Stimulation of endogenous GH and interleukin-6 receptors selectively activates different Jaks and Stats, with a Stat5 specific synergistic effect of dexamethasone

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Abstract

The interaction of GH, interleukin (IL)-6 and glucocorticoids is likely to be important in regulating the GH-insulin-like growth factor (IGF)-I axis. The signalling cascades activated by GH and IL-6 appear to be very similar, as demonstrated by studies using overexpression of the receptor and other components of the Jak-Stat and mitogen-activated protein (MAP) kinase pathways. Here we show that the human embryonic kidney cell line 293 (HEK293) expresses GH and IL-6 receptors endogenously. To determine which specific pathways might be activated by the two cytokines, at physiological levels of all components, we studied GH and IL-6 mediated signal transduction both under basal conditions and in the presence of overexpressed receptors and Stat proteins. Our results suggest a receptor specificity of Jak2 for GH receptors, and Jak1 for IL-6 receptors. Stat activation in response to GH and IL-6 was determined by reporter gene induction. Both GH and IL-6 were able to induce the reporter gene containing the Stat5 responsive element (LHRE) but the IL-6 response appeared to be mediated mainly through

Stat3 activation. In contrast, the reporter gene containing the Stat3 responsive element (SIE) was IL-6 specific. The levels of gene induction by GH and IL-6 were not altered by the co-stimulation with GH and IL-6, suggesting that there is little cross-talk at the Jak-Stat activation level between the two cytokines. Neither GH nor IL-6 activated the MAP-kinase responsive serum response element (SRE), unless GH receptors or gp130 were overexpressed. Transfection of Stat3 or Stat5 expression vectors enhanced the response to GH and IL-6. Stimulation with dexamethasone synergistically enhanced GH activation of the LHRE reporter gene but had no effect on the IL-6 activation of the same reporter or on the SIE reporter gene. Thus, our studies suggest that while each cytokine, GH and IL-6, may activate various members of the Jak-Stat pathway in overexpression studies, specific activation of Stat3 by IL-6 and of Jak2 and Stat5 by GH can be observed in HEK293 cells and that in this system the synergistic effect of dexamethasone appears specific for Stat5.

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Introduction

Growth hormone (GH) plays an essential role in childhood growth and is important in maintaining normal adult body composition, acting either directly on target tissues, or indirectly by regulating insulin-like growth factor (IGF)-I levels. Conditions with increased protein catabolism, as can be found in critically ill, septic or post-surgical patients, are often accompanied by GH resistance, and abnormally low IGF-I levels (Ross *et al.* 1991). It has been proposed that the levels of inflammatory cytokines could cause this cachectic state (Argiles & Lopez-Soriano 1999) and hence might also be responsible for the GH resistance observed at the GH receptor (GHR) or post receptor level

(Jenkins & Ross 1996). Recently interleukin (IL)-6 has been shown to decrease IGF-I levels and impair growth in mice (De Benedetti *et al.* 1997), and IL-1 β and tumour necrosis factor α (TNF α) reduce IGF-I mRNA levels in primary hepatocyte cultures (Thissen & Verniers 1997). Other potential inhibitors of GH actions are glucocorticoids which reduce IGF-I secretion from rat chondrocytes (Jux *et al.* 1998) and which are known to have a growth retarding effect in children. Dexamethasone (Dex) a synthetic glucocorticoid increases IL-6 receptor (IL-6R) expression (Mori *et al.* 1998) and enhances several cell responses to IL-1 and IL-6, such as the induction of the acute-phase protein and alpha-1-acid glycoprotein (Watanabe *et al.* 1999, Mejdoubi *et al.* 1999). The latter

induction is inhibited by GH, indicating a complex system of interaction and cross-talk between the cytokine activated receptor pathways.

Despite the different biological actions of GH and IL-6, they activate very similar sets of proteins, both having been shown to stimulate Jak1, Jak2, Stat1, Stat3, Stat5, MAP-kinase, and SHP2 (Guschin *et al.* 1995, Narazaki *et al.* 1994, Lai *et al.* 1995, Sotiropoulos *et al.* 1996, Moutoussamy *et al.* 1998b, Hirano 1998). For both the GHR and the IL-6R, activation is dependent on the binding of Jaks to the Box 1 motif in the cytoplasmic domain of the GHR and gp130 respectively, and the tyrosine phosphorylation of Jak and the receptor (reviewed in Hirano 1998, Moutoussamy *et al.* 1998a). Furthermore, it has been shown that the ligand occupied glucocorticoid receptor can effect gene induction by IL-6 and prolactin by interacting with Stat3 and Stat5 respectively (Stöcklin *et al.* 1996, Takeda *et al.* 1998).

Our aim was to compare the signalling pathways activated by GH and IL-6 and to investigate how Dex affects them, in a human cell line endogenously expressing both the GHR and IL-6R, in order to identify a potential target for IL-6 in GH resistance. The majority of studies on GH and IL-6 signalling have been carried out in murine tissue or mouse and rat cell lines (Hirano 1998, Gronowski *et al.* 1995, Smit *et al.* 1996), or by transfecting one of the receptors and one or more of the signalling molecules (Sotiropoulos *et al.* 1996, Takeda *et al.* 1998, Wang *et al.* 1995). This makes it difficult to analyse the relative induction of different pathways and to account for the distinct biological effects of GH and IL-6 in humans. The human embryonic kidney cell line, HEK293, contains many active signal transduction pathways, and has been widely used in overexpression studies. We show here that it also endogenously expresses both the GHR and IL-6R at physiological levels. Stimulation with GH and IL-6 resulted in the phosphorylation of Jak kinases and the induction of luciferase reporter genes, at low but significant levels. We show that (i) the two cytokines appear to activate distinct subsets of Jak and Stat proteins, (ii) dexamethasone enhanced Stat5 mediated gene activation by GH, but not IL-6 mediated Stat3 activation, (iii) co-stimulation with IL-6 did not down-regulate GH mediated gene induction, and that GH had no effect on the IL-6 induced luciferase levels. It is unlikely that cross-talk, at the level of the activation of the Jak-Stat pathways by the two cytokines, could explain the interference of IL-6 on GH signalling.

Materials and Methods

Reagents

Recombinant human GH (Genotrophin) was obtained from Pharmacia & Upjohn (Milton Keynes, UK) and human IL-6 and sIL6R were obtained from R&D

(Abingdon, UK). The Jak1 and Jak2 specific antibodies were from UBI TCS (Buckingham, UK). The reporter plasmids pUC18-lactogenic hormone response element (LHRE)/TK-luciferase with four copies of the LHRE: CTGCAGTGTGGACTTCTTGGGAATTAAGGGCTT TTGCTGCAG, pUC18-SIE m67/TK-luciferase with three copies of the m67: CTGCAGTCGACATTTCC CGTAAATCGTCGACTGCA (Sotiropoulos *et al.* 1996) and pUC18-SRE/TK-luciferase with one copy of the serum response element (SRE): CTAGAGGATGTCCA TATTAGGACATCTGGATCTAG (Moutoussamy *et al.* 1998b), and the expression vector pcDNAI/Amp-GHR β (Ross *et al.* 1997) have all been described previously. The other plasmids were generous gifts, the β -galactosidase (β -gal) reporter IEP- β gal-CMV from Gerald Clesham, (Papworth Hospital, Cambridge, UK) the expression vectors for gp130 from Yannick Jacques (INSERM U463, Nantes), Stat3 from James Darnell (Rockefeller University, NY, USA), and Stat5 (MGF) from Bernd Groner (Institute of Experimental Cancer Research, Freiburg, Germany).

Cell culture and transfection

HEK293 cells were grown in Dulbecco's modified Eagle's medium–nutrient mix F12 (DMEM Nut F12), supplemented with 10% fetal calf serum (FCS), 10 IU and 10 UG/ml penicillin–streptomycin, and 2 mM L-glutamine (all Gibco BRL, Paisley, UK). For transfection 2.4×10^6 cells were plated out in six-well plates with 12 ml medium total, and left overnight. Cells were then incubated in 60% DMEM Nut F12, 30% DMEM 4.5 g/l glucose (Gibco BRL) and 10% FCS, for 6 h. Transfection was carried out using the calcium phosphate transfection kit (Gibco) as previously described (Ross *et al.* 1997), using 3 μ g LHRE, *c-sis* inducible element (SIE), Stat3, Stat5, 1 μ g IEP- β gal-CMV and carrier DNA up to a total of 20 μ g DNA. After an overnight incubation, the medium was changed to FCS free DMEM Nut-F12. 293 GHRhi, a stable clone, expressing high levels of the human GHR was generated by cotransfecting pcDNAI/Amp-GHR β and pcDNA3 and selection by 400 μ M G418 (Gibco).

Receptor detection

RT-PCR experiments for IL-6R and gp130 involved 30 cycles of PCR carried out using the primers 5'cccggatc cattttctggaagtgaggcc and 5'ccggtttaacgtagaatctcttccacatg for GHR, 5'tattcaccactcaccctc and 5'aaaagatgcttctcact gcc for IL-6R and 5'ccatccccaccttcac and 5'ctgctgtgactt tcaagc for gp130, 1.5 mM Mg²⁺, and 58 °C annealing temperature, after reverse transcription. Negative controls included PCR of RNA preparations that had not been reverse transcribed, and samples lacking template (water). Binding studies were carried out by incubating cells in six-well plates with 100 000 c.p.m./ml ¹²⁵I-GH in PBS

for 4 h at room temperature. Non-specific binding was determined in the presence of 4 µg/ml unlabelled GH. Fluorescence activated cell sorter (FACS) analysis was conducted using a rabbit polyclonal IL-6 antibody, or a preimmune isotype control (R&D) under the conditions as published by Hargreaves *et al.* 1998.

Western blotting

HEK293 cells were starved overnight in serum free medium, and then stimulated for 15 min at 37 °C, with 500 ng/ml GH or 200 ng/ml IL-6. Cells were lysed in PBS-TDS (PBS, 1% Triton X100, 12 mM sodium desoxycholate, 3.5 mM SDS and 4.7 nM sodium orthovanadate) and 1 mg protein was precipitated with the relevant antibody (Jak1 1:250, Jak2 1:250) and 20 µl protein A-agarose (Sigma; Poole, UK). Precipitated proteins were separated on a 10% SDS-polyacrylamide gel and after blotting onto PVDF, phosphorylation was detected with an anti-phosphotyrosine antibody (1:2500) (4 G10, UBI) and the enhanced chemiluminescence (ECL) system (Amersham; Little Chalfont, UK).

Reporter assays

Transfected 293 cells were transferred to FCS free medium and stimulated with GH (100 ng/ml), IL-6 (200 ng/ml), sIL-6R (1000 ng/ml), dexamethasone (0.5 µM), or a combination of them. Twenty-four hours later luciferase levels were measured using the GenGlow luciferase detection system (Labtech Int. Uckfield, UK), according to the manufacturer's protocol. Briefly, cells were lysed in 0.5 ml somalyse and 100 µl of lysate was mixed with equal volumes of luciferin and ATP. β-Gal levels were measured using the β-galactosidase enzyme assay system (Promega; Southampton, UK), by incubating 50 µl of the somalyse cell lysate with 50 µl assay buffer, according to manufacturer's recommendations. The luciferase data obtained were corrected for transfection efficiency, by dividing them by the absorbency values of the β-gal assay. All functional tests were carried out in triplicate, and repeated at least twice. Statistical analysis was performed on corrected and normalised results, using the balanced one way ANOVA, in conjunction with Tukey's multiple comparison test. The ANOVA general linear model was used to ascertain the effects of multiple treatments and their interactions. All calculations were carried out using the statistical analysis package Minitab, and significance was accepted as $P < 0.05$.

Results

Demonstration of GHR and IL-6R expression

HEK293 cells have been used repeatedly in the past to study the GHR and other cytokine receptors, using

transient transfection (Sotiropoulos *et al.* 1996, Winston & Hunter 1995), but the expression of endogenous receptor at physiological levels has not been described. Using RT-PCR we demonstrated the presence of GHR mRNA in HEK293 cells (Fig. 1A). To demonstrate GHR expression on the cell surface, we incubated HEK293 cells with ¹²⁵I-hGH in the presence or absence of unlabelled GH. Specific binding (around 1–2% of the total amount of ¹²⁵I-hGH added in the assay) was observed, indicating that the cells express low, but detectable, levels of GHR (Fig. 1B). These levels were insufficient to determine the receptor number directly by Scatchard analysis. Specific binding was increased significantly in the HEK293 cells stably expressing GHR (named 293 GHRhi for HEK293 cells expressing high levels of GHR); Scatchard analysis indicated that expression levels were $100\,000 \pm 20\,000$ receptors per cell in the clone which we used. Using RT-PCR, we also demonstrated the expression of IL-6R and gp130 mRNA (Fig. 1A). Low levels of surface expression for IL-6R were confirmed by FACS analysis (Fig. 1C).

Activation of Jak1 and Jak2 by GH and IL-6

Activation and tyrosine phosphorylation of the receptor associated Jak kinases is one of the first events after ligand binding to the GHR or the IL-6R-gp130 complex (Argetsinger *et al.* 1993, Narazaki *et al.* 1994, Guschin *et al.* 1995). To demonstrate that the endogenous receptors in HEK293 cells were functional, cells were stimulated with hGH or hIL-6 and the cell lysates immunoprecipitated with Jak1 or Jak2 specific antibodies. Phosphorylated proteins were detected by Western blotting, using anti-phosphotyrosine antibodies. GH stimulated the tyrosine phosphorylation of JAK1 in parental HEK293 cells and in HEK293 cells overexpressing GHR, however, the induced signal was not enhanced by the overexpression of GH receptors (Fig. 2A). In contrast, while we detected a tyrosine phosphorylated band corresponding to Jak2 in GH stimulated HEK293 cells, Jak2 phosphorylation was markedly increased in the stable clone 293 GHRhi (Fig. 2B). IL-6 stimulation activated Jak1 phosphorylation in HEK293 cells and this activation was slightly enhanced by the addition of sIL-6R (Fig. 2A), whereas we did not observe any Jak2 tyrosine phosphorylation by IL-6 in these cells, even with the addition of sIL-6R (Fig. 2B).

GH and IL-6 independently activate Stat and MAPK pathways

An important function of IL-6 and GH is the activation of gene transcription via Stat3, Stat5 and the MAP-Kinase signalling pathways. To characterise the activity of the endogenous receptors in HEK293 cells, we tested their ability to induce gene transcription via Stat and MAP-kinase responsive enhancers. Stat5 activation was detected

Figure 1 GH and IL-6 receptor detection in HEK293 cells. A, detection of GHR, IL-6R and gp130 mRNAs by RT-PCR in HEK293, HepG2, B-lymphocytes and IM-9 cells. The amplified products are visualised with ethidium bromide; the empty lane is a negative control (water). B, ^{125}I -GH binding to HEK293 and 293GHRhi cells in the absence (black) or presence (white) of excess unlabelled GH. C, FACS analysis using FITC labelled anti IL-6R antibody (darker peak) or isotype control (light peak) antibody binding in HEK293 cells.

using the lactogenic hormone response element (LHRE) fused to the luciferase gene (Moriggl *et al.* 1996) (Fig. 3A). In HEK293 cells transfected with this construct, GH increased luciferase activity by 70%, compared with non-stimulated controls, while IL-6 only weakly activated the reporter construct (Fig. 3A, left panel). We hypothesised that IL-6 could inhibit the cell response to GH. Co-stimulation (Fig. 3A) or pre-treatment with IL-6 for up to 12 h before stimulation with GH (data not shown) had no inhibitory effect on the induced luciferase levels. This suggests that IL-6 is not negatively regulating GH signalling at the level of Stat5 activation. In cells overexpressing GHR (293 GHRhi), basal luciferase expression by the LHRE reporter gene was enhanced (Fig. 3A, right panel), and GH stimulated activation was greatly increased (about 10-fold induction). 3L-6 stimulated luciferase values were also enhanced but the fold stimulation was unchanged.

Both GH and IL-6 have been reported to activate Stat3 in various cell models (Zhong *et al.* 1994, Sotiropoulos *et al.* 1996). To test if the innate receptors of HEK293 cells could also activate Stat3, we transfected a luciferase reporter vector containing the c-sis-inducible element m67 (SIE) that is Stat1 and Stat3 responsive (Wagner *et al.* 1990) (Fig. 3B). GH had no effect on SIE mediated luciferase production in either HEK293 cells or the stable clone 293 GHRhi (Fig. 3B, left and right panel), suggest-

ing that the GHR does not activate either Stat1 or Stat3 under these conditions. IL-6 stimulation of HEK293 cells induced the SIE/TK luciferase reporter gene two-fold (Fig. 3B, left and right panel). Cotransfection with gp130 and co-stimulation with sIL-6R further enhanced this induction (Fig. 3B, right panel). Both the effect of IL-6 alone and the combination with sIL-6R and gp130 were statistically significant ($P < 0.05$). The activation of SIE by IL-6 was not altered by co-stimulation with GH (Fig. 3B, left panel).

The MAP-kinase responsive reporter vector, containing the serum response element (SRE) was not induced by GH or IL-6 in HEK293 cells (data not shown). However, when the stable line 293 GHRhi was used or gp130 was cotransfected, both GH and IL-6, in conjunction with sIL-6R, induced a two-fold induction (Fig 3C).

Effects of cotransfection of Stat3 and Stat5 on GH and IL-6 signalling

To investigate further the differences in activation of Stat3 and Stat5 by IL-6 and GH, we transfected HEK293 cells with Stat3 or Stat5 expression vectors and either the Stat5 (LHRE) or the Stat1/Stat3 (SIE) reporter construct. Induction of the LHRE by GH was more than doubled by the cotransfection of Stat5 (Fig. 4A). Stat3 cotransfection

(A) IP Jak1 **(B) IP Jak2**

Figure 2 Tyrosine phosphorylation of Jak1 and Jak2 by GH and IL-6. 293, GHRhi and HEK293 cells were non-stimulated (C), or stimulated with 500 ng/ml GH, 200 ng IL-6 alone or 200 ng IL-6 and 1 mg/ml sIL-6R. Cell lysates were immunoprecipitated with anti Jak1 antibody (A) or anti Jak2 antibody (B) and probed for phosphorylated tyrosines (upper panel) or the precipitating antibody (lower panel). Arrowheads indicate the positions of Jak1 and Jak2, respectively.

had no effect on LHRE (Fig. 4A) or SIE (Fig. 4B) induction by GH, supporting the notion that Stat3 is not activated by GH at endogenous levels of GHR in HEK293 cells.

IL-6 stimulated induction of the LHRE was increased more than three-fold by cotransfection of Stat5 (Fig. 4A). The cotransfection of Stat3 had an even more pronounced effect on IL-6 mediated LHRE induction (Fig. 4A). Activation of the SIE reporter plasmid by IL-6 was increased by Stat3, but not Stat5 cotransfection (Fig. 4B). The results suggest that IL-6 activates mainly Stat3, but is also able to signal via Stat5 if the latter is overexpressed. Furthermore they demonstrate that the LHRE is not Stat5 specific, at least in the presence of artificially increased levels of Stat3.

Neither Stat3 nor Stat5 cotransfection made the SIE reporter plasmid GH responsive in HEK293 cells (Fig. 4B). However, in the stable clone 293 GHRhi, GH induced SIE mediated luciferase transcription when Stat3, but not Stat5, was cotransfected (Fig. 4C), indicating that

SIE is Stat3 specific. The results therefore demonstrate, that supra-physiological levels of GHR and Stat3 are required for the activation of Stat3 to take place in response to GH.

Effects of dexamethasone on GH and IL-6 signalling

The down regulation of GHR binding sites by dexamethasone suggested an inhibitory effect of Dex on GH target cells (Gabrielsson *et al.* 1995, King & Carter-Su 1995, Jux *et al.* 1998). We examined if this effect was also observed in HEK293 cells. Increasing concentrations of Dex did not significantly decrease the amount of GHR binding that we measured in either HEK293 cells or in HEK293 cells overexpressing GHR (293 GHRhi) (Fig. 5).

It has also been reported, that Stat5 mediated gene transcription can be synergistically enhanced by Dex (Stöcklin *et al.* 1996). We analysed whether this is a general phenomenon of Stat mediated gene transcription or whether it is Stat5 specific. For this purpose, we determined the effect of Dex on the activation of LHRE and SIE reporter genes by GH and IL-6, with or without overexpressing Stat3 or Stat5 (Fig. 6). Activation of the LHRE reporter, by endogenous GH in HEK293 cells was synergistically enhanced by Dex (Fig. 6A) with a statistically significant effect ($P < 0.001$). We also investigated a potential interaction of Dex with Jak2 activation in 293 GHRhi cells by Western blotting. Dex did not effect Jak2 phosphorylation and did not modify the GH mediated effect Jak2 phosphorylation; these data support our observation that Dex does not change receptor amounts in these cells (Fig. 6B). We also tested a potential effect of Dex on Stat3 mediated gene transcription in our system. 293 GHRhi cells were transfected with SIE/TK luciferase alone or with either Stat3 or Stat5, and then stimulated with GH, Dex or a combination of the two (Fig. 6C). As shown above, GH only induced the SIE in the presence of overexpressed Stat3. Costimulation with Dex did not alter GH mediated SIE induction either in the presence or absence of high levels of Stat3 (Fig. 6C). These data confirm that the SIE is not Stat5 responsive and suggest that the synergistic effect of Dex is Stat5 specific.

Costimulation of the LHRE reporter gene with IL-6 and Dex did not increase the luciferase induction significantly, compared with IL-6 stimulation alone (Fig. 6D). Costimulation with Dex also did not affect the IL-6 mediated LHRE gene induction when Stat3 or Stat5 were cotransfected (data not shown). Similarly SIE induction by IL-6 was not enhanced by Dex (Fig. 6E) either in the absence or presence of overexpressed Stat3 (data not shown). This suggests that the synergistic effect of Dex is Stat5 specific, and further indicates that the LHRE induction in response to IL-6, is probably mainly Stat3 mediated.

A) LHRE/TK luc **B) SIE/TK luc** **C) SRE/TK luc**

Figure 3 Activation of the LHRE, SIE and SRE enhancers by GH and IL-6. A, HEK293 cells or 293 GHRhi cells were transfected with LHRE/TK-luc and stimulated with 100 ng/ml GH, 200 ng/ml IL-6 or both as indicated and luciferase levels determined. B, HEK293 cells or 293 GHRhi cells were transfected with SIE/TK-luc alone or in conjunction with gp130 and stimulated with 100 ng/ml GH or 200 ng/ml IL-6, 200 ng/ml IL-6 and 1000 ng/ml sIL-6R, or the combination of GH and IL-6 as indicated. C, 293 GHRhi cells transfected with the SRE/TK-luc and Jak2 or gp130 were stimulated with 500 ng/ml GH or 200 ng/ml IL-6 and 1000 ng/ml sIL-6R. Asterisks denote significant difference from control ($P < 0.05$).

Discussion

These studies demonstrate the presence of functional, endogenous receptors for GH and IL-6 and their ability to activate Jak1, Jak2, Stat3 and Stat5 in the human cell line HEK293. It was therefore possible to study the profile of

Jak and Stat proteins activated by GHR and IL-6R at physiological concentrations of all signalling components, and to determine the effect of IL-6 and Dex on GH mediated gene induction.

GHR mRNA was detectable in HEK293 cells by RT-PCR. 125 I-GH specific binding could be detected,

Figure 4 Enhancement of GH or IL-6 mediated LHRE/TK-luc and of SIE/TK-luc induction by cotransfection with Stat3 and Stat5. A, HEK293 cells were transfected with LHRE/TK-luc alone or in conjunction with Stat3 or Stat5 and stimulated with either 100 ng/ml GH or 200 ng/ml IL-6. B, Transfection and stimulation of HEK293 cells as in A, except using SIE/TK-luc. C, Transfection and stimulation of 293 GHRhi as in B. Asterisks denote significant effect of Stat3 or Stat5 cotransfection ($P < 0.05$).

albeit at low levels but was similar to that reported for hepatoma cell lines (Hep3B and HuH-7) and human liver (Hocquette *et al.* 1990, Esposito *et al.* 1994). Although the levels were insufficient for quantification by Scatchard analysis, comparison with the binding levels of several clones stably expressing GHR, suggests that the parental cell line expressed less than 1000 receptors per cell. The IL-6R levels were comparable to the human plasma cell leukaemia cell line ARH-77, which is often used to study the IL-6R (Hargreaves *et al.* 1998), and expresses approximately 500 receptors per cell.

Figure 5 Dexamethasone does not effect ¹²⁵I-hGH binding in 293 cells. 293 (left panel) or 293 GHRhi cells (right panel) were incubated for 18 h with the concentration of Dex indicated. Receptor levels were determined by measuring the binding of ¹²⁵I-hGH in the absence (white bars) or presence (black bars) of unlabeled GH.

GH and IL-6 have been reported to activate Jak1 and Jak2 in several cell lines, possibly in a tissue specific manner (Guschin *et al.* 1995). While GH was essentially reported to activate Jak2, some Jak1 activation was observed in 3T3-F442A cells and in COS cells transfected with the murine GHR (Carter-Su *et al.* 1996, Smit *et al.* 1996). Similarly it has been described that IL-6 can also activate several members of the Jak kinase family (Narazaki *et al.* 1994, Guschin *et al.* 1995). In this paper we compare in the same cell line, the relative induction of Jak1 and Jak2 by GH and IL-6 and determine how the relative amount of each receptor could regulate the activation of each Jak kinase. Jak1 phosphorylation could be detected in HEK293 cells stimulated with both GH and IL-6, but while the IL-6 response was increased by the addition of soluble receptor, higher expression of GHR (in 293 GHRhi) did not enhance the GH induced signal. This suggests that the phosphorylation of Jak1 in response to GH might involve additional factors that are limiting. In contrast, Jak2 phosphorylation could be detected after GH stimulation in HEK293 cells, and was markedly increased in the 293 GHRhi cells demonstrating a clear correlation between the number of GHRs and the intensity of the Jak2 activation. We did not detect any activation of Jak2 by IL-6 even when s-IL-6R was added. Our experiments, using the same recipient cells to analyse the two signalling pathways, demonstrate that the respective specificity of Jak1 for IL-6 and of Jak2 for GH is not linked to a difference in the expression of the Jak molecules, but to differences in the relative specificity of each receptor complex for a particular Jak. Exclusive activation of Jak1 by IL-6 is in agreement with the findings recently observed in Jak1 and Jak2 knockout mice (Neubauer *et al.* 1998, Parganas *et al.* 1998, Rodig *et al.* 1998), which show that Jak1 is essential for IL-6 specific signals while Jak2 is not required.

The profiles of Stat molecules activated by GH and IL-6 also appear to be cell line and species specific. It has been reported that GH activates Stat3 *in vivo* in rat liver (Gronowski *et al.* 1995, Ram *et al.* 1996), although the GHR lacks a Stat3 binding domain. Jak2 is therefore thought to act as an adapter protein (Sotiropoulos *et al.*

Figure 6 Synergistic action of Dex on Stat5 but not Stat3 mediated transcription. A, HEK293 cells were transfected with LHRE/TK-luc and stimulated with either 100 ng/ml GH, or 0.5 μ M Dex, or a combination of the two. B, 293 GHRhi cells were stimulated with either 500 ng/ml GH, 0.5 μ M Dex or the combination of the two. Lysates were immunoprecipitated with anti-Jak2 antibody and proteins detected with anti -P-Tyr (upper panel) or anti-Jak2 antibodies (lower panel). C, 293 GHRhi cells were co-transfected with SIE/TK-luc alone or with Stat3 and Stat5 and stimulated with 100 ng/ml GH or 0.5 μ M Dex, or both. D and E, HEK293 cells transfected with LHRE/TK-luc (D) or SIE/TK-luc (E) were stimulated with 200 ng/ml IL-6, 0.5 μ M Dex, or a combination of the two. Results, are expressed as fold induction over control. Asterisks denote significant difference from GH or IL-6 treatment alone ($P<0.005$).

1996). Transfected rodent GHR was less effective at activating Stat3, in the human cell line 2fTGH, than the endogenous receptor in the murine cell line 3T3-F442A (Smit *et al.* 1996), supporting the notion of species specificity. The earlier reports of Stat3 activation by rabbit GHR in HEK293 (Sotiropoulos *et al.* 1996) and COS-1

cells (Wang *et al.* 1995) might also be due to species specificity, or could be an artefact of GHR and Stat3 overexpression. In our cell system, we show that the inability of GH to mediate the activation of the Stat1 and Stat3 responsive SIE is not due to a lack of Stat3; IL-6 is able to induce the SIE reporter gene using endogenous

receptor and Stat protein levels. Indeed we could detect some GH induction of the Stat3 responsive SIE when both GHR and Stat3 were overexpressed, a requirement in both the other studies reporting GH activation of Stat3 (Wang *et al.* 1995, Sotiropoulos *et al.* 1996). Stat5 overexpression had no effect on the induction of the SIE by GH or IL-6, demonstrating the specificity of this enhancer. We therefore suggest that in this human cell line GH activates Stat5 but not Stat3, a finding similar to a report by Freeth *et al.* (1998) that showed GH dependent phosphorylation of Stat5 but not Stat3 in human fibroblasts. In HEK293 cells this specificity is lost by the overexpression of GHR and Stat3, suggesting that the original cell line is a more physiological model.

IL-6, unlike GH, was able to induce both the Stat1/Stat3, and the Stat5 responsive luciferase reporter vectors. Cotransfection of Stat5 indicated that the SIE is not inducible by Stat5, whereas the LHRE induction was enhanced both by Stat3 and Stat5 overexpression. The enhancement by Stat3 cotransfection was about two-fold higher than that by Stat5, suggesting that mainly Stat3 and only to a lesser extent Stat5 are responsible for the IL-6 mediated LHRE induction under physiological concentrations of the Stat proteins. Nevertheless, our data support those of Lai *et al.* (1995), who show that IL-6 is able to activate low levels of Stat5.

Despite the overlap of the signalling pathways employed by GH and IL-6, there was no interaction between the two cytokines, when cells were stimulated with both simultaneously. This suggests that acquired GH insensitivity is not due to an effect of IL-6 on GH mediated Jak–Stat activation.

Glucocorticoids are involved in the acute phase response and may downregulate GH responsiveness of target tissues and decrease IGF-I synthesis (Bang *et al.* 1993, Gabrielson *et al.* 1995, Jux *et al.* 1998). In our cell system, we did not observe any significant effect of Dex on the amount of either endogenous or transfected GHR. In contrast, Dex synergistically enhances Stat5 mediated gene induction in response to prolactin (Stöcklin *et al.* 1996). IL-6 mediated gene induction has also been reported to be synergistically enhanced by Dex, although this effect seems to depend on the promoter regions surrounding the Stat3 responsive element (Takeda *et al.* 1998). This hypothesis is in agreement with the findings by Kordula & Travis (1996) who have shown that a C/EBP site in the vicinity of the IL-6 response element is required for the effect of Dex on IL-6 induced gene transcription, while Kim & Baumann (1997) suggest a direct interaction of Stat3 with the glucocorticoid receptor similar to that observed with Stat5 (Stöcklin *et al.* 1997).

We therefore determined if Dex can enhance the effects of GH and IL-6 on LHRE and SIE induction in HEK293 cells described above. Dex synergistically enhanced the LHRE induction by GH, but did not effect the induction of either reporter construct by IL-6. This supports the

notion that the LHRE induction by IL-6 is mainly mediated by Stat3 and suggests that the synergistic actions of Dex on Stat5 and Stat3 mediated gene induction, reported by Stöcklin *et al.* (1996) and Takeda *et al.* (1998), respectively, are mediated by different mechanisms, since the effects on Stat5 are independent of other response elements in the promoter, whereas those on Stat3 are not. Interestingly, it has recently been reported that the Stat5 mediated β -casein induction by IL-2 is also synergistically enhanced by Dex (Chida *et al.* 1998). As described for the enhancement of the induction of the IL-6RE, the synergistic effect on the β -casein promoter was dependent on additional sequences apart from the Stat5 binding element. Thus it is unclear at present how the two mechanisms to enhance Stat5 activity differ, and why the direct interaction of Stat5 and the glucocorticoid receptor described by Stöcklin *et al.* (1997) does not take place in response to IL-2 activation of Stat5.

In conclusion, although the receptor signalling pathways of GH and IL-6 appear to be very similar, we show that in the same cell line they activate different sets of Stat proteins, thereby eliciting their specific biological effects. We observed little or no cross-talk between the Jak–Stat pathways activated by the two cytokines. Dexamethasone synergistically enhanced the GH mediated LHRE induction by Stat5, but not the SIE induction by Stat3, nor did it affect the induction of either response element by IL-6. It is likely that there are at least two different mechanisms by which the interaction of the glucocorticoid receptor with Stat proteins can enhance gene induction, one being dependent on a Stat binding element only, the other requiring other enhancer sequences.

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