The membrane proximal region of the cytoplasmic domain of the growth hormone receptor is involved in the activation of Stat 3

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Abstract Growth hormone receptor (GHR) signaling involves activation of the Janus Kinases (Jak) and of Stat proteins (signal transducers and activators of transcription). Growth hormone (GH) induces transcriptional activation of c-*fos* gene and the c-sis inducible element (SIE) of its promoter was shown to bind the Stat proteins. Using cells co-transfected with GHR and Stat 3 expression vectors, we directly demonstrate that GH induces tyrosine phosphorylation of Stat 3 and its binding to the SIE probe. We showed, using mutant forms of GHR, that only the cytoplasmic membrane proximal domain of the receptor, including a conserved proline rich region (box 1), is required for this effect.

Key words: Growth hormone; c-*fos*; Stat 3; Cytokine receptor

1. Introduction

Growth hormone (GH) regulates the expression of many genes involved in growth, metabolism and cellular differentiation [1]. The first step of GH action is the binding of the hormone to its receptor (GHR) which belongs to the growth hormone/prolactin hematopoietic receptor superfamily [2]. Unlike receptor tyrosine kinases, receptors of this family do not have kinase domains and show only limited similarity in their cytoplasmic regions consisting in box 1 and box 2 motifs [3]. Binding of GH induces dimerisation of its receptor [4], activation of the GHR associated kinase Jak 2 [5], and initiation of a cascade of protein tyrosine phosphorylations [6]. Jak 2 is also involved in the signaling pathway of several receptors of the family (review in [7]). Recently a direct coupling pathway was discovered between cytokine receptors and transcription [8]. The 'direct effector' mechanism proposed comprises a rapid activation by membrane receptorassociated tyrosine kinase(s) of Stat transcription factors (signal transducers and activator of transcription) [9]. Thus, after dimerization, the Stat proteins are translocated to the nucleus where they bind to target nucleic acid sequences. In several cell lines, GH regulates the expression of the proto-oncogene c-*fos* [10]. Two responsive elements have been described in the c-*fos* promoter region: the serum response element (SRE) [11] and the c-sis-inducible element (SIE) [12]. Previous studies have shown that the SRE can mediate induction of c-*fos* by GH [13]. It has also been shown that transcriptional complexes containing proteins antigenically related to Stat 1 and Stat 3 were activated after GH stimulation, and were able to bind the SIE [14].

In this paper, we used CHO cells stably expressing GHR and COS-7 or 293 cells transiently cotransfected with GHR and Stat 3 [15] expression vectors to demonstrate: (i) that Stat 3 is part of the GH-inducible complex which binds to a SIE related probe m67 [12], (ii) that GH induces tyrosine phosphorylation of Stat 3. Furthermore, using CHO cells expressing mutated forms of GHR, we demonstrate that the amino-terminal half of the GHR cytoplasmic domain is sufficient for Stat 3 activation. In addition, the carboxy-terminal part of the receptor could play a role in the down regulation of the GH-induced transcriptional complex associated with the SIE probe.

2. Experimental

2.1. Construction of expression plasmids

Plasmids containing the wild type GHR (GHR_{wt}), the mutant GHR_{454} in pECE and the mutant $GHR_{4P/A}$ in pLM108 were described previously [16–18].

2.2. Cell culture and transfections

Establishement of CHO stable clones, binding assays and cell culture conditions for CHO, COS-7 and 293 cells were performed as described [17,19]. COS-7 cells, plated in 100 mm dishes, were transiently transfected at 70% confluence by a DEAE-dextran–chloroquine procedure [18] with 5 μg of expression vector containing rabbit GHR cDNA and 5 μg of expression vector containing Stat 3 or Stat 1 cDNA (a generous gift from Pr. J. Darnell).

2.3. Western blot analysis

The human 293 clone expressing Jak 2 has been transfected as described [19] with 4 μg of GHR_{wt} and 4 μg of Stat 3 expression vectors. Forty-eight hours after transfection, cells were stimulated 30 min with 20 nM hGH. Cell lysates were immunoprecipitated with anti-Stat 3 (Santa Cruz Biotechnology) antibody and then subjected to Western blot analysis using anti-pTyr (UBI) or anti-Stat 3 antibody [19].

2.4. Electrophoretic mobility shift assay (EMSA)

Confluent cells (COS-7 cells transiently transfected or CHO stable clones) were incubated with or without 20 nM hGH for various times. Cells were lyzed using hypotonic buffer (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1 mM Na_3VO_4, 20 mM NaF, 1 mM phenylmethylsulfonylfluoride, 5 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin) and nuclear extracts were prepared in high salt buffer (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM Na_3VO_4, 20 mM NaF, antiproteases).

The sequence of the high affinity c-sis element (SIE) m67 (12) used in the gel shift assay is: top strand, 5′-GTCGACATTTCCCGTAAATCGTCGA-3′ and bottom strand, 5′-TCGACGATTTACGGG-3′. The sequences of oligonucleotides (GAS, SRE, m34) used in competition experiments are the ones described [12,13,20]. Double-stranded

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Abbreviations: GH, growth hormone, hGH, human growth hormone; GHR, growth hormone receptor; Jak, Janus kinases; Stat, signal transducer and activator of transcription; SIE, c-sis-inducible element; SRE, serum-inducible element; SIF, c-sis-inducible factor; IFN, interferon; GAS, IFNγ activation site; PRL, prolactin; EGF, epidermal growth factor; PDGF, platelet derived growth factor; LIF, leukaemia inhibitory factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; p-Tyr, phosphotyrosine.

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probe was labeled using the Klenow fragment of *E. coli* DNA polymerase I. Nuclear extracts (8 to 10 μg) were incubated with 2 μg of poly (dI-dC) in 10% glycerol, 0.5 mM EDTA, 0.5 mM DTT, 10 mM HEPES-KOH pH 7.9. ^{32}P-labeled m67 probe (40,000 cpm, approximatively 0.5 ng) was added to the incubation for 20 min at room temperature. Fifty- and hundred-fold molar excess of unlabeled competitor oligonucleotide was added as indicated. For supershift experiments, incubations were carried out with 1 μl of anti-Stat 1 (Transduction Laboratories), anti-Stat 3 or 1 μg of anti-pTyr antibodies 20 min prior to the incubation with the labeled probe. The reaction was resolved on a 5% acrylamide gel containing 0.25 × TBE buffer for 2 h at 150 V. The gel was dried and exposed at −80°C to film (Kodak).

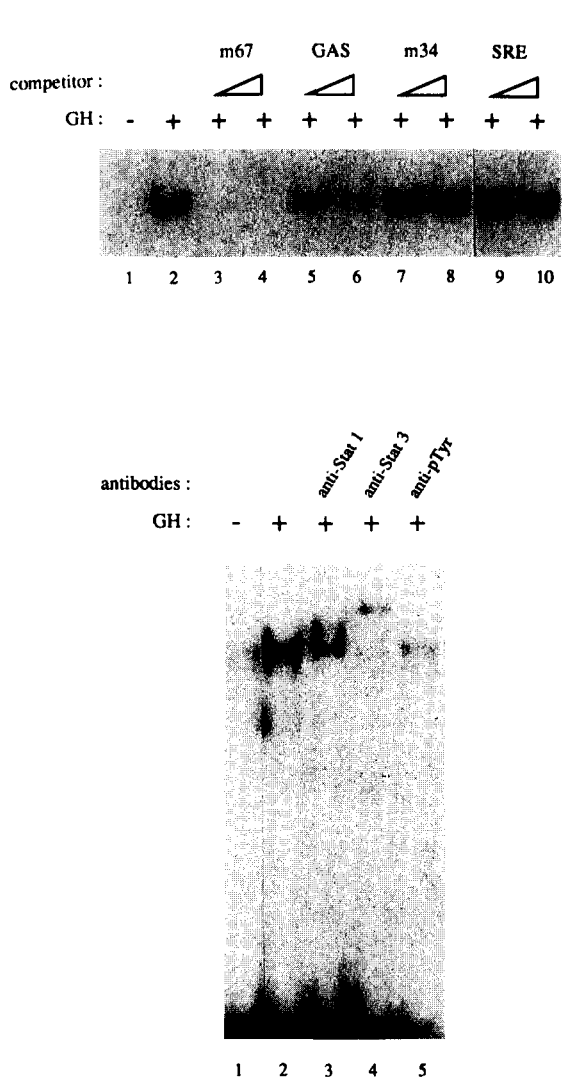

Fig. 1. GH induces a SIE-binding complex containing Stat 3. (A) CHO cells expressing GHR_{wt} were incubated for 30 min without (lane 1) or with 20 nM hGH (lanes 2–10). Electrophoretic mobility shift assays (EMSA) were performed using high affinity SIE labeled probe (m67) as described in section 2. For competition experiments, nuclear extracts were incubated with a 50-fold excess of unlabeled m67, GAS, mutated SIE sequence (m34) or SRE (lanes 3,5,7,9, respectively) or 100-fold excess (lanes 4,6,8,10) of the same probes. (B) Nuclear extracts from CHO cells expressing GHR_{wt} treated (lanes 2–5) or not (lane 1) with 20 nM hGH, were preincubated with anti-Stat 1 (lane 3), anti-Stat 3 (lane 4) or anti-pTyr (lane 5) antibody. EMSA were performed using m67 labeled probe.

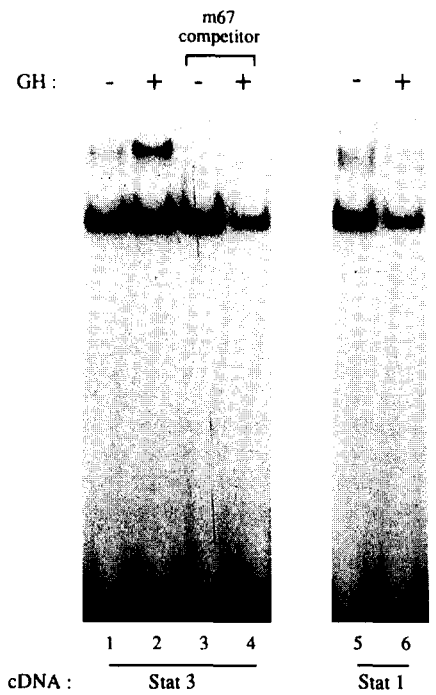

Fig. 2. Reconstitution of Stat 3 activation in COS-7 cells. COS-7 cells were transiently co-transfected with GHR and Stat 3 (lanes 1–4) or Stat 1 (lanes 5,6) expression vectors as described in section 2. Forty-eight hours after transfection, cells were treated (lanes 2,4,6) or not (lanes 1,3,5) with 20 nM hGH for 30 min. The binding of nuclear extracts to labeled m67 probe was performed with (lane 3,4) or without a 100-fold excess of m67 unlabeled probe.

3. Results

In order to analyse the GH-inducible SIE binding complex, gel-mobility shift assays were performed. Nuclear extracts from CHO cells stably transfected with the rabbit GHR_{wt} expression vector were incubated with a ^{32}P-labeled high affinity SIE oligonucleotide (m67). As shown in Fig. 1A, GH rapidly induced the appearance of a DNA-binding complex which was inhibited by an excess of unlabeled SIE competitor and to a lesser degree by an unlabeled GAS oligonucleotide derived from the Ly-6E gene. No competition was observed with a mutated SIE probe (m34) unable to bind c-sis inducible factors (SIF) or with a SRE probe, which was reported to bind proteins unrelated to the SIF complexes. Antibodies to Stat 1 and Stat 3 were used to determine the composition of the GH regulated m67 binding complex (Fig. 1B). Preincubation of the nuclear extracts with anti-Stat 3 antibody resulted in a supershift while an incomplete supershift was observed with anti-Stat 1 antibody, a reduced signal was seen with anti-pTyr antibody.

The presence of Stat 3 in the complex was further confirmed by experiments in which COS-7 cells were cotransfected with both GHR and Stat 3 cDNAs (Fig. 2). We clearly find under these conditions, a GH induced m67 binding complex, whereas only a constitutive complex was detected in co-transfection experiments with Stat 1 and GHR cDNAs. To investigate the ability of GH to induce tyrosine phosphorylation of Stat 3, 293 cells were cotransfected with GHR and Stat 3 cDNAs. Immunoprecipitations with anti-Stat 3 followed by Western blots with anti-pTyr or anti-Stat 3 antibodies demonstrated GH-

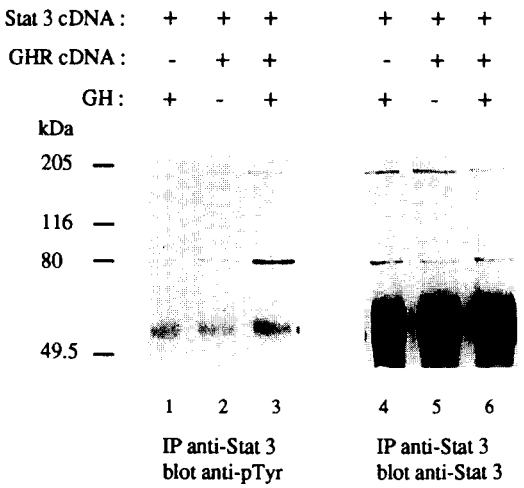

Fig. 3. GH induces tyrosine phosphorylation of Stat 3. A human 293 clone expressing Jak 2 has been transfected with 4 μg Stat 3 cDNA alone (lanes 1,4) or with GHR cDNA (lanes 2,3,5,6). Forty eight hours after transfection, cells were incubated 30 min with 20 nM hGH. Cell lysates were immunoprecipitated with anti-Stat 3 antibody and then subjected to Western blot analysis using anti-pTyr or anti-Stat 3 antibody.

induced tyrosine phosphorylation of Stat 3 (Fig. 3). No signal was detected in untransfected 293 cells (data not shown).

To identify the GHR domains involved in Stat 3 activation, different CHO cells expressing mutant forms of the GHR were used. As depicted in Fig. 3A, the mutant GHR_{454} lacks the C-terminal half of the cytoplasmic domain and the mutant $GHR_{4P/A}$ contains alanine substitutions of the four prolines in the conserved box 1 region. Scatchard analysis showed that the GH binding characteristics were equivalent (K_d values were respectively 0.45, 0.35 and 0.8 nM for CHO cells expressing GHR_{wt}, GHR_{454} and $GHR_{4P/A}$, while the number of sites ranged from 90,000 to 120,000 per cell). Western blots using anti-GHR antibodies also confirmed the expected size of the mutant receptors (data not shown). The m67 binding complexes formed with nuclear extracts from GHR_{wt} or from GHR_{454} expressing cells were similar in mobility and followed the same kinetics with a maximum binding at 15–30 min (Fig. 3B). However, it should be noted that the intensity of the complex observed with nuclear extracts from cells expressing the GHR_{454} was much higher than that observed with cells expressing the wild type receptor. In contrast, no DNA-binding complex was observed in cells expressing the $GHR_{4P/A}$ mutant. Supershift studies with the mutant GHR_{454} suggested that the complex also contained essentially Stat 3 (Fig. 3C).

4. Discussion

Part of cytokine signaling to the cell nucleus operates through recruitement of Stat molecules in the activated receptor complex. These factors are subsequently phosphorylated on specific tyrosine residues precluding their dissociation from the receptor complex, their homo or heterodimerisation and their migration to the nucleus where they bind specific sequences of cytokine responsive genes. The activation of Stat 1 and/or Stat 3 molecules has been shown to be promoted by several ligands: EGF, PDGF, LIF, IFN, GH, PRL [15,21–23], and this observation raised the question of ligand induced transcriptional specificity. Specificity could be due to differential expression of the proteins (such as receptors and Stat family members) in different cells types or to the involvement of other molecules that could add post transcriptional modifications to the Stats molecules or associate with them to form an active transcriptional complex. In fact, GH appears to activate a protein antigenically related to Stat 3 in liver (24), in 3T3 F442A preadipocytes [14] but not in IM-9 cells [25]. In this study, we directly show, using the cDNA encoding Stat 3, that the Stat 3 protein is activated by GH in COS-7 cells. In contrast, the involvement of Stat 1 in our experimental conditions is less clear: we observe

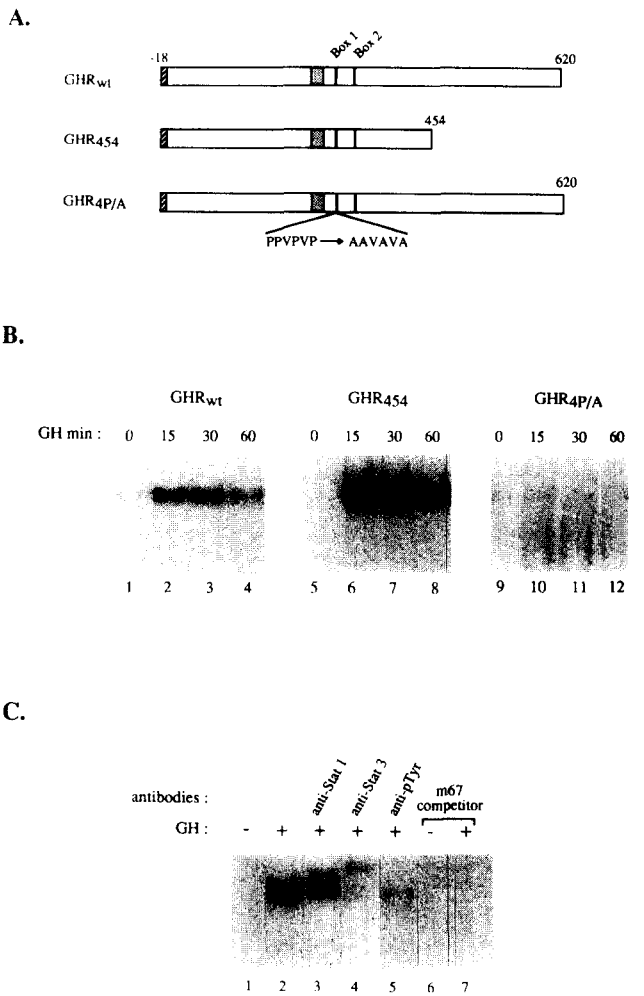

Fig. 4. Activation of m67-binding complex by GHR mutants. (A) Schematic representation of wild type and mutated forms of GHR. Mutant forms of the receptor are shown below the wild type (wt); numbers indicate amino-acid (aa) residues. Residue 1 is the first aa of the mature protein. Boxes 1 and 2 are indicated as solid lines, the peptide signal sequence as a hatched rectangle, and the transmembrane domain as stippled rectangle. The four proline residues substituted in the box 1 region (17) of the $GHR_{4P/A}$ mutant are indicated. (B) Kinetic characteristics of m67-binding complexes induced by GH in CHO cells expressing wild type and mutant forms of GHR. CHO clones were stimulated or not for the indicated times with 20 nM hGH. Then the nuclear extracts were subjected to EMSA using m67 labeled probe. (C) The m67-binding complex induced by GH in CHO cells expressing GHR_{454} contains Stat 3. Gel shift experiments were performed with nuclear extracts from CHO cells expressing GHR_{454} stimulated or not with 20 nM hGH for 30 min. For competition experiments, nuclear extracts were incubated with a 100-fold excess of m67 unlabeled probe.

in CHO cells a slight supershift of the m67 binding complex with anti-Stat 1 antibodies and a partial competition with the GAS probe, but no GH-inducible complex in COS-7 cells co-transfected with Stat 1 and GHR cDNAs. We thus hypothesize that Stat 1 can probably not form homodimers in response to GH stimulation but could participate in the formation of heterodimers with Stat 3.

The analysis of GH signaling with different mutant forms of GHR allowed us to identify the region of the receptor required for the activation of Stat 3. The mutant $GHR_{4P/A}$ has been previously shown to be unable to activate Jak 2 [26] and unpublished personal observation). We show, in the present experiments, that this mutant is also unable to activate Stat 3, since no m67 binding complex was detected in cells expressing this modified form of the receptor. In contrast, the GHR_{454} mutant was fully able to induce GH dependent activation of Stat 3 as well as phosphorylation of Jak 2 and MAP kinase activation [17]. We conclude that the membrane proximal region of the GHR, containing the box 1, is essential and sufficient for Stat 3 activation and that this activation occurs likely in a Jak 2 dependent pathway. This finding is in agreement with recent observations for GH [27] and GM-CSF [28] activation of Stat 1 and Stat 5 in Baf3 cells. It is also consistent with the 'direct effector' model in which the Stat proteins are directly activated by membrane receptor tyrosine kinase complexes. A Stat 3-specifying YXXQ motif has been recently identified as a specific target of the Stat 3 SH2 domain in the intracellular region of gp130 or of the LIF receptor [29]. This sequence is not present in the GHR and we previously showed that in our system, the GHR_{454} mutant was not or very weakly tyrosine phosphorylated [17]. So, Stat 3 does not seem to bind to the GHR through a phosphorylated tyrosine. Specific sites for Stat 3 recruitement could be present in other proteins associated with the receptor complex. The Jak 2 kinase contains the YXXQ sequence and could perhaps serve as a docking molecule for Stat 3 in GHR signaling.

The increased intensity of the m67-binding complex for the GHR_{454} mutant compared to the signal observed with the wild type receptor is puzzling. Both CHO clones have comparable binding characteristics and similar levels of Jak 2 activation. It could be possible that GHR_{454} lacks a critical domain for the binding of molecules involved in negative regulation of Stat 3. We have previously shown that C-terminal tyrosines are preferentially phosphorylated after GH binding [17], one of these could be involved in the interaction with the SH2 domain of such regulatory proteins. A negative regulatory domain for Stat 5 activation has also recently been reported in the carboxy-terminal region of the GM-CSF receptor [28].

Our results clearly show that the induction of Stat 3 binding to m67 by GH is mediated by the proximal region of the cytoplasmic domain of GHR, but additional molecules (such as a phosphatase) might be associated to the C-terminal region of the receptor and could down regulate the binding activity of Stat 3 to SIE. This hypothesis is currently under investigation.

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