Overexpression of the Short Form of the Growth Hormone Receptor in 3T3-L1 Mouse Preadipocytes (43739)

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Abstract. **In rodents, the gene for the growth hormone receptor (GHR) gives rise to two mRNA transcripts encoding two proteins: a larger membrane spanning receptor (GHR_L) and a smaller isoform, GHR_S that consists of the extracellular domain and a unique hydrophilic carboxyl terminus. We examined the hypothesis that GHR_S may contribute to cellular binding of GH and play a role in growth hormone (GH) signaling. Rat cDNA encoding GHR_S was ligated into the mammalian expression vector pcDNA-I/neo and stably transfected into mouse 3T3-L1 preadipocytes which have endogenous GH receptors and, when differentiated into adipocytes, have the biochemical machinery to express the various GH effects. Sixteen of 24 neomycin resistant clones secreted at least twice as much GHR_S in the growth medium as cells transfected with the vector alone, and in nine of these, GH binding was increased 2- to 4-fold. The amount of GHR_L in extracts of these cells was unchanged, indicating that increased binding could not be accounted for by effects on formation or degradation of GHR_L. The transfected cDNA for GHR_S directs the synthesis of a 50 kDa protein. Cross-linking of [^{125}I]hGH to transfected 3T3-L1 cells indicated a 3.5-fold increase in a 72 kDa GHR_S[^{125}I]hGH complex. In the presence of 10% newborn calf serum, incorporation of [^{35}S]methionine into cellular proteins was similar in transfected clones and control cells. Deprivation of serum for 2 hr decreased protein synthesis by ~70% in control cells, but in the transfected cells, protein synthesis was reduced only by ~50% or 30% in cells exhibiting 2× or 3× increases in GH binding. In all cells, 1 n*M* IGF-1 restored protein synthesis to the serum replete level. Similarly, although ^{3}H-2-deoxy-D-glucose (2DG) uptake was comparable in all cells after 2 hr of serum deprivation, 18 hr of serum deprivation decreased uptake by ~70% in control cells, but only by ~30% in cells with increased GH binding. One nanomolar IGF-1 restored 2DG uptake to levels seen after 2 hr of serum deprivation. IGF-1 had no effect after only 2 hr of serum deprivation. Measurement of IGF-1 secreted into the medium, revealed that clones which overexpress GHR_S produce greater amounts of IGF-1 than control cells or transfected clones that failed to overexpress GHR_S. We conclude that GHR_S contributes to GH binding and may therefore be a functional receptor. In addition, overexpression of GHR_S in 3T3-L1 cells altered cell function in the absence of GH.**
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Two products of the growth hormone receptor (GHR) gene are found in all mammals studied to date (1). The GHR is a large membrane-spanning protein that consists of ~620 amino acids (2) and is found on the surface of a variety of cell types. Upon binding to growth hormone (GH), this protein is thought to initiate a phosphorylation cascade that presumably results in expression of many, if not all, of the responses to GH (3). The other product of the GHR gene is the circulating GH-binding protein (GHBP), which, in humans and rabbits, is thought to arise by proteolytic cleavage of the GHR that releases the extracellular hormone-binding domain from cells allowing it to find its way into the circulation (4). In rodents, the GHBP arises exclusively from synthesis directed by an alternatively spliced transcript of the GHR gene

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(5). The circulating GHBP of rodents, like its counterpart in other species, consists largely of the extracellular hormone binding domain of the GHR. Alternate splicing replaces the transmembrane and intracellular domains at the carboxyl end with a unique hydrophilic peptide. Because this isoform of the GH receptor is retained in many of its cells of origin, we refer to it as the short form of the GH receptor (GHR_S).

Studies of the physiology of the GHBP in nonrodent species have been limited largely to characterization of its dynamics in blood (1). The fact that rodents express two mRNA transcripts for the GHR, one of about 4.5 kb and one of about 1.2 kb, facilitates identification of the cells that produce the GHR_S in these species. Similarly the unique carboxyl terminus allowed development of antisera specific for the short and long isoforms of GHR, and hence, permits study of these proteins in rodents in a manner not possible in other species (6–9). Studies of GHR_S indicate that both the 1.2 kb mRNA and its protein product are expressed in a wide variety of GH responsive cells including fat and muscle, as well as liver (10–16). Because its production is so widespread, we became interested in the possibility that GHR_S may have some function in the cells that produce it, perhaps as the hormone recognition component of a receptor complex. It has been known for some time that GH produces a wide variety of effects and, in adipocytes, for example, it is difficult to reconcile the range of actions with a single interaction between hormone and receptor.

One way to explore the function of GHR_S is to transfect cells with the cDNA that encodes it and observe the cellular consequences. Transfection of GHR_S into COS cells, which do not otherwise express the GHR gene, resulted in release of GHR_S into the culture medium, with none retained on the cell surface (6). We reasoned, however, that cells that do not normally express GH receptors may also not express other proteins related to transduction of the GH signal, and therefore may not be able to retain GHR_S on their surfaces or to carry out the biological responses GHR_S might transduce. We therefore introduced the cDNA for rat GHR_S into 3T3-L1 preadipocytes which express GHR (17) and a variety of responses to GH (18).

The cDNA that we had prepared previously from a rat adipocyte library (8) was ligated into the mammalian expression plasmid pcDNAI/neo and transfected into 3T3-L1 preadipocytes using the calcium phosphate method (19). Native pcDNAI/neo was used for control transfections. The cells were grown in the presence of G-418 (neomycin) to select for stable transfectants. After 14 days of selection, 24 neomycin resistant clones harboring the cDNA for GHR_S, and a control clone, were isolated.

The ability of each clone to express GHR_S was evaluated by measuring the accumulation of GHR_S in the growth medium. The assay depends upon an antiserum (AS1615), raised in rabbits against a synthetic 17 residue peptide corresponding to the unique carboxyl terminus of the rat GHR_S. To quantitate GHR_S, 500 μl of growth medium was incubated for 16 hr with 2 ng [^{125}I]hGH and AS1615 at a dilution of 1:1000. The antibody hormone complex was harvested on agarose beads coated with protein A and counted. Although the GHR_S in the mouse has 10 more amino acids in its carboxyl terminus than the rat GHR_S, it nevertheless reacts with AS1615. In two days, the release of GHR_S into the medium by 10^6 control cells transfected with only the vector was sufficient to bind ~25 fmol of hGH. The release of GHR_S was unchanged in eight of the 24 transfected clones, but the GH-binding activity in growth medium from the remaining 16 clones, ranged from ~50 to ~125 fmol/10^6 cells.

GH binding to confluent cells was determined in 24 well-culture plates. The cells were incubated for 30 min at 4°C with 5 ng/ml of [^{125}I]hGH. After removal of the binding medium, the cells were washed with ice cold buffer to remove unbound hormone, and then solubilized and counted. Control cells bound ~38 fmol of GH/10^6 cells. In 10 of the 16 clones that secreted increased amounts of GHR_S, binding of [^{125}I]hGH to the cell surface ranged from ~60 to 160 fmol/10^6 cells, but was unchanged from control in all of the clones that showed no increase in GHR_S secretion. Figure 1 illustrates the behavior of two clones that overexpress GHR_S. Both surface binding and secretion of GHR_S were increased about 4-fold in Clone 14 compared with control cells. Clone 20 secreted five times as much GHR_S as control cells, while binding to the surface of

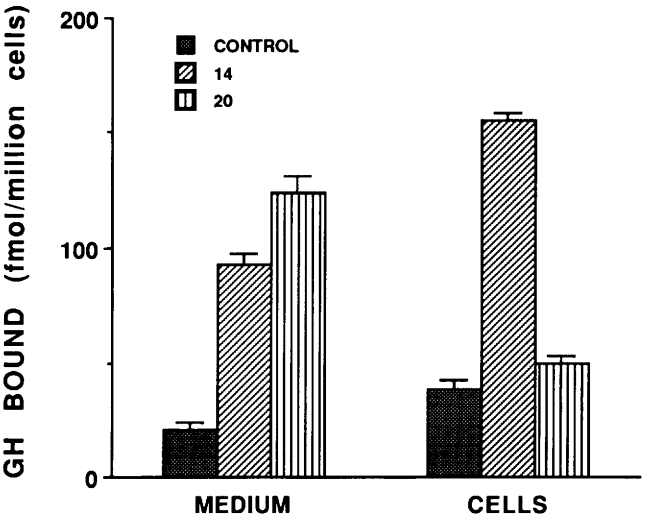

Figure 1. Effects of transfection with cDNA coding for GHR_S on the specific binding of [^{125}I]hGH in the incubation medium and to the surfaces of two transfected clones (14 and 20) and control 3T3-L1 cells. Each bar is the mean of triplicate observations. Brackets indicate SEM.

these cells was virtually unchanged. We do not understand why some clones retain the transfected GHR_S on their surface while others do not.

The increase in surface binding seen in cells like those of Clone 14 could be due to increased expression of GHR_S or to a disruption of the normal receptor turnover that might lead to an accumulation of GHR on the cell surface. To evaluate this possibility, we measured GHR and GHR_S in cell-free extracts prepared from each clone, using the same immunofunctional assays as described above. To measure GHR, AS1615 was replaced with AS2941 which was raised against the intracellular domain of GHR and is therefore specific for GHR (9). Control and transfected cells all contained sufficient GHR to bind ~85 fmol of GH/10^6 cells. Binding of GHR_S ranged from ~60 to nearly 400 fmol/10^6 cells. Figure 2 illustrates these data for the control and two representative clones.

Scatchard analysis of the binding data revealed that the affinities of the transfected GHR_S and the endogenous mouse GHR_S for [^{125}I]hGH were indistinguishable, as evidenced by the linearity of the plots. In four independent experiments control cells had a calculated maximum binding capacity of 6,800 ± 1,360 sites/cell, and cells of Clone 14 had 24,500 ± 4,370 sites/cell ($P < 0.01$). The affinity constants were 3.5 ± 0.9 × 10^9 M^{-1} for control cells and 2.2 ± 0.7 × 10^9 M^{-1} for cells of Clone 14.

To further characterize the transfected GHR_S, cells were incubated in the presence of [^{35}S]L-methionine, and the extracted proteins were immunoprecipitated with AS1615 prior to separation by polyacrylamide gel electrophoresis. AS1615 specifically immunoprecipitated a band corresponding to 50 kDa. Since the amino acid sequence of GHR_S, as predicted from the nucleotide sequence, would yield a polypeptide of ~31 kDa, it is likely that the GHR_S is heavily glycosidated. We covalently cross-linked [^{125}I]hGH to specific binding sites on transfected and control 3T3-L1 cells using the water soluble cross-linking agent bis(sulfosuccinimidyl) suberate. The cross-linked complexes were then subjected to polyacrylamide gel electrophoresis and visualized by autoradiography. Two labeled bands corresponding to 122 kDa and 72 kDa were present in each lane except when cross-linking was carried out in the presence of 5 μg/ml of unlabeled hGH. The principal band corresponds to GHR (~100 kDa) cross-linked with hGH (~22 kDa). The 72-kDa band which corresponds to GHR_S (~50 kDa) coupled with hGH is about 8% as intense as the 122-kDa band in densitometric scans of the control lane, and increases to 25% of the intensity of the GHR band in Clone 14. These data as a whole strongly support the idea that GHR_S is responsible for the increase in [^{125}I]hGH binding seen in transfected cells.

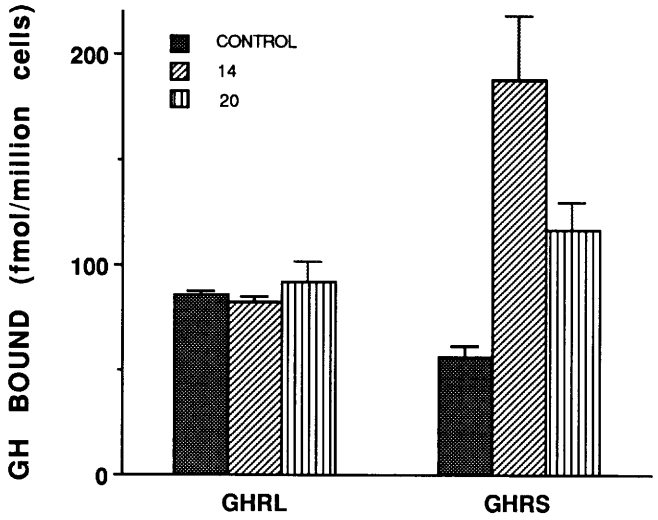

Figure 2. GH receptor isoforms in cell-free extracts of control and transfected 3T3-L1 cells. Each bar is the mean of triplicate observations. Brackets indicate SEM.

Mere participation in GH binding does not provide insight into what biological role, if any, GHR_S might play. GHR_S might serve as a component of a signal transducing element that mediates some of GH's actions, or alternatively it may act as a decoy for GH and inhibit transmission of the GH signal. Finally, it is possible that GHR_S is irrelevant to the GH-signaling pathway. To approach this issue, we sought first to determine if GH affects protein synthesis in these cells, and if so, whether or not GHR_S affects the process. Because the growth medium, which contains 10% fetal calf serum, was designed to optimize conditions for growth and protein synthesis, addition of GH would be expected to produce little effect. We therefore examined the effects of GH on protein synthesis in control and transfected cells that had been deprived of serum overnight. Cells grown to confluence in six-well plates were rinsed with phosphate buffered saline and incubated for 18 hr in Dulbecco's modified Eagle's medium containing 2% bovine serum albumin. The cells were then pulse-labeled with 10 μCi/ml of [^{35}S]L-methionine for 2 hr. After washing, the cells were dissolved in SDS. Total proteins were precipitated on filter paper with 50% trichloroacetic acid and counted. GH produced a small, concentration-dependent increase in the incorporation of ^{35}S into proteins in both control cells, and cells of Clone 14 (Fig. 3). While there was no obvious difference between cells that overexpressed GHR_S and control cells in the magnitude of the response to GH, it was evident that the basal rate of protein synthesis was 2- to 3-fold higher in the transfected cells.

A 2- to 4-fold greater rate of protein synthesis in transfected cells, as compared with control cells, is difficult to reconcile with the observation that all of the cells grow at a similar rate. In exploring this phenomenon further, we found that rates of protein synthesis

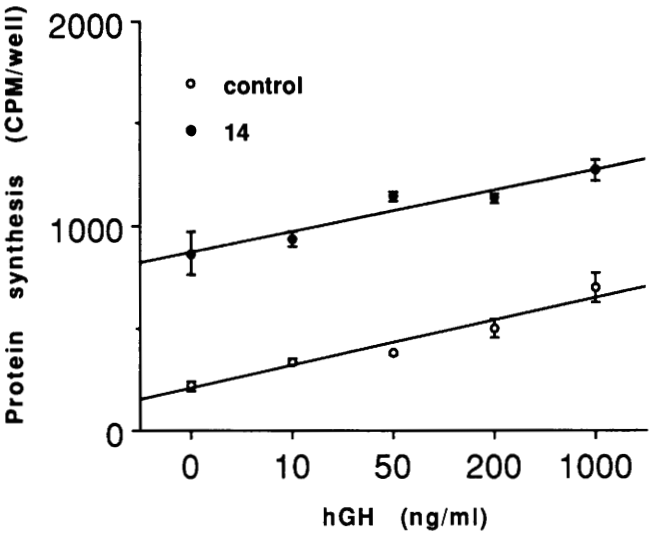

Figure 3. The effects of incubation with GH for 2 hr on the incorporation of ^{35}S-methionine into protein in control and transfected clones of 3T3-L1 cells. Each point is the mean of triplicate observations. Brackets indicate SEM.

in control and transfected cells were virtually identical when protein synthesis was measured in cells grown in the complete medium containing 10% calf serum. However, protein synthesis in control cells is exquisitely sensitive to some factor(s) present in the serum. Incubation of control cells without calf serum for 18 hr, decreased the incorporation of ^{35}S into protein by 95%. Incubation of control cells without calf serum for only 2 hr decreased protein synthesis by 65%. Clones that expressed increased amounts of GHR_S on their cell surface, however, were considerably less sensitive to the effects of serum starvation. After 2 hr of serum starvation, protein synthesis declined by only 30% in cells of Clone 14, and only by 45% in cells of Clone 9. By 18 hr after removal of serum, protein synthesis in these clones declined further to 15% or 10% of the serum replete level, but these rates of protein synthesis were 2- to 3-fold greater than seen in control cells or cells of Clone 22 which, though transfected with the cDNA for GHR_S, failed to express any increase in GH binding. In all, we have examined four transfected clones of 3T3-L1 cells that do not overexpress GHR_S on their surfaces, and four clones of transfected 3T3-L1 cells in which GH binding was increased 2- to 3-fold. All of the clones incorporated similar amounts of [^{35}S]methionine when serum was present in the growth medium immediately prior to measuring protein synthesis. The rates of incorporation of ^{35}S into protein were significantly decreased in all eight clones when measured 2 hr after serum deprivation, but the four clones that expressed increased GHR_S on their surfaces were dramatically less affected by serum starvation.

To gain insight into what factors in serum might be required to maintain protein synthesis in these cells, we attempted replacement with known compounds. Once again, incubation of cells without serum for 2 hr prior to measurement of ^{35}S incorporation into protein reduced protein synthesis by about 70% in cells that expressed normal amounts of GHR_S and only by about 30% in three different clones that retained increased amounts of GHR_S on their surfaces. Neither hGH (100 ng/ml) nor insulin (10^{-9} *M*) restored the rates of protein synthesis to control levels either in transfected cells or in control cells. In contrast, recombinant human IGF-I (10^{-9} *M*) restored protein synthesis to the serum replete level in all cells. IGF-I, however, had no influence on the rate of protein synthesis when added to cells that had been incubated with calf serum (Fig. 4).

These observations suggested that IGF-I might be the element in serum needed to maintain protein synthesis, and raised the question of whether the clones that express increased amounts of GHR_S on their surfaces might also produce increased amounts of IGF-I. Using a cDNA probe generously provided by Dr. Peter Rotwein, we noted by Northern blot analysis that control and transfected 3T3-L1 cells express the mRNA for IGF-I. To determine if, indeed, transfected cells that overexpress GHR_S produce increased amounts of IGF-I, 3T3-L1 cells were grown to confluence in six-well plates in DMEM that contained 10% fetal calf serum. The medium was removed and replaced with DMEM supplemented with 0.1% bovine serum albumin instead of serum, and the cells were allowed to incubate overnight. Aliquots were then

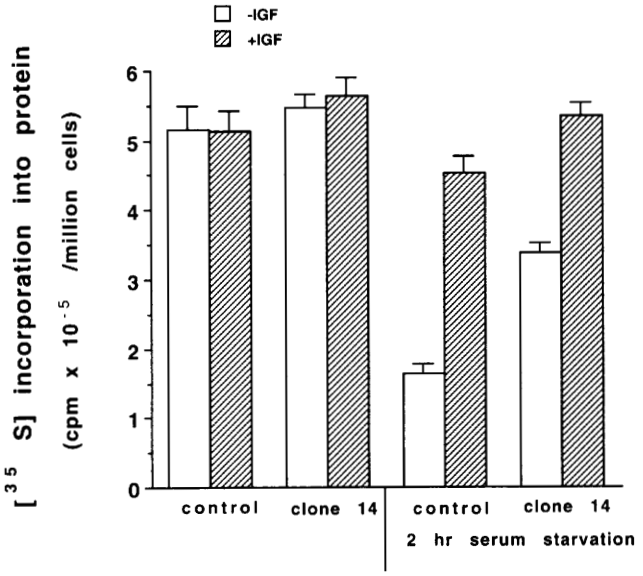

Figure 4. Effects of serum starvation and IGF-I (10^{-9} *M*) on protein synthesis in control and transfected 3T3-L1 cells. Each bar is the mean of triplicate observations. Brackets indicate SEM. Serum starvation significantly decreased protein synthesis in both clones ($P < 0.01$) but the decline was greater in the control clone than in Clone 14 ($P < 0.01$). IGF-I restored protein synthesis in the serum starved cells.

taken for measurement of IGF-I by radioimmunoassay, using the procedure described by Underwood *et al.* (20). Clones overexpressing GHR_S on their cell surfaces invariably produced greater amounts of IGF-I than control cells or transfected clones that failed to overexpress GHR_S.

The data indicate that expression of rat GHR_S in mouse 3T3-L1 cells can result in significant increases in the capacity of these cells to bind [^{125}I]hGH. Furthermore, they strongly suggest that GHR_S is attached to the cell surface in a manner that permits it to interact with GH in the extracellular medium. Although we observed no changes in the response to GH, overexpression of GHR_S was associated with changes in the physiology of these cells such that they became less dependent on serum supplementation to maintain their rates of protein synthesis. In addition, these cells produced increased amounts of IGF-I. While it is tempting to speculate that the increased availability of GHR_S on the cell surface somehow signaled increased expression of IGF-I, the data show only correlation, not causation. Similarly, while it is tempting to speculate that the decreased dependence on serum for maintenance of protein synthesis was the result of increased production of IGF-I, we cannot rule out the alternative that increased IGF-I production was a nonspecific consequence of the higher rate of protein synthesis in transfected cells incubated without serum.

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