Growth hormone induces insulin-like growth factor-I gene transcription by a synergistic action of STAT5 and HNF-1α

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Received 4 December 1998; received in revised form 11 January 1999

Abstract Salmon insulin-like growth factor-I (sIGF-I) expression is, as in mammals, induced by growth hormone (GH). To elucidate the mechanism by which GH stimulates the transcription of the IGF-I gene, we transiently transfected Hep3B cells expressing the rat GH receptor with a sIGF-I promoter-luciferase reporter construct. Activation of the construct by GH added to the medium of the transfected cells was observed when two specific transcription factors, STAT5 and HNF-1α, were simultaneously overexpressed in these cells. This finding demonstrates for the first time a GH-dependent activation of an IGF-I promoter construct in an immortalized laboratory cell line.
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Key words: IGF-I; Transcription activation; GH; STAT5; HNF-1

1. Introduction

Growth hormone (GH), a pleiotropic hormone secreted by the adenohypophysis, exerts a variety of effects on growth, development and intermediary metabolism [1,2]. The growth-promoting actions of GH, according to the longstanding somatomedin hypothesis, are mediated by insulin-like growth factor-I (IGF-I), which is secreted into the circulation by the liver as a target of GH action [3]. The existence of this hypothetical GH-IGF-I axis has been confirmed in numerous experiments since then. A single intraperitoneal injection of GH rapidly stimulates hepatic IGF-I gene transcription in vivo in hypophysectomized rats [4]. In cell cultures, it has been shown that GH strongly stimulates the transcription of the two promoters of the mammalian IGF-I gene in primary hepatocytes [5,6], as well as in Ob1771 mouse adipocytes endogenously expressing IGF-I, of which the secreted IGF-I probably serves local auto- and paracrine purposes [7,8].

Good progress has recently been made in the unraveling of the signal transduction pathway triggered by GH binding to its specific membrane-bound receptor (GH-R). Following the interaction between GH and GH-R, the homodimerized receptor associates with and specifically activates the intracellular tyrosine protein kinase JAK2 [9]. Among other effects, stimulation of JAK2 results in the tyrosyl phosphorylation of the GH-R and JAK2 itself, along with several members of the signal transducer and activator of transcription (STAT) family of transcription factors, STAT1, STAT3 and STAT5 [10–13]. Upon phosphorylation, the STATs dimerize and translocate from the cytosol to the nucleus, where they recognize and bind to short palindromic γ-interferon-activated sequence (GAS)-like DNA elements, thereby activating the transcription of specific genes. These new insights have resulted in the identification of specific GH response elements in a number of genes. STAT1 and STAT3 have been found to be involved in the GH-induced stimulation of the c-*fos* gene promoter [10,14], while STAT5 is involved in the enhancement of transcription of the β-casein gene by prolactin [15], and of the serine protease inhibitor 2.1 (Spi2.1) [16,17] and insulin [18] genes by GH. Although the role of GH as the strongest secretagogue of IGF-I is undisputed at present, the mechanism by which GH succeeds in stimulating the transcription rate of the IGF-I gene is still unresolved.

In salmon, as in mammals, liver IGF-I mRNA and protein levels become elevated after GH administration [19–21]. Since the liver is the main endocrine source of circulating IGF-I, studies on regulation of IGF-I expression have mainly been focused on the role of liver-specific and liver-enriched transcription factors. Hepatocyte nuclear factor-1α (HNF-1α), a liver-enriched transcription factor, was found to bind to and strongly stimulate the activity of the salmon IGF-I gene promoter [22], as has also been described for the homologous IGF-I promoter P1 in the human IGF-I gene [23]. Nevertheless in salmon, as in mammals, the molecular link between GH action and IGF-I upregulation has not been elucidated to date. Dissection of the molecular mechanism involved is hampered by the fact that IGF-I promoter activation by GH is not observed in tumor-derived, de-differentiated cells in culture after transfection. This even holds true for cultured cells still able to endogenously express IGF-I in significant amounts (e.g. SK-N-MC and OvCar-3 cells). Possibly, these laboratory cell lines lack sufficient amounts of GH-R or other components of the GH signal transducing system to effectuate upregulation of the activity of the IGF-I promoter, be it endogenous or brought into the cells by transfection. In the present study, we tried to reconstitute the GH signaling pathway leading from the GH-R to the IGF-I promoter in cultured hepatoma-derived Hep3B cells and to identify the components needed for GH-dependent promoter activation.

2. Materials and methods

2.1. Recombinant DNA constructs

The sIGF-I reporter construct p261M is derived from constructs

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Abbreviations: GAS, γ-interferon-activated sequence; GH, growth hormone; GH-R, growth hormone receptor; HNF, hepatocyte nuclear factor; IGF, insulin-like growth factor; JAK, Janus kinase; Luc, luciferase; MEM, modified essential medium; Spi, serine protease inhibitor; STAT, signal transducer and activator of transcription; TK, thymidine kinase

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PII: S0014-5793(99)00064-2

p385D and p105M, which have been described previously [22]. The *Hin*dIII-*Pvu*II fragment of p385D was cloned into *Hin*dIII/*Pvu*II digested p105M, resulting in a promoter construct harboring the *Hin*dIII to *Mst*I fragment of the sIGF-I promoter. The sIGF-I p163M reporter construct was made by polymerase chain reaction (PCR) amplification of the region between positions −122 to +41 relative to the transcription start site. To this end, we made use of a forward oligonucleotide (5′-CGTTATTTAAGCTTGTGCCCAAAATCCT-TAATGAATAATTTAGG-3′), extending from positions −122 to −90 and containing a 5′-anchor sequence with a *Hin*dIII site (underlined), together with a reverse oligonucleotide (5′-GGCGTCTT-CCATGGCAGGCTCGTTTTGG-3′) between positions +27 to +41 with a 5′-anchor sequence containing a *Nco*I site (underlined). The PCR product was cloned into the *Hin*dIII/*Nco*I digested luciferase reporter plasmid. The constructs were checked by dideoxy-sequencing (Pharmacia T7 sequencing kit, Amersham Pharmacia Biotech, Uppsala, Sweden). The reporter construct 6×Spi-TK-Luc used as a positive GH response control in transient transfection experiments has been described previously [24]. Briefly, it contains six tandem repeat copies of the 45 bp GHRE-II element of the Spi2.1 gene promoter, extending from −145 to −102, fused to a minimal basal TK promoter (−104 to +51) and cloned in front of the firefly luciferase reporter gene. The expression plasmid encoding full-length rat GH-R, prRXba 6.5, was constructed by cloning the *Bgl*I/*Bam*HI cDNA fragment into the *Spe*I/*Xba*I site of pcDNA I (Invitrogen, Leek, The Netherlands). The 6×Spi-TK-Luc and prRXba 6.5 constructs were kindly made available to us by Dr. G. Norstedt (Stockholm). As an internal control for transfection efficiency a plasmid containing the Rous Sarcoma Virus promoter and enhancer directing the expression of the β-galactosidase reporter gene (RSV-LacZ) was co-transfected with all constructs. The expression plasmids encoding full-length sheep STAT5 (pXM-MGF STAT5, [15]) and mouse hepatocyte nuclear factor-1α (CMV-HNF-1α, [25]) were kindly provided by Dr. B. Groner (Freiburg) and Dr. G.R. Crabtree (Stanford), respectively.

2.2. Cell culture and transfection

The human hepatoma-derived cell line Hep3B (ATCC HB 8064) was cultured in α-modified essential medium (α-MEM), supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 mg/ml streptomycin and 300 mg/ml glutamine. The cells were grown at 37°C in 5% CO_2. Hep3B cells were transfected using the calcium phosphate coprecipitation method [26] at 45–50% confluence in 6-well plates. Cells were transiently transfected with 4 μg of the reporter construct, 800 ng of the expression vector encoding rat GH-R, and (unless stated differently) with 500 ng of the expression vector encoding full-length sheep STAT5 and 400 ng of the mouse HNF-1α expressing construct. To correct for variations in transfection efficiency, 200 ng of RSV-LacZ were included in each transfection. The total amount of DNA added to the cells was kept constant at 6 μg by the addition of carrier DNA. Four h after addition of the precipitate, cells were shocked in 10% dimethyl sulfoxide in serum-free α-MEM for 2 min. Fresh serum-free α-MEM supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin, 300 mg/ml glutamine and with or without 400 ng/ml human GH (Norditropin, Novo Nordisk, Copenhagen, Denmark) was added. The cells were harvested 16 h later, washed in phosphate-buffered saline and incubated for 15 min in 300 μl of lysis buffer (100 mM potassium phosphate buffer pH 7.8, 8 mM $MgCl_2$, 1 mM dithiothreitol, 15% glycerol, 1% Triton X-100). Luciferase measurements and β-galactosidase assays were performed essentially as described [27,28]. Briefly, cellular debris was removed by centrifugation at 13 000×*g*. Luciferase activity of 100 μl of clear supernatant was measured after addition of 100 μl of 0.5 mM luciferin (Sigma, St. Louis, MO, USA) in lysis buffer supplemented with 0.5 mM ATP. Peak light emission was recorded on a LUMAC/3M Biocounter M2010A. β-Galactosidase activity of 30 μl of the clear lysate was measured after addition of 3 μl 100×Mg solution (0.1 M $MgCl_2$, 4.5 M β-mercaptoethanol), and 66 μl of 1×ONPG (4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside in 0.1 M sodium phosphate, pH 7.5). After incubation at 37°C (usually 30 min to 1 h) and addition of 500 μl of Na_2CO_3, the intensity of the yellow color was determined by its optical density at 420 nm.

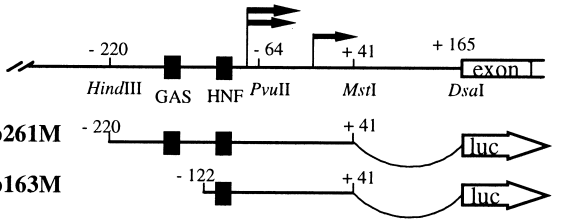

Fig. 1. Schematic representation of the sIGF-I promoter constructs p261M and p163M. The top part represents the genomic organization of the 5′-untranslated region and first exon of the sIGF-I gene. The double arrow indicates two transcription start sites which are situated very close to each other. The single arrow indicates the downstream transcription start site, to which position number +1 in the sequence has been assigned. Black boxes represent the reported HNF-1 site and the GAS-like consensus sequence.

3. Results

3.1. Overexpression of STAT5 is not sufficient for GH-dependent activation of the sIGF-I promoter

The effect of GH on the promoter of the sIGF-I gene was studied in Hep3B cells transiently transfected with sIGF-I promoter-luciferase constructs (Fig. 1). Since Hep3B cells lack significant levels of the GH-R, an expression plasmid encoding the full-length rGH-R was brought into the cells together with the promoter constructs by co-transfection. After transfection, cells were incubated in the presence or absence of 400 ng/ml GH for 16 h, after which the cells were lysed and luciferase activities in the lysates were measured. As a control for GH activation the construct 6×Spi-TK-Luc, containing six tandem repeat copies of the GH responsive element of the Spi2.1 gene in front of the TK promoter [24], was used. As can be seen in Table 1, GH treatment of Hep3B cells transiently transfected with the 6×Spi-TK-Luc construct resulted in about 8-fold activation of the TK promoter. However, no activation by GH was observed for the sIGF-I construct p261M under identical experimental conditions. Since the 6×Spi-TK-Luc construct contains six copies

Table 1
Effect of GH on promoter activity of the 6×Spi-TK-Luc control plasmid and the p261M sIGF-I reporter construct

	6×Spi-TK-Luc		p261M sIGF-I	
	−GH	+GH	−GH	+GH
No factor	1.00 ± 0.13	7.87 ± 2.33	1.00 ± 0.09	1.09 ± 0.22
STAT5	3.22 ± 0.76	388.52 ± 7.11	1.04 ± 0.20	1.39 ± 0.24
HNF-1	1.41 ± 0.01	3.05 ± 1.04	15.16 ± 0.71	18.06 ± 1.84
STAT5/HNF-1	3.61 ± 0.06	386.69 ± 34.38	15.15 ± 2.82	35.91 ± 3.51

Luciferase values measured for the basal promoter activities were in the range of 7500 light units for the 6×Spi-TK-Luc construct and 600 light units for the sIGF-I construct. The background value of the luciferase assay without addition of lysate is approximately 15 light units. The data represent mean ± S.E.M. values of two independent duplicate experiments for the sIGF-I promoter construct and one duplicate experiment for the 6×Spi-TK-Luc construct.

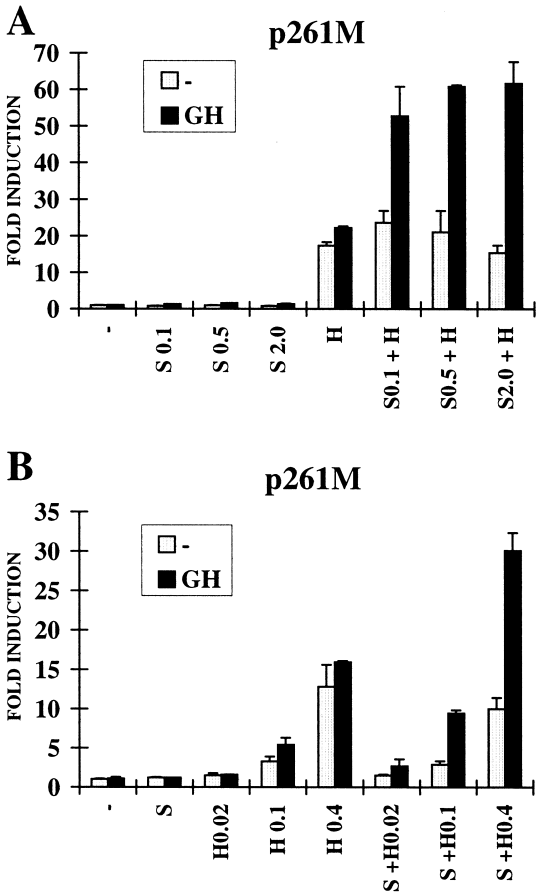

Fig. 2. Effect of GH treatment in Hep3B cells transiently transfected with the p261M sIGF-I promoter construct, the rGH-R construct, and varying amounts of expression plasmids encoding STAT5 (panel A) and HNF-1α (panel B).The basal promoter activity of p261M was set at 1. The data in A and B represent the mean ± S.E.M. values of two independent experiments performed in duplicate.

of the GH response element and thus may be more sensitive to GH treatment, we tried to enhance the sensitivity of the system by overexpressing STAT5 in the transfected cells. STAT5 overexpression caused the 6×Spi-TK-Luc construct to be activated much more efficiently by GH, the 8-fold induction being enhanced about 50 times to levels of around 400-fold activation (Table 1). Even in a system with such high sensitivity for GH, no significant activation of the sIGF-I promoter construct by GH was observed (Table 1).

3.2. GH-dependent activation of the sIGF-I promoter by co-expression of STAT5 and HNF-1α

sIGF-I promoter activity is known to be stimulated by the liver-specific transcription factor HNF-1α [22]. Since efficient promoter activation often requires the synergistic effect of two or more transcription factors, we tested whether simultaneous overexpression of STAT5 and HNF-1α resulted in sIGF-I promoter activation to levels higher than those observed with HNF-1α alone. In the presence of both HNF-1α and STAT5, GH-dependent activation of the sIGF-I promoter was indeed observed. In the absence of GH, the p261M sIGF-I promoter construct (Fig. 1) is activated about 15-fold by HNF-1α alone, and not by STAT5 alone. The combination of STAT5 and HNF-1α also resulted in 15-fold activation of the sIGF-I promoter, as observed with HNF-1α on its own. When GH was added to the medium of the transfected cells, the individual effects of STAT5 and HNF-1α separately did not change significantly, but their combined presence resulted in about 35-fold activation of the sIGF-I construct (Table 1). From this we conclude that the activity of the sIGF-I promoter can be induced by GH in cultured transformed cells after reconstruction of the GH signaling system by co-expression of the GH-R, STAT5 and the liver-specific transcription factor HNF-1α.

As described previously, the level of activation of the sIGF-I promoter by HNF-1α is rather variable, ranging from 16- to 34-fold [22]. This variation seems to be caused by slight differences in cell density and growth rate of the cells at the time of transfection. However, within a single experiment further 2- to 3-fold activation by GH was always observed, to levels of 30 to 60 times higher than basal sIGF-I promoter activity. As can be seen in Fig. 2A, the maximal activation by GH is already observed when 0.1 μg of STAT5 expression vector was added to the transfection mixture, and essentially the same results were obtained using 0.4 or 2.0 μg of STAT5 expression plasmid. An independent experiment testing the effect of the level of HNF-1α expression shows that sIGF-I promoter activity increases with the amount of HNF-1α vector in the transfection mixture, ranging from 0.02 to 0.4 μg (Fig. 2B). Higher amounts of HNF-1α expression vector did not result in significantly higher activation.

3.3. A STAT5 recognition element is present in the GH responsive construct

The GH-dependent potentiation of the activation of the sIGF-I promoter by HNF-1α in the presence of STAT5 suggests that a STAT binding element is present in the promoter. Since the p261M construct still shows this effect, the putative binding site should be located within the 261 bp fragment of the promoter present in p261M (Fig. 1). A GAS-like consensus sequence (TTCTAAGAA) is indeed found within this part of the promoter at positions −159 to −151, 50 bp upstream of the HNF-1 binding site. In order to discriminate whether the region responsible for the GH-dependent activation via

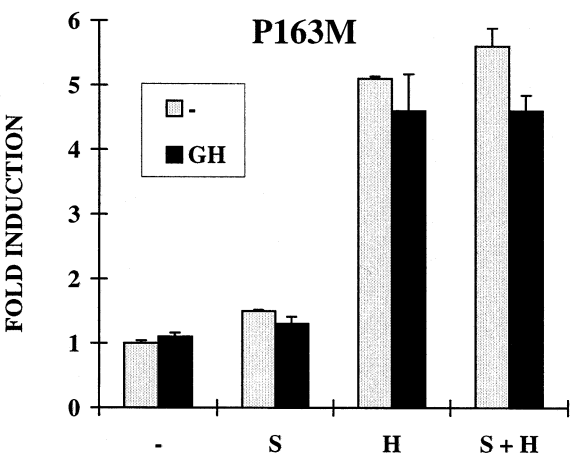

Fig. 3. Effect of GH treatment of Hep3B cells transiently transfected with the p163M salmon IGF-I promoter construct, the rGH-R construct, and expression plasmids encoding STAT5 and/or HNF-1α. The activation values represent the mean ± S.E.M. of two independent duplicate experiments.

STAT5 is indeed located upstream of the HNF-1 site in p261M, we constructed a reporter plasmid, p163M, which lacks the promoter region between positions −220 to −123 (Fig. 1). Thus p163M includes the HNF-1 binding site (−109 to −97) and the sequence downstream of this site to the *Mst*I site (+41), but not the GAS-like element (−159 to −151). HNF-1α is still able to activate the transcription rate of the p163M construct, but neither in the absence nor in the presence of GH further activation was found when the cells were co-transfected with the STAT5 encoding plasmid (Fig. 3). From this we conclude that in addition to the HNF-1 binding site a second element is involved in GH-dependent stimulation of the sIGF-I promoter, and that the STAT binding consensus sequence at positions −159 to −151 relative to the transcription start site is the most likely candidate for this role.

4. Discussion

The DNA fragment upstream of the ATG start codon of the sIGF-I gene has been shown to possess promoter activity [29]. As in other IGF-I promoters, no TATA or CAAT box-like elements have been detected in the sIGF-I promoter region, which may be the cause of the heterogeneous initiation of transcription and the low basal activity observed for the IGF-I promoters [30,31]. Multiple transcription start sites have been identified in the sIGF-I gene [29], the three most prominent of which are located within the first 250 nucleotides upstream of the ATG start codon (see Fig. 1). RNA analysis has revealed that the level of preproIGF-I mRNA is increased 6-fold in the liver of salmon injected with bovine GH [20]. The mechanism by which GH regulates IGF-I expression has been elusive, and the effects of GH observed in intact animals and in primary hepatocytes could not be reproduced using promoter constructs in transfected tumor-derived cells in tissue culture. New insights into the signal transduction pathways of the GH stimulus, however, have resulted in the identification of GH response elements in a number of genes, of which the Spi2.1 gene is one of the most widely studied and most GH sensitive examples. A 45 bp GH responsive element has been identified in the 5′ flank of this gene [32]. Reporter constructs containing six to eight repeats of the 45 bp element linked to a heterologous promoter (e.g. the thymidine kinase (TK) promoter), are clearly sensitive to GH regulation, especially in cells stably transfected with a GH-R encoding expression construct [32,33]. This sensitivity is mediated by STAT5, binding synergistically to two GAS-like elements within the 45 bp fragment [34]. In view of these findings, we tested whether combined overexpression of GH-R and STAT5 in Hep3B cells would lead to induction of responsive promoters by GH. For the 6×Spi-TK-Luc construct, harboring six copies of the 45 bp GH response element, this was indeed the case. In contrast, the activity of the sIGF-I promoter was hardly influenced by GH even under these conditions. However, by concomitant expression of HNF-1α we were able to demonstrate for the first time a distinct stimulating effect of GH on a sIGF-I promoter construct. The already rather strong activation caused by HNF-1α was enhanced 2- to 3-fold by GH stimulation in STAT5 overexpressing cells. The need for synergistic binding of transcription factors to two or more sequence elements in order to activate a promoter is frequently observed. In fact, the natural promoter of the Spi2.1 gene, harboring only one copy of the 45 bp GH response element, is also activated only very moderately by GH in a transient transfection system. Here, a second element, the so-called GAGA box at about 60 nt downstream of the STAT5 binding sites, seems to play an important role in vivo. It has been found that a high level of Spi2.1 gene transcription correlates with hypersensitivity of the promoter to DNaseI and maximal occupancy of the GAGA box. Based on these and other findings, a mechanism of Spi2.1 gene activation has been suggested in which GH-dependent chromatin remodeling caused by the recruitment of GAGA box binding proteins is the first compulsory and presumably predominant step [34]. A similar mechanism may well play a role in GH-induced activation of the sIGF-I gene promoter. Conceivably, HNF-1α and possibly other (even by itself non-stimulating) members of the HNF-1 family may bind to the response element in the promoter, and thereby enable GH-activated STAT5 to also bind to its response element. Such a model could explain the observed rapid stimulation of transcription of the sIGF-I gene in the liver upon injection of GH into the intact animal, since the activation of STAT5 by JAK2 in the hepatocytes is an immediate response to the GH signal.

The GH-IGF-I axis is a well conserved feature in vertebrate evolution, suggesting that the factors involved in this regulatory mechanism are equally well conserved. Although our studies were performed using mouse HNF-1 and sheep STAT5 in a human liver-derived cell line, studies on the corresponding transcription factors in fish indicate that both factors are indeed highly conserved during evolution. The Atlantic salmon (*Salmo salar L.*) HNF-1 cDNA clone shows high conservation with respect to other species of the homeo domain, the POU domain, and the dimerization domain. Of the three transcription activation domains, two out of three (ADI and ADIII) are also conserved, albeit to a lesser extend. The salmon HNF1 protein is able to bind specifically and with equivalent affinities to the rat and salmon albumin promoters [35]. A putative STAT molecule has been identified in the channel catfish (*Ictalurus punctatus*), and binds to the mammalian interferon-gamma activation site, a known motif of mammalian STAT binding [36]. From these and other studies, STAT molecules were concluded to have been highly conserved in vertebrate evolution.

Acknowledgements: The authors wish to thank Drs G.R. Crabtree, B. Groner, and G. Norstedt for their kind gifts of recombinant DNA constructs used in this study. This work was supported in part by a grant from the HCM Programme of the European Commission (ERBCHRX-CT94-0556).

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