Identification of Intracellular Domains in the Growth Hormone Receptor Involved in Signal Transduction (43743)

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Abstract. **The growth hormone (GH) receptor belongs to the GH/prolactin/cytokine super-family of receptors. The signal transduction mechanism utilized by this class of receptors remains largely unknown. In order to identify functional domains in the intracellular region of the GH receptor we generated a number of GH receptor mutants and analyzed their function after transfection into various cell lines. A truncated GH receptor missing 184 amino acids at the C-terminus was unable to mediate GH effects on transcription of the Spi 2.1 and insulin genes. However, this mutant was fully active in mediating GH-stimulated metabolic effects such as protein synthesis and lipolysis. Furthermore, this mutant GH receptor internalized rapidly following GH binding. Another truncated GH receptor lacking all but five amino acids of the cytoplasmic domain could not mediate any effects of GH nor did it internalize. Deletion of the proline-rich region or changing the four prolines to alanines also resulted in a GH receptor deficient in signaling. Mutation of phenylalanine 346 to alanine resulted in a GH receptor which did not internalize rapidly; however, this mutant GH receptor was capable of mediating GH-stimulated transcription as well as metabolic effects. These results indicate that the intracellular part of the GH receptor can be divided into at least three functional domains: (i) for transcriptional activity, two domains are involved, one located in the C-terminal 184 amino acids and the other in the proline-rich domain; (ii) for metabolic effects, a domain located in or near the proline-rich region is of importance; and (iii) for internalization, phenylalanine 346 is necessary.**

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Growth hormone (GH) exerts diverse effects in a number of tissues and cell types. The biological action of GH can be divided into three categories, which involve GH's effects on metabolism, proliferation and differentiation. Metabolic effects of GH have been observed in many different cell types of which the best characterized is the effect of GH on carbohydrate metabolism in adipocytes. In this cell type, GH exerts a transient insulin-like response stimulating glucose uptake and utilization as well as lipolysis (1, 2). In myocytes, GH also stimulates glucose oxidation (3), whereas in bone, GH has been shown to stimulate mineral metabolism (4). In several other cell types, GH stimulates general protein synthesis and amino acid transport. As for the mitogenic effects of GH, in primary cultures of insulin producing pancreatic β cells, GH directly stimulates cell proliferation. A 10-fold induction of the proliferative index could be observed after the cells were cultured in the presence of physiological concentrations of GH; this effect was independent of IGF-1 (5, 6). In addition, a direct proliferative response to GH has been reported in primary osteoblasts (7), germinal cells of the tibial growth plate (8), vascular endothelial cells (9), epithelia cells (10), regenerating skeletal muscle fibers (11), thymocytes (12), and B cells (13). Finally, in terms of GH's effects on differentiation, preadipocytes have been reported to differentiate into adipocytes in response to GH. Upon addition of fetal calf serum to preadipocytes which have been grown to confluence, these cells will be induced to differentiate into adipocyte-like cells.

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The active compound in serum was identified as GH, and subsequently it was shown that GH would by itself induce differentiation of preadipocytes (14). The process of differentiation was accompanied by the induction by GH of several adipocyte-specific genes as well as the induction of the c-*fos* and c-*jun* proto-oncogenes (15). Furthermore, GH has been found to affect development of thymocyte progenitor cells in the bone marrow (16) and may also be involved more generally in differentiation in the hemopoietic system (17).

Most of the effects of GH described above have been shown to be mediated by a specific GH receptor. The cDNA encoding the human and rabbit GH receptor was cloned and sequenced in 1987 (18); since then, the GH receptor cDNA from rat, mouse, cow, sheep, and pig has also been cloned. The primary structure of the GH receptor predicts a single chain protein of approximately 640 amino acids with a centrally located hydrophobic transmembrane domain. Sequence comparison has revealed a significant identity to the prolactin receptor (19) and detailed analysis showed that the location of cysteine residues in the extracellular domain of the receptors for GH, prolactin, erythropoietin, IL-3, IL-4, IL-5, and IL-6 is conserved (20). The intracellular region of the GH receptor, however, showed no sequence identity to other receptors or signaling molecules. Only a very limited identity was observed between the GH and prolactin receptors in a proline-rich domain close to the transmembrane region. Thus, the primary sequence of the GH receptor revealed little information concerning the signal transduction mechanism. In terms of the signaling pathway triggered by GH binding to its receptor, it was previously reported that GH did not use classical second messengers such as cAMP or IP_3. However, several groups reported the activation of protein kinase C by GH, but without the expected rise in intracellular IP_3 concentration. This was explained as a GH stimulation of phospholipase C hydrolyzing phospholipids other than PIP_2. It was also observed that GH could stimulate phosphorylation of several cellular proteins, including the GH receptor itself, on tyrosine residues, yet the GH receptor clearly does not belong to the class of growth factor receptors which contain intrinsic tyrosine kinase activity. It is more likely that the GH receptor binds and activates a nonreceptor tyrosine kinase. Recently, JAK2 kinase was identified as a GH receptor associated tyrosine kinase which could be activated by GH (21).

A major question in understanding GH receptor signaling is whether the diverse biological actions of GH are mediated by the same GH receptor, and, if so, whether one or several signaling mechanisms are responsible for the various effects of GH. With the development of cellular transfection methods using the cloned GH receptor cDNA (see Norstedt *et al.*, this volume), it has become possible to demonstrate that proliferative, metabolic, as well as differentiation, processes are mediated by the cloned GH receptor. With these cell transfection methods for studying signal transduction and biological actions of the cloned GH receptor, it has now become feasible to characterize functional domains of the GH receptor by site-directed mutagenesis followed by introduction of the mutated GH receptor into cultured cells. In this minireview, we will summarize the results from such transfection studies performed in several different cell lines and analyze the ability of mutated GH receptors to mediate effects of GH on both metabolism and proliferation, as well as differentiation.

Identification of GH Receptor Domains Involved in Regulating Gene Transcription

In the insulin producing cell line RIN-5AH, GH stimulates the expression of the insulin gene following binding to specific GH receptors (22). In these cells a linear relationship between GH receptor occupancy and insulin expression was observed. Transfection of RIN-5AH cells with a rat GH receptor cDNA resulted in over-expression of the receptor protein and an increased insulin expression in response to GH, which paralleled the level of GH receptor expression (23). Two GH receptor (GHR) mutants were constructed by site-directed mutagenesis, one having a stop codon at Position 295 (GHR 1–194) and the other having a stop codon at Position 455 (GHR 1–454). The transfection of RIN-5AH cells with these two GH receptor mutants showed that neither mutated receptor was able to mediate the effect of GH on insulin expression (24) (Fig. 1). This suggests that the C-terminal 184 amino acids are important for the transcriptional activity of the GH receptor. Since the RIN-5AH cells express endogenous GH receptors, these cells are not ideal for studying GH receptor mutations. A transient assay was developed by Kelly and co-workers (25) in which the mutated receptor cDNA is co-transfected with a CAT fusion gene containing a GH responsive promoter. Using this assay, the results from the RIN-5AH cells were confirmed. Furthermore, it was shown that studies using GH receptors with mutations in the proline-rich region, either by deleting or by altering the four prolines to alanines, resulted in a receptor which was unable to mediate the transcriptional activity of GH. Therefore, two domains of the GH receptor have been implicated in mediating transcriptional activity, one located in the C-terminal 184 amino acids and the other being the proline-rich domain.

Identification of GH Receptor Domains Involved in Regulating Metabolic Pathways

In CHO cells stably transfected with the rat GH receptor, GH will stimulate protein biosynthesis and

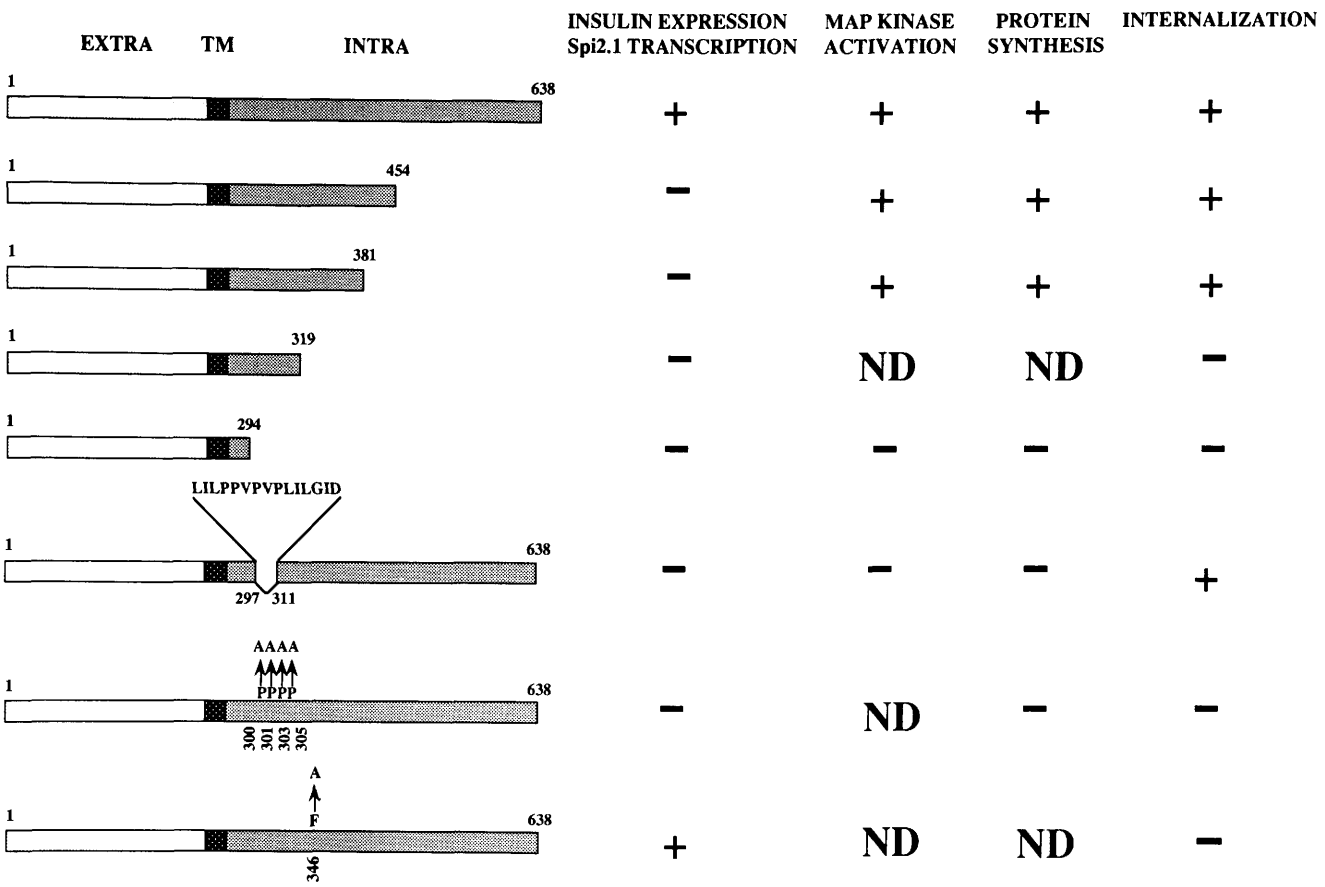

Figure 1. Activities of mutated GH receptors in transfected cells. Schematic representation of GH receptor mutants and their ability to mediate biological activities in transfected cell lines. Insulin expression was measured in RIN-5AH cells stably transfected with the mutated GH receptor as described (23). Spi 2.1 expression was measured in transiently transfected CHO cells by co-transfecting the GH receptor with a GH responsive Spi 2.1 promoter/CAT construct as described (25). MAP kinase activity and protein synthesis was measured in stably transfected CHO cells as described (27). Internalization of the GH receptor was measured both in stably transfected CHO cells and in transiently transfected COS cells by an acid wash procedure. (+), activity similar to that of the wild-type GH receptor; (−), lack of GH stimulated activity; ND, not determined.

lipid metabolism (26). Such metabolic effects have also been observed in other cell types expressing endogenous GH receptors. CHO cells transfected with the truncated GH receptor GHR 1–454 are responsive to GH in terms of protein synthesis and lipolysis. In contrast, when GHR 1–294 is expressed no activity could be observed (Fig. 1). These observations suggest that a domain located between amino acids 294 and 454 is important for signaling metabolic effects. In an effort to further characterize this domain, we focused on the proline-rich region located between amino acids 299 and 306. This domain is highly conserved in the GH receptor of various species as well as in the prolactin receptor. When GH receptors were mutated in the proline-rich domain either by deleting the entire domain or by substituting the four prolines to alanine, their ability to mediate GH effects on protein biosynthesis was lost (Fig. 1). Interestingly, when MAP kinase activity is measured in CHO cells expressing the various mutated GH receptors, it was observed that the GHR 1–454 is fully active (27) while GHR 1–294, as well as the two receptors mutated in the proline-rich domain, are inactive. This observation might suggest that activation of MAP kinase activity is an intermediate step in the signaling cascade leading to activation of metabolic pathways.

Identification of GH Receptor Domains Involved in Internalization

The GH receptor is rapidly internalized in many cell types following binding of GH. Whether this internalization is important for biological action or rather serves as a means of terminating the signaling remains controversial. In order to identify domains in the GH receptor involved in internalization, we measured GH receptor internalization in both stably transfected CHO cells and in transiently transfected COS cells. The results obtained from these two cell systems were identical, indicating that there appears to be no cell type-specific GH receptor internalization mechanisms. GH receptor mutants truncated at Position 381

were able to internalize to the same extent as the full-length GH receptor, whereas GHR 1–294 and GHR 1–318 did not exhibit high-efficiency internalization, suggesting that a domain required for internalization is located between amino acids 318 and 381. In other hormone receptors, aromatic residues have been implicated as important structures in internalization domains (28). In the region of amino acids 318 to 381 of the GH receptor, only four aromatic residues are present. When tyrosine 333 and 338, and phenylalanine 337 are mutated to alanine's lack of effect on receptor internalization was observed. However, when phenylalanine 337 was substituted for alanine, a complete loss of high-efficiency internalization resulted; in fact, this GH receptor mutant internalized as poorly as the truncated GHR 1–294.

Conclusions

By site-directed mutagenesis followed by stable or transient transfection of the GH receptor cDNA into various cell lines, it has been possible to demonstrate that the intracellular portion of the GH receptor consists of separate functional domains. The proline-rich region of the GH receptor seems to be important for most of the biological effects mediated by the receptor, indicating that this part of the receptor interacts with, or regulates, a central signaling molecule. Indeed, there is evidence to suggest that this region of the GH receptor also binds or activates the Janus kinase 2. However, this proline-rich region is clearly not sufficient on its own to activate transcriptional signaling, since the GHR 1–454 is deficient in mediating GH transcriptional effects on both the insulin and the Spi 2.1 genes. A more detailed mapping of the C-terminal domain involved in the transcriptional signaling is under way. The identification of a single amino acid, phenylalanine 346, as being important for internalization suggests there is a rather short and specific internalization sequence in the GH receptor. The fact that this mutant is fully active in all other biological systems tested so far is indicative of the fact that hormone/receptor complex internalization may not be required for signal transduction.

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