutant with an in-frame internal deletion of residues 297–406 (rbGHRdel 297–406) that thus lacks most of the membrane-proximal one-third of the receptor's cytoplasmic domain but has an intact Box 1 (JAK2-binding) region (41). rbGHRdel 297–406 binds GH normally, interacts with JAK2, and allows GH-induced JAK2 tyrosine phosphorylation (41). As seen in Fig. 1B, in CHO cells that stably express this mutant (CHO-rbGHRdel 297–406), the internally deleted receptor was easily detected by anti-GHRcyt at the molecular mass noted previously. A protein of ~43 kDa, the size expected of a remnant, was also detected. PMA treatment resulted in loss of receptor and further accumulation of the remnant protein (lane 2 versus lane 1). In other experiments (data not shown), we also observed PMA-induced receptor loss and remnant accumulation in COS-7 cells transiently expressing GH mutants lacking cytoplasmic domain residues 294–498 (rbGHRdel 294–498) and 391–620 (rbGHRdel 391–620) (both described previously in Ref. 41). Thus, neither the full cytoplasmic domain nor any particular cytoplasmic domain region (at least distal to the Box 1 element) is clearly required for PMA-induced receptor proteolysis. We do not yet know if the increased remnant present in CHO-rbGHRdel 297–406 cells even in the absence of PMA reflects an alteration in basal protease sensitivity in this mutant; such a change in protease specificity has been noted, for example, in mutants of the interleukin 6 receptor, another TACE substrate (53). In any case, the parallel findings of PMA-inducible proteolysis in the wild-type and mutant rbGHRs indicate the utility of both stably transfected CHO cell lines as model systems in the current study.

GH binding causes dimerization of the GHR, activation of the GHR-associated JAK2 tyrosine kinase, and internalization of the receptor (reviewed in Refs. 1 and 2). We showed previously that GHR proteolysis acutely affects receptor abundance...
GH Inhibition of GHR Proteolysis

Fig. 2. Concentration dependence of GH-induced receptor disulfide linkage and GH inhibition of PMA-induced receptor proteolysis in CHO-rbGHR<sub>del 297–406</sub> cells. A, serum-starved CHO-rbGHR<sub>del 297–406</sub> cells, prepared as described in the legend for Fig. 1A, were exposed to PMA (+) or the DMSO vehicle (−) for 10 min after pretreatment for 5 min with or without GH (at the indicated concentrations). Detergent extracts were resolved by SDS-PAGE under nonreducing (top panel) and reducing (bottom panel) conditions and immunoblotted with anti-GHR<sub>cyt</sub>-AL47 or anti-GHR<sub>cyt</sub>-AL47; as indicated. The disulfide-linked (dsl) and non-disulfide-linked receptors present for each treatment condition in the top panel are bracketed. The positions of the receptor and the remnant resolved under reducing conditions in the bottom panel are indicated by a bracket and an arrow, respectively. B, densitometric analysis of the immunoblot in A. Left panel, abundance of GH-induced disulfide-linked GHR<sub>del 297–406</sub> formed in response to 50, 100, and 500 ng/ml GH (maximum accumulation (100%) was seen in the presence of 500 ng/ml GH). Middle panel, fractional PMA-induced loss of non-disulfide-linked GHR<sub>del 297–406</sub> caused by PMA in the presence of 0, 50, 100, and 500 ng/ml GH (expressed for each GH concentration as the loss of signal caused by PMA divided by the signal measured for the non-PMA-treated sample). Right panel, accumulation of remnant induced by PMA in the presence of 0, 50, 100, and 500 ng/ml GH (maximum accumulation (100%) was seen in the presence of 0 ng/ml GH). The left and middle panels reflect the nonreducing immunoblot; the right panel reflects the reducing immunoblot. The experiment shown in A and B is representative of three such experiments.

(13–15) and can thereby impact upon GHR activation (15); we next explored whether GH stimulation could influence receptor proteolysis. As seen in Fig. 1, A and B, treatment with GH (500 ng/ml) for 5 min did not acutely promote GHR proteolysis (loss of GHR with accumulation of remnant) in CHO-rbGHR or CHO-rbGHR<sub>del 297–406</sub> cells (lane 3 versus lane 1). However, GH exposure did substantially diminish the ability of PMA to cause receptor proteolysis in both cell types. This was apparent in comparing the abundance of either remaining full-length GHRs or GHR remnants accumulated in response to PMA in the presence and absence of GH (Figs. 1, A and B, lane 4 versus lane 2). When several such experiments were analyzed by densitometry, relative PMA-induced loss of full-length GHRs declined on average by 66% and 99% in GH-treated CHO-rbGHR and CHO-rbGHR<sub>del 297–406</sub> cells, respectively (Fig. 1, C and D, top panels). Remnant abundance induced by PMA was diminished on average by 79% and 62% in these cells when exposed to GH before the PMA (Fig. 1, C and D, bottom panels). As indicated in Fig. 1, C and D, each of these differences was statistically significant.

The concentration dependence of the ability of GH to prevent PMA-induced GHR cleavage was examined in the experiment in Fig. 2, in which protection from proteolysis is compared with GH-induced GHR disulfide linkage. We have shown previously that GH induces the concentration- and time-dependent appearance of a high molecular weight form of the GHR detected by immunoblotting only under nonreducing conditions (32, 40, 47). CHO-rbGHR<sub>del 297–406</sub> cells were treated with 0–500 ng/ml GH for 5 min before exposure to PMA for 10 min. Cell extracts were divided into two fractions; proteins were resolved by SDS-PAGE under either nonreducing (Fig. 2A) or reducing (data not shown) conditions. Similar to our previous data using IM-9 cells, GH promoted the appearance of the disulfide-linked (dsl) form of rbGHR<sub>del 297–406</sub> when evaluated by anti-GHR<sub>cyt</sub> immunoblotting of nonreduced detergent cell extracts (Fig. 2A). The GH concentration dependence of the relative abundance of the disulfide-linked form of rbGHR<sub>del 297–406</sub> (lanes 1, 3, 5, and 7 of Fig. 2A) is displayed graphically in Fig. 2B, left panel. The subsequent treatment with PMA did not markedly affect the abundance of this GH-induced disulfide-linked form. In contrast, in the absence of GH, PMA did cause loss (roughly a 17.5% decrease in abundance by densitometry) of the non-disulfide-linked rbGHR<sub>del 297–406</sub> form (Fig. 2A, lane 2 versus lane 1; graphically displayed in Fig. 2B, middle panel). GH treatment prevented this PMA-induced loss in a concentration-dependent manner (progressively less fractional loss in the presence of 50 and 100 ng/ml GH versus 0 ng/ml GH, and no loss in the presence of 500 ng/ml GH; Fig. 2B, middle panel). We also assessed by densitometry the GH concentration dependence of inhibition of PMA-induced rbGHR<sub>del 297–406</sub> remnant formation in the reduced extracts of this same experiment. As seen in Fig. 2B, the profiles of GH inhibition of PMA-induced loss of non-disulfide-linked rbGHR<sub>del 297–406</sub> (middle panel) and appearance of the rbGHR<sub>del 297–406</sub> remi-
GH Inhibition of GHR Proteolysis

In the experiment shown in Fig. 5, human IM-9 cells were exposed to PMA or its vehicle for 10 min after a 5-min pretreatment with either vehicle, GH, G120K, or the combination of G120K and GH at a 3:1 ratio, and PMA-induced GHR loss and remnant accumulation were assessed by immunoblotting. As expected, GH substantially inhibited PMA-induced GHR proteolysis.

In the experiment shown in Fig. 5A, human IM-9 cells were exposed to PMA or its vehicle for 10 min after a 5-min pretreatment with either vehicle, GH, G120K, or the combination of G120K and GH at a 3:1 ratio, and PMA-induced GHR loss and remnant accumulation were assessed by immunoblotting. As expected, GH substantially inhibited PMA-induced GHR pro-

that this is not the explanation. GH-induced and constitutive GHR internalization is dependent on the presence of a critical phenylalanine (rat Phe-346, corresponding to rbGHR residue 327) (54), which resides in a region of the receptor that is internally deleted in rbGHRdel 297–406. This receptor, like other mutant GHRs truncated to eliminate this region (54, 55), is likely not significantly internalized in response to GH.

Another possible explanation for GH inhibition of GHR proteolysis is that GH-induced signaling either directly inhibits activation of the GHR-cleaving enzyme or impairs PMA's induction of protein kinase C activity, which is apparently required for induced GHR cleavage (13, 15). In either case, such GH-induced signaling would presumably begin with activation of JAK2. To test this possibility, we used the human fibrosarcoma cell line γ2A. This cell line was previously shown to lack JAK2 at the mRNA and protein levels (42). γ2A also lacks immunologically detectable GHR protein; we stably expressed the rbGHR in this cell by transfection to yield γ2A-rbGHR (Fig. 4A, lane 2 versus lane 1). GH stimulation of γ2A-rbGHR cells caused disulfide linkage of the transfected rbGHR (Fig. 4B), verifying that the receptor could bind GH and become dimerized. However, consistent with previous reports (56), GH treatment did not yield discernable tyrosine phosphorylation of cellular proteins, as assessed by anti-phosphotyrosine immunoblotting of cellular extracts resolved by SDS-PAGE (data not shown). This is consistent with the lack of JAK2 in these cells. Despite their lack of JAK2, γ2A-rbGHR cells responded to PMA treatment with substantial loss of full-length GHR and accumulation of the cell-associated receptor cytoplasmic domain-containing remnant protein (Fig. 4C, lane 4 versus lane 3 compared with lane 2 versus lane 1). In other experiments (data not shown), PMA-induced GHBP shedding was also easily detected in γ2A-rbGHR cell supernatants. Thus, the inducible proteolysis and shedding apparatus did not depend on the presence of JAK2. Interestingly, pretreatment with GH substantially inhibited PMA-induced GHR proteolysis in γ2A-rbGHR cells (Fig. 4C, lane 4 versus lane 2), much as we observed in the CHO transfectants and in IM-9 cells (see below). Densitometric assessment of GH inhibition of PMA-induced remnant accumulation in several similar experiments with γ2A-rbGHR cells is graphically shown in Fig. 4D. Thus, the ability of GH to inhibit inducible GHR proteolysis did not depend on either the presence of JAK2 or the ability of the hormone to promote JAK2-mediated intracellular signaling.

GH Inhibition of PMA-induced GHR Proteolysis Corresponds to the Ability of GH to Dimerize the Receptor—The data presented in Figs. 1–4 strongly suggest that the acute inhibition of inducible GHR proteolysis and GHBP shedding by GH could not be explained by either GH-induced GHR internalization or signaling. We next considered whether GH binding and/or GH-induced receptor dimerization could account for this inhibition. The GH antagonist, G120K (which is similar to the previously reported G120R), is mutated at site 2 of the human GH molecule; it thus binds normally to the GHR via the nonmutated G120K site 1 region but fails to properly dimerize the receptor because its mutated site 2 region inadequately binds to a second GHR monomer (30–32). We used G120K to explore whether GH binding versus productive GH-induced GHR dimerization is required for GH inhibition of PMA-induced GHR proteolysis.
GH inhibition of GHR proteolysis is not dependent on JAK2. A and B, reconstitution of JAK2- and GHR-deficient γ2A cells with rbGHR. γ2A (lane 1) and γ2A-rbGHR (lane 2) cells were detergent-solubilized, and extracts (the equivalent of one-half of a 100 × 20-mm dish of cells/sample) were resolved by SDS-PAGE under reducing conditions and immunoblotted with anti-GHRcyt-AL47. The stably transfected rbGHR present in γ2A-rbGHR cells is bracketed. B, serum-starved γ2A-rbGHR cells at confluence (prepared as described in A) were treated with (+) or without (−) GH (500 ng/ml) for 15 min. Detergent extracts (the equivalent of one-half of a dish of cells/sample) were resolved by SDS-PAGE under nonreducing and immunoblotted with anti-GHRcyt-AL47. The positions of the disulfide-linked (dsl) and non-disulfide-linked receptors present for each condition are bracketed. Note that GH caused disulfide linkage of the rbGHR, confirming that receptors are engaged and dimerized even in the absence of JAK2. C and D, GH inhibition of PMA-induced GHR proteolysis in γ2A-rbGHR cells. C, serum-starved γ2A-rbGHR cells (prepared as described in A and B) were exposed to PMA (+) or the DMSO vehicle (−) for 10 min after pretreatment for 5 min with or without GH (500 ng/ml, as indicated). Detergent extracts were resolved by SDS-PAGE and immunoblotted with anti-GHRcyt-AL47. The full-length GHR and the GHR remnant protein that appears in response to PMA are indicated by a bracket and an arrow, respectively. D, densitometric analysis of the effect of GH pretreatment (●) on PMA-induced accumulation of remnant in comparison with no GH pretreatment (●), as described in Fig. 1. Mean ± S.E. for each is shown for n = 3 determinations. For samples treated with PMA in the presence versus absence of GH: *, p < 0.01.

To determine whether GH inhibition of PMA-induced GHR proteolysis is due to GHR dimerization versus GHR disulfide linkage, we used our previously characterized GHR C241S cells. GH pretreatment afforded protection from PMA-induced proteolysis to the rbGHR C241S mutant (Fig. 6, lane 4 versus lane 2), similar to our findings with the wild-type rbGHR and rbGHRdel 297–406 in Figs. 1, 2, 4, and 5. These results suggest that it is the ability of GH to cause receptor dimerization rather than receptor disulfide linkage that allows inhibition of PMA-induced GHR proteolysis.

DISCUSSION

The proteolytic cleavage of the GHR with shedding of its extracellular domain is one established mechanism that is used by various species to generate soluble circulating GHBP. Whereas an end product achieved (GHBP) is functionally the same, proteolysis is a fundamentally different process than GHBP generation by alternative splicing of a common GHR mRNA precursor, the dominant GHBP production mechanism used by rodents (10–12). Both the alternative splicing and proteolysis mechanisms of GHBP generation could, in principle, allow for the observed general correlation that may exist between circulating GHBP levels and tissue GHR abundance (4, 36–38). However, in contrast to splicing, factors that cause increased GHBP by proteolysis might be expected to acutely diminish rather than increase GHR abundance. Indeed, in tissue culture model systems, we have observed just such a down-regulation of GHR abundance associated with metalloproteinase-mediated proteolytic GHBP generation in several cell types in response to treatment with PMA, N-ethylmaleimide, and serum (13–15). In the setting of GHR proteolysis induced by PMA, platelet-derived growth factor, and serum, this receptor down-regulation was accompanied by diminished GH signaling (15). Additionally, we have shown that GHR proteolysis in response to these stimuli generates a cell-associated receptor...
cytoplasmic domain-containing remnant protein (13, 15); although we have no evidence as yet of a role for it, such a cytoplasmic domain-containing fragment has been shown to affect signaling in other receptor systems (57, 58). These observations have suggested to us that, in addition to generating soluble GHBP, inducible GHR proteolysis may act in various ways to affect GHR function and GH signaling. Furthermore, although it can bind the GHR, the G120K antagonist itself was unable to protect the receptor from PMA-induced proteolysis, but it did antagonize GH's protection; thus we concluded that GH-induced GHR dimerization, rather than simply receptor occupancy, was required to inhibit the cleavage process. Finally, by comparing cells expressing the wild-type receptor (CHO-rbGHR) and a receptor mutant that is dimerized but not disulfide-linked by GH (CHO-rbGHR C241S), we determined that covalent (disulfide-mediated) receptor dimerization was not necessary to confer protection from inducible proteolysis.

Relatively little is known about the effects of GH on GHBP levels. Prolonged elevated GH levels found in acromegalics are frequently associated with reduced circulating GHBP content (33); however, it is difficult to interpret the significance of this observation with regard to its mechanism(s) because of the many adaptations to long-term elevations of GH that can occur. When investigated in normal male children, plasma GHBP levels were shown to correlate inversely with 24-h GH secretion (mean 24-h GH concentration, the sum of GH pulse amplitudes, the sum of GH pulse areas, interpulse mean GH concentration, and the number of GH pulses per 24 h) (59). Among other possibilities, this observation may suggest that a negative influence of GH on production of GHBP may have physiological relevance.

Mullis et al. (21) studied the effects of GH on GHR mRNA abundance, GHR mRNA transcription rate, and GHBP level in the supernatants of HuH7 human hepatoma cells in culture. This study showed a biphasic response, with an increase in GHR expression at low GH levels and a decrease in GHR expression at high levels. GHBP in cell supernatants declined within the first hour of GH treatment for GH concentrations of 50 ng/ml or less, remained constant in the presence of 150 ng/ml GH, and increased after a 3-h incubation in 500 ng/ml GH. These complex results led the authors to conclude that GHBP abundance did not necessarily reflect GHR mRNA abundance and can be regulated posttranscriptionally and can likely be regulated posttranslationally.

In our study, we examined the acute effects of GH on PMA-inducible GHR proteolysis in cells that both endogenously (IM-9) and heterologously (the CHO and γ2A transfectants) express GHRs and found similar results in each, likely nullifying any confounding effects of changes in GHR gene expression that might be induced by GH. Furthermore, we opted not to measure GHBP in the cell supernatants in situations in
GH-induced GHR dimerization protects against inducible receptor proteolysis and GHBP shedding. This model depicts the findings in this study and their implications for GHR down-regulation and signaling. In both A and B, the unliganded GHR is seen as a monomer (or perhaps a loosely associated dimer; A, 1 and B, 1). In the absence of GH, the transmembrane metalloprotease (likely TACE) in the vicinity of the GHR, activated by an inducer (data not shown), gains access to the receptor cleavage site in the near extracellular domain (A, 2). Metalloprotease-mediated processing yields shed GHBP and the membrane-associated GHR remnant that includes the receptor transmembrane and cytoplasmic domains (A, 3). When cells are exposed to GH before the addition of the metalloprotease inducer, GHRs undergo GH-induced dimerization: activation of associated JAK2 molecules results in tyrosine phosphorylation of the GHR and JAK2 (B, 2). As a consequence of GH-induced GHR dimerization, metalloprotease activation in this case does not result in GHR proteolysis (B, 3). The situation in A would be the same if the GH antagonist alone were present, binding via site 1 but not dimerizing the GHR, or if both GH antagonist and GH (at a ratio of 3:1 or greater) were present. In concert with our previous data (13–15), this model predicts that unliganded GHRs are more susceptible to proteolysis and shedding than liganded GHRs.

Several interesting and potentially testable questions regarding GH signaling and mechanisms of inducible GHR proteolysis also emerge from our work. The model presented in Fig. 7 is based on the data generated in this study and depicts some of these issues. For example, does GH inhibition of GHR proteolysis serve to enhance GH signaling? Such a role could be envisioned if it is assumed that, as we have observed in tissue culture (15), growth factors or other stimuli might down-regulate GHR abundance by proteolysis. In this instance, an already GH-dimerized receptor, either poised for or already engaged in signaling, would be less susceptible to down-regulation (deactivation) by these other stimuli. Receptor proteolysis generates a GHR cytoplasmic domain-containing remnant protein with an unknown signaling significance. Could dimerization-driven protection from proteolysis also serve as a mechanism by which GH prevents the generation of...
this remnant and thereby modulates the remnant's signaling (or the remnant's inhibition of signaling by the full-length GHR?)

We recently identified the transmembrane metalloprotease TACE as a GHR sheddase capable of mediating PMA-induced GHBP shedding and GHR down-regulation (14). Our current data allow us to speculate that receptor dimerization, which is known to involve apposition of the stem regions at the GHR extracellular face (28, 29), might prevent GHR proteolysis by causing a steric or conformational hindrance of TACE access to or activity at that dimerization interface. Conversely, it may be predicted that the GHR monomer is a better sheddase substrate than the dimer. Given the SDS-PAGE migration-based estimated sizes of the shed GHBP (39, 62, 63) and the GHR remnant (13, 15), this dimerization interface region is also likely to contain the locus of inducible GHR proteolysis. It will be interesting and important to test the dimerization sensitivity of interaction of this site(s) with TACE or other GHR sheddases that might be identified to further understand the structural features underlying their catalysis. As a practical matter, our results predict that proteolytic site mapping studies may be facilitated by using monomeric rather than dimerized GHRs as the substrate.

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