The regulatory mechanism by which interleukin-6 stimulates GH-gene expression in rat GH3 cells

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Abstract

The present study was performed to elucidate the effect of interleukin (IL)-6 on the human GH (hGH)-gene expression in GH3 rat pituitary tumor cells using stable transfection of the hGH promoter fused to a luciferase reporter gene. Our results showed that IL-6 (10^2 – 10^4 U/ml) stimulated GH secretion and synthesis, and promoted the luciferase expression in stably transfected GH3 cells with the maximal action of 1.99 times above the control by 10^4 U/ml IL-6. Among the inhibitors of signaling transduction pathways, mitogen-activated protein kinase kinase (MAPKK/MEK) inhibitor PD98059 (40 μ M) and p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 (5 μ M) completely blocked the stimulatory effect of IL-6. Western blot analysis demonstrated that IL-6 indeed increased the

activation of phosphorylated MEK and p38 MAPK in GH3 cells. Neither overexpression of Pit-1 nor inhibiting Pit-1 expression affected IL-6 induction of hGH-promoter activity. To identify the DNA sequence that mediated the effect of IL-6, six deletion constructs of hGH promoter were created. The stimulatory effect of IL-6 was abolished following deletion of the –196 to –132 bp fragment. In conclusion, our data show that IL-6 promotes GH secretion and synthesis by rat pituitary GH3 cells. The stimulatory effect of IL-6 on hGH-gene promoter appears to require the activation of MEK and p38 MAPK, and a fragment of promoter sequence that spans the –196 to –132 bp of the gene, but may be unrelated to Pit-1 protein.

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Introduction

Evidence accumulated over the past decades supports the existence of a neuroimmunoendocrine network. Neuro-immunoendocrine systems interact to maintain physiological homeostasis during inflammation and stress response. The function of endocrine tissue, such as the regulation of the hypothalamic–pituitary–adrenal axis (HPAA), synthesis, and secretion of hormones by the pituitary gland, is influenced by cytokines produced mainly by immunocytes (Chesnokova & Melmed 2002, Haddad *et al.* 2002). For example, a number of studies have demonstrated that interleukin (IL)-2, IL-11, ciliary neurotropic factor (CNTF), leukemia-inhibitory factor, transforming growth factor- β (TGF- β), and tumor necrosis factor- α (TNF- α) stimulate or inhibit growth hormone (GH) secretion by the pituitary gland (Arzt *et al.* 1999, Castro *et al.* 2000). Our studies recently showed that interferon- γ (IFN- γ), IL-1 β , IL-11, CNTF and TGF- β not only regulated GH secretion, but also GH synthesis (Gong *et al.* 2003a, 2003b, 2005). Furthermore, we also found the mechanism by which IFN- γ and IL-1 β promoted GH-gene expression in rat pituitary GH3 cells may be different from the classical growth hormone-releasing hormone (GHRH)-induced regulatory mechanism. The action of GHRH is

mediated mainly through the intracellular cAMP–protein kinase A (PKA) signaling pathway. Pit-1, a pituitary-specific transcription factor, plays a pivotal role in promoting the transcription of GH gene by binding to its two recognition sequences (–131 to –106 and –93 to –66 bp) within the GH-gene promoter (Bluet-Pajot *et al.* 1998), whereas the stimulatory effect of IL-1 β appears to require the intracellular mitogen-activated protein kinase kinase (MAPKK/MEK), p38 mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3-K)-dependent signaling pathways and the promoter sequence that spans the –196 to –132 bp of the gene, but is unrelated to Pit-1 protein (Gong *et al.* 2005).

IL-6 is the end product of a cytokine signaling cascade secreted by specialized immune cells in the immune processes and in the host response to infection. It has a great influence on many functions, including differentiation, stimulation, and activation of immune cells, or other cells of neuroendocrine origin. Recently, it has also been shown to affect hormone secretion, acting at different levels of HPAA (Arzt 2001, Gautron *et al.* 2003). A considerable number of studies report that IL-6 receptors (IL-6R) are expressed in rodent and human pituitary cells (Shimon *et al.* 1997, Bethin *et al.*

2000, Hanisch *et al.* 2000). The production of IL-6 protein and mRNA expression by normal rat anterior pituitary cells and human pituitary adenomas have also been demonstrated by several groups (Spangelo *et al.* 1990, Jones *et al.* 1994, Velkeniers *et al.* 1994). IL-6 stimulates the release of prolactin (PRL), GH, adrenocorticotropin (ACTH), follicle-stimulating hormone (FSH), and luteinizing hormone from normal anterior pituitary cells (Renner *et al.* 1996, Arzt *et al.* 1999), and GH and PRL release from rat pituitary GH3 tumor cells (Arzt *et al.* 1993). Moreover, IL-6 has also been demonstrated to stimulate pro-opiomelanocortin (POMC)-gene expression in human corticotrope adenoma (Pereda *et al.* 2000) and murine ArT-20 cells (Katahira *et al.* 1998).

Since IL-6 and its receptors are presented in pituitary cells, and it can also stimulate GH secretion, whether or not and by which mechanisms IL-6 regulates GH synthesis still remains unclear. To address this question, in the present study, we examined the effect of IL-6 on human GH (hGH)-gene expression in GH3 rat pituitary tumor cells using stable transfection of the hGH promoter fused to a luciferase reporter gene. Our results indicate that IL-6 promotes GH secretion and synthesis by rat pituitary GH3 cells, and that two distinct kinases, MAPKK/MEK and p38 MAPK, are essential for IL-6-induced hGH-gene expression. In addition, we also identified a fragment of the hGH promoter that appears to mediate the action of IL-6.

Materials and Methods

Plasmid constructs

Construction of the reporter pGL3-484-Luc1 (484-Luc1) was described previously (Gong *et al.* 2005). The deletion constructs: pGL3-380-Luc1 (380-Luc1), pGL3-250-Luc1 (250-Luc1), pGL3-196-Luc1 (196-Luc1), pGL3-132-Luc1 (132-Luc1), and pGL3-66-Luc1 (66-Luc1) which contained hGH-gene 5'-promoter fragments spanning -380, -250, -196, -132, -66 to +30 bp were synthesized using the parent 484-Luc1 as template in separate PCR. pGL3-484-Luc (484-Luc) contains the hGH-gene promoter from -484 to +2 bp and was constructed previously (Luan *et al.* 2000). pcDNA3.1(+) (a kind gift from Prof. De-xian Zheng, Chinese Academy of Medical Sciences) carried the neomycin-resistance gene and was used to screen stable transfection cell colonies. pSV- β -Gal (Promega) was an internal control plasmid. The Pit-1 expression plasmid, pcDNA-Pit-1cDNA, was a kind gift from H P Elsholtz (Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada). Pit-1 antisense oligonucleotides (Pit-1 OND), 5'-GAAAGTTGT-CAACTTCCCAC-3' were commercially synthesized by Bioasia Biotechnology (Shanghai, China).

Cell culture

GH3 cells, a rat pituitary tumor cell line, were purchased from American Type Culture Collection (Manassas, VA, USA), GH3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 20% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) in a 5% CO₂-95% air atmosphere at 37 °C. These cells have been used previously by ourselves and others as an accepted model for studying both GH-gene expression and the molecular mechanism involved (Tamura *et al.* 2000, Gong *et al.* 2003a, 2003b, 2005, Adriaio *et al.* 2004).

RNA preparation and reverse transcriptase (RT)-PCR

Total RNA from GH3 cells was extracted using a commercially available EZNA Total RNA kit (Omega Bio-Tek, Doraville, GA, USA). For RT, the SuperScript first-strand synthesis system kit (Invitrogen) was used. Two microliters of RT products (cDNA) were amplified with IL-6R primers as follows: IL-6R sense primer: 5'-TGCCAACCTTGTGG-TATCAGCC-3' (22 bp), IL-6R antisense primer: 5'-TGAA-GACACAGAGAAGCAATCC-3' (22 bp). These primers were based on sequences published in the literature (Zhang *et al.* 2000). The predicted product is 499 bp. The PCR system contained 5 μ l 10 $\times$ PCR buffer, 2 μ l RT product (cDNA), 4 μ l 25 mM MgCl₂, 4 μ l 2.5 mM dNTP mixture, 1.25 U Taq DNA polymerase, and 1 μ l 25 μ M sense and antisense primers in a total volume of 50 μ l. Amplification was carried out as following: 1 $\times$ (3 min at 94 °C, 1 min at 60 °C, and 3 min at 72 °C), 35 $\times$ (30 s at 94 °C, 30 s at 55 °C, and 45 s at 72 °C), and 1 $\times$ (10 min at 72 °C). A portion (10 μ l) of RT-PCR product was electrophoresed in 1.5% agarose gel in Tris-acetate-EDTA buffer. The gel was stained with ethidium bromide and photographed using Alphamager M2200 (Alpha Innotech, San Leandro, CA, USA).

Stable transfection and treatment of cells

Stably transfected GH3 cells were created by co-transfecting 484-Luc1 and pcDNA3.1(+) that carried the neomycin resistance gene, at a ratio of weight 5:1, with 1, 2-dimyristylaxypyl-3-dimethyl-hydroxy ethyl ammonium bromide and cholesterol in membrane-filtered water (DMRIE-C) transfection reagent (Life Technologies) as described previously (Gong *et al.* 2005). Monoclones that grew in media containing 300 μ g/ml Geneticin G418 (Life Technologies) were assayed for luciferase activity. The three appropriate colonies which expressed luciferase in a middle degree were chosen for the following studies.

The stably transfected GH3 cells were seeded in 24-well plates at a density of 3×10^5 cells/well and treated with different concentrations of IL-6 (10 – 10^4 U/ml, equal to 2.5–2500 ng/ml) (Promega) without or with various signal transduction inhibitors, including PD98059 (an inhibitor of

MAPKK/MEK), SB203580 (an inhibitor of p38 MAPK), LY294002 (an inhibitor of PI3-K), bisindolylmaleimide I (an inhibitor of protein kinase C, PKC), PKA inhibitor 6-22 amide (PKi) (an inhibitor of PKA), or AG82 (an inhibitor of tyrosine kinase) (Calbiochem, San Diego, CA, USA) in serum-free medium. After 4 h, the medium was harvested for assay of rat GH content, and the cells were lysed for assay of rat GH content and luciferase activity.

Protein extraction, electrophoresis, and Western blotting

Protein extraction, gel electrophoresis, and Western blot analysis were performed according to the manufacturer's instructions of the commercially available PhosphoPlus MEK1/2 and p38 MAP Kinase antibody kits (Cell Signaling Technology, Beverly, MA, USA). In brief, proteins were extracted from GH3 cells treated with IL-6 (10^3 U/ml, equal to 250 ng/ml) in serum-free medium for the indicated time in the presence or absence of MEK inhibitor PD98059 (40 μ M) or p38 MAPK inhibitor SB203580 (5 μ M), and separated by electrophoresis on 12% SDS-PAGE. Proteins were then electrotransferred to nitrocellulose membrane (Immobilon-P, Millipore, Billerica, MA, USA), and immunological detection was performed using anti-MEK, p38 MAPK, or anti-phospho-specific MEK, p38 MAPK antibodies at a 1:1000 dilution overnight at 4 °C with gentle agitation. After washing with Tris-buffered saline/Tween-20 three times, blots were further incubated for 1 h at room temperature with anti-rabbit IgG and anti-biotin (for detecting biotinylated protein markers) horseradish peroxidase (HRP)-linked antibodies at 1:2000 and 1:1000 dilution respectively. At the end of the incubation, a chemiluminescent peroxidase substrate, LumiGLO, was applied, and the membranes were exposed briefly to X-ray film according to the manufacturer's instruction.

Transient transfection

The basic process was similar to that described in stable transfection. For the study of whether Pit-1 protein is involved in the effect of IL-6 on hGH-gene promoter activity, the well-grown stably transfected GH3 cells were seeded in 12-well plates at a density of 5×10^5 cells/well, then Pit-1 expression plasmid pcDNA-Pit-1 cDNA (1.0 μ g) and/or Pit-1 OND (0.5 μ g) were transfected into these cells. For the study of the responses of various deletion constructs to IL-6, the deletion luciferase constructs (1.0 μ g each) and pSV- β -Gal (1.0 μ g, an internal control plasmid) were co-transfected into GH3 cells (8×10^5 cells/well) which were not transfected with any plasmids. The above-mentioned GH3 cells which had been transfected with the indicated plasmids were treated with 10^3 U/ml (250 ng/ml) IL-6 in serum-free medium for 4 h, then these cells were lysed for assay of luciferase and β -galactosidase activities. The difference in transfection efficiency among the constructs was corrected by β -Gal activity. The total amount of plasmid DNA was adjusted with pGL3-Basic-Luc (no luciferase expression) to keep the total DNA equal per well.

Assays

Both luciferase and β -Gal activities were measured using commercially available assay kits (Luciferase Assay System10-Pack (Cat. no. E1501) and β -Galactosidase Enzyme Assay System with Reporter Lysis Buffer (Cat. no. E2000), Promega) and performed as previously described (Gong *et al.* 2005). For rat GH assay in culture medium and cell lysates, commercially available assay kits were used (Amersham Biosciences). Each sample was measured twice. The intra- and interassay coefficients of variation were 7.8 and 10.5% respectively.

Statistical analysis

All experiments were performed in triplicate in three different colonies. Samples in each group of experiments were in triplicate or in quadruplicate. The mean luciferase activity of the control group was defined as 1; the relative luciferase activity (RLA) of the experimental groups was obtained by comparison with the control groups. Statistical analysis was performed between the control groups and the experimental groups using ANOVA, and $P < 0.05$ was considered statistically significant.

Results

Expression of IL-6R mRNA in GH3 cells

We employed RT-PCR analysis to determine whether IL-6R mRNA was expressed in rat pituitary GH3 cell line. The results showed that an expected band of 499 bp was identified in total RNA derived from GH3 cells. The PCR products were further confirmed by the digestion of Tag I and DNA sequencing (data not known).

IL-6-induced GH secretion and synthesis in stably transfected GH3 cells

After incubation the stably transfected GH3 cells with 10^3 U/ml IL-6 were trypsinized and counted in a hemocytometer. The results showed that the number of GH3 cells did not increase significantly as compared with the control values (data not shown). GH content in medium gradually increased, following incubation of GH3 cells with 10^2 – 10^4 U/ml IL-6 for 4 h. The maximal stimulatory action reached 4.4 times of the control values (Fig. 1, 1493.0 ± 134.4 vs 339.5 ± 18.0 , $P < 0.001$). This result indicates that IL-6 promoted GH secretion by GH3 cells. Meanwhile, GH content in cell lysates also gradually increased with the administration of 10^2 – 10^4 U/ml IL-6. Since the sum GH content in medium and cell lysates represents the amount of GH production by GH3 cells, these data suggest that IL-6 promoted GH synthesis, as well as GH secretion by GH3 cells.

Figure 1 Effect of interleukin (IL)-6 on growth hormone (GH) secretion and synthesis in stably transfected GH3 cells. Stably transfected GH3 cells were plated in 24-well plates at a density of 3×10^5 cells/well. After 24 h, these cells were treated with different concentrations of IL-6 (10 – 10^4 U/ml, equal to 2.5 – 2500 ng/ml) for 4 h in serum-free medium, then GH concentrations in medium (open columns) and medium plus cell lysate (solid columns) were measured by enzyme immunoassay. Data represent means $\pm$ s.d. of three separate wells in three independent experiments. * $P < 0.001$ vs control (0).

IL-6 stimulated luciferase expression in stably transfected GH3 cells

In our pre-experiment, a time-course of 1, 2, 3, 4, and 6 h for the effect of IL-6 on the hGH-gene promoter activity was observed first, and the results showed that IL-6 stimulated the hGH-promoter activity as early as 1 h after IL-6 administration, while the maximal stimulatory action was observed at 4 h (data not shown). This result is consistent with those of our previous studies on other cytokines (Gong *et al.* 2003a, 2003b, 2005). We therefore selected 4 h for further experiments. A gradual increase in the luciferase expression in stably transfected GH3 cells was observed after treating these cells with 10^2 – 10^4 U/ml IL-6 for 4 h. The maximal stimulatory action was noted to be 1.99 times above the control by 10^4 U/ml IL-6 (Fig. 2, $P < 0.001$). These findings indicate that IL-6 increases the hGH-gene promoter activity in GH3 cells in a dose-dependent manner.

Intracellular signaling pathways underlying IL-6-stimulatory action

In order to identify the possible signaling transduction pathways involved in the stimulatory action of IL-6, the stably transfected GH3 cells were treated with IL-6 in the presence or absence of a panel of specific inhibitors known to block selected pathways. These specific inhibitors and their concentrations were chosen in terms of the previously reported literature (Matthews & O'Neill *et al.* 1999, Secondo *et al.* 2003) and used in our pre-experiments. These inhibitors

proved to have no toxicity to GH3 cells in the concentrations used in the following experiments (data not shown). As shown in Fig. 3, treatment of the stably transfected GH3 cells with bisindolylmaleimide I (the PKC inhibitor, $2 \mu\text{M}$), PKi ($2 \mu\text{M}$), AG82 (the tyrosine kinase inhibitor, $20 \mu\text{M}$), SB203580 (the p38 MAPK inhibitor, $5 \mu\text{M}$), and LY294002 (the PI3-K inhibitor, $10 \mu\text{M}$) alone did not have

Figure 2 Effect of IL-6 on the luciferase expression in stably transfected GH3 cells. Stably transfected GH3 cells (3×10^5 cells/well) were plated in 24-well plates and cultured for 24 h. These cells were then treated with different concentrations of IL-6 (10 – 10^4 U/ml, equal to 2.5 – 2500 ng/ml) in serum-free medium. Four hours later, the cells were lysed and luciferase activities were assayed. Data represent means $\pm$ s.d. of three or four separate wells in three independent experiments shown as relative luciferase activity (RLA). * $P < 0.001$ vs control (0).

Figure 3 Effects of various inhibitors of signaling pathways on IL-6-induced hGH-gene promoter activity in stably transfected GH3 cells. Stably transfected GH3 cells were treated with IL-6 (10^3 U/ml, equal to 250 ng/ml) in the presence or absence of a panel of specific inhibitors (40 μ M PD98059, 5 μ M SB203580, 10 μ M LY294002, 2 μ M bisindolylmaleimide I (Bisin I), 2 μ M protein kinase A inhibitor 6-22 amide (PKi), and 20 μ M AG82) for 4 h in serum-free medium, then the luciferase activities were assayed. Data represent means $\pm$ s.d. of three or four separate wells in three independent experiments shown as relative luciferase activity (RLA). * $P < 0.001$ vs control; # $P < 0.001$ vs IL-6 group.

any effect on the basal GH-promoter activity. However, treatment of the stably transfected GH3 cells with PD98059 (the MEK inhibitor, 40 μ M) significantly inhibited the basal GH-promoter activity by 41% (Fig. 3, $P < 0.001$). After adding 10^3 U/ml IL-6 to GH3 cells plus the above inhibitors, the results demonstrated that bisindolylmaleimide I, LY294002, PKi, and AG82 did not influence the action of IL-6 on GH3 cells, whereas PD98059 and SB203580 both completely abolished the stimulatory effect of IL-6 on the luciferase expression (Fig. 3, $P < 0.001$). Concerning the action of PD98059, the results indicate that PD98059 had an inhibitory action not only on the GH-basal promoter activity, but also on the IL-6-induced promoter activation. All these findings imply that the activation of MEK and p38 MAPK is essential for IL-6 stimulation.

Activation of MEK and p38 MAPK by stimulating GH3 cells with IL-6

To further confirm that IL-6 did indeed increase the phosphorylation of MEK and p38 MAPK in GH3 cells, using Western blot technology, we tested the activities of total and phosphorylated MEK and p38 MAPK after treating these cells with 10^3 U/ml IL-6. As shown in Fig. 4a and c, there was no significant change in the total amounts of MEK and p38MAPK between the control and the IL-6-treated GH3 cells. However, when anti-phosphorylated p38 MAPK antibody was used, the increased activity of phosphorylated p38 MAPK was observed at 2, 3 and 4 h after treatment with

10^3 U/ml IL-6 (Fig. 4d). Meanwhile, after exposure of GH3 cells to IL-6 plus 5 μ M SB203580 for 4 h, the activity of phosphorylated p38 MAPK was observed to decline significantly (Fig. 4d). Similar results were observed for the activation of phosphorylated MEK (Fig. 4b). Together with the above results, this further suggests that IL-6 indeed increases the activation of phosphorylated MEK and p38 MAPK in GH3 cells, implying that the activation of phosphorylated MEK and p38 MAPK is involved in the stimulatory action of IL-6 on the hGH-gene promoter activity in GH3 cells.

Relationship between the actions of IL-6 and Pit-1 protein

As shown in Fig. 5, Pit-1 overexpression induced by transfection with Pit-1 expression plasmid pcDNA-Pit-1cDNA (1.0 μ g) into stably transfected GH3 cells increased luciferase expression 23% as compared with the control values (column 2 vs column 1, $P < 0.001$), and this stimulatory action of Pit-1 was abolished after Pit-1 OND (0.5 μ g) was used to suppress Pit-1 expression. Together with our pre-experiment results that showed the introduction of Pit-1 OND alone had an inhibitory effect on the GH-basal promoter activity in GH3 cells (data not shown), this further confirms the pivotal role of Pit-1 in basal GH transcription. To determine whether Pit-1 is also involved in the effect of IL-6 on hGH-gene expression, the stably transfected GH3 cells transfected transiently with pcDNA-Pit-1cDNA (1.0 μ g) were treated with IL-6 (10^3 U/ml). The results

Figure 4 Effects of IL-6 on MEK and p38 MAPK (total and phosphorylated) activities in GH3 cells. Stably transfected GH3 cells were stimulated with 10^3 U/ml (250 ng/ml) IL-6 in serum-free medium for the indicated time (at top of a and c) in the presence or absence of 40 μ M PD98059 (a and b) or 5 μ M SB203580 (c and d). Cells were then lysed in SDS sample buffer and proteins were separated by electrophoresis with subsequent electrotransferring to nitrocellulose membrane. Immunoblotting detection was performed using (a) anti-MEK, (b) anti-phospho-specific MEK, (c) antibodies or anti-p38 MAPK, and (d) anti-phospho-specific p38 MAPK antibodies as the primary antibodies at a 1:1000 dilution. The positions of total MEK (t-MEK), phosphorylated MEK (p-MEK), total MAPK (t-p38 MAPK), and phosphorylated MAPK (p-p38 MAPK) are indicated on the right-hand side of each panel. On the left-hand side is the protein molecular marker (kDa). The blots are representative of three independent experiments.

showed that Pit-1 overexpression further increased luciferase expression in stably transfected GH3 cells by 26% as compared with adding IL-6 alone (Fig. 5; column 5 vs column 4, $P < 0.01$), 1.76 ± 0.05 times of the control value (no Pit-1

overexpression, no IL-6 administration) (Fig. 5; column 5 vs column 1, $P < 0.001$). Interestingly, the stimulatory action of IL-6 on hGH-promoter activity still existed when Pit-1 expression was suppressed by Pit-1 OND (Fig. 5; column 6 vs

Figure 5 Relationship between Pit-1 protein and the stimulatory effect of IL-6 in stably transfected GH3 cells. Stably transfected GH3 cells were seeded in 12-well plates at a density of 5×10^5 cells/well and incubated for 24 h at 37 °C. These cells were then transfected with Pit-1 expression plasmid pcDNA-Pit-1 cDNA (1.0 μ g) and/or Pit-1 OND (0.5 μ g) using 2 μ l DMRIE-C transfection reagent for 5 h in 0.8 ml OPTI-MEM medium (Invitrogen), followed by treatment of IL-6 (10^3 U/ml, equal to 250 ng/ml) for 4 h. Finally, the cells were lysed for luciferase activity assay. Data represent means $\pm$ s.d. of three or four separate wells in three independent experiments shown as relative luciferase activity (RLA). * $P < 0.001$ vs column 1; $\Delta P < 0.01$ vs column 4.

column 1, $P < 0.001$). This finding demonstrates that Pit-1 OND abolished only the action of Pit-1, but failed to affect the induction of IL-6, suggesting that Pit-1 may not be involved in the stimulatory effect of IL-6 on hGH-promoter activity, although it plays an important role in basal GH transcription.

The critical promoter sequence that mediates the action of IL-6

Next, we searched for the *cis*-acting element(s) essentially required for IL-6-induced hGH-gene transcription by transiently transfected GH3 cells (transfection with no plasmid) with a series of deletion constructs of the hGH-gene promoter. As shown in Fig. 6, IL-6 increased the luciferase expression in GH3 cells transfected with 484-Luc, 380-Luc1, 250-Luc1, and 196-Luc1, as in GH3 cells transfected with 484-Luc1, by 81, 79, 78, and 80% respectively (comparison with absence of IL-6; $P < 0.001$), but IL-6 did not affect the luciferase expression in GH3 cells transfected with 132-Luc1 and 66-Luc1. This suggests that the critical promoter sequence that mediated the action of IL-6 may be situated between the nucleotides -196 and -132 bp of the hGH gene. In addition, although 132-Luc1 plasmid had two Pit-1 binding sites as compared with 66-Luc1, there were no differences in their responses to IL-6. Together with the former experiment, this further demonstrates that no relationship may exist between Pit-1 and the action of IL-6.

Discussion

IL-6 is a pleiotropic cytokine that plays key roles in several immune processes and in the host response to infection.

Recently, there is increasing evidence for the role of IL-6 in pituitary function, particularly in the regulation of the HPAA (Chesnokova & Melmed 2002). A few studies demonstrate that IL-6 is an afferent signal to the HPAA during local inflammation (Bethin *et al.* 2000, Turnbull *et al.* 2003), and IL-6 regulates this axis by directly targeting rat pituitary corticotropes, such as stimulating POMC transcription and ACTH release (Bethin *et al.* 2000, Gautron *et al.* 2003). Meanwhile, Bethin *et al.* (2000) found that IL-6R is present on pituitary corticotropes and adrenocortical cells. In 2003, Gautron *et al.* (2003) reported for the first time using dual *in situ* hybridization that corticotropes in the rat pituitary co-express both IL-6R and IL-6R subunit gp130, thus making these cells prone to respond to IL-6. In our present study, we found that IL-6R was also expressed in rat pituitary GH3 cells using RT-PCR analysis. Studies performed by Hanisch *et al.* (2000) and Kurotani *et al.* (2001) demonstrated IL-6 itself, in addition to its receptor, was expressed in human normal pituitary glands and pituitary adenomas. These findings suggest that IL-6, in addition to its regulatory action in HPAA, may also have a pivotal role in regulating pituitary GH secretion and synthesis.

Next, we successfully established a new *in vitro* system for investigating the effect of proinflammatory cytokine IL-6 on GH expression and the possible mechanism(s) involved. Because of some reports in the literature that IL-6 stimulated pituitary cell proliferation (Arzt *et al.* 1998), in our pre-experiment, we first observed the effect of IL-6 on the GH3 cells proliferation by cell counting. The results show that there is no stimulatory effect of IL-6 on GH3 cells number within the tested hours. This does not mean that IL-6 did not stimulate GH3 cell proliferation, and instead may be due to short-time action of IL-6.

Figure 6 The relative transcription activities of various deletion constructs of Luc-reporter and their responses to IL-6. GH3 cells (8×10^5 cells/well, transfection with no plasmid) were transiently transfected with various deletion luciferase constructs ($1.0 \mu\text{g}$ each) along with pSV- β -Gal ($1.0 \mu\text{g}$) for 5 h at 37°C . The cells were then treated with vehicle (solid columns), or 10^3 U/ml (250 ng/ml) IL-6 (open columns) in serum-free medium for 4 h. The luciferase activities were normalized by the β -Gal activity as an internal control. Data represent means $\pm$ s.d. of three or four separate wells in three independent experiments shown as relative luciferase activity (RLA). * $P < 0.001$ vs absence of IL-6. a, CRE; b, AP-1; c, Pit-1.

After incubating GH3 cells with IL-6 for 4 h, we observed that GH release was significantly enhanced by 10^2 – 10^4 U/ml IL-6. In accordance with our findings, Mainardi *et al.* (2002) and Thiele *et al.* (2003) also demonstrated that IL-6 significantly stimulated GH release from the primary cultured human somatotrope pituitary adenomas and pig pituitary cells. The GH-stimulatory potency of IL-6 was identical to, or even stronger than that of GHRH in comparative studies performed by Thiele *et al.* (2003). Reduced expression of the cytokine transducer gp130, which is required for IL-6 signal transduction, by stably transfecting gp130 antisense into GH3 cells, inhibited IL-6-induced GH secretion and cell growth (Castro *et al.* 2003). In our present study, we demonstrated for the first time that IL-6 not only promoted GH release, but also promoted its synthesis in rat pituitary GH3 cells. IL-6 stimulated the hGH-gene transcription through inducing the hGH-promoter activity.

Binding of IL-6 to its receptors, IL-6R might activate different signaling cascades in target tissue. In this study, we examined the signaling pathways underlying the stimulatory action of IL-6 using a panel of specific inhibitors known to block selected pathways. The results showed AG82, as well as bisindolylmaleimide I, PKi, and LY294002, did not affect the action of IL-6. In contrast, PD98059 and SB203580 both completely abolished the inductive role of IL-6 in hGH expression. These data suggest that the effect of IL-6 on hGH expression might be mediated by the MEK- and p38 MAPK-dependent pathways. This hypothesis was further confirmed by Western blot analysis. In this experiment, we found that IL-6 stimulated the phosphorylation of MEK and p38 MAPK in GH3 cells, and the specific inhibitors of these two kinases could block the IL-6-induced phosphorylation. Studies performed recently by Meng *et al.* (2005) and Chang *et al.* (2005) found that IL-6 activated serum and glucocorticoid kinase stimulate cell proliferation via phosphorylation and activation of p38 MAPK. Legendre *et al.* (2005) also found that IL-6 upregulated matrix metalloproteinase-gene expression by a MAPK-dependent pathway.

Recently, it has been shown that MAPK-dependent pathways are present in GH3 cells (Pearson *et al.* 2001). MAPK pathways activated by insulin-like growth factor-I (IGF-I), GHRH, thyrotropin-releasing hormone, and epidermal growth factor, not only regulate the expression of GH and PRL genes in rat pituitary GH3 cells (Castillo & Aranda 1997, Kievit *et al.* 2001, Kanasaki *et al.* 2002), but also promote cellular growth and proliferation of GH3 cells (Lee *et al.* 2001, Lewis *et al.* 2002). Secondo *et al.* (2003) also reported that PRL upregulated the expression of both nitric oxide synthase α and nitric oxide synthase β proteins via PI3-K, MAPK, and protein kinase B (PKB) signaling transduction components in GH3 cells. The similar signaling pathways activated by IFN- γ and IL-1 β to regulate the hGH-gene transcription in B lymphocyte IM-9 and GH3 cells have been observed in our previous studies (Luan *et al.* 2001, Gong *et al.* 2003a, 2005). Taken together, these results indicate that MEK, p38 MAPK, and PI3-K are all present and active in

GH3 cells as well as cAMP-PKA, and are involved in gene transcription regulation stimulated by some hormones and factors. To our knowledge, in the present study we are the first to report that MEK and p38 MAPK-dependent pathways in rat pituitary GH3 cells are essential for the inductive action of IL-6 on hGH-gene promoter activity.

Knall *et al.* (1996) reported that IL-8 caused activation of the MAPK pathway in human neutrophils and that this activation was dependent on PI3-K activity. Studies performed by Onger *et al.* (2005) also showed that FSH stimulation of insulin-like growth factor-binding protein-3 is mediated by the cAMP-PKA pathway, but requires PI3-K and MAPK activation. In our study, whether MEK and p38 MAPK respectively mediated the induction of IL-6 on hGH-gene expression or interacted with each other remains to be determined.

Delidow *et al.* (1991) and Tamura *et al.* (2000) found that the regulatory mechanism of GH and PRL-gene transcription by activin and TGF- β was independent of Pit-1 protein in GH3 cells. In this study, we also found that Pit-1 may not be involved in the stimulatory effect of IL-6 on hGH-promoter activity, although a significant number of studies showed that Pit-1 plays a pivotal role in basal and GHRH-induced GH-gene transcription. We have come to the same conclusion when we investigated the effect of IFN- γ , IL-1 β , IL-11, CNTF, TNF- α , and TGF- β on hGH-gene expression in GH3 cells (Wang *et al.* 2000, Gong *et al.* 2003a, 2003b, 2005). In addition, using a series of deletion constructs of hGH-gene promoter, we found that the critical promoter sequence which mediated the action of IL-6 was located between nucleotides -196 and -132 bp, which does not contain the binding sites of Pit-1 protein. This further demonstrates that Pit-1 protein(s) may not be an important transcription factor in the stimulatory effect of IL-6 on hGH-gene promoter.

Our deletion analysis revealed that the effect of IL-6 on hGH-gene expression was mediated by the sequence from -196 to -132 bp within the hGH promoter. This sequence also mediated the induction of IL-1 β on hGH-gene expression (Gong *et al.* 2005). We analyzed this sequence using DNassist software (<http://209.196.36.47/dnaassist.zip>) and found that there were some consensus sequences for cAMP-response element-binding protein, activator protein (AP)-2, and AP-1 in this segment. However, whether or not these transcription factors that bind to the relevant response elements in the -196/-132 hGH promoter are involved in the induction of IL-6 on GH-gene expression is not known, and further studies are needed.

In conclusion, our results show that IL-6 promotes GH secretion and synthesis in rat pituitary GH3 cells. The stimulatory effect of IL-6 on the hGH-gene promoter appears to require the activation of MEK and p38 MAPK, and a fragment of the promoter sequence that spans the -196 to -132 bp of the gene, but may be unrelated to Pit-1 protein. These findings confirm that cytokines are involved in the regulation of pituitary function and support the current concept that the endocrine and immune systems interact to

maintain homeostasis. On the other hand, these findings also suggest that the intracellular regulatory mechanism of GH-gene transcription to cytokines differs from the classical GHRH regulatory mechanism, including signaling transduction pathway(s), transcription factor(s), and the response elements in the GH-gene promoter.

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