Growth Hormone Signaling in Human T47D Breast Cancer Cells: Potential Role for a Growth Hormone Receptor-Prolactin Receptor Complex

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GH receptor (GHR) and prolactin (PRL) receptor (PRLR) are structurally similar cytokine receptor superfamily members that are highly conserved among species. GH has growth-promoting and metabolic effects in various tissues in vertebrates, including humans. PRL is essential for regulation of lactation in mammals. Recent studies indicate that breast tissue bears GHR and PRLR and that both GH and PRL may impact development or behavior of breast cancer cells. An important facet of human GH (hGH) and human PRL (hPRL) biology is that although hPRL interacts only with hPRLR, hGH binds well to both hGHR and hPRLR. Presently, we investigated potential signaling effects of both hormones in the estrogen receptor- and progesterone receptor-positive human T47D breast cancer cell line. We found that this cell type expresses ample GHR and PRLR and responds well to both hGH and hPRL, as evidenced by activation of the Janus kinase 2/signal transducer and activator of transcription 5 pathway. Immunoprecipitation studies revealed specific GHR-PRLR association in these cells that was acutely enhanced by GH treatment. Although GH caused formation of disulfide-linked and chemically cross-linked GHR dimers in T47D cells, GH preferentially induced tyrosine phosphorylation of PRLR rather than GHR. Notably, both a GHR-specific ligand antagonist (B2036) and a GHR-specific antagonist monoclonal antibody (anti-$GHR_{ext\text{-}mAb}$) failed to inhibit GH-induced signal transducer and activator of transcription 5 activation. In contrast, although the non-GHR-specific GH antagonist (G120R) and the PRL antagonist (G129R) individually only partially inhibited GH-induced activation, combined treatment with these two antagonists conferred greater inhibition than either alone. These data indicate that endogenous GHR and PRLR associate (possibly as a GHR-PRLR heterodimer) in human breast cancer cells and that GH signaling in these cells is largely mediated by the PRLR in the context of both PRLR-PRLR homodimers and GHR-PRLR heterodimers, broadening our understanding of how these related hormones and their related receptors may function in physiology and pathophysiology. **(*Molecular Endocrinology* 25: 597–610, 2011)**

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Abbreviations: BS^3, Bis-(sulfosuccinimidyl) suberate; dsl, disulfide linkage; GHR, GH receptor; G120R, hGH-G120R; G129R, hPRL-G129R; hGH, human GH; hPRL, human PRL; JAK2, Janus kinase 2; LNCaP-PRLR, LNCaP cells stably transfected with expression vector directing expression of hPRLR; LNCaP-vector, LNCaP cells stably transfected with expression vector only; PRL, prolactin; pY, phospho-tyrosine; STAT5, signal transducer and activator of transcription 5.

GH is a 22-kDa protein produced largely by the anterior pituitary that potently induces multiple growth promoting and metabolic effects (1, 2). The GH receptor (GHR) is a single membrane-spanning glycoprotein that is a member of the cytokine receptor superfamily (3). GHR is expressed in many tissues, most prominently in liver, muscle, and fat, but it is also found in breast under certain conditions, and GH affects mammary development (4–7). Indeed, GH is produced locally in the mammary gland and its expression is increased in some human mammary proliferative disorders (8, 9). Forced GH expression in human breast or endometrial cancer cells yields more aggressive behavior of explants in mice (7, 10). Notably, rodents that are either GH- or GHR-deficient exhibit greatly reduced incidence and aggressiveness of experimentally induced cancers, including breast and prostate, suggesting that the GH axis may potentiate such cancers (11–14).

Current information suggests that GHR is present at the cell surface as a homodimer that changes in conformation in response to GH binding to its extracellular domain, triggering activation of the intracellular domain-associated Janus kinase 2 (JAK2) tyrosine kinase and signaling via the JAK2/signal transducer and activator of transcription 5 (STAT5) pathway, among others (4, 15–19). The GH-induced conformational changes in the GHR correlate with GH-induced covalent disulfide linkage (dsl) between receptor dimer partners mediated by the only unpaired cysteine (C241) in the GHR extracellular domain (19–22). Both GH signaling and GH-induced GHR dsl are blocked by GH antagonists and by a conformation-specific anti-GHR extracellular domain antibody, but formation of GHR C241-C241 dsl is not absolutely required for GH signaling (21, 23). This suggests that GH-induced dsl is a reflection of, rather than a prerequisite for, enhanced GH-induced noncovalent association between receptor dimer partners in the vicinity of the extracellular subdomain 2 and stem regions just outside of the plasma membrane.

Prolactin (PRL) is of similar size and overall structure to GH. In humans, the two hormones [human GH (hGH) and human PRL (hPRL)] share 16% sequence identity. Like GH, PRL emanates mainly from the anterior pituitary, but its expression has been detected in mammary cells (24, 25). Like GHR, PRLR is a cytokine receptor family member. Human GHR and PRLR share homology (32% extracellular domain identity; less in the intracellular domain) (26). PRL has multiple effects but has particularly important roles in breast development and lactation (27, 28). Furthermore, PRL may have a role in human breast cancer by virtue of endocrine and/or autocrine/paracrine effects (29–31). Importantly, PRL signaling shares features with GH signaling, including utilization of the JAK2/STAT5 pathway (32–35). One fascinating feature of hGH/PRL biology relates to interactions between these ligands and their receptors. In humans, hGH binds not only the GHR but also the PRLR; the physiological consequences of hGH-hPRLR interaction are incompletely known but may diversify GH's role in humans (36–39). Distinct hGH amino acids are critical in determining hGHR *vs.* hPRLR specificity. In contrast, hPRL binds hPRLR but does not interact with hGHR.

PRLR likely exists as a preformed homodimer, and PRL engagement of the PRLR homodimer underlies PRL signaling (40, 41). In contrast to GHR homodimers in response to GH, covalently linked PRLR homodimers have not been observed. Some cells, such as breast and breast cancer cells, coexpress GHR and PRLR (5, 35, 42), but the issue of GHR-PRLR heterodimerization has been only minimally explored. Notably, previous reconstitution studies suggest that ovine GHR and PRLR can heterodimerize, at least in response to placental lactogens (43, 44). Furthermore, our recent work suggests that the human forms of these receptors may also heterodimerize (42), but biochemical evidence for such heterodimerization has been lacking, and whether GH or PRL can work through such a heterodimer is unknown.

In the present study, we found that the estrogen receptor- and progesterone receptor-positive human T47D breast cancer cell expresses ample GHR and PRLR and responds well to both hGH and hPRL, as evidenced by activation of the JAK2/STAT5 pathway. Immunoprecipitation studies revealed specific GHR-PRLR association in these cells that was acutely enhanced by GH treatment. Although GH caused formation of dsl and chemically cross-linked GHR dimers in T47D cells, GH preferentially induced tyrosine phosphorylation of PRLR rather than GHR. Notably, both a GHR-specific ligand antagonist (B2036) and a GHR-specific antagonist monoclonal antibody (anti-$GHR_{ext\text{-}mAb}$) failed to inhibit GH-induced STAT5 activation. In contrast, although the non-GHR-specific GH antagonist (G120R) and the PRL antagonist (G129R) individually only partially inhibited GH-induced activation, combined treatment with these two antagonists conferred greater inhibition than either alone. These data indicate that endogenous GHR and PRLR associate (possibly as a GHR-PRLR heterodimer) in human breast cancer cells and that GH signaling in these cells is largely mediated by the PRLR in the context of both PRLR-PRLR homodimers and GHR-PRLR heterodimers, broadening our understanding of how these related hormones and their related receptors may function in physiology and pathophysiology.

Results

Like PRL, GH activates the JAK2/STAT5 pathway in T47D human breast cancer cells

We previously demonstrated that human T47D breast cancer cells respond to GH and PRL (35, 42). In the current study, we first compared GH and PRL signaling in T47D cancer cells (Fig. 1). Activation and resultant tyrosine phosphorylation of JAK2, a receptor-associated cytoplasmic tyrosine kinase, is a critical step in ligand-induced activation of both GHR and PRLR. To assess JAK2 tyrosine phosphorylation, serum-starved T47D cells were exposed to vehicle, GH (500 ng/ml) (Fig. 1A), or PRL (500 ng/ml) (Fig. 1B) for 10 min before detergent extraction. Extracted proteins were immunoprecipitated with our anti-$JAK2_{AL33}$ serum, and eluates were separated by SDS-PAGE and sequentially immunoblotted with antiphospho-tyrosine (pY) and anti-$JAK2_{AL33}$. Both GH and PRL acutely caused tyrosine phosphorylation of JAK2 in these cells.

STAT5 activation is also reflected by tyrosine phosphorylation. We assessed STAT5 tyrosine phosphorylation in response to GH and PRL in T47D cells in concentration dependence (Fig. 1C) and time-course (Fig. 1D) experiments. Treatment for 10 min with as little as 20 ng/ml GH allowed detectable tyrosine phosphorylation of STAT5. STAT5 phosphorylation reached its maximum with 500 ng/ml GH. At this GH concentration, STAT5 activation was sustained, persisting for at least 60 min. PRL treatment produced a similar pattern of STAT5 activation.

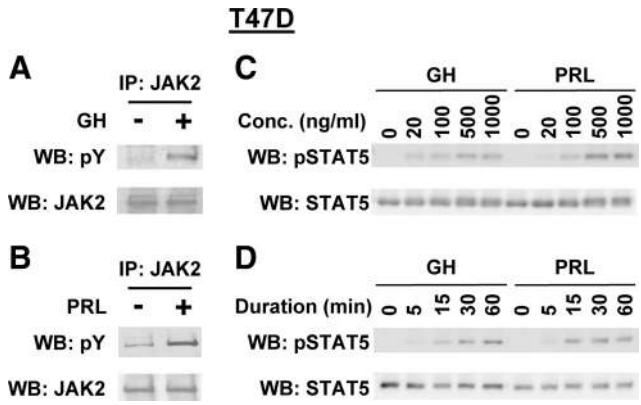

FIG. 1. Both hGH and hPRL cause JAK2 and STAT5 tyrosine phosphorylation in T47D human breast cancer cells. A and B, JAK2 activation. Serum-starved T47D cells were treated without or with GH (A), or PRL (B) (500 ng/ml each for 10 min), after which detergent cell extracts were immunoprecipitated with anti-$JAK2_{AL33}$. Eluates were separated by SDS-PAGE and immunoblotted with the anti-pY antibody, 4G10. The blot was stripped and reprobed with anti-$JAK2_{AL33}$ as a loading control. C, STAT5 phosphorylation concentration dependence. Serum-starved T47D cells were treated without or with the indicated concentrations of GH or PRL for 10 min. Detergent cell extracts were resolved by SDS-PAGE and immunoblotted with an antibody that recognizes tyrosine phosphorylated STAT5 (pSTAT5). The blot was stripped and reprobed with antibody for total STAT5 (STAT5) as a loading control. D, STAT5 phosphorylation time course. Serum-starved T47D cells were treated without or with 500 ng/ml GH or PRL for the indicated durations. Immunoblotting of detergent cell extracts for pSTAT5 and STAT5 was performed, as in C. Data shown are representative of three experiments for each set of conditions. Conc, Concentration; WB, Western blot.

GH engages GHR in T47D cells

In humans, GH can engage both GHR and PRLR. We explored further whether GHR was present and, if so, was engaged by GH in T47D cells (Fig. 2). We first assessed whether GHR was immunologically detectable and underwent GH-induced dsl, which reflects GH-induced GHR conformational changes usually associated with GHR signal generation (19–22). T47D cells were treated briefly with GH, and detergent extracts were resolved by SDS-PAGE under nonreduced conditions before Western transfer and blotting with anti-$GHR_{cyt\text{-}AL47}$, a rabbit serum that specifically reacts with the GHR intracellular domain (Fig. 1A). GHR (non-dsl GHR) was easily detected under these conditions and migrated at the expected position previously observed for hGHR in other cell types (20, 45, 46). Furthermore, GH promoted the appearance of the dsl form of the GHR that migrates at roughly twice the molecular mass of monomeric (non-dsl) GHR under these conditions. Thus, GH was capable of binding and engaging GHR in these human breast cancer cells, as seen previously in other (human and nonhuman) cell types. This was further explored by covalent chemical cross-linking experiments (Fig. 2B), using methods we previously developed (20). In this case, T47D cells were briefly treated with GH, and detergent extracts were treated with a noncleavable chemical cross-linker, bis-(sulfosuccinimidyl) suberate (BS^3). After quenching the cross-linking, the extracts were resolved by SDS-PAGE under reduced conditions, and GHR was immunoblotted. This analysis also revealed that GH induced formation of a covalently cross-linked form of GHR, the migration of which paralleled that of the dsl form observed under nonreduced conditions in the noncross-linked samples in Fig. 2A. Notably, PRL treatment did not yield either the dsl form of GHR (data not shown) or the covalently cross-linked form of GHR (Fig. 2B).

GHR-specific GH antagonists fail to block GH-induced STAT5 activation in T47D cells

Because GH both induced GHR dsl and promoted the ability of GHR to be covalently chemically cross-linked, we explored effects of GHR-specific GHR antagonists on GH signaling in T47D cells. Anti-$GHR_{ext\text{-}mAb}$ is a conformation-specific monoclonal antibody that recognizes the extracellular domain of rabbit and hGHRs via subdomain 2 (19, 22, 23, 47) (data not shown). Brief pre-

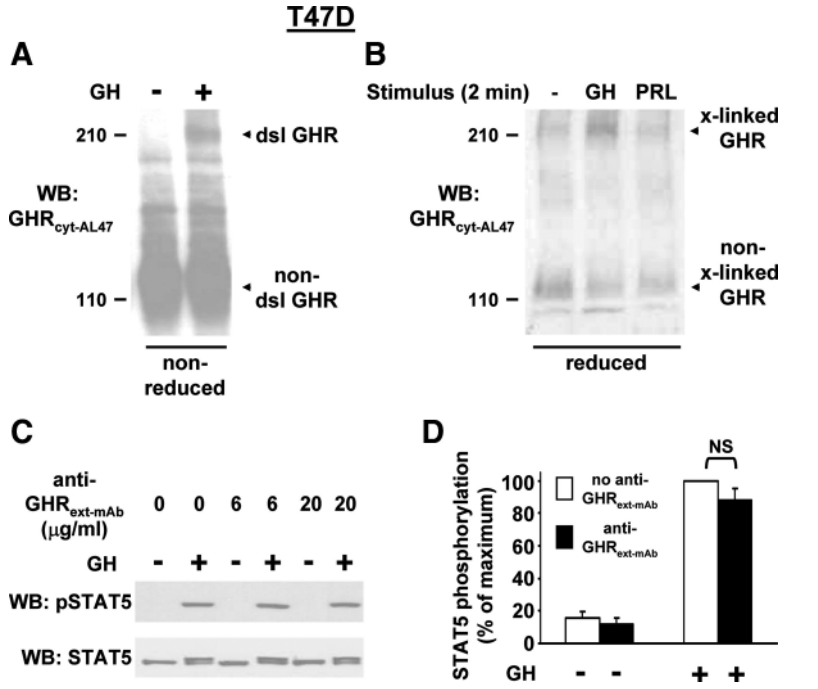

FIG. 2. GHR is engaged by GH in T47D cells, and anti-$GHR_{ext\text{-}mAb}$ fails to inhibit GH-induced STAT5 tyrosine phosphorylation in T47D cells. A, GHR dsl. Serum-starved T47D cells were treated without or with 500 ng/ml GH for 10 min. Detergent cell extracts were resolved by nonreduced SDS-PAGE before immunoblotting with anti-$GHR_{cyt\text{-}AL47}$. The positions of the 110- and 210-kDa molecular mass markers are indicated, as are the positions of dsl and non-dsl forms of GHR. B, GHR cross-linking. Serum-starved T47D cells were treated with vehicle, GH, or PRL (500 ng/ml each) for 2 min. Detergent cell extracts were treated with 1 mM BS^3, as in *Materials and Methods*, and resolved by reduced SDS-PAGE before immunoblotting with anti-$GHR_{cyt\text{-}AL47}$. and anti-STAT5. D, Densitometric analysis of data from three separate experiments, including that presented in C. The pSTAT5 level induced by GH in the absence of antibody pretreatment is considered 100%. Data are expressed as mean ± SE. NS, No significant difference.The positions of the 110- and 210-kDa molecular mass markers are indicated, as are the positions of cross-linked (x-linked) and noncross-linked (non-x-linked) forms of GHR. The immunoblots shown in A and B are representative of three such experiments for each condition. C, Serum-starved T47D cells were treated without or with anti-$GHR_{ext\text{-}mAb}$ at the indicated concentrations for 15 min before treatment with GH (500 ng/ml) or vehicle for 10 min. Detergent extracts were sequentially immunoblotted with anti-pSTAT5. pSTAT5, PhosphoSTAT5; WB, Western blot.

treatment of GH-responsive cells that express rabbit GHR dramatically and specifically inhibits GH-induced GHR dsl and GH-induced signaling (19, 22, 23), indicating that anti-$GHR_{ext\text{-}mAb}$ is a GHR-specific GH antagonist.

To test its effects on T47D cells, serum-starved cells were pretreated with of anti-$GHR_{ext\text{-}mAb}$ (6 or 20 μg/ml) for 15 min before treatment with GH for 10 min. Surprisingly, GH-induced STAT5 activation was unaltered by antibody pretreatment (Fig. 2, C and D). In other experiments (data not shown), pretreatment with even 40 μg/ml of $GHR_{ext\text{-}mAb}$ failed to inhibit GH signaling in T47D cells. As a control, we examined another GH-responsive human cell line, the LNCaP prostate cancer cell. As expected, acute GH treatment promoted dose-dependent STAT5 activation in these cells (Fig. 3A). In contrast to T47D cells, GH-induced STAT5 phosphorylation in LNCaP cells was quite sensitive to anti-$GHR_{ext\text{-}mAb}$ pretreatment (Fig. 3, B and C). These results indicate that the inability of anti-$GHR_{ext\text{-}mAb}$ to inhibit GH signaling was selective for T47D cells.

B2036 is a recombinantly produced mutated form of hGH that displays enhanced binding site 1 affinity (attributed to eight mutations in that site) and markedly diminished site 2 affinity (due to mutation of glycine-120 to lysine) (48). B2036 binds GHR, but not PRLR; thus, like anti-$GHR_{ext\text{-}mAb}$, B2036 is a GHR-specific GH antagonist (49). Interestingly, B2036 had no effect on GH-induced STAT5 activation in T47D cells (Fig. 4, A and B) but nearly completely inhibited GH-induced STAT5 activation in LNCaP cells (Fig. 4, A and C).

GH-induced activation of GHR and PRLR in T47D cells

The lack of inhibition of GH-induced signaling in T47D by the GHR-specific antagonists, B2036 and anti-$GHR_{ext\text{-}mAb}$, could, in principle, be due to an inability of those antagonists to interact for unknown reasons with GHR in these cells. We explored this possibility further by assessing the impact of each antagonist on GH-induced GHR dsl in these cells (Fig. 5A). Notably, pretreatment with either B2036 or anti-$GHR_{ext\text{-}mAb}$ inhibited GH-induced GHR dsl. These findings strongly suggest that both antagonists had ample access to the GHR in T47D cells and prevented the GH-induced conformational changes in the GHR that are associated with activation but yet failed to inhibit GH signaling.

These findings in T47D cells may indicate that, although it can cause GHR conformational changes, the degree to which GH signals through the GHR *per se* is uncertain. To further address this issue, we asked whether GH caused acute tyrosine phosphorylation of GHR *vs.* PRLR in these cells (Fig. 5, B and C). Serum-starved T47D cells were treated with GH (500 ng/ml) for 0–10 min, as indicated, and detergent extracts were immunoprecipitated with our GHR-specific anti-GHR serum that recognizes the receptor intracellular domain. Although blotting of the SDS-PAGE-resolved eluates from these precipitates with the same antibody revealed ample GHR, reprobing of the same blots with antiphosphotyrosine antibodies

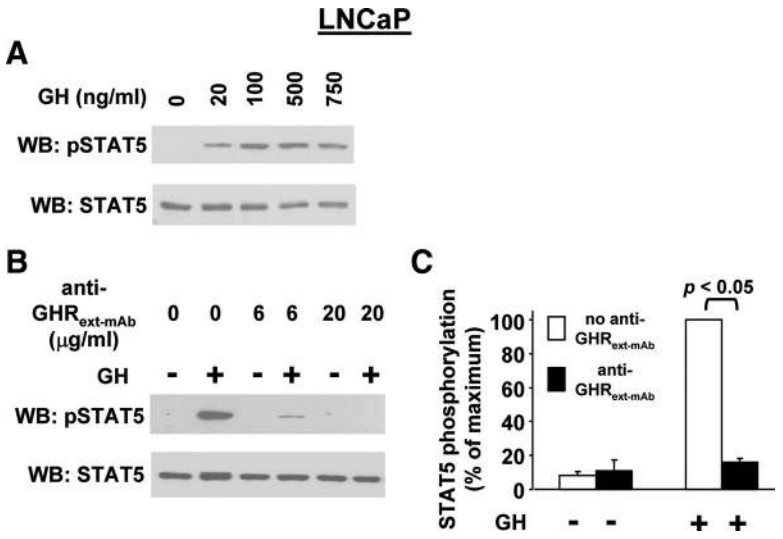

FIG. 3. Anti-GHR$_{ext-mAb}$ inhibits GH-induced STAT5 tyrosine phosphorylation in LNCaP cells. A, GH induces STAT5 tyrosine phosphorylation in LNCaP cells. Serum-starved LNCaP cells were treated without or with the indicated concentrations of GH for 10 min. Detergent cell extracts were resolved by SDS-PAGE and serially immunoblotted with anti-pSTAT5 and anti-STAT5. The blot shown is representative of three independent experiments. B, Effects of anti-GHR$_{ext-mAb}$. Serum-starved LNCaP cells were treated without or with anti-GHR$_{ext-mAb}$ at the indicated concentrations for 15 min before treatment with GH (500 ng/ml) or vehicle for 10 min. Detergent extracts were sequentially immunoblotted with anti-pSTAT5 and anti-STAT5. C, Densitometric analysis of data from three separate experiments, including that presented in B. The pSTAT5 level induced by GH in the absence of antibody pretreatment is considered 100%. Data are expressed as mean ± SE. *P* value is indicated. pSTAT5, PhosphoSTAT5; WB, Western blot.

revealed little, if any, GH-induced GHR tyrosine phosphorylation (Fig. 5B, *left four lanes*). As a control, the same experiment was performed using LNCaP cells; in contrast to T47D cells, there was ample acute GH-induced GHR tyrosine phosphorylation in LNCaP cells (Fig. 5B, *right four lanes*), indicating that the protocol and reagents used are capable of detecting this modification.

In the same fashion, we tested whether GH induced PRLR tyrosine phosphorylation in T47D cells (Fig. 5C). In this case, PRLR was immunoprecipitated, and GH- or PRL-induced PRLR tyrosine phosphorylation was assessed by antiphosphotyrosine blotting. As expected, PRL treatment acutely promoted substantial PRLR tyrosine phosphorylation. Notably, GH-induced PRLR tyrosine phosphorylation was also easily detected, in contrast to the findings regarding GH-induced GHR tyrosine phosphorylation in the same cells. Both GH and PRL also caused appearance of another tyrosine phosphoprotein in the PRLR precipitates (Fig. 5C, *asterisk*). This phosphoprotein comigrated with JAK2 (data not shown) and presumably represents PRLR-associated tyrosine phosphorylated JAK2.

The findings in Fig. 5, B and C, suggested receptor use (GHR *vs.* PRLR) in response to GH differs in T47D *vs.* LNCaP cells. To examine this further, we compared the two cell types in terms of the abundance of the two receptors (Fig. 5D). Extracts of LNCaP and T47D cells containing equal amounts of total protein were resolved by SDS-PAGE and blotted for GHR and PRLR. This revealed the expected ample GHR in both cells. In contrast, T47D harbored abundant PRLR, but no PRLR was detected in LNCaP cells. This is consistent with a previous report (50), in which PRLR mRNA levels, as determined by real-time RT-PCR, were high in T47D cells and low in LNCaP cells.

We further addressed the potential impact of PRLR on GHR by expressing hPRLR in LNCaP cells. We isolated pools of LNCaP cells stably transfected with either expression vector only (LNCaP-vector) or an expression vector directing expression of hPRLR (LNCaP-PRLR). PRLR expression was assessed by immunoprecipitation and immunoblotting (Fig. 5E). As expected, the control LNCaP-vector cells, like parental LNCaP, exhibited no detectable PRLR. In contrast, PRLR was specifically detected in LNCaP-PRLR cells. Notably, acute treatment with GH yielded reduced GHR tyrosine phosphorylation in the LNCaP-PRLR cells *vs.* LNCaP-vector cells (Fig. 5F).

A PRLR-specific antagonist only partially inhibits GH-induced STAT5 activation in T47D cells

Collectively, the data in Figs. 1–5 indicate that GH causes STAT5 signaling in T47D cells and that GH engages GHR, causing it to undergo conformational changes typically associated with its participation in signaling. However, GHR-specific antagonists fail to block GH signaling in these cells. Furthermore, comparison with PRLR-deficient LNCaP cells and LNCaP cells stably expressing PRLR suggests that the presence of PRLR in T47D cells and in LNCaP-PRLR cells lessens coupling of GHR engagement to its propagation of signaling. Because GH is known to also engage PRLR, we considered whether GH signaling in T47D cells might be accounted for by the GH-PRLR interaction by employing the PRLR-specific antagonist, hPRL-G129R (G129R) (Fig. 6) (51). As expected, G129R itself over a broad concentration range failed to activate STAT5 signaling in T47D cells; further, PRL-induced STAT5 activation (at 500 ng/ml) was completely inhibited by coincubation with a relatively low concentration (5×) of G129R (Fig. 6, A and B). In contrast, GH-induced STAT5 activation (at 500 ng/ml) was only partially (~50%) inhibited by G129R, even at a

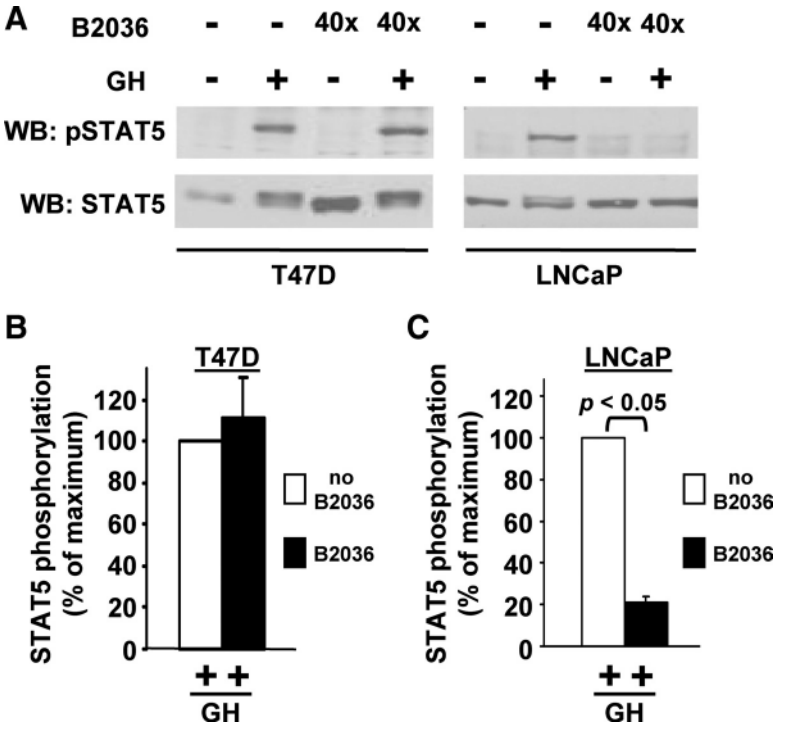

FIG. 4. Effects of GHR-specific GH antagonist, B2036, on GH-induced STAT5 tyrosine phosphorylation in T47D and LNCaP cells. A, Serum-starved T47D or LNCaP cells, as indicated, were treated with vehicle or GH (500 ng/ml; 10 min) in the presence or absence of B2036 [40-fold molar excess to GH (40×)]. Detergent cell extracts were resolved by SDS-PAGE and serially immunoblotted with anti-pSTAT5 and anti-STAT5. B and C, Densitometric analysis of data for T47D (B) and LNCaP (C) from three separate experiments, including that presented in A. The pSTAT5 level induced by GH in the absence of B2036 is considered 100% in each case. Data are expressed as mean ± SE. *P* value for LNCaP is indicated. There was no significant effect of B2036 cotreatment for T47D cells. pSTAT5, PhosphoSTAT5; WB, Western blot.

concentration 40-fold in excess of GH. Thus, a PRLR-specific antagonist failed to completely block GH signaling. In concert with the finding that GHR-specific antagonists have no inhibitory effect, these findings imply that GH signaling in GHR- and PRLR-expressing T47D cells may be conveyed by other than GHR homodimers (which would be inhibited by GHR-specific antagonists) or PRLR homodimers (which would be inhibited by PRLR-specific antagonists).

GHR and PRLR specifically coprecipitate in T47D cells

To pursue other potential GHR and PRLR arrangements that might foster GH signaling, we asked whether GHR associated with PRLR in T47D cells (Fig. 7). Serum-starved cells were solubilized in Triton X-100-containing lysis buffer, and these extracts were subjected to immunoprecipitation with anti-GHR serum or, as a specificity control, a nonimmune serum (Fig. 7A). Eluates were sequentially immunoblotted for GHR and PRLR. As anticipated, GHR was specifically precipitated by the anti-GHR serum. Notably, however, ample PRLR was also specifically detected in the anti-GHR precipitate, indicating that GHR and PRLR associate in T47D cells, even in the absence of treatment with GH or PRL. PRL treatment did not affect GHR-PRLR association (data not shown); however, GH substantially augmented coimmunoprecipitation (Fig. 7, B and C). T47D cells were treated with GH (500 ng/ml; 2 min) or vehicle, after which detergent extracts were precipitated with either anti-GHR (Fig. 7B) or anti-PRLR (Fig. 7C). GH treatment did not affect the abundance of directly precipitated GHR (assessed by anti-GHR immunoblotting of anti-GHR precipitates in Fig. 7B) or PRLR (assessed by anti-PRLR immunoblotting of anti-PRLR precipitates in Fig. 7C). In contrast, more PRLR was detected in anti-GHR precipitates of GH-treated *vs.* vehicle-treated samples (Fig. 7B), and the same was found for GHR detected in anti-PRLR precipitates of GH-treated samples (Fig. 7C). The data in Fig. 7 indicate that endogenous hGHR and PRLR specifically associate in T47D cells and that GH augments this association. Of note, specific GHR-PRLR coimmunoprecipitation was also observed in LNCaP-PRLR cells in response to GH (Fig. 7D).

Enhanced inhibition of GH signaling in T47D cells by the combination of a non-GHR-specific antagonist and a PRLR-specific antagonist

Unlike B2036, hGH-G120R (G120R) is a GH antagonist that binds to both GHR and PRLR and can antagonize signaling from both receptors, although its affinity for GHR is likely greater than for PRLR (49). Because B2036 failed to inhibit GH signaling and G129R only partially inhibited GH signaling in T47D cells, we tested the effects of G120R in both T47D and LNCaP cells (Fig. 8). In PRLR-deficient LNCaP cells, cotreatment with GH and G120R revealed that G120R at low concentrations (3- or 5-fold molar excess compared with GH) abolished GH signaling (Fig. 8, A *last four lanes* and C). In contrast, G120R at the same concentrations (3× and 5× relative to GH) that strongly inhibited GH signaling in LNCaP had much more modest effects (<50% inhibition) on GH signaling in T47D cells (Fig. 8, A *first four lanes* and B). Higher G120R concentrations (10× and 40×) had further inhibitory effects with nearly complete inhibition at 40×. As above, this is in contrast to the G129R, in which

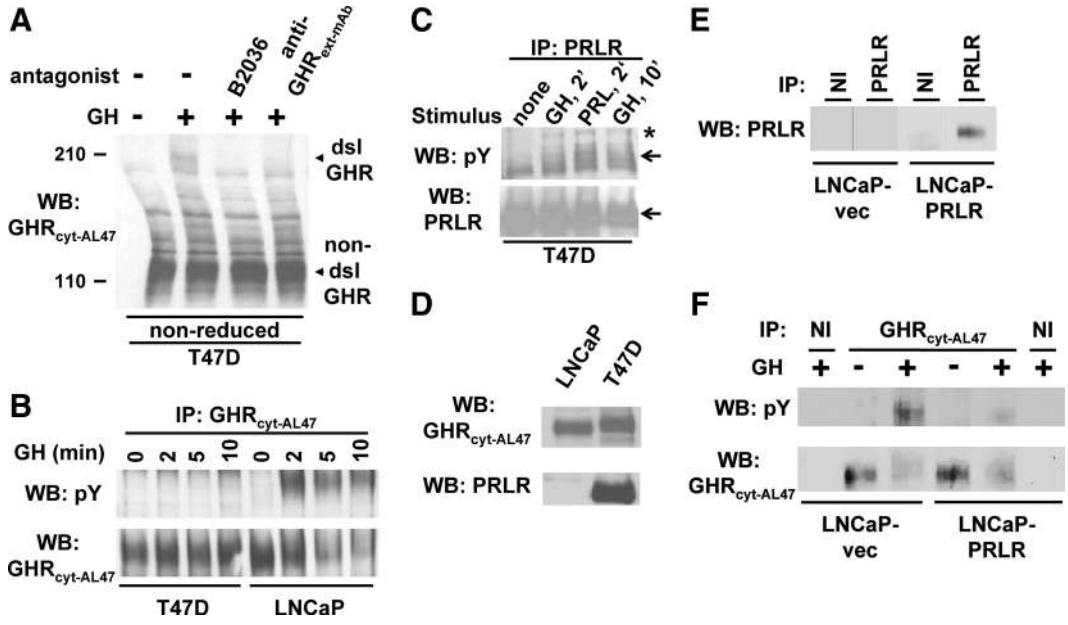

FIG. 5. Despite engaging GHR, GH causes tyrosine phosphorylation of PRLR, rather than GHR, in T47D cells. A, GHR-specific antagonists block GH-induced GHR dsl in T47D cells. Serum-starved T47D cells were treated with vehicle or GH (500 ng/ml; 10 min) in the presence or absence of B2036 (40×) or anti-GHR$_{ext-mAb}$ (20 μg/ml), as indicated. Detergent extracts were resolved by nonreduced SDS-PAGE and immunoblotted with anti-GHR$_{cyt-AL47}$. The positions of the 110- and 210-kDa molecular mass markers are indicated, as are the positions of dsl and non-dsl forms of GHR. Note that both GHR-specific antagonists prevent GH-induced GHR dsl. B, GH induces GHR tyrosine phosphorylation in LNCaP, but not T47D. Serum-starved T47D or LNCaP cells, as indicated, were treated without or with GH (500 ng/ml) for the indicated durations. Detergent extracts were immunoprecipitated with anti-GHR$_{cyt-AL47}$, and eluates were resolved by SDS-PAGE and serially immunoblotted with anti-pY and anti-GHR$_{cyt-AL47}$. C, GH induces PRLR tyrosine phosphorylation in T47D. Serum-starved T47D cells were treated without or with GH or PRL (500 ng/ml each) for the indicated durations. Detergent extracts were immunoprecipitated with anti-PRLR, and eluates were resolved by SDS-PAGE and serially immunoblotted with anti-pY and anti-PRLR. *Arrowhead* indicates the position of PRLR. *Asterisk* indicates expected position of JAK2. D, Relative GHR and PRLR abundance in T47D and LNCaP. Serum-starved LNCaP or T47D cells, as indicated, were detergent extracted. Proteins were resolved by SDS-PAGE and serially immunoblotted with anti-GHR$_{cyt-AL47}$ and anti-PRLR. Note the presence of both GHR and PRLR in T47D but only GHR in LNCaP. The immunoblots shown in A–D are representative of three such experiments for each condition. E, Generation of pools of LNCaP cells that stably express hPRLR or vector only. LNCaP cells were transfected and selected in G418, as in *Materials and Methods*. Equal aliquots of serum-starved LNCaP-vector or LNCaP-PRLR cells were immunoprecipitated with either control (NI) or anti-PRLR antibody. Eluates were immunoblotted with anti-PRLR. Note that hPRLR is specifically detected only on LNCaP-PRLR cells. F. Expression of PRLR in LNCaP cells reduces GH-induced GHR tyrosine phosphorylation. Equal aliquots of serum-starved LNCaP-vector or LNCaP-PRLR cells were stimulated with or without GH (500 ng/ml; 10 min), as indicated. Detergent extracts were immunoprecipitated with NI or anti-GHR$_{cyt-AL47}$ antibody, as indicated, and eluates were resolved by SDS-PAGE and transferred to nitrocellulose for sequential immunoblotting with anti-pY and anti-GHR, as indicated. NI, Nonimmune; IP, immunoprecipitation; WB, Western blot.

the 40× condition only partially inhibited GH signaling (Fig. 6). Thus, G120R (which can inhibit both GHR and PRLR) at high concentration is more effective than G129R (which is PRLR specific) at inhibiting GH signaling in T47D cells. In concert with our findings that GHR-specific antagonists (B2036 and anti-GHR$_{ext-mAb}$) are ineffective at inhibiting GH signaling in these cells, the data in Figs. 6 and 8 may suggest that receptors other than the homodimeric GHR and homodimeric PRLR contribute substantially to GH signaling in T47D; the GHR-PRLR heterodimer observed in Fig. 7 may be such a candidate receptor.

To pursue this further, we compared the impact of the G120R and G129R antagonists alone or in combination. In the representative experiments shown in Fig. 9, T47D cells were treated with GH (500 ng/ml). As expected, cotreatment with G120R alone or G129R alone, each at either a 10× (Fig. 9A) or 5× (Fig. 9B) molar excess compared with GH, partially reduced GH-dependent STAT5 activation. Notably, however, cotreatment with the combination of G120R and G129R, each at a 5× molar excess (so as to collectively comprise a 10× molar ratio) (Fig. 9A) or at a 2.5× molar excess (so as to collectively comprise a 5× molar ratio) (Fig. 9B), inhibited GH-induced STAT5 activation more effectively than either antagonist individually. Similarly, GH-induced JAK2 tyrosine phosphorylation was inhibited by the combination of the two antagonists to a greater degree than by either alone (Fig. 9C). These data in Fig. 9 collectively indicate a greater antagonistic effect on GH signaling in T47D cells by the combination of G120R and G129R compared with each alone.

Discussion

There has been substantial progress over the past three decades concerning mechanisms by which GH, PRL, and

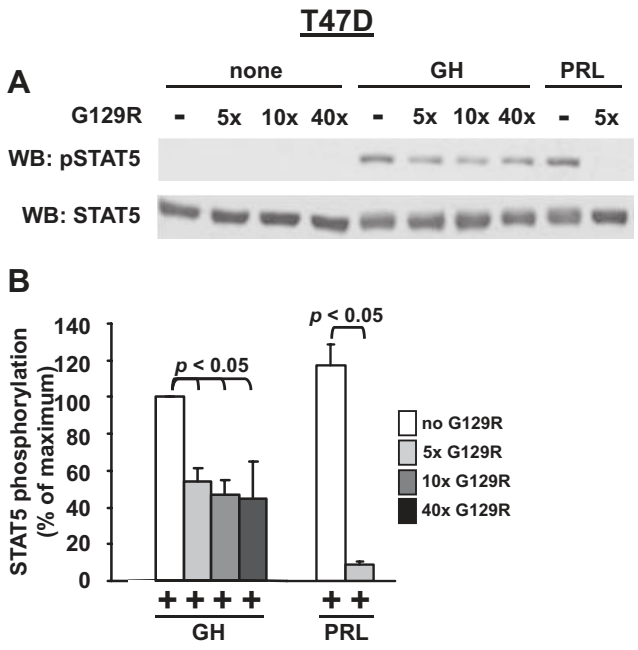

FIG. 6. The PRL antagonist, G129R, partially blocks GH-induced STAT5 tyrosine phosphorylation in T47D cells. A, Serum-starved T47D cells were treated with vehicle, GH, or PRL (500 ng/ml each; 10 min) in the presence or absence of G129R (fold molar excess, as indicated, to GH or PRL). Detergent cell extracts were resolved by SDS-PAGE and serially immunoblotted with anti-pSTAT5 and anti-STAT5. B, Densitometric analysis of data from three separate experiments, including that presented in A. The pSTAT5 level induced by GH in the absence of G129R is considered 100% in each case. Data are expressed as mean ± SE. *P* values for comparisons are as indicated. Note partial inhibition of GH-induced STAT5 tyrosine phosphorylation by G129R, even at high molar excess, whereas low molar excess of G129R strongly inhibits PRL-induced STAT5 tyrosine phosphorylation. pSTAT5, PhosphoSTAT5; WB, Western blot.

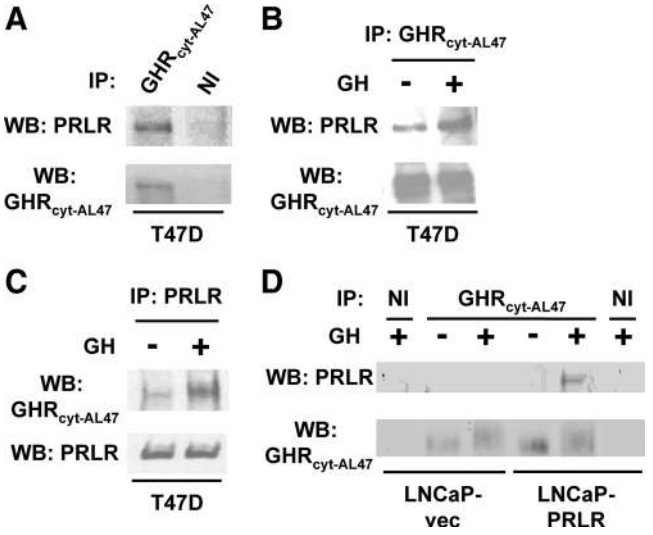

FIG. 7. Specific coimmunoprecipitation of GHR and PRLR in T47D cells and LNCaP-PRLR cells. A, Serum-starved T47D cells were detergent extracted and subjected to immunoprecipitation with anti-$GHR_{cyt\text{-}AL47}$ or nonimmune serum (nonimm), as indicated. Eluated proteins were resolved by SDS-PAGE and serially immunoblotted with anti-PRLR and anti-$GHR_{cyt\text{-}AL47}$. Note specific coimmunoprecipitation of GHR and PRLR. B and C, Serum-starved T47D cells were treated without or with GH for (500 ng/ml; 2 min). Detergent extracts were immunoprecipitated with anti-$GHR_{cyt\text{-}AL47}$ (B) or anti-PRLR (C). Eluted proteins were resolved by SDS-PAGE and serially immunoblotted with anti-PRLR and anti-$GHR_{cyt\text{-}AL47}$, as indicated. Note GH-induced increase in coimmunoprecipitation of GHR and PRLR. D, Serum-starved LNCaP-vector or LNCaP-PRLR cells were treated without or with GH for (500 ng/ml; 10 min). Detergent extracts were immunoprecipitated with anti-$GHR_{cyt\text{-}AL47}$ or NI. Eluted proteins were resolved by SDS-PAGE and serially immunoblotted with anti-PRLR and anti-$GHR_{cyt\text{-}AL47}$, as indicated. Note GH-induced increase in coimmunoprecipitation of GHR and PRLR in LNCaP-PRLR. NI, Nonimmune; IP, immunoprecipitation; WB, Western blot.

related cytokines and hormones activate their receptors to accomplish their biological actions. Despite this progress, several fundamental aspects of GHR and PRLR triggering remain uncertain. For example, early work suggested that GH promoted homodimerization of GHR by virtue of a sequential binding mechanism whereby site 1 on GH bound to one GHR, which then facilitated binding to a second GHR via site 2 on the GH molecule and formation of the active tripartite complex of 1:2 GH:GHR stoichiometry (52–54). However, recent observations suggest that at least a fraction of unliganded GHRs already exist as dimers (*i.e.* they are "predimerized"), findings also seen for some other cytokine receptors, including PRLR (16–19, 40, 41, 55, 56). For GHR, this prehomodimerization may be mediated by the receptor's transmembrane domain and/or residues in subdomain 2 of the extracellular domain (16, 18, 19). The degree to which GH-induced GHR dimerization *vs.* GH-induced conformational change within an already predimerized GHR is responsible for GHR activation remains uncertain but may have implications in agonist and antagonist drug design. In addition to these important uncertainties about the GHR (and PRLR) activation mechanisms, the role of GH interaction with PRLR in allowing GH-dependent signals in relevant human cellular contexts also remains unclear. The potential clinical significance of these issues is underscored by observations that cancer cells, in particular human breast cancer cells, coexpress both GHR and PRLR and by emerging data that implicate both GH and PRL as players in breast cancer biology (5, 11, 12, 57, 58).

In this study, we reveal findings in the human T47D breast cancer cell line that shed light on some of these issues. We find that both human GH and PRL activate JAK2 and STAT5 phosphorylation in these cells. Despite GH's ability to cause dsl of GHRs (taken as an indication of formation of a signaling-competent GHR homodimer), GH fails to cause substantial GHR tyrosine phosphorylation and instead acutely causes PRLR tyrosine phosphorylation. Notably, GHR-specific GH antagonists (B2036 and anti-$GHR_{ext\text{-}mAb}$) fail to block GH signaling, but both inhibit GH-dependent GHR dsl. We find that GHR and PRLR in T47D cells are specifically coimmunoprecipitated and that the degree of association of the

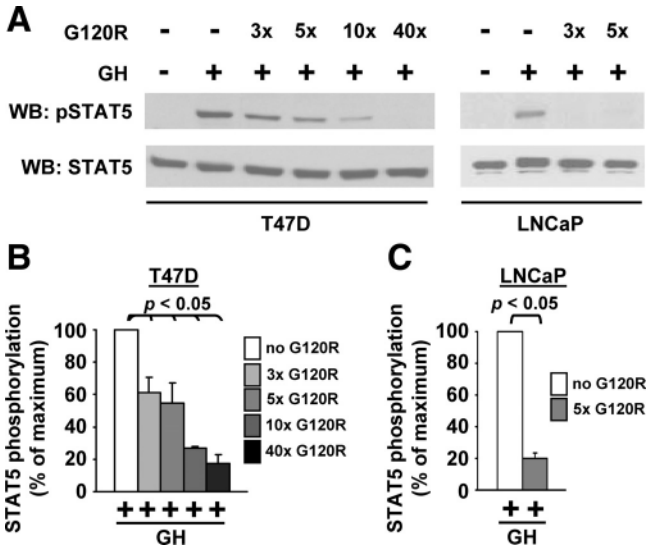

FIG. 8. Effects of GHR and PRLR antagonist, G120R on GH-induced STAT5 tyrosine phosphorylation in T47D and LNCaP cells. A, Serum-starved T47D or LNCaP cells, as indicated, were treated with vehicle or GH (500 ng/ml; 10 min) in the presence or absence of G120R (fold molar excess to GH, as indicated). Detergent cell extracts were resolved by SDS-PAGE and serially immunoblotted with anti-pSTAT5 and anti-STAT5. B and C, Densitometric analysis of data from three separate experiments, including that presented in A for T47D (B) and LNCaP (C). The pSTAT5 level induced by GH in the absence of G120R is considered 100% in each case. Data are expressed as mean ± SE. *P* values for comparisons are as indicated. Note partial inhibition of GH-induced STAT5 tyrosine phosphorylation by G120R in T47D cells at low concentrations and near complete inhibition at 40×, but near complete inhibition of GH-induced STAT5 tyrosine phosphorylation by G120R at low concentration in LNCaP cells. pSTAT5, PhosphoSTAT5; WB, Western blot.

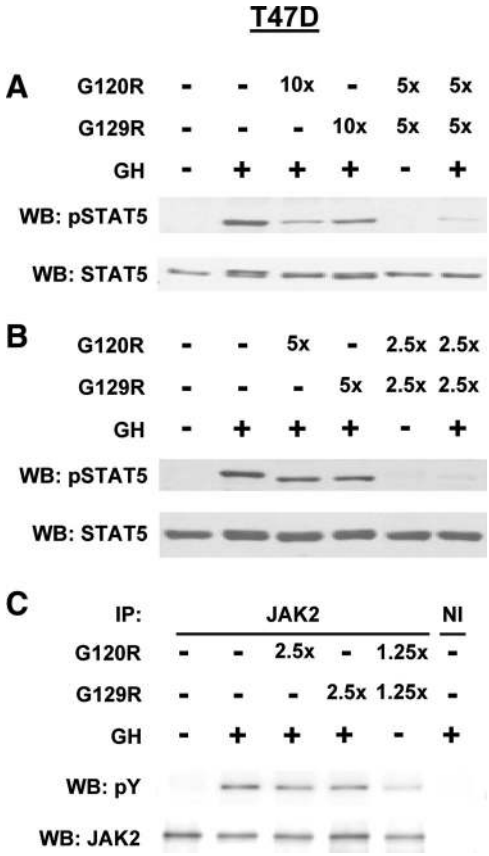

FIG. 9. Enhanced inhibition of GH-induced STAT5 and JAK2 tyrosine phosphorylation in T47D cells by combined treatment with G120R and G129R. A–C, Serum-starved T47D cells were treated with vehicle or GH (500 ng/ml; 10 min) in the presence or absence of G120R and/or G129R (fold molar excess to GH, as indicated in A–C). Detergent cell extracts were resolved by SDS-PAGE and serially immunoblotted with anti-pSTAT5 and anti-STAT5 (A and B) or immunoprecipitated with anti-JAK2 or NI control antibody and serially immunoblotted with anti-pTyr and anti-JAK2. pSTAT5, PhosphoSTAT5; WB, Western blot; NI, nonimmune; IP, immunoprecipitation.

two receptors is acutely enhanced by treatment of the cells with GH. This GH-induced association is also detected in human LNCaP cancer cells stably transfected with hPRLR. In contrast to GHR-specific GH antagonists, the PRLR-specific antagonist, G129R, blunts GH-induced STAT5 activation, but only partially, and the GHR- and PRLR-interacting GH antagonist, G120R, blocks GH signaling in T47D cells, but only at relatively high concentrations. Finally, low concentrations of G120R and G129R together better antagonize GH signaling under conditions in which each individually is less effective.

Human GHR and PRLR are structurally similar members of the cytokine receptor superfamily (3, 26). Their extracellular domains are roughly one-third identical and form the binding sites for their respective ligands. However, differences in their abilities to share each others' ligands (PRL binds PRLR, GH binds both GHR and PRLR) and the lack of a similarly positioned unpaired cysteine in the stem region (present at cysteine-241 in GHR, absent in the stem region of PRLR) also underscore important differences in their extracellular domains. Some other cytokine receptors are known to form heterodimers or hetero-oligomers with each other, but such associations have rarely, if at all, been reported for family members most similar to GHR and PRLR. Although our prior studies have suggested that a hGHR-PRLR heterodimer might form (42) and that ovine GHR and PRLR can associate in response to placental lactogen in a reconstitution system (43, 44), definitive evidence for such receptor assemblages and the effects of GH on GHR-PRLR complexes has been lacking. In the current study, we find that GHR and PRLR specifically coimmunoprecipitate and that GH impressively augments the degree of such coimmunoprecipitation. These biochemical findings are interesting in the context of the predimerization *vs.* ligand-enhanced dimerization issue raised above for GHR and PRLR homodimers. Our data may indicate either that GHR and PRLR exist in a "loosely" associated preheterodimer that is made more stable by GH engagement and/or that GH in fact promotes increased numbers of GHR-PRLR heterodimers to form from previously monomeric receptors. Indeed, the same possibilities may ap-

ply to GHR and/or PRLR homodimers. For both the homodimers and heterodimers, we do not yet know either the fraction of receptors preengaged or the propensity of the ligands to augment receptor-receptor affinity *vs.* dimer abundance. However, our findings of GHR-PRLR association in human breast cancer cells are important in that they prompt us to ask questions concerning what region(s) of each receptor confer their association, whether associated molecules (*e.g.* JAK2) are required, and whether association between GHR and PRLR competes with each receptor's homodimerization. Each of these questions will be approachable with reconstitution and mapping studies. It is also important to emphasize, as above, that the specifically coprecipitated GHR and PRLR may exist in a complex of receptor homodimers rather than as a heterodimer *per se*. We cannot yet discriminate these possibilities completely.

Our data indicate that GHR homodimers are detected in response to GH in both T47D and LNCaP cells. However, unlike the situation in LNCaP cells, GHR homodimers in T47D cells do not appear to transduce signals in response to GH, as evidenced by the lack of GH-induced GHR tyrosine phosphorylation in T47D cells and the inability of GHR-specific antagonists to block GH-induced STAT5 activation in those cells. This was despite the ability of these antagonists to block GH-induced GHR dsl. Thus, GH engagement of GHR homodimers is selectively uncoupled from productive GHR signaling in T47D cells (compared with LNCaP cells). The mechanism(s) underlying this uncoupling in T47D cells is as yet unknown. However, it is reasonable to hypothesize that the presence of PRLR in T47D (but not in LNCaP) is involved in uncoupling. Indeed, forced expression of PRLR in LNCaP reduced GHR signaling. Because both GHR and PRLR associate with and use JAK2 for signal transduction, it is conceivable that PRLR in T47D or LNCaP-PRLR cells either qualitatively or quantitatively competes for JAK2 association with GHR, such that GHR-JAK2 coupling is relatively ineffective. Similarly, in T47D cells, the propensity of GHR and PRLR to interact, coupled with abundant PRLR expression, could favor formation of GHR-PRLR complexes over GHR-GHR homodimers, thereby contributing to ineffective GHR-GHR homodimer signaling. These and other intriguing possibilities can be approached in future studies by experiments in which PRLR in T47D cells is silenced, asking what is the impact on GH-dependent GHR tyrosine phosphorylation and downstream signaling. If the degree to which PRLR and GHR are coexpressed varies among different cancer cell types or within a cancer cell type between individuals, a mechanistic understanding of the impact of PRLR on GHR signaling could prove clinically useful, in particular if GHR antagonism is a therapeutic goal.

Along these lines, it is also intriguing that the PRLR-specific antagonist, G129R, only partially inhibits GH-dependent STAT5 activation in T47D cells. Indeed, the concentration dependence of G129R's effects (Fig. 6B) is of interest. Coincubation with GH and a 5-fold molar excess of G129R reduces GH-induced STAT5 signaling by roughly 50%, whereas, as expected, the same degree of molar excess of the antagonist nearly completely inhibits PRL-induced STAT5 activation. Thus, G129R is intact and functional but not as potent regarding GH signaling as compared with PRL signaling. We interpret these data to indicate that a component of GH signaling in T47D cells is poorly inhibited by a PRLR-specific antagonist and also not inhibited by GHR-specific antagonists (as above). Thus, it might be mediated by a receptor form (perhaps a GHR-PRLR heterodimer) that is not inhibited by either GHR-specific or PRLR-specific antagonists alone. Our findings with the pan-GHR/PRLR antagonist, G120R, are thus also notable and possibly consistent with this hypothesis. At low concentrations, G120R might be expected to inhibit GHR homodimers (which are apparently not operative in response to GH in these cells but are operative in LNCaP cells that lack PRLR) and PRLR homodimers, and indeed, a 5-fold molar excess of G120R does inhibit GH-induced STAT5 activation by roughly one-half. In contrast, however, higher G120R concentrations (10–40×) further antagonize GH signaling. If GH signaling in T47D cells is mediated in part by GHR-PRLR heterodimers, one might envision that G120R, unlike G129R, is able to block such heterodimer activity, at least at high antagonist concentrations.

When viewed through this prism, the enhanced inhibition of GH signaling in T47D cells achieved by coincubation of G120R plus G129R (compared with either alone) may be understandable. Together, submaximal concentrations of G120R and G129R robustly inhibit GH signaling, whereas such concentrations of either antagonist alone are less inhibitory. These findings lead us to hypothesize that the substantial PRLR (homodimer) antagonism afforded by a submaximal G129R concentration allows the submaximal concentration of G120R to more effectively antagonize remaining PRLR and GHR (in GHR-PRLR complexes). We note that other mechanisms might exist to explain these novel findings. However, one model would predict that in the absence of GH or PRL, GHRs and PRLRs exist in T47D cells as monomers or loosely (or transiently) associated dimers (homo- or heterodimers). It is possible that the GHR-PRLR is the preferred dimer (or hetero-oligomer) in these cells, either because of a high intrinsic affinity of one receptor for the other or because

of the relative abundance of each receptor in these cells. Thus, GHR and PRLR are coimmunoprecipitable, even in the absence of ligand in T47D cells. However, in response to GH, we predict that GHR-PRLR heterodimers (as well as GHR homodimers and PRLR homodimers) are stabilized and thereby achieve an activated conformation. In contrast, PRL is viewed in this model as stabilizing only PRLR homodimers.

This model of GHR-GHR and GHR-PRLR association conceptualizes our findings in T47D cells. However, these findings may also suggest that the same model applies in cells that express GHR without PRLR. Thus, we envision a scenario similar to earlier views of GH signaling, in which receptor monomers are dimerized by virtue of each monomer binding to a single GH molecule. However, as informed by observations of GHR predimerization, we refine this model to suggest that the unliganded monomeric GHRs can form GHR homodimers, at least transiently, in the absence of GH. According to our findings and those of others, these transient GHR homodimers may be detected in the endoplasmic reticulum, in the secretory pathway, and at the cell surface and are mediated by the receptor transmembrane domain and/or the dimerization interface in the extracellular domain subdomain 2 (16, 18, 19). As opposed to being fixed dimers, however, we view these homodimers, at least as they exist at the cell surface, as in equilibrium with receptor monomers, such that ligand binding favors dimer formation and acquisition of the activated GHR conformation. Further study will be required to test whether this model, driven by our observations of GHR-PRLR complex formation, is applicable to GHR-GHR homodimer signaling as well.

We note also that our current observations of potentially functionally relevant interaction of GHR with PRLR may further reflect a tendency of GHR to interact in important ways with other receptors to convey GH signals. For example, our recent work in GH-responsive mouse preadipocytes, mouse osteoblasts, and human LNCaP cells indicates that GH induces formation of a complex that includes GHR, JAK2, and the IGF-I receptor and that knockdown of IGF-I receptor dampens GH-dependent STAT5 activation and downstream gene transcription (59, 60) (our unpublished data). Similarly, others have detected potentially relevant GH-dependent association between GHR and epidermal growth factor receptor (61). In concert with our current observations, these findings broaden our concept of how GH and its receptor may function to allow a range of physiological and pathophysiological activities, in part by partnering with other receptors and signaling systems.

Finally, we are intrigued by the clinical implications of our findings in human breast cancer cells with three existing antagonists (B2036, G120R, and G129R) and our GHR antagonist antibody (anti-$GHR_{ext\text{-}mAb}$). GHR and PRLR antagonists are viewed as potentially relevant therapeutics in breast cancer and other malignancies (51, 62). In the setting of breast cancer, their utility may reside in the ability to block GHR and/or PRLR signals that arise from the effects of circulating hormones and/or hormones produced in an autocrine/paracrine fashion by the tumor cells or other nearby cell types. We observe enhanced inhibition of acute GH-induced STAT5 activation when G120R and G129R are employed simultaneously. Furthermore, pure GHR antagonists are unable to block GH-mediated signaling in T47D cells but are strongly inhibitory in PRLR-deficient LNCaP cells. Collectively, these data suggest the compelling possibility that the responses of cancer cells to GH and/or PRL may vary depending on their relative complements of GHR and PRLR. This possibility will require more extensive *in vitro* and *in vivo* testing of the signaling characteristics and behaviors of various cancer cell lines in terms of the abundance of each receptor, the response to GH *vs.* PRL, and the effect of the various antagonists. We note an important caveat in our analysis is that our experiments were carried out in the absence of added zinc. Because others have observed clear effects of added zinc to increase the affinity of hGH for hPRLR (37), we are mindful of the potential impact of the ambient zinc concentration on the effects of GH antagonists as well. Nevertheless, one outcome of our current studies may be that there is utility in combining antagonists (either by adding each together or by producing a drug that incorporates both antagonists joined together) for certain cancers depending on their GHR/PRLR phenotypes.

Materials and Methods

Materials

Routine reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO) unless otherwise noted. Fetal bovine serum, gentamicin sulfate, penicillin, and streptomycin were purchased from BioFluids (Rockville, MD). Recombinant hGH was kindly provided by Eli Lilly and Co. (Indianapolis, IN). Recombinant hPRL was obtained from the National Hormone and Pituitary Program. B2036 was obtained from Pfizer, Inc. (New York, NY). Recombinant G120R and recombinant G129R were produced and prepared as previously described (22, 42).

Antibodies

Polyclonal antiphospho-STAT5 was purchased from Zymed Laboratories (San Francisco, CA). Anti-STAT5, and anti-PRLR (monoclonal or polyclonal), were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal anti-pY

(4G10) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal anti-$GHR_{cytAL-47}$ against the intracellular domain of GHR (45), anti-$JAK2_{AL33}$ (63), and monoclonal anti-$GHR_{ext-mAb}$ against the extracellular domain of GHR (23) were described previously.

Cell culture and stable transfection

T47D and LNCaP cells were purchased from American Type Culture Collection (Manassas, VA). T47D was cultured in RPMI1640 medium, supplemented with 10% fetal bovine serum, 50 μg/ml gentamicin sulfate, 100 units/ml penicillin, and 100 μg/ml streptomycin. LNCaP was cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 1.5 g/liter sodium bicarbonate, 1.0 mM sodium pyruvate, 50 μg/ml gentamicin sulfate, 100 U/ml penicillin, and 100 μg/ml streptomycin. To obtain LNCaP cells that express hPRLR, transfection of LNCaP cells with an expression vector encoding hPRLR (pEF-hPRLR; a gift of C. Clevenger, Northwestern University, or vector only as a control) was performed using Lipofectamine Plus (Invitrogen Life Technologies, Inc., Carlsbad, CA) according to the manufacturer's instructions. Stably transfected pools were selected by growth on G418-containing medium.

Cell starvation, cell stimulation, and protein extraction

Serum starvation of cells was accomplished by substitution of 0.5% (wt/vol) BSA (fraction V; Roche Molecular Biochemicals, Indianapolis, IN) for fetal bovine serum in the culture medium for 16–20 h before experiments. Pretreatments and stimulations were carried out at 37 C in serum-free medium. Stimulations were terminated by washing the cells once with ice-cold PBS supplemented with 0.4 mM sodium orthovanadate and then harvested in lysis buffer (1% Triton X-100, 150 mM NaCL, 10% glycerol, 50 mM Tris-HCL, 100 mM NaF, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM benzamidine, 5 μg/ml aprotinin, and 5 μg/ml leupeptin). Cells were lysed for 30 min at 4 C in lysis buffer before centrifugation at 15,000 × *g* for 10 min at 4 C. The protein concentration was determined, and equal aliquots of protein extracts (supernatant) were subjected to immunoprecipitation or were directly electrophoresed and immunoblotted as indicated below.

For chemical cross-linking in detergent lysates, fresh BS^3 (Pierce, Rockford, IL) was dissolved in PBS in a 100 mM stock concentration. Directly after the addition of detergent lysis buffer to cells and vortexing, BS^3 (final concentration, 1 mM) or PBS (untreated samples) was added for 10 min on ice. Cross-linking was terminated by the addition of 10 mM (final concentration) ammonium acetate for 5 min on ice before processing as described above.

Immunoprecipitation and Western immunoblot analysis

For immunoprecipitation, 0.5–1 mg protein was incubated with antibody against JAK2, GHR, or PRLR overnight at 4 C. Protein A-agarose (fast flow; Pharmacia Biotech, Providence, RI) was then added, and incubations continued for 4 h at 4 C. Immunoprecipitated proteins were resolved by sodium dodecyl sulfate, 7.5% PAGE, and transferred to nitrocellulose paper. Western transfers were immunoblotted with anti-pY (4G10), anti-$JAK2_{AL33}$, anti-$GHR_{cytAL-47}$, or anti-PRLR antibodies.

For Western blotting, 30 μg protein per lane was resolved by 7.5% SDS-PAGE and transferred to nitrocellulose membrane (Amersham Biosciences, Pittsburgh, PA). Western transfers were immunoblotted with primary antibodies, after which horseradish peroxidase-conjugated secondary antibody (Pierce Chemical Co., Rockford, IL) was added for detection of bound antibody by Supersignal Femto Maximum Sensitivity Substrate Reagent (Pierce Biotechnology, Rockford, IL). Membrane stripping was performed according to the manufacturer's instructions (Amersham Biosciences).

Densitometric and statistical analysis

Immunoblots were scanned using a high-resolution scanner (Hewlett-Packard Co., Palo Alto, CA). Densitometric quantification of images was performed using an image analysis program, ImageJ (developed by WS Rasband Research Services Branch, National Institute of Mental Health, Bethesda, MD). Pooled data of densitometry assays from several experiments are displayed as mean ± SE. The significance (*P* value) of differences of pooled results was estimated using unpaired *t* tests.

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