**Growth Hormone (GH) and a GH Antagonist Promote GH Receptor Dimerization and Internalization**

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It has previously been shown that a human growth hormone (hGH) analog, hGH-G120R, acts as a GH antagonist (Chen, W. Y., Wight, D. C., Wagner, T. E., and Kopchick, J. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5061–5065; Chen, W. Y., White, M. E., Wagner, T. E., and Kopchick, J. J. (1991) Endocrinology 129, 1402–1408; Chen, W. Y., Chen, N.-Y., Yun, J., Wang, X. Z., Wagner, T. E., and Kopchick, J. J. (1994) J. Biol. Chem. 269, 15892–15897). In this study, we report the ability of hGH and hGH-G120R to be internalized by GH receptor expressing cells. Additionally, results of chemical cross-linking experiments revealed that both native hGH and hGH-G120R form complexes similar in size to that expected for hGH when bound to recombinant hGH-binding protein (bp). The molecular mass of the complex was determined to be approximately 280 kDa which is consistent with multiple receptors interacting with the ligand. The predominant radiolabeled band detected was a complex of approximately 140 kDa which probably represents one GH molecule bound to one GH receptor. The cross-linked complexes were not detected in the presence of excess unlabeled hGH or hGH-G120R and were not observed in cells which do not express detectable levels of GH receptors. Also, GH induced tyrosine phosphorylation of a complex of proteins of approximately 95 kDa in these cells whereas hGH-G120R did not. Thus, we have separated the hGH or hGH-G120R/GHR binding and internalization capabilities from the ability to stimulate tyrosine phosphorylation of intracellular proteins.

Growth hormone receptor (GHR) activation is mediated by the binding of GH. The GHR/GH interaction ultimately results in alterations of lipid, nitrogen, mineral, and carbohydrate metabolism as well as cellular differentiation (5–12). The mechanism(s) by which these biological signals are elicited are not yet fully understood. However, it has been demonstrated that a GHR/GH activated tyrosine kinase (13–21), protein kinase C (22, 23), and mitogen-activated protein or extracellular signal-regulated kinase (24–28) are involved in the intracellular signaling mechanism of GH.

Recently, the crystal structure of hGH with the extracellular domain of the hGH-binding protein (hGHbp) was solved. It was found to exist as a hGH-(hGHbp)2 complex (4, 29). Additionally, GH dependent dimerization of this Escherichia coli expressed hGHbp was found to occur sequentially (29). In this model, hGH was shown to bind one hGHbp molecule through site 1 on hGH and then a second hGHbp molecule binds through site two of hGH subsequently establishing a hGH-(hGHbp)2 complex. It was suggested that formation of the 1-ligand-2-receptor dimeric complex may be important in the GH-induced signal transduction system (29, 30). This type of GH/GHR signal transduction mechanism is analogous to that used by many tyrosine kinase receptors, such as epidermal growth factor and platelet-derived growth factor, in which binding of one hormone molecule to its receptor is thought to induce formation of a dimer, through a 2-hormone-2-receptor complex (31). Binding of these growth factors to their cognate receptors activate tyrosine kinases and receptor autophosphorylation. Therefore, receptor dimerization may be a critical step in mediating biological activities for a number of growth factors.

It has clearly been demonstrated that the third α-helix of GH is critical for its biological activity (1–3). In a structure-function study of this helical region, bGH-Gly119 and hGH-Gly120 were identified as amino acids critical for growth promotion (2, 3). Transgenic mice expressing these GH antagonist genes possessed a dwarf phenotype (1, 32, 33). In fact, substitution of several amino acids (except alanine) of this Gly residue resulted in GH antagonists which were found to be active both in vitro and in vivo (2, 3, 12, 33).

Similarly, a hybrid receptor containing the extracellular binding domain of the hGH linked to the transmembrane and intracellular domains of the murine granulocyte colony-stimulating factor receptor was generated and expressed in a myeloid leukemia cell line, FDC-P1 (34). Treatment of these cells with hGH resulted in cellular proliferation. However, treatment of these cells with hGH-G120R, which contains a functional site 1 but a sterically blocked site 2, failed to promote cellular proliferation. Therefore, it was suggested that hGH-G120R acted as a GH antagonist, presumably by its inability to dimerize GHRs (34).

It has recently been reported that hGH down-regulates GHRs in IM-9 lymphocytes whereas hGH-G120R does not (30). This result may imply that hGH and hGH-G120R are "recognized" differently by the cell and suggests that hGH-G120R is not internalized following binding to GHRs.

In order to test the ability of hGH-G120R to dimerize and internalize, hGH-G120R was purified and iodinated. The iodinated forms of hGH and hGH-G120R were analyzed by binding and cross-linking to cells expressing the GHRs. Additionally,
the ability of the GH antagonist to be internalized was determined.

MATERIALS AND METHODS

Iodination of Hormones—Human GH and a GH antagonist, hGH-G120R, were purified as described previously (35) and were labeled with Na125I by the lactoperoxidase method to a specific activity of 80–105 μCi/μg (36). Briefly, 1.0 mCi of Na125I was added to 1.0 mg of hGH or hGH-G120R. Lactoperoxidase (10 μg dissolved in 10 μl of 0.4 mol/liter phosphate buffer, pH 5.6) and H2O2 (5 μl of 1.76 mmol/liter) were then added. After 30 min, the reaction was terminated by the addition of 100 μl of transfer buffer (0.47 mol/liter sucrose, 0.06 mol/liter K1, sodium azide 0.02%, pH 7.6). Radiolabeled hGH was then separated by Sephadex G-100 chromatography.

Radioimmunoassay—A radioimmunoassay kit (Hybritech, San Diego, CA) was used in order to determine the concentrations of hGH and hGH-G120R. However, only hGH was detected by the radioreceptor assay kit (3). hGH-G120R was not detectable by radioimmunoassay but was recognized by immunoblotting with a polyclonal hGH antibody (kindly provided by Eli Lilly, IN). Therefore, the epitope for the monoclonal antibody from the Hybritech radioimmunoassay kit may be near Gly120 or in the third helical region. A second hGH radioimmunoassay kit (Nichols Institute Diagnostics) was able to recognize both hGH as well as hGH-G120R in order to determine GH concentrations (3).

GH/GHR Internalization—An acid extraction procedure (37) was used to differentiate surface bound 125I-hGH and internalized 125I-hGH-G120R. Briefly, mouse L cells (MLCs), pGHR-W10 (MLCs which express the porcine GHR), and IM-9 cells were maintained in DMEM or RPMI 1640 plus 10% fetal bovine serum. For the internalization studies the cells were grown to confluence in six-well culture plates, depleted of serum, and incubated with 100,000 cpm of 125I-hGH or 125I-hGH-G120R for 24 h at 4°C. Subsequently, the cells were incubated at 37°C for 0, 5, 10, 15, 30, and 120 min during which time GH was internalized. Each point on the graph represents the mean value of three experiments performed in duplicate. See "Materials and Methods" for details.

RESULTS

Internalization of 125I-hGH and 125I-hGH-G120R—In order to determine whether hGH-G120R was able to be internalized, experiments were conducted using cell lines which express GHR, i.e. pGHR-W10, and human IM-9 lymphocytes. Previous results have shown that pGHR-W10 cells were able to internalize approximately 85% of the specific bound 125I-pGH (38, 39). Results of the internalization assay of hGH and the hGH antagonist are shown in Fig. 1 (A and B). Both the 125I-hGH and 125I-hGH-G120R were internalized by pGHR-W10 and IM-9 lymphocytes with radioactive activity increasing from 5 to 65% within 30 min at 37°C. Approximately 85% of the bound radiolabeled ligand was internalized by 1 h. MLCs did not significantly internalize hGH or hGH-G120R (data not shown).

Cross-linking of 125I-hGH and 125I-hGH-G120R—In the presence of 125I-hGH or 125I-hGH-G120R and a chemical cross-linker, BS3, radiolabeled bands with molecular masses of approximately 70, 140, and 280 kDa were observed in cells expressing the pGHR (Fig. 2, lanes 5 and 11). These radiolabeled complexes were not seen in MLCs which do not express pGHRs (Fig. 2, lanes 2–4 and 8–10). Additionally, the observed complexes were able to be specifically competed with unlabeled 125I-hGH and 125I-hGH-G120R, indicating that the complexes were not due to nonspecific binding.
linking to 125I-hGH or 125I-hGH-G120R. Both the 75- and 150-kDa complex of 150 kDa was observed with pGHR-TR1 upon cross-linking with 125I-hGH-G120R, without competing GH, with unlabeled hGH, and with unlabeled hGH-G120R, respectively. Lanes 11–13 represent identical conditions as lanes 8–10, but in pGHR-W10 cells. Lanes 1 and 14 represent molecular weight markers. The arrows on the left represent radiolabeled bands specifically competed with excess unlabeled hGH or hGH-G120R.

hGH and hGH-G120R (500 ng/ml) (Fig. 2, lanes 6, 7, 12, and 13).

A radiolabeled band with a molecular mass of approximately 75 kDa was observed for pGHR-TR1 when cross-linked to either 125I-hGH or 125I-hGH-G120R (Fig. 3, lanes 5 and 11). Upon subtraction of the molecular mass of hGH, 22 kDa, from the complex, pGHR-TR1 was found to possess a mass of approximately 53 kDa. Additionally, a larger radiolabeled complex of 150 kDa was observed with pGHR-TR1 upon cross-linking to 125I-hGH or 125I-hGH-G120R. Both the 75- and 150-kDa complexes were able to be specifically competed with unlabeled hGH or hGH-G120R (Fig. 3, lanes 6 and 12). Also, a radiolabeled complex of approximately 140 kDa was detected in pGHR-W10 cells (Fig. 3, lanes 3 and 9) that were specifically competed with excess hGH or hGH-G120R (Fig. 3, lanes 4 and 10). No complexes were seen in MLCs (Fig. 3, lanes 1, 2, 7, and 8).

pp95 Induction Assay—The ability of hGH or hGH-G120R to induce tyrosine phosphorylated proteins of approximately 95 kDa in pGHR-W10 cells was examined. hGH and pGHR were able to induce pp95 in pGHR-W10 cells (Fig. 4, lanes 2 and 4, respectively). No pp95 induction was observed when pGHR-W10 cells were incubated with hGH-G120R (Fig. 4, lane 3).

DISCUSSION

It has been established that E. coli-derived hGHbp is able to form a dimer when incubated with GH (4, 29). This dimeric complex consists of one hGH molecule bound to two hGHbp with hGH containing two binding sites for the hGHbp. It was proposed that GH site 1 binds with one hGHbp molecule and then a second GHbp interacts with GH site 2 (29). De Vos et al. (4) have demonstrated that the hGH site 2 is found on the exposed sides of helices 1 (amino acids Phe1, Ile16, and Arg9) and 3 (Asp116) (4). Additionally, hGH-G120R, which retains a functional site 1 but a sterically blocked site 2, has been shown to inhibit proliferation of FDC-P1 cells transfected with a hybrid receptor that contained the extracellular domain of the hGHR linked to the transmembrane and intracellular domain of the murine granulocyte macrophage colony-stimulating factor receptor (34). It was hypothesized that once hGH-G120R is bound to its receptor, it could not dimerize and induce a GH signal. Therefore, these results suggest that GHR dimerization is critical for GH-induced signal transduction (19, 34).

GH treatment of NIH 3T3-F442A fibroblasts resulted in tyrosine phosphorylation of GHRs as a result of association with an associated tyrosine kinase, JAK2, a non-receptor associated tyrosine kinase (13, 14, 18, 20). It has been identified as a GH-activated tyrosine kinase responsible for self-phosphorylation and tyrosine phosphorylation of GHRs and possibly other intracellular proteins (20, 42).

A 95–96-kDa protein (pp95) of unknown identity has been shown to be induced in MLCs stably transfected with the pGHR (pGHR-W10 cells) and 3T3-F442A cells upon treatment with physiological concentrations of GH (21, 38, 41, 43). Addi-
tionally, the GH antagonists, bGH-M8 and hGH-G120R, did not induce tyrosine phosphorylation of pp95 (3, 21). These results suggest that GH-induced tyrosine phosphorylation of pp95 plays an integral role in GH signal transduction.

GH treatment of a human lymphocyte cell line (IM-9), which contain endogenous GHRs, results in tyrosine phosphorylation of two proteins with molecular masses of approximately 93 and 120 kDa (19). Additionally, treatment of IM-9 cells with hGH analogs (nM concentrations) directed toward site 1 of two proteins with molecular masses of approximately 93 and 120 kDa (19). Incubation of IM-9 lymphocytes with 0.5 nM recombinant hGH stimulated tyrosine phosphorylation of the 93-kDa protein but stimulated low level tyrosine phosphorylation of the 120-kDa protein (19). Treatment of IM-9 lymphocytes with 0.5 nM recombinant hGH stimulated the ability of hGH to stimulate tyrosine phosphorylation. It was concluded that hGH-G120R antagonized tyrosine phosphorylation due to its inability to dimerize because of a defective site 2. High concentrations (μM) of hGH inhibited tyrosine phosphorylation, presumably by inhibiting dimer formation and favoring the monomeric form (e.g., GH-GHR complex) (19, 34). It should be noted that hGH-G120R treatment of IM-9 lymphocytes was able to stimulate tyrosine phosphorylation of the 120-kDa protein (19). This protein may possibly be JAK2 (20) which would be internalized by use of cells which express GHRs. We hypothesized that we would see receptor dimerization and internalization with hGH but would not detect receptor dimerization and internalization with hGH-G120R. Surprisingly, both 125I-labeled hGH and hGH-G120R were able to be internalized following binding to GHR. Our results indicated that 75% of the bound hormone was internalized within 40 min. Therefore, it appears that there is no significant difference in the internalization process between hGH and hGH-G120R (19, 34). It may be that 1) GHR internalization is constitutive independent of GH binding or 2) once GH is bound to its receptor, the GH-GHR complex is internalized. Regardless of the internalization pathway, we found that hGH-G120R was internalized in a manner similar to that of hGH.

Additionally, we detected a 70-kDa band, which may represent the GH-GHR complex. The GHbp may be the extracellular domain of the full-length pGHR generated by proteolytic cleavage. The 246-amino acid extracellular domain would produce a protein of approximately 25 kDa. We and others have shown that the GHR is heavily glycosylated on asparagine residues (39, 48). N-Linked glycosylation comprised approximately 24% of the total molecular mass of the pGHR (39). Therefore, cross-linking of GH or G120R to the extracellular domain (i.e., GHbp) would result in a complex of approximately 70 kDa which can be seen in Fig. 2.

Together, our results indicate that the GH antagonist, hGH-G120R, is able to bind, dimerize, and internalize GHRs in a similar manner as hGH. However, the dimeric complex formed with hGH-G120R is not functional as is the hGH:GHR dimer. Perhaps the hGH-G120R-GHR dimeric complex is not biologically equivalent to the native GH:GHR dimer and may be unable to associate with yet to be discovered intracellular elements in order to elicit GH's signal transduction system. Another possible explanation for the separation of GH binding and internalization from subsequent intracellular signaling is the hypothesis that the intracellular "trafficking" pattern is different for hGH-receptor versus hGH-G120R-receptor complexes. Ultimately, the data suggests that GHR internalization and dimer formation are not positively correlated with GH induced intracellular signaling.

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