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Transregulation of Leukemia Inhibitor Factor Receptor Expression and Function by Growth Factors in Neuroblastoma Cells

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Abstract

The cytokines that signal through the leukemia inhibitory factor (LIF) receptor are members of the neurotrophic cytokine family and have varied and numerous roles in the nervous system. In this report we have determined the effects of growth factor stimulation on LIF receptor (LIFR) expression and signal transduction in the human neuroblastoma cell line NBFL. We show here that stimulation of NBFL cells with either epidermal growth factor or fibroblast growth factor decreases the level of LIFR in an extracellular signal-regulated kinase (Erk)1/2-dependent manner and that this downregulation is due to an increase in the apparent rate of lysosomal LIFR degradation. Growth factor-induced decreases in LIFR level inhibit both LIF-stimulated phosphorylation of signal transducers and activators of transcription 3 (STAT3) and LIFR-mediated gene induction. We also show that Ser1044 of LIFR, which we have previously shown to be phosphorylated by Erk1/2, is required for the inhibitory effects of growth factors. Neurons are exposed to varying combinations and concentrations of growth factors and cytokines that influence their growth, development, differentiation and repair *in vivo*. These findings demonstrate that LIFR expression and signaling in neuroblastoma cells can be regulated by growth factors that are potent activators of the mitogen activated protein kinase pathway, and thus illustrate a fundamental mechanism that underlies cross-talk between receptor tyrosine kinase and neurotrophic cytokine signaling pathways.

Keywords

leukemia inhibitory factor; gp130; growth factor; MAP Kinase; cytokine

The leukemia inhibitory factor receptor (LIFR), together with glycoprotein 130 (gp130), acts as a signal transducing receptor for a group of related cytokines that include leukemia

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¹The abbreviations used in the text are: CaMK, Ca²⁺/calmodulin-dependent kinase; CLC, ciliary neurotrophic factor; CNTF, ciliary neurotrophic factor; CNTFR, ciliary neurotrophic factor receptor; CT-1, ciliary neurotrophic factor-1; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; FGF, fibroblast growth factor; G-CSF, granulocyte colony stimulating factor; G-CSFR, G-CSF receptor; gp130, glycoprotein 130; IL, interleukin; JAK, Janus kinase; LIF, leukemia inhibitory factor; LIFR, leukemia inhibitory factor receptor; MAPK, mitogen activated protein kinase; MEK, MAPK kinase; NGF, nerve growth factor; OsM, Oncostatin M; PERK, phosphorylated ERK; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PIAS, protein induced by activated STATs; PMA, phorbol 12-myristate 13-acetate; PY-STAT3, Tyr705-phosphorylated STAT3; SDS, sodium dodecyl sulphate; SHP, src homology 2 domain-containing phosphatase; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; Tfr, transferrin receptor; TNF, tumor necrosis factor; VIP, vasoactive intestinal peptide.

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inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), oncostatin M (OsM) and neuropoietin, which are all members of the gp130-family of cytokines (Taga and Kishimoto 1997; Elson et al. 2000; Turnley and Bartlett 2000; Heinrich et al. 2003; Derouet et al. 2004). The cytokines in this family all signal through heteromeric complexes involving gp130 and at least one other receptor component (Taga and Kishimoto 1997). CNTF, CLC and neuropoietin all use an additional extracellular receptor, CNTF receptor (CNTFR) for initial binding to the cell surface and formation of the signal transducing receptor complex (Ip et al. 1993; Lelievre et al. 2001; Derouet et al. 2004). CT-1 is also believed to require a third receptor for signaling that is not CNTFR (Robledo et al. 1997). LIF requires only LIFR and gp130 for binding and signal transduction but human OsM can signal through a heteromer consisting of gp130 paired with either LIFR or the related OsM receptor (Turnley and Bartlett 2000). Additional gp130-family members which signal through complexes that include gp130 but not LIFR include interleukin (IL)-6, IL-11 and IL-27 (Heinrich et al. 1998; Pflanz et al. 2004).

When the genes encoding LIF, CNTF, OsM and CT-1 are knocked out individually or in combination, the mutant mice exhibit relatively mild phenotypes (Heinrich et al. 1998; Oppenheim et al. 2001; Morikawa et al. 2004; Holtmann et al. 2005). However the importance of the cytokine family as a whole is demonstrated by the embryonic and perinatal lethality of knockouts of the genes for either gp130, LIFR or CNTFR (Heinrich et al. 1998). In fact, cytokines signaling through the LIFR have been shown to have diverse effects both inside and outside the nervous system, playing roles in biological functions as varied as blastocyst implantation, cardiac hypertrophy and neuronal survival (Heinrich et al. 1998; Sharkey 1998; Li et al. 2004). Even within the nervous system, the effects of LIF, CNTF, CLC, OsM and CT-1 are pleiotropic. The LIFR acts in conjunction with gp130 within the peripheral and central nervous systems to mediate signals involved in survival and regeneration of various neuron types as well as differentiation and development of neurons and glial cells (Turnley and Bartlett 2000; Barnabe-Heider et al. 2005; Alfonsi et al. 2008). Cytokines signaling through the LIFR are able to sustain the pluripotentiality of neuronal stem cells (Halvorsen 2006; Bauer et al. 2007). In addition, the specific effect a LIFR-mediated cytokine will elicit varies with stage of neuronal development (Bauer et al. 2007) and is very likely influenced by the presence of other cytokines and growth factors.

Cytokines signaling through the LIFR activate the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway and the mitogen activated protein kinase (MAPK) pathway (Turnley and Bartlett 2000). These pathways are negatively regulated by several constitutive and inducible inhibitors. Phosphatases such as src homology 2 domain-containing phosphatases (SHPs) in the cytosol and additional phosphatases in the nucleus inhibit signaling by acting on the receptors themselves or on activated STATs. However, SHP-2 also functions to link gp130 and LIFR to the MAPK pathway and thus positively regulates MAPK activation (Heinrich et al. 1998; Bartoe and Nathanson 2000). Protein inhibitors of activated STATs (PIAS) inhibit signaling through the LIFR by facilitating sumoylation of STATs. Suppressors of cytokine signaling (SOCS) proteins are induced by cytokines signaling through the LIFR and inhibit that signaling by either binding to LIFR and gp130 themselves or by interacting with receptor-associated JAKs. SOCS proteins also recruit E2 ubiquitin transferase, resulting in the ubiquitination and degradation of signaling components (Heinrich et al. 2003; Yoshimura et al. 2007).

Another way to negatively control signaling through the LIFR is by decreasing the number of receptors available to bind to cytokines and transduce signals. In a number of non-neuronal cell types, cytokines acting through the LIFR are able to downregulate their own receptors (Blanchard et al. 2000; Zvonic et al. 2005; Yu et al. 2007). It has also been shown in non-neuronal cells that other factors signaling independently of LIFR, such as insulin,

epithelial growth factor (EGF) or tumor necrosis factor (TNF) are able to cause a decrease in the amount of LIFR expressed (Blanchard et al. 2000; Yu et al. 2007). Because regulatory mechanisms can be dramatically different in different cell types and because of the vital role played by cytokines that rely on the LIFR in the nervous system, we chose to investigate the potential for transregulation of the LIFR by growth factors in neuroblastoma cells.

In this paper we show that growth factors cause a decrease in LIFR levels in NBFL neuroblastoma cells to varying degrees that correlated with the extent and duration of activation of extracellular signal-regulated kinase (Erk1/2). We also show that the decrease in LIFR is due to an increase in the rate of protein degradation and is blocked by inhibitors of lysosomal but not proteasomal degradation. Growth factor-mediated LIFR downregulation regulates LIF signaling, inhibiting both LIF-stimulated STAT3 tyrosine phosphorylation and LIFR-mediated stimulation of neuropeptide gene transcription.

Experimental procedures

Materials

Rat CNTF, human fibroblast growth factor (FGF), murine EGF, human OsM and human granulocyte-colony stimulating factor (G-CSF) were all obtained from Peprotech (Rocky Hill, NJ, USA). Human LIF and mouse nerve growth factor (NGF) were purchased from Alomone Labs (Jerusalem, Israel). Mouse anti-transferrin receptor (TfR) and mouse anti-p42/p44 (Erk1/2) were from Zymed (San Francisco, CA, USA). Rabbit anti-phosphorylated STAT3 (Tyr 705), rabbit anti-STAT3 and mouse anti-phosphorylated Erk1/2 (Thr 202/Tyr 204) were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-LIFR (C-19) and anti-gp130 (M-20) came from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Secondary antibodies (HRP-conjugated sheep anti-mouse and donkey anti-rabbit) were from Amersham Biosciences/GE Healthcare Biosciences Corporation (Piscataway, NJ, USA). PD98059 and MG132 were purchased from Calbiochem (San Diego, CA, USA). Cycloheximide and chloroquine were from Sigma-Aldrich (St. Louis, MO, USA). Dithiothreitol (DTT) was purchased from Bio-Rad (Hercules, CA, USA). Dulbecco's Modified Eagle Medium (DMEM) was from GIBCO Invitrogen Corporation (Grand Island, NY, USA) and fetal bovine serum (FBS) was obtained from HyClone (Logan, UT, USA). D-Luciferin came from BD Biosciences (San Jose, CA, USA) and Emerald and Galacton-Plus reagents were purchased from Tropic (Bedford, MA, USA). The expression vector for EF- β -galactosidase was generously provided by Dr. Aviva Symes (Pitts et al. 2001).

Cell culture

NBFL cells were obtained from Dr. Aviva Symes and were maintained in DMEM supplemented with 10% heat-inactivated FBS, 5% heat-inactivated horse serum, 100 units/mL penicillin G and 0.1 mg/mL streptomycin sulphate in 10% CO₂ at 37° C.

Assays of receptor levels and STAT3 and ERK1/2 phosphorylation

For assays of LIFR and gp130 levels or STAT3 or ERK1/2 phosphorylation, NBFL cells were plated at a density of 6×10^4 cells per mL on 6-well tissue culture plates (2 mL per well). Cells were grown for 2 days and serum-starved overnight. Cytokines and growth factors were dissolved in phosphate-buffered saline (PBS). PD98059 was dissolved in 100% dimethyl sulfoxide (DMSO), chloroquine in water, while cycloheximide and MG132 were both in 100% ethanol. PD98059 or DMSO vehicle was added 30 min prior to stimulation by growth factors or cytokines. Cycloheximide or ethanol vehicle treatment was concurrent with indicated cytokine or growth factor treatment. MG132 or ethanol vehicle was added 1 h before subsequent growth factor stimulation, while chloroquine or water vehicle was added

45 min before stimulation with growth factors. After stimulation for times indicated, cells were immediately collected in 100 μ L of 2X sample buffer (100 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS), 0.1 M DTT, 20% glycerol and 0.02% bromophenol blue), sonicated at setting 2, duty cycle 20% for 12 pulses with a Branson Sonifier 250 and boiled for 5 min or stored at -20°C for later use. Boiled lysates were separated by SDS-10%-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF transfer membrane. When blotting for receptors, membranes were divided so that multiple proteins could be detected from one gel without stripping and re-probing. For analysis of phosphorylated proteins, samples were run on duplicate gels to avoid stripping and re-probing. Duplicate gels were loaded with lysate samples of equal protein content and were all transferred at 90V for 2 hours to ensure equivalent protein levels on paired blots. Films were scanned and bands quantified using ImageJ software.

DNA constructs and transient cell transfection

The constructs used in this study have been described previously: the vasoactive intestinal peptide (VIP)-Luciferase construct (Johnson and Nathanson 1994; Bartoe and Nathanson 2002), the chimeric G-CSF receptor (G-CSFR)/LIFR constructs (both WT and S1044A) (Schiemann et al. 1995) and the EF- β -gal construct (Pitts et al. 2001). NBFL cells were plated in 24-well tissue culture plates (0.5 mL/well) at a density of 6×10^4 cells per mL and kept in a 10% CO_2 incubator at 37°C . 24 h post-plating, cells were transiently transfected using the calcium phosphate method (Johnson and Nathanson 1994). Transfection was done using 750 ng of total DNA per well, consisting of 200 ng each for cDNAs that encode the G-CSFR/LIFR WT or S1044A chimeras and the VIP-luciferase reporter gene, 100 ng of EF- β -galactosidase and 250 ng of empty pGEM4 or pCD vector in 50 μ L of transfection solution. Cesium chloride- or Qiagen-purified DNA in TE buffer was added to 0.25 M CaCl_2 to make up half the volume of the transfection mixture. The CaCl_2 /DNA mixture was then added drop-wise to an equivalent volume of 2X HEPES buffered saline (50 mM HEPES, 280 mM NaCl, 10 mM KCl, 1.5 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 12 mM dextrose, pH 7.05) with vortexing. The transfection mixture was incubated at room temperature for 15 min before resuspending and adding to wells of NBFL cells. After addition of DNA precipitates, cells were placed in a 5% CO_2 incubator at 37°C .

Chemiluminescence assays

24 h after transfection of cells, the media was removed and replaced with serum-free DMEM with 1X penicillin-streptomycin solution and returned to the 5% CO_2 incubator for approximately 16 h. Cells were pretreated with 10 ng/mL EGF for 4 h or left untreated before stimulating with 10 ng/mL GCSF, 10 ng/mL LIF or an equivalent volume of PBS (vehicle) for 3 h (all at 37°C). After stimulation, the media was removed and 100 μ L of lysis buffer (10 mM ATP, 6 mM MgSO_4 , 1 mM DTT, 0.1% TX-100 and 100 mM KPO_4 , pH 7.8) was added to each well. Cells were frozen at -80°C before thawing on ice for assays. For luciferase assays, 25 μ L of cell lysate was added to 350 μ L of luciferase assay buffer (10 mM ATP, 6 mM MgSO_4 and 100 mM KPO_4 , pH 7.8). Assay tubes were loaded into an AutoLumat Plus LB 953 Luminometer (Berthold Technologies) where 100 μ L of 1.0 mM D-luciferin was added to each tube before measuring and integrating the luminescence created over 30 s. To measure β -galactosidase activity, 25 μ L from each cell extract was placed in tubes on ice and 100 μ L of Galactolight buffer (100 mM NaPO_4 , pH 8.0, 1 mM MgCl_2 and 1% v/v Galacton-Plus) was added to each tube at 10 s intervals and then incubated on ice for 1 h. 100 μ L of Emerald luminescence amplifier was added to each tube in the luminometer and luminescence produced was integrated over 5 s. All experiments were repeated 3 times with 6 – 9 samples from similarly treated replicate culture wells per experiment. 25% of all culture wells in each experiment were left untransfected to measure the levels of luciferase and β -galactosidase luminescence that were not due to transfected

reporters. Average values from untransfected cells were subtracted from values for transfected cells and then corrected luciferase values for transfected cells were divided by corrected β -galactosidase levels from the same sample to normalize for transfection efficiency and cell number. Fold induction due to LIF or G-CSF stimulation was calculated by dividing normalized LIF- or G-CSF-stimulated values by the average of normalized values from similarly treated unstimulated lysates.

Results

Effect of growth factor and cytokine stimulation on LIFR levels in neuroblastoma cells

We first tested if stimulation of NBFL cells with factors that bind the LIFR as well as factors that signal independently of LIFR would cause a change in LIFR levels. NBFL cells were plated and prepared as described in Materials and Methods and then stimulated for 5 h with 100 ng/mL of CNTF, OsM, LIF, FGF, 10 ng/mL EGF or 100 ng/mL NGF. After stimulation, cell lysates were separated by SDS-PAGE and analyzed for expression of LIFR and for expression of the control protein, TfR, which should not be altered by any of the chosen stimulations (Fig 1). None of the cytokines which signal through LIFR caused a significant decrease in the amount of LIFR. However, two of the tested growth factors, EGF and FGF, which do not bind or signal through LIFR, both caused a large decrease (81 and 68% respectively) in LIFR levels while NGF did not cause a significant decrease (Fig. 1).

Decreases in LIFR level and activation of Erk1/2

It has been demonstrated in hepatic cells that transregulation of LIFR by insulin is dependent on the factor's ability to activate the MAPK pathway (Schiemann et al. 1995; Blanchard et al. 2000). While all factors tested in our experiments were able to activate Erk1/2 as measured by phosphorylation of Erk1/2 at Thr202 and Tyr204 (Fig. 2a), LIF, OsM, CNTF and NGF caused a modest degree of Erk 1/2 activation that returned to basal level after 30 to 60 min of stimulation. Conversely, EGF and FGF stimulation resulted in relatively robust phosphorylation of Erk1/2 that lasted at least 3 h, suggesting that a strong and extended activation of Erk1/2 may be required for growth factor-induced downregulation of LIFR. To verify the role of Erk1/2 phosphorylation in LIFR downregulation we treated cells with 50 μ M PD98059, a MAPK kinase (MEK) inhibitor, before stimulating with EGF. As shown in Fig. 2b, pretreatment of cells with PD98059 blocked EGF-induced ERK1/2 phosphorylation so that no P-ERK1/2 was detectable after either 10 or 60 minutes of EGF-stimulation of PD98059-treated cells. PD98059 also significantly inhibited the downregulation in LIFR seen after 5 h of EGF stimulation (Fig. 2c and 2d). Stimulation of cells with EGF led to a 45% decrease in LIFR while EGF stimulation of cells pretreated with PD98059 did not cause a significant decrease in LIFR (Fig. 2d). Treatment of IMR-32 neuroblastoma cells with NGF or FGF also caused prolonged activation of Erk1/2 and decreased levels of LIFR.²

Additive effects of cycloheximide and growth factor stimulation

In order to determine if the observed effect of EGF or FGF stimulation on LIFR in NBFL cells was due to inhibition of LIFR synthesis, we treated cells with 10 μ g/mL cycloheximide, which inhibits synthesis of new proteins, before adding EGF. After treating for times indicated, cell lysates were separated by SDS-PAGE and analyzed for expression of LIFR, gp130 and TfR (Fig. 3). There was no effect of vehicle treatment on levels of either LIFR or gp130 over the 5 h time course. EGF treatment alone caused a significant decrease in LIFR level over 5 h but had no effect on the level of gp130. While cycloheximide treatment caused a steady decline in the amount of LIFR and gp130, the combination of cycloheximide and EGF further enhanced decreases of LIFR and gp130 over cycloheximide alone. With cycloheximide only, the half-life of LIFR was 3.6 ± 1.7 h and it was decreased

to 1.2 ± 0.1 h with the addition of EGF. The half-life of gp130 was 2.8 ± 1.2 h when treated with cycloheximide and was further decreased by the addition of EGF to 1.4 ± 0.3 h. These results suggest that EGF increases the apparent rate of degradation of both LIFR and gp130.

The role of Erk1/2 in protein synthesis-independent downregulation of LIFR

Our observation that EGF stimulation caused an additional decrease in gp130 over that induced by inhibition of protein synthesis was particularly intriguing because EGF alone had no effect on gp130 steady-state levels. To test the potential role of Erk1/2 activation in the apparent increased degradation of both gp130 and LIFR, cells were treated with 50 μ M PD98059 before addition of cycloheximide and EGF. Cells were then collected and blotted for LIFR, gp130 and TfR (Fig. 4a). Cells treated with cycloheximide and EGF yielded half-lives for gp130 and LIFR that were very close to similarly treated cells from the previous experiment (1.4 and 1.7 h for gp130 or 1.2 and 1.3 h for LIFR). However, PD98059 pretreatment caused a significant increase only in the half-life of LIFR (1.3 ± 0.2 h to 2.2 ± 0.8 h) while the half-life of gp130 was essentially unchanged (1.7 ± 0.5 h to 2.0 ± 0.7 h). These data indicate that Erk1/2 activation is involved in EGF-mediated decreases in LIFR but not in the EGF-mediated increase in gp130 degradation.

Involvement of the proteasome and lysosome in EGF-mediated LIFR downregulation

To determine if EGF stimulation was causing degradation of LIFR we investigated the role of the proteasome and lysosome in EGF-mediated LIFR downregulation. To determine if the proteasome was necessary for the observed decreases in LIFR, we treated cells with indicated concentrations of MG132 ($K_i = 4$ nM), a proteasome inhibitor, before adding EGF for 5 h. After treatment, cells were collected and cell lysates were analyzed for LIFR and TfR expression (Fig. 5a). Concentrations of MG132 >200-fold higher than its K_i did not significantly inhibit EGF-stimulated decreases in LIFR levels, suggesting that the proteasome is not required for the observed downregulation. To investigate the potential involvement of the lysosome, cells were treated with 50 μ M chloroquine, an inhibitor of lysosomal function, before stimulating with EGF. Following EGF stimulation cell lysates were analyzed for LIFR and TfR by western blotting (Fig. 5b). Chloroquine pretreatment completely blocked EGF-mediated decreases in LIFR levels, indicating a critical role for the lysosome in this trans-stimulated downregulation of LIFR (Fig. 5c). The effects of chloroquine were independent of any inhibition of Erk1/2 activation as phosphorylation levels of Erk1/2 were unchanged by this drug treatment (Fig. 5d). The effect of chloroquine suggests that the decreases in LIFR observed following EGF treatment are due to increased rates of lysosomal degradation. Addition of chloroquine to the cell media also significantly increased basal levels of LIFR (2.2 fold), implying that the lysosome is also involved in steady-state regulation of LIFR.

The effect of transregulation of LIFR on LIFR-mediated signaling events

To test the effects that EGF-mediated downregulation of LIFR has on signaling initiated through the LIFR, we pretreated NBFL cells with EGF (10 ng/mL) for 5 h before stimulating the cells briefly with LIF (10 ng/mL). Cell lysates were collected and blotted for LIFR, Tyr705-phosphorylated STAT3 (PY-STAT3) and STAT3 (Fig. 6a). There was no detectable increase in STAT3 tyrosine phosphorylation following treatment with EGF alone, consistent with earlier research demonstrating a lack of STAT3 activation by EGF in PC12 cells (Wu and Bradshaw 1996). EGF pretreatment caused a decrease in LIFR and also strongly inhibited LIF-induced tyrosine phosphorylation of STAT3 as compared to control lysates. When PD98059 was added to cells before treatment with EGF, the decrease observed in LIFR due to EGF was less and there was significantly less inhibition of STAT3 tyrosine phosphorylation (Fig. 6b).

The Role of Ser1044 of LIFR in EGF-mediated regulation of LIF signaling

Serine 1044 in the cytoplasmic tail of LIFR is a target of phosphorylation by MAPK and has been shown to be critical for insulin-mediated transregulation of LIFR in hepatoma cells (Schiemann et al. 1995; Blanchard et al. 2000). To determine if the observed inhibition of LIF signaling requires Ser1044 of LIFR, we used a chimeric receptor, composed of the extracellular domain of the G-CSF receptor fused to the cytosolic domain of LIFR containing either the WT sequence or a mutation of Ser 1044 to Ala (S1044A) (Schiemann et al. 1995). This system allows stimulation of the transfected receptor with G-CSF while the response of the endogenous receptors to LIF can be determined in the same cells. The chimeric receptors were transiently transfected into NBFL cells with a VIP promoter controlling a luciferase reporter gene and constitutively expressed β -galactosidase to control for minor variance in transfection levels. G-CSF stimulation of cells transfected with the chimeric receptors and VIP-Luciferase reporter resulted in a significant increase in luciferase activity over cells transfected with the VIP-Luciferase reporter alone². Transfected cells were treated with EGF (10 ng/mL) or left untreated for 4 h before stimulating either with G-CSF (10 ng/mL) which acts through the transfected chimeric receptors, or LIF (10 ng/mL) which binds endogenous LIFR and gp130 to initiate its signal transduction. After 3 h of cytokine stimulation, cells were collected and assayed for luciferase and β -galactosidase activity. The results of a representative experiment are shown in Table 1. LIF-stimulated luciferase activity was greatly inhibited by EGF pretreatment, with EGF treatment resulting in luciferase levels that were $48.5\% \pm 17.8$ of maximal LIF-induced levels in cells expressing WT GCSFR/LIFR and $51.4\% \pm 17.2$ in cells expressing G-CSFR/LIFR (S1044A). In cells expressing WT chimeric receptors, pretreatment with EGF caused an inhibition of G-CSF-stimulated VIP-luciferase expression that was similar to the inhibition by EGF of LIF stimulated activity. EGF treatment resulted in luciferase levels that were $54.1\% \pm 7.3$ of maximal G-CSF stimulated luciferase levels. In contrast G-CSF-induced luciferase activity was not significantly inhibited by EGF pretreatment in S1044A chimera transfected cells, with EGF pretreatment resulting in $91.9\% \pm 15.1$ of maximal G-CSF stimulated luciferase levels (Fig. 7). This result indicates that Ser 1044 of LIFR is required for EGF-mediated downregulation of LIFR signaling in NBFL neuroblastoma cells.

Discussion

During development, growth, differentiation and injury neurons can be exposed to cytokines and growth factors, as well as combinations of factors that will doubtlessly alter and influence the signaling of each other due to various forms of crosstalk. Some interactions among neuronal factors have already been identified. Both LIF and EGF have been implicated in the differentiation of cortical progenitor cells into astrocytes (Bauer et al. 2007). One report showed that the change in progenitor response to LIF stimulation that leads to astrocyte differentiation is due to an increase in the expression of EGF receptor (Viti et al. 2003). Several studies have investigated the relationship between LIF and NGF in the changes occurring in damaged neurons, showing that NGF can inhibit LIF-stimulated signaling and protein upregulation (Rajan et al. 1998; Shadiack et al. 1998; Ozturk and Tonge 2001). Our study examined one kind of potential crosstalk between LIFR-signaling cytokines and EGF or other strong activators of the MAPK pathway in neuroblastoma cells.

Although PD98059 inhibition of Erk1/2 phosphorylation strongly inhibited EGF-mediated LIFR decreases, our data suggest that activation of Erk1/2 alone is not sufficient to downregulate LIFR. All of the factors and cytokines tested resulted in phosphorylation of Erk1/2, but only those growth factors producing strong and extended Erk1/2 phosphorylation caused significant decreases in LIFR levels. While EGF and FGF treatment of NBFL cells resulted in robust activation of Erk1/2 that lasted 3 h, NGF-induced Erk1/2 activation was reduced to basal levels within 30 min. EGF and FGF treatment also caused

strong downregulation of LIFR while NGF did not. Erk1/2 activation by LIF, OsM and CNTF more closely resembles that of NGF, ending within 30 to 60 min of initial treatment and all of these cytokines also failed to cause a decrease in LIFR levels.

Erk-dependent downregulation of LIFR has been observed in non-neuronal cells (Blanchard et al. 2000) but the lack of downregulation by cytokines which bind and signal through the LIFR was surprising because in many other systems LIF, OsM, CNTF and other cytokines of the same family have been shown to cause downregulation of their own receptors (Blanchard et al. 2000; Blanchard et al. 2001; Zvonic et al. 2005; Yu et al. 2007). An *in vivo* study found evidence for CNTF-stimulated reductions in CNTFR in skeletal muscle, but did not investigate LIFR and gp130 levels (DiStefano et al. 1996). Another group found that LIF and CNTF treatment of neurons can inhibit subsequent binding of CNTF, providing evidence of reduced receptor surface expression, but they did not examine total receptor levels (Wong et al. 1995). Our observation that cytokines that signal through the LIFR do not cause its downregulation might be explained by cell type-specific differences in mechanisms of LIFR regulation.

We found that EGF and cycloheximide co-treatment causes a faster rate of LIFR decrease than cycloheximide alone. This observation indicates that the primary means by which EGF downregulates LIFR is not inhibition of the synthesis of LIFR but is instead likely to be an increased rate of degradation. Interestingly, although EGF alone did not appear to decrease steady state gp130 levels, EGF with cycloheximide co-treatment resulted in a faster rate of gp130 decrease when compared to cycloheximide alone. It is possible that EGF treatment activates pathways that simultaneously stimulate the rates of both degradation and synthesis of gp130, the latter being inhibited with addition of cycloheximide. Gp130 downregulation has also been observed in macrophages where treatment with pro-inflammatory stimuli led to degradation of gp130 (Kiu et al. 2007).

We used experiments where PD98059 was added to cells before treating with cycloheximide and EGF to confirm the role of Erk1/2 in EGF-mediated decreases in LIFR. The experiments also clearly showed that the EGF-induced increase in degradation of gp130 is not Erk1/2 dependent. Gibson *et al* demonstrated that gp130 could be phosphorylated on Ser782 of its cytoplasmic tail by 3T3-L1 lysates stimulated with LIF, EGF or phorbol 12-myristate 13-acetate (PMA) and that Erk was not responsible for this phosphorylation (Gibson et al. 2000). They also showed that this residue appeared to be involved in regulation of surface expression of the receptor. It was later shown that inhibition of phosphatase 2A caused an increase in phosphorylation of Ser 782 of gp130 and resulted in the downregulation of cellular gp130 levels via the proteasome (Mitsunashi et al. 2005). Ca²⁺/calmodulin-dependent kinase (CaMK)II and CaMKIV have been shown to be able to phosphorylate gp130 at Ser782 in an Erk-dependent manner though there is evidence that there are additional kinases that might contribute to this phosphorylation (Gibson et al. 2005). It is possible that the EGF-mediated decreases in gp130 that we observed in the presence of cycloheximide were due to EGF-induced activation of one or more of these kinases.

Our findings that EGF-induced LIFR downregulation was blocked by a lysosomal inhibitor is in agreement with several observations made regarding induced decreases in LIFR in non-neuronal cell types (Blanchard et al. 2000; Zvonic et al. 2005; Yu et al. 2007). There is some evidence that the proteasome is involved in the constitutive, non-stimulated degradation of LIFR in fibroblasts (Blanchard et al. 2001). Although we did not observe any decrease in EGF-stimulated LIFR-downregulation during MG132-mediated proteasomal inhibition, we cannot rule out the potential dependence of LIFR-degradation on proteasomal functions not blocked by MG132. Yu *et al* also made the interesting observation in endothelial cells that a

functional proteasome is necessary for the stability of LIFR and that inhibitors of the proteasome are themselves able to downregulate LIFR (Yu et al. 2007). However, we did not see any evidence of this requirement in the neuroblastoma cells used in our study as MG132 did not cause an appreciable decrease in LIFR basal levels. It was recently discovered that SOCS3 is involved in the ubiquitination of G-CSFR on a lysine near the box1 domain, which was required for targeting to the lysosome but not for endocytosis of the receptor. This lysine is conserved in several related cytokine receptors, including gp130 and LIFR, so it may have implications for degradation of LIFR as well (Irandoost et al. 2007).

We found that the ability of LIF to stimulate tyrosine phosphorylation of STAT3 was diminished when cells were first treated with EGF and that this effect was partially reversed when Erk signaling was inhibited. It is not surprising that the amount of LIF-stimulated STAT3 phosphorylation with EGF and PD98059 treatment was less than in control cells because we also observed that PD98059 itself, without EGF treatment, caused a strong decrease in the amount of tyrosine phosphorylation of STAT3. However, comparing LIF-stimulated PY-STAT3 following EGF treatment and following EGF and PD98059 treatment, it is clear that PD98059 significantly inhibited the EGF-mediated decreases in STAT3 phosphorylation. These results indicate that EGF-mediated, Erk1/2-dependent downregulation of LIFR has the ability to alter signaling by cytokines which signal through the LIFR.

It has been reported that treatment of adipocytes with cytokines which signal through the LIFR inhibited tyrosine phosphorylation of STAT3 by other cytokine family members that use the LIFR, apparently due to decreased LIFR levels (Zvonic et al. 2005). Our findings are also in agreement with observations that LIF-stimulated STAT3 phosphorylation is inhibited by insulin treatment in hepatocytic cells (Blanchard et al. 2000) and by TNF treatment in endothelial cells (Yu et al. 2007). In contrast to these findings, a recent report has shown that pretreatment of fibroblasts with insulin can inhibit IL-6-induced STAT3 tyrosine phosphorylation in an Erk-dependent manner, but even a brief treatment with insulin can inhibit IL-6-induced STAT3 transcriptional activity (Andersson et al. 2007). The authors did not investigate effects on gp130 levels but did find that insulin, when used together with IL-6, led to increased levels of SOCS3 and activation of SHP-2 both of which can inhibit STAT3 activation by LIFR and gp130 (Bartoe and Nathanson 2002; Heinrich et al. 2003). Thus, there are multiple mechanisms which can contribute to growth factor-mediated inhibition of gp130 cytokine signaling in different cell types. While mechanisms of regulation other than receptor degradation cannot be ruled out, our observation that Ser1044 of LIFR is necessary for maximal EGF-induced decreases in LIF signaling supports the theory that degradation of LIFR is the primary cause of inhibition of LIF signaling. Schiemann *et al* (1995) demonstrated that Ser1044 of LIFR was a substrate for Erk1/2 phosphorylation and was also necessary for transregulation of LIFR by insulin in H-35 cells. It has also been shown that Ser1044 is required for insulin- and LIF-induced decreases in LIFR in H-35 cells (Blanchard et al. 2000). We observed that LIF-stimulated VIP-luciferase activity mediated by chimeric receptors lacking Ser1044 was significantly less affected by EGF treatment than VIP-luciferase activity stimulated via wild type chimeric receptors. This result shows that transregulation of LIFR by EGF in NBFL cells is sufficient to not only decrease levels of LIFR but also to significantly disrupt LIF-stimulated signaling at the level of induction of neuropeptide gene transcription.

This paper is the first to show the effects of growth factor treatment on LIFR expression and LIF-mediated signaling in neuroblastoma cells. All of our results together suggest a model of EGF and LIF crosstalk in neuroblastoma cells where EGF stimulation causes a strong and extended phosphorylation of Erk1/2 which in turn phosphorylates Ser1044 of LIFR. This

phosphorylation of LIFR increases its rate of degradation by the lysosome leading to decreased LIF signaling and LIF-mediated gene induction. These studies provide a better understand of how signaling generated through the LIFR can be regulated when neurally-derived cells are exposed to multiple stimuli.

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Fig. 1. EGF and FGF stimulation of neuroblastoma cells causes a decrease in the amount of LIFR

NBFL cells were treated with 100 ng/mL of CNTF (C), OsM (O), LIF (L), FGF (F) or NGF (N) or 10 ng/mL EGF (E) or left untreated (-). After 5 h the cells were collected and LIFR and TfR levels measured by western blot analysis of lysates (a). Bands were quantified and LIFR measurements were normalized to the levels of TfR to calculate the percentage change in LIFR amounts following stimulation (b). Data in graph are averages $\pm$ SD of 3 separate experiments. P-values were calculated by ANOVA followed by a Dunnett post-test, comparing individual cytokine or growth factor treatments to untreated cells. * $p < 0.01$

Fig. 2. Decreases in LIFR are correlated with robust and extended activation of Erk1/2
 NBFL cells were stimulated with 100 ng/mL of CNTF, OsM, LIF, FGF or NGF or 10 ng/mL EGF for times indicated. Cells were collected and phospho-Erk1/2 (P-Erk1/2) and total Erk1/2 were measured by western blot analysis (a). Other cells were pretreated with 50 μ M PD98059 for 30 minutes before stimulating with 10 ng/mL EGF for indicated times. Cells were collected and lysates blotted for P-Erk1/2 and total Erk1/2 (b) or LIFR and TfR (c). Blots shown are representative of 3 separate experiments (a and c) or 2 separate experiments (b). Data represented by the blot in 2c were quantified and are shown in graph (d). Graph shows mean $\pm$ SEM of LIFR levels following treatment with DMSO alone (closed bar), DMSO + 5h EGF (darkly shaded bar), PD98059 alone (open bar) or PD98059 + 5h EGF (lightly shaded bar). P-values were calculated by ANOVA followed by a Student-Newman-Keuls post-test. *p < 0.001.

Fig. 3. EGF-mediated decrease in LIFR is due to an increase in apparent rate of degradation
 NBFL cells were treated with EGF (10 ng/mL), 10 μ M cycloheximide (cyc), both EGF and cycloheximide or left untreated for the times indicated. Cells were collected and LIFR, gp130 and TfR levels were measured by western blotting (a and b). LIFR and gp130 levels were quantified by densitometry and measurements were normalized to TfR levels to calculate the percentage change in receptor levels following stimulation. Levels of LIFR (c) and gp130 (d) were plotted on logarithmic scales and lines of best fit were calculated for cycloheximide treated ($\circ$) and cycloheximide + EGF treated ($\blacklozenge$) cells. Data in line graphs are averages of 4 or 5 separate experiments. Error bars show SD. Bar graph (e) shows averages $\pm$ SD for half-lives of LIFR and gp130 when treated with cycloheximide alone (shaded bar) or cycloheximide plus EGF (open bar). P-values were calculated by student's t-tests comparing cells treated with cycloheximide plus EGF to cycloheximide alone. * $p < 0.05$

Fig. 4. Inhibition of MEK blocks EGF-induced increase in apparent rate of degradation for LIFR but not gp130

NBFL cells were pretreated with 50 μ M PD98059 or vehicle for 30 min before adding 10 μ g/mL cycloheximide and EGF (10 ng/mL) for the time indicated. Cell lysates were collected and western blotted for LIFR, gp130 and TfR (a). Bands were quantified and LIFR and gp130 measurements were normalized to TfR levels to calculate the percentage change in receptor levels following stimulation with or without PD98059 pretreatment. Levels of LIFR and gp130 were plotted on logarithmic scales and lines of best fit were calculated for PD98059 treated and untreated. Data compiled is from 5 independent experiments. Bar graph (b) shows averages $\pm$ SD for half-lives of LIFR and gp130 when left untreated before

cycloheximide and EGF stimulation (shaded bars) or when pretreated with PD98059 (open bars). P-values were calculated by student's t-tests comparing cells pretreated with PD98059 with non-pretreated cells. * $p < 0.05$

Fig. 5. Inhibition of the lysosomal but not proteasomal pathway blocks EGF-mediated decreases in LIFR levels

NBFL cells were pretreated with indicated concentrations of MG132 for 1 h before adding EGF (10 ng/mL) for 5 h. Cell lysates were collected and western blotted for LIFR and TfR (a). Other plates of NBFL cells were pretreated with 50 μ M chloroquine (chlor.) for 45 minutes or not pretreated before addition of EGF for indicated times. Cells were collected and lysates analyzed by Western Blotting for levels of LIFR and TfR (b) or phospho-Erk1/2 (P-Erk1/2) and total Erk1/2 (d). Bands for LIFR and TfR were quantified by densitometry and LIFR levels were normalized to TfR levels. Bar graph (c) shows the levels of LIFR without EGF treatment (shaded bars) or with EGF treatment (open bars), with or without chloroquine pretreatment. Data shown are the averages $\pm$ SD of 10 replicate samples from 3 separate experiments. P-values were calculated by student's t-tests comparing cells treated with EGF to non-EGF treated cells. * $p < 0.0001$

Fig. 6. EGF-mediated activation of MEK inhibits LIF-induced tyrosine phosphorylation of STAT3

NBFL cells were pretreated with 50 μ M PD98059 (PD) or vehicle for 30 minutes and then treated with EGF (10 ng/mL) or left untreated for 5 h. Cells were then stimulated with LIF (10 ng/mL) or left unstimulated for 15 minutes. Cell lysates were collected and western blotted for LIFR, PY-STAT3 and STAT3 (a). Bands for PY-STAT3 and STAT3 were quantified and PYSTAT3 was normalized to STAT3 to calculate relative levels of tyrosine phosphorylation of STAT3 following LIF stimulation and no pretreatment (filled bar), EGF treatment (darkly shaded bar), pretreatment with PD98059 (open bar) and treatment with both PD98059 and EGF (lightly shaded bar) (b). Data shown are averages $\pm$ SD of 2

separate experiments. P-values were calculated by ANOVA followed by a Student-Newman-Keuls post-test. * $p < 0.05$

Fig. 7. EGF-mediated inhibition of LIF signaling is dependent on Ser 1044 of LIFR

NBFL cells were transiently transfected with chimeric receptors containing the extracellular domain of the G-CSF receptor fused to the cytoplasmic domain of either wild type LIFR (WT) or LIFR with serine 1044 mutated to alanine (S1044A). Cells were treated with EGF (10 ng/mL) for 4 h or left untreated before stimulating with G-CSF (10 ng/mL), LIF (10 ng/mL) or vehicle for 3 h. Cell lysates were assayed for induced luciferase activity and constitutive β -galactosidase activity. Fold inductions by G-CSF and LIF stimulation were calculated by dividing luciferase/ β -galactosidase numbers by values from similarly treated (with or without EGF) control lysates. Graph shows the fold induction of EGF-treated cells, stimulated with G-CSF or LIF, as a percentage of fold induction achieved in cells not treated with EGF. Open bars show percentages for WT chimeric transfections while filled bars represent data for S1044A chimeric transfections. Percentages shown are averages $\pm$ SEM of three independent experiments with 6 to 9 replicate samples in each experiment. P-values were calculated by student's t-tests comparing cells treated with EGF to non-EGF treated cells. * $p < 0.005$

Table 1

NBFL cells were transfected with GCSFR/LIFR chimeric receptors with either a WT version of the cytoplasmic domain of LIFR or a cytoplasmic domain with serine 1044 mutated to alanine (S1044A). Where indicated cells were pretreated with 10 ng/mL EGF for 4 h before stimulating with GCSF (10 ng/mL), LIF(10ng/mL) or vehicle. After 3 h of stimulation cells were collected in lysisbuffer and then assayed for luciferase and β -galactosidase activity. Numbers shown for luciferase/ β -galactosidase values are averages $\pm$ SD ($\times 10^3$) for a representative experiment with 6 replicate samples.

	WT chimerictransfection		S1044A chimeric transfection	
Stim.	No pretreatment	EGF pretreatment	No pretreatment	EGF pretreatment
	Luciferase/ β -galactosidase ($\times 10^3$)			
none	0.79 $\pm$ 0.14	1.29 $\pm$ 0.34	0.70 $\pm$ 0.13	1.14 $\pm$ 0.48
LIF	18.20 $\pm$ 1.77	10.99 $\pm$ 3.10	19.76 $\pm$ 6.75	14.23 $\pm$ 4.80
GCSF	5.51 $\pm$ 1.14	4.14 $\pm$ 0.82	6.25 $\pm$ 1.51	9.23 $\pm$ 2.36
	Fold induction over unstimulated			
none	1	1	1	1
LIF	23.04	8.52	28.23	12.48
GCSF	6.97	3.21	8.93	8.10