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Introduction of exogenous growth hormone receptors augments growth hormone-responsive insulin biosynthesis in rat insulinoma cells

(transfection/gene expression/hormone binding/pancreatic β cells)

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ABSTRACT **The stimulation of insulin biosynthesis in the pancreatic insulinoma cell line RIN5-AH by growth hormone (GH) is initiated by GH binding to specific receptors. To determine whether the recently cloned rat hepatic GH receptor is able to mediate the insulinotropic effect of GH, we have transfected a GH receptor cDNA under the transcriptional control of the human metallothionein promoter into RIN5-AH cells. The transfected cells were found to exhibit an increased expression of GH receptors and to contain a specific GH receptor mRNA that was not expressed in the parent cell line. The expression of GH receptors in one clone (1.24) selected for detailed analysis was increased 2.6-fold compared to untransfected cells. The increased GH receptor expression was accompanied by an increased responsiveness to GH. Thus, the maximal GH-stimulated increase of insulin biosynthesis was 4.1-fold in 1.24 cells compared to 1.9-fold in the nontransfected RIN5-AH cells. The expression of the transfected receptor was stimulated 1.6- and 2.3-fold when cells were cultured in the presence of 25 or 50 μM Zn^{2+}, respectively. The increased expression of the GH receptor by Zn^{2+} was associated with an increased magnitude of GH-stimulated insulin biosynthesis. A close stoichiometric relationship between the level of receptor expression and the level of GH-stimulated insulin biosynthesis was observed. We conclude from these results that the hepatic GH receptor is able to mediate the effect of GH on insulin biosynthesis in RIN5-AH cells.**

Growth hormone (GH) exerts a wide variety of actions in several tissues. Some of these effects, such as the stimulation of cartilage and bone growth (1), are partially mediated by the local production of somatomedins (2). GH also has several direct actions, including insulin- and anti-insulin-like effects on adipocytes (3, 4) and stimulation of proliferation of smooth muscle cells (5), erythroid progenitor cells (6), and insulin-producing pancreatic β cells (7).

Although receptors for GH are present in many cell types, an important question is whether a single GH receptor mediates the multiple effects of GH or whether different receptors exist. Recently, GH receptor cDNA clones have been isolated and sequenced from the livers of rabbit and human (8), rat (9), and mouse (10). From the deduced amino acid sequence these clones have been demonstrated to encode a protein of 638–650 amino acids containing one centrally located hydrophobic transmembrane domain. Sequence analysis did not reveal homology to other known proteins, with the exception of the prolactin receptor, which was found to be 33% identical (11). A second class of cDNA clones representing GH binding proteins has also been described. These encode a protein identical to the extracellular domain of the liver GH receptor, but with a short, hydrophobic carboxyl-terminal region replacing the transmembrane and intracellular domains (10, 12). This short form of the GH receptor is generated by alternative splicing of transcripts from the GH receptor gene and is believed to be a secreted GH serum binding protein.

Recent evidence suggests that the cloned GH receptor is involved in mediating the proliferative effect of GH on cartilage growth. A subset of patients suffering from Laron-type dwarfism has various mutations in the GH receptor gene, including large deletion(s) of exons corresponding to the extracellular domain (13) and a point mutation resulting in a phenylalanine to serine substitution at position 96 in the extracellular domain (14). These mutations are believed to produce a functionally defective GH receptor, resulting in impaired somatic growth of the affected individual.

In pancreatic islet β cells, GH stimulates proliferation and insulin biosynthesis through an insulin-like growth factor 1-independent mechanism (15). Insulin biosynthesis, and to a lesser extent proliferation, is also stimulated by GH in the insulin-producing tumor cell line RIN5-AH (16, 17). We have recently shown that these cells express GH receptors similar to the long form of the cloned hepatic GH receptor (18). Studies examining the dose-dependent stimulation of insulin biosynthesis by GH in RIN5-AH cells revealed a close stoichiometric relationship between GH receptor occupancy and the level of insulin biosynthesis, indicating the absence of spare receptors (16). To determine whether the cloned hepatic GH receptor is able to mediate the insulinotropic effect of GH we have isolated RIN5-AH cells that were transfected with the rat hepatic GH receptor cDNA and have analyzed their GH responsiveness in terms of insulin production.

MATERIALS AND METHODS

Construction of Expression Plasmid. The plasmid pE2.9, containing the full-length rat GH receptor cDNA (9), was digested with *Spe* I and partially digested with *Bgl* II, and the 2.4-kilobase (kb) fragment containing the coding sequence was isolated and ligated into the plasmid pMTGH (S. Nilsson, Huddinge, Sweden), which had been digested with *Bam*HI and *Xba* I. The latter plasmid contains the human metallothionein IIa promoter and simian virus 40 enhancer as well as transcription termination sequences from the human GH gene cloned into the vector pUC8.

Cell Culture and Transfection. RIN5-AH cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 units of penicillin per ml, and 100 μg of streptomycin per ml at 37°C in a humidified atmosphere containing 5% CO_2 in air as described (16). For

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Abbreviations: GH, growth hormone; hGH, human GH.

transfection with the expression vector pLM108, RIN5-AH cells were detached from the cell culture flask by treatment with trypsin (0.05% trypsin in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution containing 0.3 mM EDTA), and the cells (10^7) were resuspended in 0.5 ml of phosphate-buffered saline solution. The cell suspension was then placed in a sterile plastic microcuvette containing 40 μg of pLM108 and 10 μg of pRSVneo and exposed to a single voltage pulse (200 V, 500 mF) as described (19). After 3 days the cells were cultured in the presence of 250 μg of G418 per ml for 6 days to select for stable transfectants. Further selection was performed in the presence of 125 μg of G418 per ml. After 12–14 days in culture, G418-resistant colonies were picked individually and cultured for further analysis.

^{125}I-Labeled Human GH (^{125}I-hGH) Receptor Binding. For screening of ^{125}I-hGH binding in transfected cells and for experiments evaluating the effect of Zn^{2+} on binding, RIN5-AH cells or transfected cells were seeded in six-well culture dishes and allowed to reach confluence. Monolayers were then washed in binding buffer [10 mM Hepes (pH 7.4) containing 0.13 M NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.24 mM $MgSO_4$, 2.5 mM NaH_2PO_4, and 1% human serum albumin]. ^{125}I-hGH, prepared as described (16) (≈20,000 cpm; specific activity = 80 mCi/mg; 1 Ci = 37 GBq) in binding buffer, was added in a total volume of 1 ml. The dishes were then incubated at room temperature for 90 min, after which binding was terminated by washing the cells five times in ice-cold binding buffer. The cells were then harvested by trypsinization and transferred to tubes for measurement of bound radioactivity. Nonspecific binding (^{125}I-hGH bound in the presence of 10 μg of hGH per ml) was subtracted from total binding to calculate specific binding. For competition experiments ^{125}I-hGH binding was measured to cells in suspension. Cells were detached from the culture flask by trypsinization and allowed to recover in spinner culture overnight. Cells were then washed and resuspended in binding buffer and binding was performed at room temperature for 90 min. Binding was terminated by centrifugation of the cell suspension through dibutyl phthalate as described (18).

RNA Analysis. Total RNA was isolated from cultured cells by guanidine isothiocyanate homogenization followed by ethanol precipitation (20). For analysis of GH receptor expression, 15 μg of total RNA was separated by electrophoresis in 1% agarose/formaldehyde gels followed by transfer to nylon membranes. Membranes were then pretreated and hybridized with a ^{32}P-labeled rat GH receptor cDNA probe. The GH receptor probe was an 864-base-pair (bp) *Bam*HI fragment of the rat GH receptor cDNA (9); this fragment corresponds to the extracellular domain of the receptor. Membranes were then washed under high stringency conditions and exposed to x-ray film at −80°C without intensifying screens.

Insulin Content. Cells (5×10^4) were seeded in 24-well cell culture dishes in RPMI 1640 medium containing 10% fetal calf serum. After 24 hr the medium was changed to RPMI 1640 containing 1% fetal calf serum and the hormone to be tested. After 3 days in culture the cells were washed in phosphate-buffered saline and cellular insulin content was measured in cell homogenates by radioimmunoassay as described (16). The protein content in cell homogenates was measured using the Bio-Rad protein assay kit and bovine serum albumin as the standard. All insulin concentrations are expressed as insulin per mg of total cellular protein.

RESULTS

The plasmid pLM108 (Fig. 1*A*) containing the entire coding region of the rat GH receptor was cotransfected with a neomycin resistance marker into RIN5-AH cells by electroporation (19). After selection for 2 weeks in G418, 68 resistant colonies were isolated and analyzed for ^{125}I-hGH binding activity. Nine of the 68 clones showed significantly elevated binding (50–300%) when compared to nontransfected RIN5-AH cells. One clone, designated 1.24, was selected for detailed analysis. Competition experiments using trace concentrations (4 pM) of ^{125}I-hGH showed that the binding capacity of the 1.24 cells was 2.6-fold higher than that of the nontransfected RIN5-AH cells, but with a similar IC_{50} of 4–6 ng of hGH per ml (Fig. 1*B*). Analysis of the binding data by

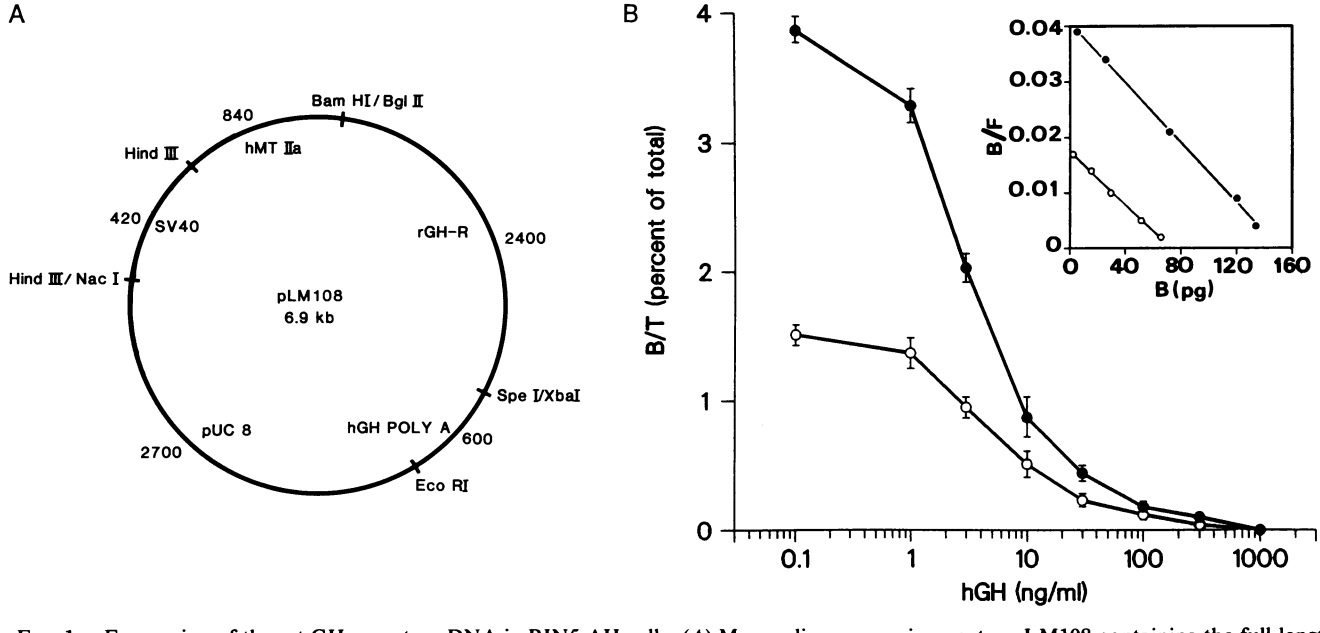

FIG. 1. Expression of the rat GH receptor cDNA in RIN5-AH cells. (*A*) Mammalian expression vector pLM108 containing the full-length rat GH receptor cDNA under the transcriptional regulation of the human metallothionein IIa promoter. (*B*) Competition of ^{125}I-hGH binding with hGH in untransfected RIN5-AH cells (○) and GH receptor transfected 1.24 cells (●). Cells (2.5×10^6) in 1 ml of binding buffer were incubated with ^{125}I-hGH (50,000 cpm) in the presence of the indicated concentration of hGH. After a 90-min incubation the binding was terminated by centrifugation of 400-μl samples through dibutyl phthalate. Specific binding is plotted against the concentration of unlabeled hGH. The mean ± SD of three experiments is shown. B/T, bound/total. (*Inset*) Scatchard plot of data from one representative experiment. B/F, bound/free.

the method of Scatchard yielded parallel plots and a calculated receptor number in the nontransfected and transfected cells of 1800 and 4600, respectively. To verify that the increased receptor number in 1.24 cells was a result of expression of the transfected GH receptor cDNA, we performed Northern blot analysis of RNA from RIN5-AH and 1.24 cells using a rat GH receptor-specific cDNA probe. In 1.24 cells a specific 3.0-kb transcript was observed in addition to the 1.3- and 4.8-kb transcripts found in nontransfected cells (Fig. 2). Furthermore, all of the 9 clones originally found to exhibit increased ^{125}I-hGH binding contained a GH receptor transcript of 3.0–3.2 kb that was not present in the parent cell line.

To investigate whether the transfected GH receptor was able to mediate the insulinotropic effect of GH we analyzed the effect of GH on insulin biosynthesis in RIN5-AH and 1.24 cells. We have previously shown that GH stimulates insulin biosynthesis in RIN5-AH cells (17), resulting in accumulation of insulin (16), and that this effect is due to increased insulin gene expression. Furthermore, a linear relationship was observed between the level of insulin biosynthesis and GH receptor occupancy in the RIN5-AH cells, indicating the absence of so-called spare receptors. In the 1.24 cells, hGH stimulated insulin biosynthesis maximally (4.1-fold) at 100 ng/ml, with an EC_{50} of 10 ng/ml. In contrast, in RIN5-AH cells insulin biosynthesis was only increased 1.9-fold at 100 ng of hGH per ml (Fig. 3). There was no significant difference in basal insulin content between the two cell lines.

Due to the presence of the metallothionein promoter upstream of the GH receptor cDNA in the expression plasmid, it was possible to increase the expression of the transfected receptor by culturing 1.24 cells in the presence of Zn^{2+}. After 24 hr in the presence of 25 or 50 μM Zn^{2+}, ^{125}I-hGH binding capacity of the 1.24 cells was increased 1.6- and 2.3-fold, respectively (Fig. 4). Culturing RIN5-AH cells in the presence of Zn^{2+} did not affect GH receptor expression. Scatchard plot analysis of binding data from competition experiments using 1.24 cells cultured in the presence of various concentrations of Zn^{2+} showed that the increased binding capacity was caused by an increase of receptor number (data not shown). At Zn^{2+} concentrations >50 μM significant cell death occurred. The effect of hGH on insulin biosynthesis in 1.24 cells cultured in the presence of various concentrations of Zn^{2+} was analyzed. Increased expression of the transfected GH receptor was associated with increased GH responsiveness (Fig. 5). A 4.9- and 8-fold stimulation of insulin biosynthesis was observed in 1.24 cells cultured in medium supplemented with 25 and 50 μM Zn^{2+}, respectively. No effect of Zn^{2+} on hGH-stimulated insulin biosynthesis in nontransfected RIN5-AH cells was observed. Correlation analysis showed that the increased GH responsiveness, as reflected by insulin biosynthesis, was proportional to the number of GH receptors expressed on the cells.

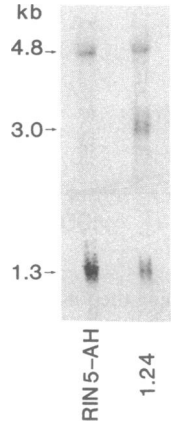

FIG. 2. Northern blot analysis of GH receptor expression in RIN5-AH and 1.24 cells. Fifteen micrograms of total RNA was applied to each lane. The GH receptor transcripts were identified using a 564-bp *Bam*HI fragment of the cloned rat GH receptor cDNA as a probe. After hybridization the filter was washed under high stringency conditions and placed in opposition to x-ray film for 14 days at −70°C without intensifying screens. The arrows point to the transcripts found in transfected cells. The sizes of the GH receptor transcripts in RIN5-AH cells (in kb) are indicated.

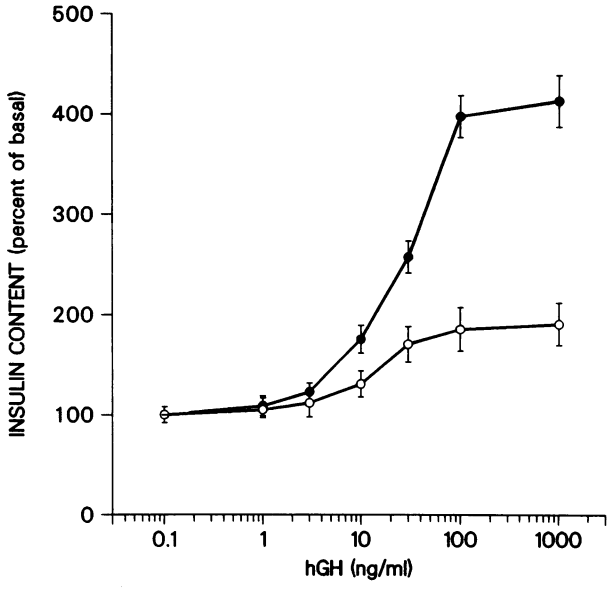

FIG. 3. GH stimulation of insulin content in RIN5-AH and 1.24 cells. RIN5-AH (○) and 1.24 (●) cells were cultured for 3 days in the presence of the indicated concentration of hGH. The insulin content per μg of cellular protein, expressed as percentage of basal, is shown. The mean ± SD for three experiments is shown.

DISCUSSION

Transfection of RIN5-AH cells with a full-length rat liver GH receptor cDNA expression vector resulted in the generation of several clones exhibiting increased ^{125}I-hGH binding. Evidence that the increased binding is caused by expression of the transfected receptor was obtained from two sets of experiments. (*i*) All of the clones found to exhibit increased binding of ^{125}I-hGH also contained a 3.0- to 3.2-kb GH receptor transcript detected by Northern blot analysis. This transcript was not observed in untransfected RIN5-AH cells. The size of this mRNA agrees well with the predicted size of

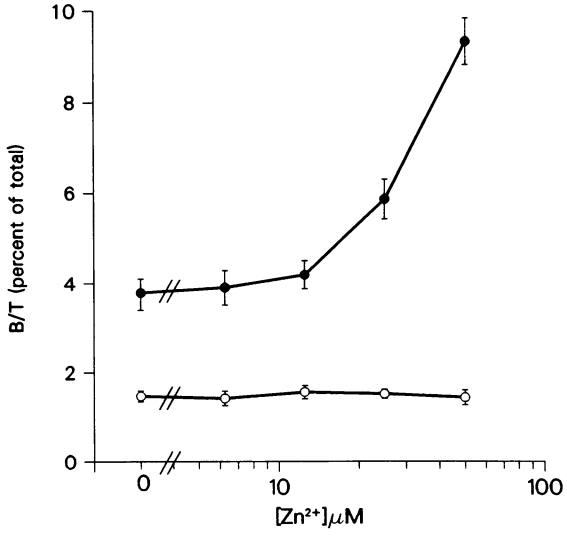

FIG. 4. Effect of Zn^{2+} on GH receptor expression in RIN5-AH and 1.24 cells. Total binding of ^{125}I-hGH was measured to RIN5-AH (○) and 1.24 (●) cells after an overnight culture in the presence of the indicated concentration of Zn^{2+}. Specific ^{125}I-hGH binding is plotted against the concentration of Zn^{2+}. The mean ± SD for three experiments is shown. B/T, bound/total.

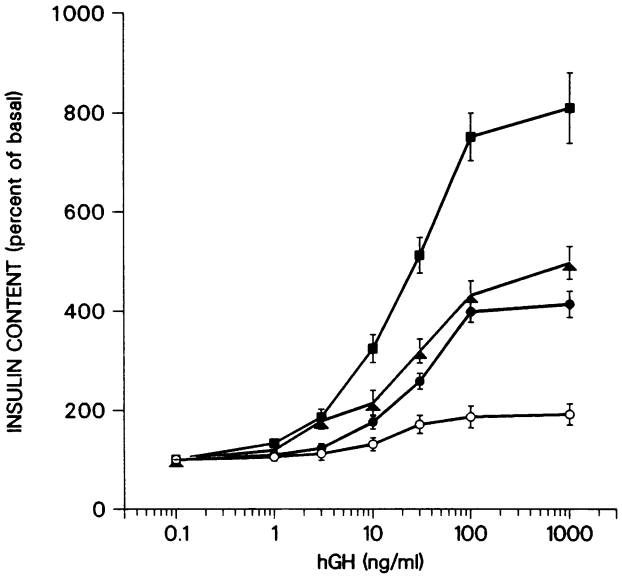

FIG. 5. Effect of Zn^{2+} on hGH-stimulated insulin biosynthesis in RIN5-AH and 1.24 cells. Insulin content was measured in cells cultured in the presence of the indicated concentration of hGH. RIN5-AH cells (○) were cultured in medium without Zn^{2+}; 1.24 cells were cultured in medium without Zn^{2+} (●), with 25 μM Zn^{2+} (▲), and with 50 μM Zn^{2+} (■). The insulin content per μg of cellular protein expressed as the percentage of basal is shown. The mean ± SD for three experiments is shown.

a transcript from the GH receptor expression plasmid. (*ii*) Because the GH receptor cDNA was fused to the human metallothionein promoter, expression of the GH receptor could be stimulated by divalent cations such as Zn^{2+}. In 1.24 cells there was a dose-dependent effect of Zn^{2+} on ^{125}I-hGH binding, with a maximal 2.3-fold stimulation observed at 50 μM Zn^{2+}. Taken together these observations strongly indicate that the increased binding observed in 1.24 cells is due to expression of the transfected receptor. Competition experiments and Scatchard plot analysis of the binding data revealed that the affinity of the transfected receptor was indistinguishable from that of the endogenous receptor and that the increased binding capacity was caused by an increased receptor number.

In RIN5-AH cells GH stimulates the biosynthesis of insulin without affecting insulin release (16). The effect on insulin biosynthesis is reflected by an increased cellular content of insulin. Dose–response studies on the effect of GH on insulin biosynthesis revealed a close correlation between GH receptor occupancy and the magnitude of GH-stimulated insulin content in RIN5-AH cells, indicating the absence of spare receptors. This observation led us to the hypothesis that an increased level of GH receptor expression should result in increased GH-stimulated insulin biosynthesis. In GH receptor transfected 1.24 cells it was observed that the expression of the transfected GH receptor was associated with an increased GH responsiveness. Furthermore, when the expression of the transfected receptor was stimulated with Zn^{2+}, the GH responsiveness increased accordingly. The close association between the level of GH receptor expression and insulin biosynthesis suggests that the transfected GH receptor is able to mediate the insulinotropic effect of GH. Thus the cloned hepatic GH receptor can be regarded as a true receptor since it is able to specifically bind GH and transmit a biological response. Since the transfected rat GH receptor only binds GH and not prolactin, and since we found that bovine GH had the same effect as hGH on the insulin biosynthesis (N.B., unpublished observation), we conclude that a somatogenic receptor is responsible for the insulinotropic effect of hGH.

Little is known about the mechanism by which GH exerts its effects. GH receptors have been found to be tyrosine phosphorylated by a GH-dependent mechanism (21); however, the amino acid sequence of the GH receptor does not reveal any homology to known tyrosine kinases. This suggests that the receptor is not itself a tyrosine kinase but rather that it is able to stimulate a receptor-associated tyrosine kinase activity either directly or indirectly. In preadipocytes GH stimulates c-*fos* expression by a protein kinase C-dependent mechanism as well as the rapid formation of diacylglycerol (22). No accumulation of inositol phosphates was observed, however. These findings indicate that GH activates phospholipase C-dependent hydrolysis of phospholipids other than phosphatidylinositol phosphate, but the nature of such phospholipids remains obscure. In adipocytes GH has also been reported to stimulate lipogenesis by a protein kinase C-dependent pathway (23, 24), even though the GH receptor clearly does not belong to the family of receptors normally associated with phospholipase C activation, such as the angiotensin receptor (25). The fact that the cloned GH receptor cDNA encodes for a protein with a M_r of 80,000 and the fact that the predominant GH receptor found in RIN5-AH cells has a M_r of 115,000 (18) suggest that the GH receptor may be associated with another protein possessing catalytic activity as described above.

As we have found evidence for an insulin-like growth factor 1-independent mitogenic effect of GH and prolactin in primary rat pancreatic β cells (N.B. and J.H.N., unpublished data), it is hypothesized that the long forms of the receptors expressed in certain tissues are able to transmit direct mitogenic signals, whereas the shorter forms may transmit other metabolic activities—e.g., induction of insulin-like growth factor 1. Alternatively, the stimulation of insulin production in the pancreatic β cell may be analogous to the induction of insulin-like growth factor 1 in other cell types. The present *in vitro* cell culture system might prove useful for structure/function analysis of the GH receptor and for studying the GH receptor signal transduction mechanism by expressing altered GH receptors in RIN5-AH cells.

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