Signal Transduction by the Growth Hormone Receptor (43745)

MICHAEL J. WATERS,*[,1] SCOTT W. ROWLINSON,* RICHARD W. CLARKSON,* CHANG-MIN CHEN,* PETER E. LOBIE,† GUNNAR NORSTEDT,† HICHEM MERTANI,‡ GERARD MOREL,‡ ROSS BRINKWORTH,* CHRISTINE A. WELLS,* STAN BASTIRAS,§ ALAN R. ROBINS,§ GEORGE E. MUSCAT,* AND ROSS T. BARNARD*

Departments of Physiology and Pharmacology, and Molecular Biology and Drug Design Centres, University of Queensland, 4072, Australia; Centre for Biotechnology,† Karolinska Institute, Novum, S-14152 Huddinge, Sweden; CNRS URA 1454,‡ Neuroendocrinology, Lyon-Sud School of Medicine, Oullins, France; and Bresatec Ltd.,§ Adelaide, South Australia, 5000, Australia*

Abstract. **It has been proposed that dimerization of identical receptor subunits by growth hormone (GH) is the mechanism of signal transduction across the cell membrane. We present here data with analogs of porcine GH (pGH), with GH receptors (GHR) mutated in the dimerization domain and with monoclonal antibodies to the GHR which indicate that dimerization is necessary but not sufficient for transduction. We also report nuclear uptake of GH both *in vivo* and *in vitro*, along with nuclear localization of the receptor and GH-binding protein (GHBP). This suggests that GH acts directly at the nucleus, and one possible target for this action is a rapid increase in transcription of C/EBP delta seen in 3T3-F442A cells in response to GH. This tyrosine kinase-dependent event may be an archetype for induction of other immediate early gene transcription factors which then interact to determine the programing of the subsequent transcriptional response to GH.** [P.S.E.B.M. 1994, Vol 206]

The growth hormone receptor (GHR) was the first of the cytokine receptors to be cloned (1). However, it was not until 1988 that we (2) and the Djiane/Kelly group (3) showed that the prolactin receptor was homologous, thereby defining the first members of this new class of cytokine receptors. Although the subsequent recognition of other members of this class by Bazan (4), Cosman *et al.* (5), and others raised the possibility of common mechanistic features for signal transduction within this new family, such features were not discernible from the primary sequences. It was not until the elucidation of the crystal structure of the human growth hormone (hGH) (extracellular GHR)$_2$ complex by De Vos *et al.* (6) that progress towards understanding the mechanism of signal transduction occurred. The novel realization that the hormone induces dimerization of two receptor subunits, and the proof that this occurs in solution (7) provided a means for transducing the hormone signal by dimerization of critical cytoplasmic regions. This mechanism accounts for the antagonistic activity of the G120R mutant of growth hormone (GH), demonstrated first in transgenic mice by Kopchick's group (8). From the crystal structure, it is clear that an arginine substitution for glycine at this location in the third helix would disrupt binding to tryptophan 104 of the second receptor subunit and hence ability to dimerize receptor subunits. The development of *in vitro* assays for GH based on the expression of a hGHR (extracellular region)/G-CSF hybrid receptor in FDC-P1 cells (9, 10) provided a system to test the hypothesis that hormone-induced receptor dimerization is the initial step in signal transduction. Using this hybrid receptor, Fuh *et al.* (9) were able to show that three monoclonal antibodies to the GHR supported FDC-P1 myeloid cell replication when bivalent, but not when univalent. Moreover, the G120R mutant was shown to be an hGH antagonist in this assay system and its antagonistic potency could be increased by raising Site 1 affinity with additional mutagenesis.

These observations led to the idea (9, 11) that hormone Site 1, mapped in the original alanine scanning

[1] To whom requests for reprints should be addressed at Department of Physiology & Pharmacology, The University of Queensland, Qld 4072, Australia.

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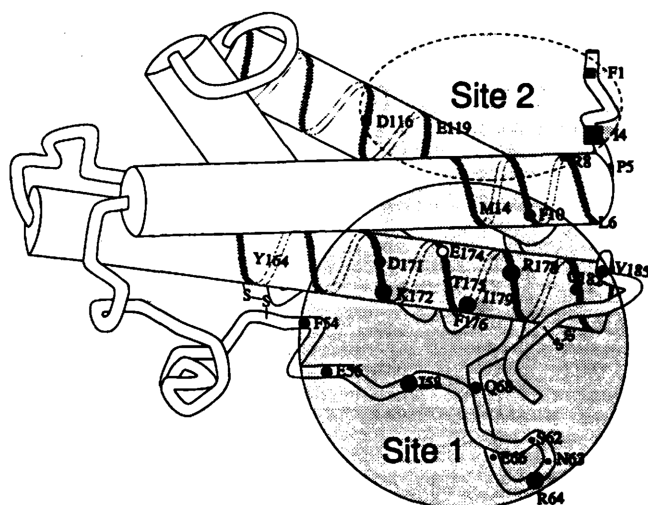

Figure 1. Site 1 and 2 of human GH as defined by alanine scanning mutagenesis (reproduced from Cunningham *et al.* [7] with permission).

mutagenesis studies of Cunningham and Wells (12), could be used to alter affinity as measured by Scatchard analysis, but could not be used to increase bioactivity. Site 2 on the other hand, was seen as the region promoting dimerization, hence bioactivity (Fig. 1).

Accordingly, a bell-shaped dose response curve is seen for GHR activation because at high GH concentrations all receptor molecules take part in Site 1 interaction with the hormone and none are then available to undertake dimerization via Site 2.

A similar mechanism has been imputed for the Nb2 prolactin receptor based on a bell-shaped dose response curve with hGH and the ability of G120R hGH to inhibit prolactin action (13). This same analog was used by Silva *et al.* (14) to support the hypothesis that receptor dimerization is needed by the human IM9 lymphocyte receptor for tyrosine kinase activation and tyrosine phosphorylation of a 93-kDa protein subunit. The role of third helix mutants is not restricted to antagonism of GH action, however, one such mutant has been found with significantly increased galactopoietic activity in cows (conversion of leucine 127 to valine L127V, [15]). Such mutants will be of obvious economic relevance.

Site 2 Alone Does Not Determine Bioactivity

Strictly equating Site 2 residues with modulation of biological function turns out to be incorrect. Our mutagenesis studies with porcine GH (pGH) mutants have identified a region of the molecule involved in binding to Receptor 1, located at the carboxy terminus of helix 1 (residues 30–34[2]). Identification of these residues stemmed from our earlier studies on the divalent cation dependence of hGH binding to rabbit GHR (16, 17), and we have recently shown that glu 34 and a nearby residue, asp 170, account for a large part of this cation dependence, partly because substitution of these human residues into pGH renders its binding strongly cation dependent.[3] It is clear from the crystal structure that hGH residues gln 30, glu 34 and asp 170 sit adjacent to residues glu 220 and glu 126, 127 of receptor 1, and that glu 126 and 127 are located in the four residue link region between receptor beta barrel domains 1 and 2. In pGH, the interacting hormone residues are lys 30, arg 34 and his 170, so there are no repulsive interactions with E126 and 127 and E220 of the receptor, but in human, E34 and D170 require neutralization by bound divalent ions.[3] Substitution of Position 30, 34 and 170 of pGH with glu (30, 34) or asp (170) leads to a small increase in affinity (K30E R34E pGH, 2.4-fold, Table I) or no change (H170D) when divalent ions are present, as expected.[3] However, the bioactivity of these mutants is markedly decreased in the FDC-P1 proliferation assay using cells stably transfected with rabbit GHR (Table I).

Table I. Receptor Binding and Bioassay Characteristics of pGH Mutants

Analogue	Affinity relative to wild type ($n \geq 3$, ±SEM)	Bioactivity relative to wild type ($n \geq 3$, ±SEM)
P6S	0.73 ± 0.05	0.49 ± 0.07 (2.0)
L16R	1.33 ± 0.18	0.72 ± 0.11
K30Q R34E	0.90 ± 0.23	0.20 ± 0.02 (5.0)
K30E R34E	2.38 ± 0.39	0.13 ± 0.01 (7.7)
E33K	2.10 ± 0.25	1.10 ± 0.18
H170D	0.81 ± 0.16	0.33 ± 0.07 (3.0)

This finding is unexpected in terms of the simple dimerization model of Fuh *et al.* (9) and can only be accommodated if electrostatic interactions in the link region, or the beta strand adjacent to E220 are influencing the angle of the dimerization domain (β barrel domain), hence the ability to dimerize. Since the α-helical link region was proposed by De Vos *et al.* (6) to be responsible for orientation of domain 1 and 2, a scissor effect (small movement in the link region manifesting as a large change in domain 2 orientation) is a plausible mechanism for such a decrease in hormone bioactivity.

A more striking departure from support for simple dimerization as the initiator of signal transduction is

[2] Using the numbering system of Abdel Meguid.

[3] Rowlinson SW, Barnard R, Bastiras S, Robins AJ, Senn C, Wells JRE, Brinkworth R, Waters MJ. Evidence for involvement of the carboxy terminus of helix 1 of GH in receptor binding: Use of charge reversal mutagenesis to account for the Ca^{2+} dependence of binding and for design of higher affinity analogues. Biochemistry (accepted).

provided by our results with monoclonal antibodies to the GHR. Every one of six monoclonal antibodies were either inhibitory or without effect on signal transduction through the full length rabbit GHR stably expressed in FDC-P1 cells. However, two of these (MAbs 263 and 43) were able to act as agonists with the FDC-P1 line expressing the GHR-GCSF receptor hybrid used by Fuh *et al.* (9) to demonstrate that three monoclonal antibodies (including 263) could act as agonists. Clearly with the homologous system, simple dimerization is insufficient to trigger signal transduction.

Effect of Mutagenesis of Residues in the Receptor Dimerization Domain on Signal Transduction

We therefore decided to determine the importance for signal transduction of residues within the dimerization domain by mutation of residues within that domain. As a bioassay we used CHO K1 cells transiently cotransfected with a pECE expression vector containing the rabbit GHR and with the c-*fos* promoter 5′ of a luciferase reporter gene in the pLUC expression vector.

Residues identified as contributing to association of dimerization domains in the GH (receptor)$_2$ complex (6) were mutated to alanines as a first step. Simultaneous alanine conversion of asparagine 144, serine 146, leucine 147, and threonine 148 (mutant 4A) had minimal effect on signal transduction, as did conversion of histidine 151 and aspartate 153 to alanines in addition to the 4A mutations. However, mutation of tyrosine 200 and serine 201 to alanines abolished signal transduction (mutant 4A-150A-200A). Scatchard analysis of these mutants demonstrated no significant difference in affinity of these mutants for hormone relative to wild type rabbit GHR (all values 1.5–1.7×10^{10} M^{-1}). Levels of expression were also similar. Accordingly, we conclude that interaction of residues between receptor dimerization domains is essential for signal transduction to the c-*fos* promoter.

Signal Transduction to the Nucleus

GH increases the transcription of a number of genes independently of IGF 1. Examples include Spi 2.1 (18, 19), c-*fos* (20), lipoprotein lipase (21), somatostatin and insulin (22), and cytochrome P450 11C13 (23). This process may be mediated through activation of plasma membrane associated (JAK2) tyrosine or ser/thr kinases, with some means of transmission of a phosphorylation signal to the relevant nuclear transcription factor. Alternatively, it is possible that GH enters the nucleus and generates a local transcriptional activation signal, either by direct interaction or by nuclear kinase activation through nuclear GHR. The homologous hormone, prolactin, is known to localize to the nucleus in T-lymphocytes, and this is required for interleukin 2 mitogenic effects (24). We present here data supporting a direct nuclear action for GH, although we acknowledge the likelihood of plasma membrane mediated nuclear actions such as those mediating γ-interferon actions, as well.

Nuclear Uptake of GH

Earlier, we showed that GH-binding protein (GHBP) is present in rabbit and rat nuclei by immunohistochemistry (IHC) and by direct nuclear isolation studies (25, 26). As a second step, we have now shown nuclear uptake of GH both *in vivo* and *in vitro*.

For the *in vivo* demonstration, 10 to 12-week-old Lewis dwarf rats were injected intracardially with 20 μCi of [^{125}I]recombinant bovine GH (bGH) ± 100 μg cold hormone, and killed over a subsequent 2-hr period. By quantitative electron microscopy, [^{125}I]label is first seen on the plasma membrane, then receptorsomes and nuclear membrane/matrix, and, finally, golgi and lysosomes. Maximal uptake into the nuclear fraction was seen at 30 min after injection, with some nuclear membrane localization seen as early as 5 min.

These *in vivo* studies are supported by *in vitro* studies with CHO cells expressing full length rat GHR or receptor deletion mutants. About 20% of internalized [^{125}I]hGH enters the nucleus, as determined by direct nuclear isolation, and this peaks around 50 min after hormone addition. Total internalization peaks later, around 100 min after [^{125}I]hGH addition. No nuclear uptake was seen in untransfected CHO cells, and none was seen in cells expressing the GHBP, emphasizing the need for membrane anchorage and/or an internalization consensus sequence for hormone uptake to the nucleus.

Surprisingly, we have recently been able to show that full-length GHR is located in the nucleus. This was accomplished by IHC using a polyclonal antibody raised against a cytoplasmic GHR/glutathione-S-transferase fusion protein expressed in the pGEX system. Localization was demonstrated at the EM level, and confirmed with receptor mutants expressed in CHO cells. Full length receptor expressed in CHO cells can be found in isolated nuclei, even after washing with 2% Triton X100 to remove all EM visualizable nuclear membrane. On the other hand, nuclear localization was not seen with a CHO line expressing a membrane anchored receptor deletion mutant truncated just below the membrane (at residue 255). This mutant provides a good control for microsomal membrane contamination of the nuclear fraction.

Accordingly, we can postulate a nuclear GH/GHR/GHBP system which may regulate gene transcription, presumably by local activation of JAK 2 or phospholipase C. We have explored the possibility that the GHBP interacts directly with promoter elements of GH responsive genes (Spi 2.1, *fos,* IGF 1),

but have been unable to demonstrate this by gel super shift or immunoprecipitation approaches.

In order to investigate this problem further, we have studied two transcription factors which are rapidly induced by GH. These two were identified in a scan of potentially GH regulated transcription factors in nuclear extracts of GH sensitive 3T3-F442A preadipocytes ± bGH treatment. [^{32}P]end-labeled oligo probes for the DNA binding sites of AP1, C/EBP, CREB, NF1, SIF, SRF, LFA-1 and EF2 were used in quantitative gel shifts to determine the ability of GH to induce specific DNA binding activities. We found that only AP1 and C/EBP binding activity increased in response to 40 ng/ml bGH when measured with the AMBIS scanner.

Since c-*fos* and c-*jun* are known to be induced by GH (27, 28), the increased AP1 activity was not unexpected. However, the increase in C/EBP was novel, although discovered independently in rat liver (29). We were able to show that C/EBP induction occurs within 30 min of GH addition and can be blocked by the tyrosine kinase inhibitor herbimycin A. Prolonged treatment with PMA blocked the response, indicating the phorbol ester sensitive C kinases are also involved. Phosphorylation of C/EBP is not the reason for the increased binding however, since gel shifts were unaffected by treatment of nuclear extracts with alkaline phosphatase. Induction could be blocked with cycloheximide, indicating *de novo* protein synthesis. Furthermore, we were able to show induction of only the delta isoform of C/EBP by GH in Northerns. Thus, transcription activation of the C/EBP gene is a very early nuclear event in GH action on 3T3 preadipocytes, and appears to involve a kinase transduction mechanism.

It is of interest that we have identified C/EBP elements in the promoter regions of 12 of 18 potentially or actually GH-responsive genes. Paquereau *et al.* (19) have shown that a C/EBP element is located within the Spi 2.1 promoter and is required for GH induction in liver cells. We find Spi 2.1, c-*fos* and c-*jun* promoter oligos give two bands in gel shifts with nuclear extracts from GH stimulated 3T3-F442A cells, and one of these is competed by C/EBP oligonucleotide. This band increases during GH induction of C/EBP in 3T3-F442A cells.

We propose, therefore, that a member of the C/EBP family is involved in the GH induction of many GH specific genes, and is induced by GH itself. It is likely that this immediate early transcription factor operates in conjunction with other such factors (e.g., AP1) in determining the program of the cellular transcriptional response to GH.

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