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Research Article

We recently described the expression of leukemia inhibitory factor (LIF) in human fetal and murine corticotrophs. LIF and the related cytokine oncostatin M induced basal, and corticotropin-releasing hormone (CRH) induced proopiomelanocortin (POMC) mRNA and ACTH secretion in AtT20 cells. LIF signaling and regulation of POMC gene transcription were therefore tested. Dexamethasone inhibited both basal- and LIF-induced ACTH secretion ($P < 0.05$) and LIF induction of ACTH was also attenuated by immunoneutralization of either the LIF receptor (35%, $P < 0.05$) or the gp130 affinity converter (41%, $P < 0.05$). These antisera also attenuated basal ACTH secretion in the absence of added ligand ($P < 0.05$). To examine intrapituitary LIF signaling, phosphorylation of post-receptor substrates was measured. 1 nM LIF rapidly induced tyrosyl phosphorylation of STAT 1 and STAT 3 proteins, as well as tyrosyl phosphorylation of a 115-kD protein, coimmunoprecipitated with STAT 1. The transfected rat POMC promoter -706/+64, fused to the luciferase reporter gene, was induced by LIF, which exerted strong (18-fold) synergy with CRH. Deletion of the major CRH responsive region in POMC (-323/-166) abolished CRH induction of transcription and severely limited LIF synergy. Although 8 bromo cAMP or forskolin modestly enhanced POMC transcription (2.8-fold), LIF markedly potentiated (7.4-fold) these cAMP activators. These results demonstrate that corticotroph LIF action is receptor mediated and involves activation of STAT signaling pathways. LIF potentially synergizes with both CRH and [...]

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Leukemia Inhibitory Factor (LIF) Stimulates Proopiomelanocortin (POMC) Expression in a Corticotroph Cell Line

Role of STAT Pathway

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Abstract

We recently described the expression of leukemia inhibitory factor (LIF) in human fetal and murine corticotrophs. LIF and the related cytokine oncostatin M induced basal, and corticotropin-releasing hormone (CRH) induced proopiomelanocortin (POMC) mRNA and ACTH secretion in AtT20 cells. LIF signaling and regulation of POMC gene transcription were therefore tested. Dexamethasone inhibited both basal- and LIF-induced ACTH secretion ($P < 0.05$) and LIF induction of ACTH was also attenuated by immunoneutralization of either the LIF receptor (35%, $P < 0.05$) or the gp130 affinity converter (41%, $P < 0.05$). These antisera also attenuated basal ACTH secretion in the absence of added ligand ($P < 0.05$). To examine intrapituitary LIF signaling, phosphorylation of post-receptor substrates was measured. 1 nM LIF rapidly induced tyrosyl phosphorylation of STAT 1 and STAT 3 proteins, as well as tyrosyl phosphorylation of a 115-kD protein, coimmunoprecipitated with STAT 1. The transfected rat POMC promoter $-706/+64$, fused to the luciferase reporter gene, was induced by LIF, which exerted strong (18-fold) synergy with CRH. Deletion of the major CRH responsive region in POMC ($-323/-166$) abolished CRH induction of transcription and severely limited LIF synergy. Although 8 bromo cAMP or forskolin modestly enhanced POMC transcription (2.8-fold), LIF markedly potentiated (7.4-fold) these cAMP activators. These results demonstrate that corticotroph LIF action is receptor mediated and involves activation of STAT signaling pathways. LIF potently synergizes with both CRH and cAMP induction of POMC transcription. This novel intrapituitary signaling mechanism may mediate a neuroimmune pituitary interface. (*J. Clin. Invest.* 1996. 97:1852–1859.) Key words: ACTH • cytokines • pituitary signaling • pituitary gene regulation • leukemia inhibitory factor

Introduction

The pituitary is an abundant source of several growth factors which also act as mediators of in vitro hormone production. In-

terleukins 1, 2, and 6 are detectable both in rodent and human pituitary tissue and in human pituitary tumors (1–3). Development and function of differentiated pituitary neuroendocrine cells are regulated both by hypothalamic trophic hormones and by intrapituitary cytokines and growth factors. These cytokines have been shown to stimulate the hypothalamic–pituitary–adrenal axis in vivo and pituitary ACTH production (4–6).

The leukemia inhibitory factor (LIF)¹, originally isolated as an inhibitor of mouse-myeloid M1 cells, has been shown to be secreted by bovine pituitary monolayer cells (7) and we have recently demonstrated LIF gene expression in human fetal, adult, and murine pituitary cells (8). We have also shown that human fetal pituitary cells express high-affinity LIF binding (8). Murine pituitary AtT20 cells also express LIF binding sites and respond to LIF by increasing steady state levels of proopiomelanocortin (POMC) mRNA and ACTH peptide secretion (8). Expression of LIF and its binding sites varies among the human fetal pituitary cell populations, with the highest proportion of LIF-immunopositive cells being corticotrophs (8). In addition to these observations in human fetal pituitary, LIF immunoreactivity and receptor expression was also observed in normal and adenomatous pituitary tumor tissue removed at hypophysectomy (8). These data suggest an important role for LIF, both in normal pituitary development and in pituitary tumor pathogenesis.

LIF exerts its effects by activating specific target receptors. LIF receptors share a common receptor subunit, gp130, with the closely related oncostatin M (OSM) and also with ciliary neurotrophic factor, interleukin 11, interleukin 6, and cardiotrophin 1 (9–13). In addition, LIF, ciliary neurotrophic factor, and OSM also share the structurally related low affinity LIF-receptor beta subunit (14) which heterodimerises with gp130 to form a high affinity binding site (15). Ciliary neurotrophic factor signaling also requires an alpha subunit, as does IL-6. This subunit has a limited tissue distribution, thus determining selective tissue action (16). These diverse cytokines therefore retain their specificity of downstream intracellular action despite sharing common transmembrane receptor subunits.

The LIF-related cytokines likely act by stimulating activity of the JAK family of transmembrane receptor-associated tyrosine kinases (17). Subsequently, phosphorylation of signal transduction and activation of transcription (STAT) family members occurs (18, 19), which appears to be followed by translocation to the nucleus and participation in the assembly of a variety of transcription factor complexes (20–22). This JAK–STAT pathway has been linked to signaling by growth

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1. *Abbreviations used in this paper:* CNTF, ciliary neurotrophic factor; CRH, corticotropin-releasing hormone; LIF, leukemia inhibitory factor; LIF-R, LIF-receptor; OSM, oncostatin M; POMC, proopiomelanocortin; STAT, signal transduction and activation of transcription.

hormone (23), prolactin (24), erythropoietin, (25) and the interferons (21, 26–30).

LIF is a differentiation factor in a number of tissues, so its presence in fetal pituitary corticotrophs, and its previously demonstrated stimulation of ACTH production, suggest it may have a role in pituitary development. LIF and related cytokines exert divergent actions in diverse target tissues, raising the possibility that different intracellular substrates may be involved in signaling. The best characterized signaling molecules for this class of ligands are STAT 1 and STAT 3 and it is likely that additional STAT family members exist. Cytokine signaling typically leads to gene induction through specific cytokine response elements, but the POMC gene does not contain such consensus sequences. We have therefore investigated intracellular signaling triggered by LIF, measured LIF-induced changes in POMC gene transcription, and determined the POMC gene elements required for cytokine action.

Methods

Cell culture. AtT20 cells, obtained from the American Type Culture Collection (Rockville, MD), were grown in DME supplemented with 10% FCS, 2 mM L glutamine, streptomycin, and penicillin (all supplied by GIBCO BRL, Gaithersburg, MD). Human IgE myeloma cells, U266, were also obtained from American Type Culture Collection and were cultured in RPMI with 15% FCS, 2 mM glutamine, streptomycin, and penicillin. Bacterially expressed, recombinant LIF and OSM were obtained from R & D Systems, Inc. (Minneapolis, MD) and recombinant IL-6 was obtained from GIBCO BRL. Goat anti-human gp130 IgG and anti-LIF IgG were obtained from R & D Systems, Inc. Polyclonal, rabbit anti-LIF (LIFR) receptor serum, raised against the soluble, extracellular portion of the LIFR was obtained from Immunex Corp. (Seattle, WA). ACTH assay was performed using a RIA kit purchased from Diagnostic Products Corp. (Los Angeles, CA). Intraassay variation was 4.9% at 152 pg/ml, inter-assay variation was 6.4% at 134 pg/ml.

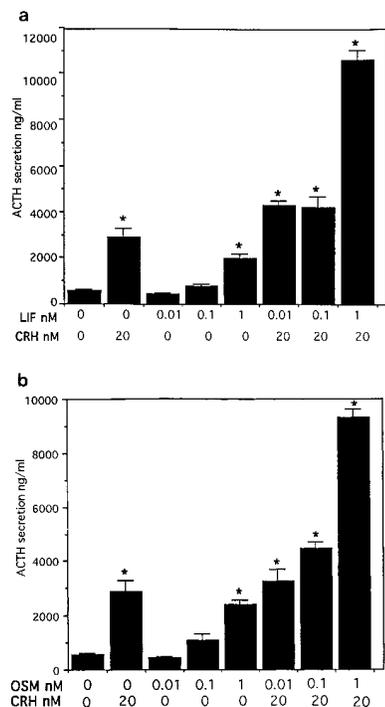


Figure 1. Effects of LIF, OSM, and CRH on ACTH secretion. AtT20 cells were serum starved for 24 h before being incubated for 24 h with the indicated concentrations of CRH and LIF (a) or CRH and OSM (b). Representative experiment performed in triplicate. *indicates $P < 0.05$ compared to control by ANOVA.

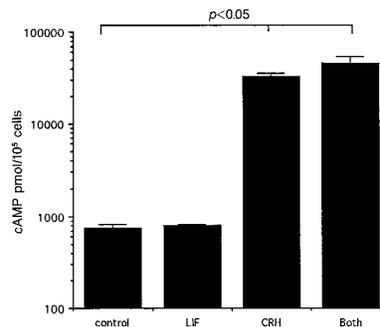


Figure 2. Effects of LIF and CRH on intracellular cAMP levels in AtT20 cells. Cells were serum starved for 24 h, and then treated with 1 nM LIF, 10 nM CRH, or both in the presence of 0.9 nM isobutylmethylxanthine. Cells were harvested after 15 min and extracted with 95% ethanol:0.1 N HCl.

cAMP was measured by RIA. The results are expressed as pmol cAMP/100,000 cells. Statistical comparison is by ANOVA, followed by Bonferonni t test.

cAMP assay. AtT20 cells were cultured in serum-free medium for 24 h before treatment with LIF, corticotrophin-releasing hormone (CRH), or both in the presence of 0.9 nM isobutylmethylxanthine for 15 min. Cells were washed in ice cold PBS and then extracted twice in ice cold 95% ethanol: 0.1 N HCl. The extracts were dried, then dissolved in assay buffer. The cAMP was measured using an RIA kit, Biotrak from Amersham Corp. (Arlington Heights, IL), according to the manufacturer's recommendations.

Immunoprecipitation and Western blotting. For immunoprecipitation with anti-ISGF3, cells were lysed in 1% Triton X100 in buffer containing Tris 50 mM, 150 mM NaCl, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1 mM phenylmethylsulfonylfluoride (PMSF). Lysate was precleared with protein A-Sepharose (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) for 1 h at 4°C, then incubated with antiinterferon stimulated gene factor 3 (ISGF3) (Transduction Laboratories, Lexington, KY) overnight at 4°C. Immune complexes were precipitated by addition of 5 μ g rabbit anti-mouse (Dako Corp., Carpinteria, CA) and 30 μ l of 50% protein A-Sepharose. The pellet was washed three times in ice cold lysis buffer and then eluted in 30 μ l 2 \times SDS-PAGE loading buffer by boiling for 5 min.

For immunoprecipitation with antiSTAT 3, cells were lysed in boiling 1% SDS, 10 mM Tris (pH 7.4). Lysate was then diluted 1:10 in immunoprecipitation buffer consisting of 1% Triton X100, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM PMSF, and 0.5% NP-40. 1 μ g of anti-STAT 3 was added and the tube rocked at room temperature for 1 h. Immune complexes were precipitated with the addition of 5 μ g rabbit anti-mouse IgG (Dako Corp.) and 50 μ l 50% protein A-Sepharose. The pellet was washed three times in immunoprecipitation buffer, and then eluted in SDS-PAGE sample buffer.

The supernatant was loaded onto a 7.5% SDS-PAGE gel for electrophoresis. The proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore Corp. Waters Chromatography, Milford, MA) in 25 mM Tris (pH 8.3), 192 mM glycine, and 20% vol/vol methanol at 400 mA for 1 h. The membrane was blocked in 3% grade III ovalbumin (Sigma Chemical Co., St. Louis, MO), 150 mM NaCl, 10 mM Tris (pH 8.0), and 0.05% Tween. Antiphosphotyrosine detection used biotinylated PY20 in blocking buffer for 14 h at 4°C. The membrane was washed in 150 mM NaCl, 10 mM Tris (pH 8.0), and 0.05% Tween. Streptavidin-peroxidase conjugate (Amersham Corp.) was incubated with the membrane at 1:1,500 dilution with blocking buffer, and then, after further washes, detection was accomplished using ECL reagent as suggested by the manufacturer (Amersham Corp.).

To reprobe membrane, antibodies were stripped using 100 mM betamercaptoethanol, 2% SDS, and 62.5 mM Trip (pH 6.7) at 50°C for 30 min.

Plasmids. Full length POMC-luc (-706/+64) was the kind gift of

Dr. M. Low (Vollum Institute, OR). The (-323/-34)POMC-luc deletion was created by cutting the parent plasmid with Stu1 and Pst1 (GIBCO BRL), polishing the ends with Klenow (GIBCO BRL), and self-ligating with T4 ligase (GIBCO BRL). The (-323/-166)POMC-luc deletion was created by cutting the parent plasmid with Stu1 and BssH11 (GIBCO BRL), polishing the ends with Klenow (GIBCO BRL), and self-ligating with T4 ligase (GIBCO BRL).

Transfection. Cells were plated in growth medium and allowed to adhere. Cells were then washed in serum and antibiotic-free medium and overlaid with DNA/lipofectamine (GIBCO BRL) mix. Routinely, 2 µg DNA were used per well. Cells were incubated with DNA for 5 h, then the medium was changed to DME supplemented with glutamine 2 mM and BSA 0.1%. Treatments were added after 16 h, and cells harvested 6 h after that. Cells were washed in ice cold PBS, and then lysed in 25 mM Tris phosphate (pH 7.8), 10 mM MgCl₂, 0.1% BSA, 15% glycerol, 1% Triton X100, and 1 mM EDTA. 180 µl of cleared cell lysate was used in a luciferase assay. Luciferase activity was measured in a Berthold Lumat LB 9501 luminometer (Wallac, Inc., Gaithersburg, MD) in the presence of 0.8 mM ATP and 0.3 mM D-luciferin. Integrated light emission over 15 s was measured. It was found in preliminary work that transfection efficiency varied less than 15% within a given experiment, as reported by others (31). Thus cotransfection of another reporter to control for transfection efficiency was not necessary. All transfections were repeated at least three times.

Results

LIF related cytokines and CRH synergise to stimulate ACTH secretion in AtT20 cells. To determine the interaction of CRH with LIF, AtT20 cells were incubated for 24 h in the presence of increasing doses of LIF with CRH. As shown in Fig 1a, LIF induced ACTH secretion in a dose-dependent manner. 1 nM LIF caused a fourfold induction in ACTH secretion after 24 h. Although CRH (20 nM) alone induced the expected sevenfold induction of ACTH, combination of the two compounds was synergistic with maximal doses of both agents together stimulating ACTH production 20-fold. Similarly, OSM induced

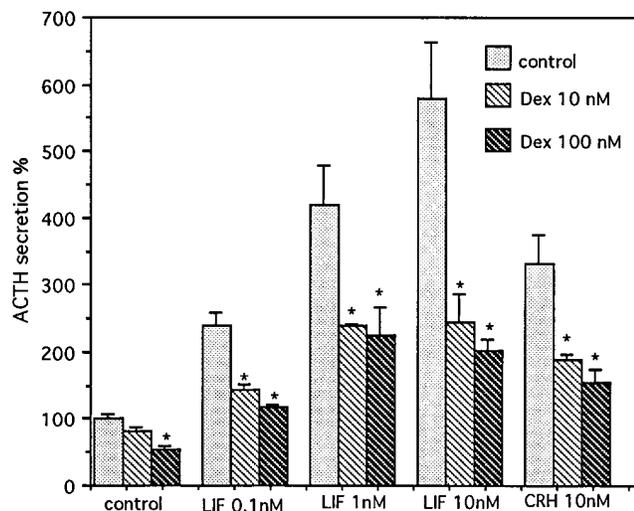


Figure 3. Dexamethasone inhibits LIF induction of ACTH secretion. AtT20 cells were serum starved for 24 h before being treated with dexamethasone for 48 h, followed by addition of indicated peptide for another 24 h. Results are expressed as percentage change relative to normalized control. LIF was used at 1 nM, CRH at 10 nM. Representative experiment performed in triplicate. *indicates $P < 0.05$ compared to control by ANOVA.

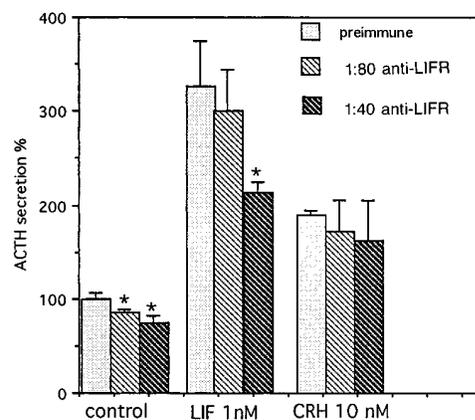


Figure 4. LIF-R antiserum attenuates LIF effects on ACTH secretion. AtT20 cells were incubated with rabbit LIF-receptor antiserum or preimmune rabbit serum with or without the indicated compounds for 24 h. Results are expressed as percent change over normalized control. Representative experiment performed in triplicate. *indicates $P < 0.05$ compared to preimmune serum by ANOVA.

ACTH secretion 6-fold and, together with CRH, resulted in a synergistic 18-fold induction of peptide release (Fig. 1b). Furthermore, although cAMP levels increased over 40-fold within 15 min in response to CRH, LIF did not alter cAMP accumulation in these cells (Fig. 2). These results suggest that the synergy between CRH and LIF signaling on POMC does not occur at the level of cAMP accumulation.

Glucocorticoid inhibits LIF induction of ACTH secretion. As glucocorticoids suppress ACTH release and inhibit POMC gene transcription in pituitary corticotrophs (32), we examined modulation of ACTH secretion by LIF in the presence of dexamethasone. Pretreatment of AtT20 cells with 10 or 100 nM dexamethasone effectively blocked 1 nM LIF mediated induction of ACTH secretion and also inhibited 10 nM CRH stimulation of ACTH as expected (Fig. 3). The inhibition of LIF action by dexamethasone was not overcome by 10 nM LIF.

LIF activation and ACTH secretion. To determine whether the LIF effects on ACTH secretion were mediated by the low affinity LIF-transmembrane receptor, a series of incubations were performed in the presence of either LIF-R antiserum or preimmune rabbit serum. As expected, 1 nM LIF significantly induced stimulation of ACTH during 24 h of incubation. In the absence of added LIF, the addition of LIF-R antiserum (1:80-1:40) significantly inhibited basal ACTH concentrations ($P < 0.05$), consistent with apparent interruption of an autocrine LIF-positive regulatory effect on ACTH secretion (Fig. 4). LIF-R antiserum (1:40) also attenuated 1 nM LIF induction of ACTH production by approximately 35% ($P < 0.05$). There was a modest, but not significant, effect of the specific antiserum on CRH induction of ACTH.

gp130 activation and ACTH secretion. To clarify the role of the gp130 receptor component in pituitary LIF signaling, we tested the effects of goat anti-human gp130 IgG. Due to limited cross-reactivity with the murine receptor, high concentrations of this IgG were used. Induction of ACTH secretion by both LIF and OSM, which are known to require gp130, was inhibited by coincubation with gp130 antibody (Fig. 5). The antiserum attenuated LIF- and OSM-induced ACTH secretion by 41 and 38%, respectively ($P < 0.05$). Under these conditions,

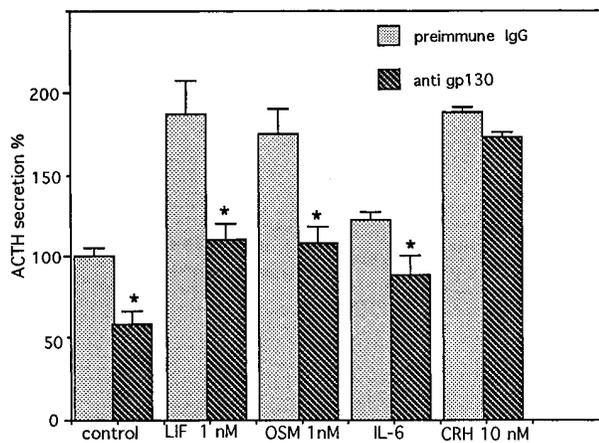


Figure 5. Anti-gp130 inhibits ACTH secretion. AtT20 cells were incubated with goat anti-gp130 IgG or normal goat IgG at 250 μ g/ml and the indicated peptides for 24 h. Results are expressed as percent change over normalized control. Representative experiment performed in triplicate. *indicates $P < 0.05$ compared to normal IgG control by ANOVA.

and in the absence of added cytokine, basal ACTH release was inhibited by 42% ($P < 0.05$), again consistent with an autocrine LIF effect on ACTH. In the presence of IL-6, which also signals through gp130, the antiserum also attenuated ACTH secretion by 28%. A small and statistically insignificant (8%) reduction in CRH-induced stimulation by anti-gp130 possibly reflects loss of endogenously derived LIF synergy with exogenous CRH.

STAT 1 α tyrosyl phosphorylation. As LIF signaling may be mediated by STAT proteins, we tested the activation of these substrates in LIF-treated AtT20 cells. LIF-induced tyrosyl phosphorylation of proteins immunoprecipitated with anti-STAT 1 was examined using the antiphosphotyrosine monoclonal antibody PY20 (Fig. 6 a). In these cells, LIF-induced phosphorylation of two principal proteins, one with the expected electrophoretic mobility of STAT 1 α and the other migrating with a predicted molecular mass of 115 kD. A

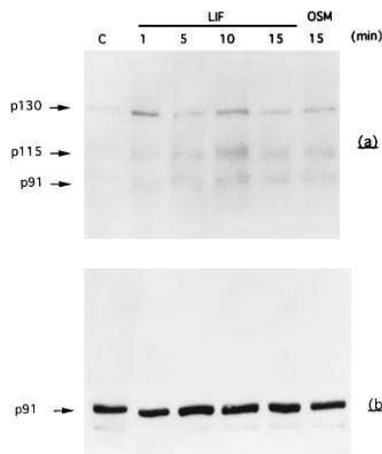


Figure 6. LIF induces tyrosyl phosphorylation of STAT 1 and p115 proteins in AtT20 cells. AtT20 cells were serum starved for 24 h before being treated with LIF (1 nM) or OSM (1 nM) for the indicated times before lysis and immunoprecipitation with anti-STAT 1, SDS-PAGE, and Western blotting with antiphosphotyrosine (a). The membrane was stripped and reprobed with anti-STAT 1 (b) to

verify equal protein loading. Molecular weight was estimated against a prestained standard ladder from Amersham Corp. and is indicated.

tyrosyl phosphorylated protein sized at approximately 130 kD was also consistently observed and appeared to be phosphorylated under basal condition (Fig. 6, 7). Since JAK kinases migrate to approximately this position and are known to interact closely with STAT 1 α , we sought to examine whether p130 was a member of this kinase family. However, reprobing of the blots with antibodies to JAK-1, JAK-2, or Tyk-2 failed to detect this band (data not shown). Others have previously observed this band and attributed it to coimmunoprecipitated gp130 (18, 19).

After probing with PY20, the membranes were stripped and reprobed with anti-ISGF3, which recognizes both splice variants of the STAT 1 gene, STAT 1 α and 1 β . This showed equal loading of STAT 1 protein in each lane and confirmed the identity of the faster migrating band as STAT 1 α (p91) (Fig. 6 b). However, the p115 protein, although immunoprecipitated by anti-ISGF3, was not recognized by the same antibody after denaturation. The rapid induction of STAT phosphorylation observed by 1 min, waned after 10 min. The time course for phosphorylation of both STAT 1 α and p115 occurred in parallel.

To confirm that the observed changes in STAT protein phosphorylation were due to the specific activity of LIF, we attempted to block this effect with anti-LIF serum. We used the titer of antiserum previously shown by us to block the effects of LIF on ACTH production (1:160) (8). The addition of LIF and preimmune serum caused a small, variable, additional induction of STAT protein phosphorylation, while the specific anti-LIF serum not only blocked this effect but reduced the phosphorylation state of both STAT 1 α protein and p115 to that of quiescent, unstimulated cells (Fig. 7).

As OSM shares the gp130 subunit with LIF, and also stimulated POMC expression in AtT20 cells, the effects of OSM on STAT protein phosphorylation were examined. After 10 min, OSM induced comparable induction of STAT 1 phosphorylation similar to that seen with LIF (Fig. 6, 7). In addition, the related p115 protein was similarly induced by OSM.

STAT 3 activation. LIF-R signaling is reported to result in phosphorylation of STAT 3 protein in addition to STAT 1 α (18, 19). STAT 3 (or acute phase response factor was originally cloned as the 92-kD protein tyrosyl phosphorylated in response to interferon (33). After treatments, cell lysis under denaturing conditions was performed since the STAT 3 antibody

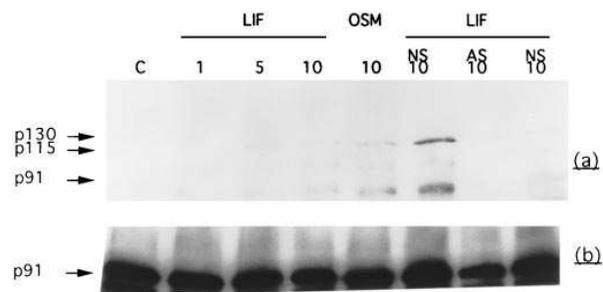


Figure 7. LIF induces tyrosyl phosphorylation of STAT 1 and p115 proteins in AtT20 cells. AtT20 cells were serum starved for 24 h before being treated with LIF (1 nM) or OSM (1 nM) either with or without normal IgG (NS) or antiLIF IgG (AS) before lysis, immunoprecipitation with anti-STAT 1, and Western blotting with antiphosphotyrosine (a). The membrane was stripped and reprobed with anti-STAT 1 (b). Estimated molecular weights are indicated.

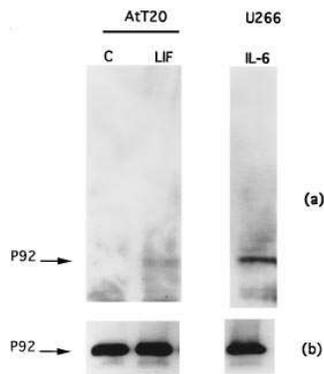


Figure 8. LIF induces tyrosyl phosphorylation of STAT 3 in AtT20 cells. AtT20 cells and myeloma cells U266 were serum starved for 24 h before treatment of AtT20 cells with LIF (1 nM) and U266 cells with IL-6 (50 ng/ml) for 5 min before lysis, immunoprecipitation with anti-STAT 3, SDS-PAGE, and Western blotting with anti-phosphotyrosine (a). The membrane was stripped and re-probed with anti-STAT 3 (b) to confirm the identity of the phosphorylated band and to confirm equal loading. Estimated molecular weights are indicated.

does not appear to recognize the native conformation of the molecule. LIF induced rapid tyrosyl phosphorylation of a protein band in AtT20 cells, which was also observed in the human, IL-6-responsive, myeloma cell line U266 (Fig. 8 a). The identity of this band was confirmed by re-probing the blot with anti-STAT 3, depicted in Fig. 8 b.

POMC transcription. To directly examine the effects of LIF on POMC gene transcription, a fragment of the rat POMC gene -706/+64 fused to the luciferase reporter gene was transfected into AtT20 cells using lipofectamine. This region of POMC contains the elements required for pituitary expres-

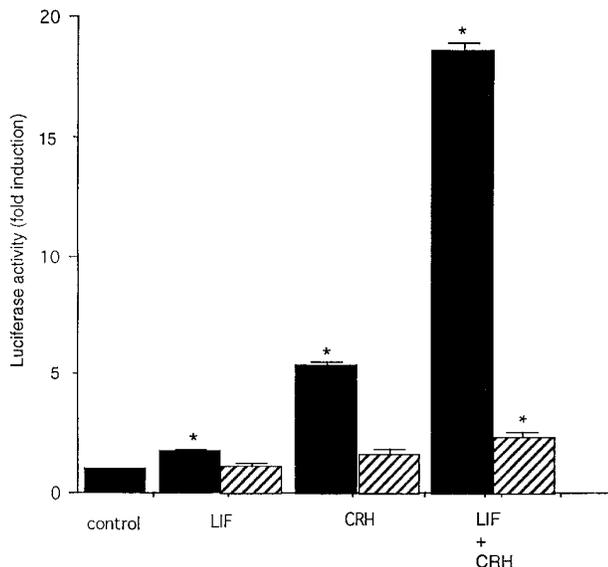


Figure 9. LIF and CRH effects on POMC transcription. POMC-luc (-706/+64) (solid bars) or SV40-luc (diagonal striped bars) were transfected into AtT20 cells using lipofectamine. Cells were treated with vehicle (control), LIF (1 nM), CRH (10 nM), or both LIF 1 nM and CRH 10 nM in serum-free medium for 6 h. Cells were then washed and harvested into lysis buffer. Results are expressed as fold induction of luciferase activity over control, mean of three wells \pm SD. * indicates $P < 0.05$ compared to control.

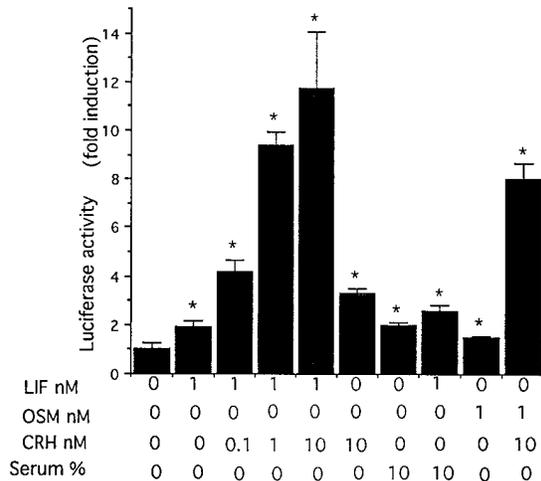


Figure 10. LIF, OSM, CRH, and serum effects on POMC transcription. POMC-luc (-706/+64) was transfected into AtT20 cells using lipofectamine. Cells were treated with LIF, OSM, CRH, or serum in the indicated concentrations in serum-free medium for 6 h before harvest. Results are expressed as fold induction of luciferase activity over control, mean of three wells \pm SD. * indicates $P < 0.05$ compared to control.

sion of the gene and regulation both by CRH and glucocorticoid (31, 34). 1 nM LIF exerted modest effects on luciferase expression, typically a twofold induction (Fig. 9-12). This effect was not significantly enhanced by the addition of serum to the incubation medium. CRH, as expected, stimulated expression of the reporter (5.4-fold). LIF exerted striking synergy with 10 nM CRH in inducing reporter gene activity, with 1 nM LIF enhancing the reporter from 5.4-18.6-fold (Fig. 9). This effect was seen over a range of CRH concentrations from 0.1-10 nM (Fig. 10). The related cytokine oncostatin M at 1 nM concentration also was a weak agonist of POMC transcription, enhancing reporter gene activity 1.6-fold (Fig. 10), but, together with 10 nM CRH, increased reporter activity 10-fold.

The major CRH-responsive region in the POMC reporter is located between -133 and -236 (35). Deletions of part (-323/-166) and all (-323/-34) of this region were performed in an attempt to more clearly delineate the element responsible for LIF action. Both deleted constructs were weaker than the intact promoter in driving POMC expression (Fig. 11). The more severe deletion (-323/-34) had only 23% of wild-type activity under basal, untreated conditions, while the more conservative deletion had activity reduced to 85% of wild type. Both constructs exhibited markedly reduced LIF (1 nM) responsiveness, 5 and 54% induction, respectively, and CRH (10 nM) responsiveness, 41 and 85% induction, comparable to a minimal SV40 promoter in the same plasmid (Fig. 9). However, both of these constructs exhibited enhanced transcription in response to the combination of CRH (10 nM) and LIF (1 nM) (160 and 270%, respectively) (Fig. 11).

To determine whether CRH synergy with LIF was dependent on activation of cAMP pathways, transfectants were treated with either 8 bromo cAMP or forskolin. Expression of the full-length POMC reporter was only induced 2.7-fold by forskolin 5 μ M, compared to 2.2-fold with LIF 1 nM and 3.7-fold with CRH 10 nM. Forskolin and 8 bromo cAMP were

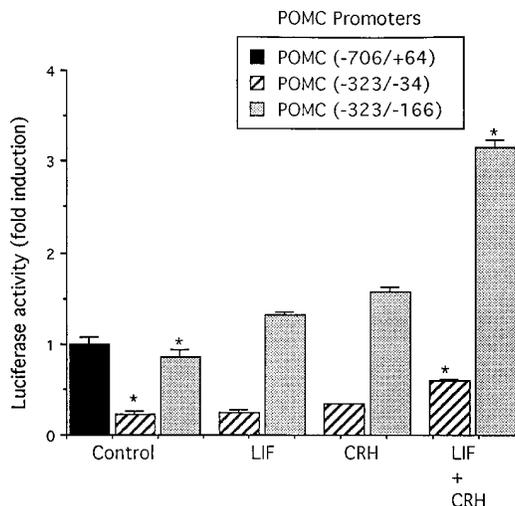


Figure 11. Basal activity and LIF responses of (–323/–34)POMC-luc and (–323/–166)POMC-luc. Full-length POMC-luc (–706/+64) [solid bar], (–323/–34)POMC-luc [diagonal striped bars] or (–323/–166)POMC-luc [speckled bars] were transfected into AtT20 cells. Cells were grown in serum-free medium and harvested after 6 h. Basal activity of the two deleted POMC constructs were compared to that of the full-length promoter (control). Also, the responses of the two deletion mutants to 1 nM LIF (LIF), 10 nM CRH (CRH), or both LIF and CRH (LIF+CRH) were measured and are compared to basal activity of the two constructs. Results are expressed as fold induction over control, mean of three wells \pm SD. * indicates $P < 0.05$. POMC (–323/–34) and POMC (–323/–166) differ significantly from POMC (–706/+64) under control conditions. Combined LIF and CRH significantly enhanced both POMC (–323/–34) and POMC (–323/–166) transcription.

consistently weaker agonists than CRH in this assay. However, 1 nM LIF enhanced the effect of 5 μ M forskolin to 7.4-fold induction over control. Under the same conditions, 1 nM LIF enhanced the stimulation by 10 nM CRH from 3.7-fold over control to 14.6-fold over control (Fig. 12). Again, the cAMP agonists were consistently weaker than CRH even after LIF 1 nM addition.

Discussion

We originally identified LIF gene expression in human fetal pituitary tissue and demonstrated that LIF binding and immunoreactivity was predominantly associated with corticotroph cells (8). We have also shown LIF immunostaining in functional pituitary adenomas and demonstrated that murine AtT20 cells also express LIF mRNA and respond to LIF by increasing POMC gene expression (8). We now extend these observations by demonstrating that both LIF and OSM exert synergistic effects with CRH on POMC transcription. This induction of POMC by LIF is not explicable by augmenting CRH induction of cAMP. We also show that dexamethasone is a potent inhibitor, not only of CRH induction of ACTH secretion, but also of LIF action, suggesting distal convergence of these three signaling pathways, possibly at the level of the POMC gene.

The LIF-R is required to mediate LIF effects in AtT20 cells as evidenced by the immunoneutralization data shown above. These experiments also support the hypothesis that LIF is an

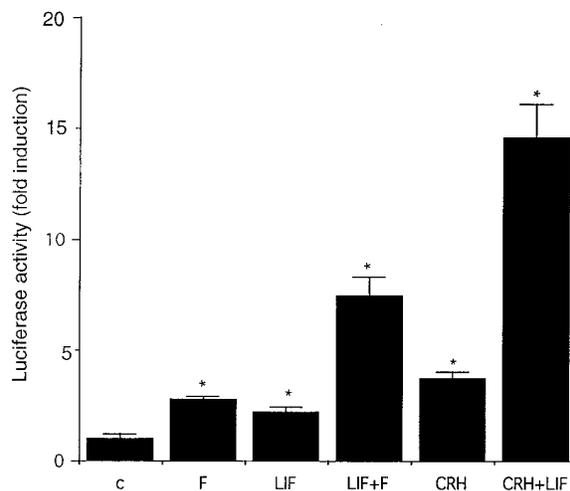


Figure 12. LIF, CRH, and forskolin effects on POMC transcription. Full-length POMC-luc (–706/+64) was transfected into AtT20 cells. Cells were treated with 5 μ M forskolin (F), 1 nM LIF (LIF), combination of forskolin and LIF (LIF+F), 10 nM CRH (CRH), or CRH and LIF (CRH+LIF). Results are expressed as fold induction of luciferase activity over vehicle-treated control cells, mean of three wells \pm SD. Representative experiment performed in triplicate. * indicates $P < 0.05$ compared to control.

important autocrine factor for sustaining basal ACTH production in AtT20 cells. Prolonged incubations with receptor antiserum exerted only slight effects on cell proliferation, excluding a direct cytotoxic effect, but LIF itself modestly inhibits cell number at concentrations greater than 1 nM (data not shown). Further immunoneutralization experiments support the importance of the shared gp130 subunit in LIF and OSM signaling in AtT20 cells and also in mediating IL-6 effects. Both anti-LIFR and anti-gp130 caused small decreases in CRH stimulated ACTH production in the absence of added LIF. As discussed above, this latter observation probably reflects loss of autocrine LIF effects on ACTH secretion.

The LIF-related cytokine IL-6 has been shown to powerfully stimulate ACTH release in vivo (36), at least in part by a hypothalamic mechanism. It is possible that part of this observed effect may result from IL-6 stimulation of local pituitary LIF production.

The activated LIF-R complex is thought to recruit and activate two of the three JAK kinase family (17) and subsequently induce tyrosyl phosphorylation of STAT members (18). We now demonstrate a time-dependent induction of STAT 1 α phosphorylation using antiphosphotyrosine antibody and also detect a larger protein species that was immunoprecipitated by the anti-ISGF3 under nonreducing conditions. This larger protein may be coimmunoprecipitated and so represent a partner for STAT 1 or possibly reflect a shared epitope that is not recognized by the antibody after denaturation. As the epitope on STAT 1 is recognized by this antibody in both native and denatured conformations, the more probable hypothesis is that p115 is a discreet protein which coimmunoprecipitated with STAT 1 α under these conditions. Furthermore, we also demonstrate STAT 3 tyrosyl phosphorylation induced by LIF in the AtT20 cells, supporting a hypothesis that multiple STAT family members are involved in mediating LIF effects in AtT20 cells.

We demonstrate that LIF exerts a transcriptional effect on the $-706/+64$ POMC gene fragment. It is possible that another cytokine-responsive element lies further upstream of the promoter fragment used (-706). In the presence of low concentrations of CRH, LIF exerts powerful effects on gene transcription driven by this promoter. Our data suggest that more than one region in the promoter may be responsible for mediating this effect. Certainly, abolishing CRH action by deletion of the principal CRH responsive element severely blunts the synergy of LIF with CRH, (from 18-fold induction to 3.6-fold), but it does not completely ablate it. This suggests that LIF/CRH synergy may occur elsewhere within the gene. A functional AP-1 site has recently been described within the non-coding exon 1 of POMC (+44) (37). It is possible that LIF/CRH action is partly mediated by this element, previously shown to be CRH responsive.

In summary, the murine AtT20 cells provide a useful model for studying LIF action on the pituitary corticotroph. Both gp130 and LIF-R components are required to mediate LIF and OSM actions on POMC expression in AtT20 cells. LIF signaling involved tyrosyl phosphorylation of STAT 1 α , STAT 3, and a novel p115 protein. These results, in conjunction with our earlier observations (8), ascribe a mechanism for cytokine-mediated regulation of ACTH. Thus, in addition to CRH and glucocorticoid, the LIF-related cytokines appear to participate in regulating POMC gene expression.

Recently, increasing evidence supports the existence of a network of distinct cytokines interfacing between the endocrine and immune systems and participating in neuroendocrine stress responses (38). Although we have also shown that LIF stimulates ACTH secretion in primary cultures of mouse pituitary (data not shown), it is unclear yet whether pituitary LIF action is solely paracrine or whether endocrine-derived LIF also participates in this immunopituitary interface. We have recently shown that *in vivo* pituitary LIF expression is regulated by LPS stress (Wang, Z., S.-G. Ren, and S. Melmed, manuscript submitted for publication). Thus, regarding neuroendocrine stress responses, if intracellular signaling events similar to those observed in AtT20 cells were to occur *in vivo*, they would provide a mechanistic explanation for continued activation of the hypothalamic-pituitary-adrenal axis in conditions of stress. The continued activation of this axis may be achieved through the LIF-mediated potentiation of CRH action so as to overcome increased glucocorticoid negative feedback at the level of the hypothalamus and pituitary. This may also explain the paradoxical fall in hypothalamic CRH concurrent with an increase in adenohypophysal POMC observed in an animal model of chronic inflammatory disease (39).

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