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Zvonic, S., Baugh, J., Arbour-Reily, P., Mynatt, R., & Stephens, J. (2005). Cross-talk among gp130 cytokines in adipocytes. *Journal of Biological Chemistry, 280* (40), 33856-33863. https://doi.org/10.1074/jbc.M508020200

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 40, pp. 33856–33863, October 7, 2005
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Cross-talk among gp130 Cytokines in Adipocytes*

Received for publication, July 22, 2005 Published, JBC Papers in Press, August 11, 2005, DOI 10.1074/jbc.M508020200

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The interleukin-6 (IL-6) family of cytokines is a family of structurally and functionally related proteins, including IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1). These proteins are also known as gp130 cytokines because they all share gp130 as a common transducer protein within their functional receptor complexes. Several of these cytokines (LIF, OSM, CNTF, and CT-1) also utilize the LIF receptor (LIFR) as a component of their receptor complex. We have shown that all of these cytokines are capable of activating both the JAK/STAT and p42/44 mitogen-activated protein kinase signaling pathways in 3T3-L1 adipocytes. By performing a variety of preincubation studies and examining the ability of these cytokines to activate STATs, ERKs, and induce transcription of SOCS-3 mRNA, we have also examined the ability of gp130 cytokines to modulate the action of their family members. Our results indicate that a subset of gp130 cytokines, in particular CT-1, LIF, and OSM, has the ability to impair subsequent signaling activity initiated by gp130 cytokines. However, IL-6 and CNTF do not exhibit this cross-talk ability. Moreover, our results indicate that the cross-talk among gp130 cytokines is mediated by the ability of these cytokines to induce ligand-dependent degradation of the LIFR, in a proteasome-independent manner, which coincides with decreased levels of LIFR at the plasma membrane. In summary, our results demonstrate that an inhibitory cross-talk among specific gp130 cytokines in 3T3-L1 adipocytes occurs as a result of specific degradation of LIFR via a lysosome-mediated pathway.

The interleukin 6 (IL-6)[2] cytokine family is a group of functionally and structurally related proteins including IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1) (1, 2). All of these cytokines possess a similar helical structure and signal via structurally related receptor complexes. All of the IL-6 family cytokines were originally identified as factors with distinct and unique properties (3–8), yet it is currently known that these cytokines can function in a pleiotropic and redundant manner (9, 10). In fact, members of the IL-6 cytokine family play pivotal roles in the immune, hematopoietic, nervous, cardiovascular, and endocrine systems, as well as in bone metabolism, inflammation, and acute phase responses (11–21).

All functional receptor complexes for the IL-6 cytokines use glycoprotein 130 (gp130) as a component required for both ligand binding and signal transduction (15, 22–24). Hence, IL-6 family cytokines are often referred to as gp130 cytokines. Several gp130 cytokines (LIF, OSM, CNTF, and CT-1) also require the gp130-related protein LIFR (LIF receptor) as a part of their functional receptor complexes (25–29). In fact, these cytokines have very low affinity to gp130 until it becomes a part of the LIFR/gp130 heterodimer. Apart from the shared functional receptor components, IL-6, IL-11, OSM, and CNTF also have cytokine-specific receptor components, often referred to as the α-receptors (30–32).

Although LIF and CT-1 signal through a LIFR/gp130 heterodimer, other gp130 cytokines have more distinct receptor complex structures. IL-6 first binds to its α-receptor, IL-6R, and then two of these IL-6·IL-6R complexes associate with gp130 each, allowing the formation of a gp130 homodimer (33, 34). A similar pattern has also been proposed for the formation of IL-11 functional receptor complexes (31). OSM can either signal through the LIFR/gp130 heterodimer (26, 35) or through its α-receptor OSMR associated with a gp130 subunit (36, 37). Structurally, OSMR is closely related to both LIFR and gp130. However, neither OSMR nor LIFR can bind OSM in the absence of gp130 subunit. The α-receptor for CNTF, CNTFRα, is structurally closely related to extracellular region of IL-6R but is anchored to the cell membrane via a glycosylphosphatidylinositol linkage. CNTF binds to CNTFRα, followed by the recruitment of gp130 and LIFR by two of these dimers, resulting in a hexameric receptor complex (38). The α-receptors for IL-6, IL-11, and CNTF can also be found in a soluble form, and these soluble receptors can bind their ligands in a manner identical to their membrane-associated forms (39, 40). These soluble receptors have the potential to confer cytokine responsiveness to cells expressing gp130 and LIFR that do not express membrane-associated forms of the α-receptors (41, 42).

The ubiquitous expression of gp130 in every cell type examined explains the pleiotropic nature of gp130 cytokine action, whereas shared usage of gp130 by all these cytokines in part explains the redundancy of their actions. Unlike gp130, LIFR and the specific α-receptors exhibit a more tissue-specific expression (43) and therefore contribute to the specificity of gp130 cytokine actions. Spatial and temporal expression of the individual cytokines is another factor that contributes to the specificity of their actions (44).

Functionally, it is the formation of the gp130/gp130 homodimer or the LIFR/gp130 heterodimer that is essential for the downstream signal transduction of gp130 cytokines. The function of the α-receptor is to recruit the ligand and aid in the formation of signal-transducing dimers in response to the ligands that are themselves incapable of doing so. After stimulation by gp130 cytokines, target cells undergo a wide variety of fates: growth promotion, growth arrest, differentiation, or the expression of specific genes. The specificity of cytokine actions in a given cell type arises not only from the differences in the receptor and cytokine expression, but also from the differences in downstream signaling pathways activated by the cytokine.

* This work was supported by Grant R01DK52968-02 from the National Institutes of Health (to J. M. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: IL-6, interleukin-6; CNTF, ciliary neurotrophic factor; CNTFRα, α-receptor for CNTF; CT-1, cardiotrophin-1; ERK, extracellular signal-regulated kinase; GH, growth hormone; LDM, low density microsomes; LIF, leukemia inhibitory factor; LIFR, LIF receptor; MAPK, mitogen-activated protein kinase; OSM, oncostatin M; SOCS-3, suppressor of cytokine signaling-3; STAT, signal transducers and activators of transcription.

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Upon stimulation by gp130 cytokines, the gp130 receptor itself undergoes homo- or heterodimerization governed by the phosphorylated tyrosine residues on the cytoplasmic region of the protein. This dimerization triggers the activation of cytoplasmic protein kinases associated with gp130 receptor. In the case of gp130, those kinases are JAK1, JAK2, and TYK2 (45–50). Activated JAKs phosphorylate tyrosine residues in the distal part of gp130 and LIFR and provide docking sites for Src homology 2 domain-containing proteins such as STATs. Even though the STAT family consists of 7 members, it is generally recognized that gp130 cytokines activate STAT 3 and, to a lesser extent, STAT 1 (51, 52). Once recruited to the receptor, STATs are phosphorylated and form dimers that translocate to the nucleus, bind DNA, and modulate transcription. Stimulation by gp130 cytokines can also lead to the activation of the p42/44 MAPK pathway (53).

Recent studies have shown that three different gp130 cytokines activate both the JAK/STAT and p42/44 MAPK signaling pathways in fat cells (54–57). Because these cytokines utilize many of the same signaling proteins, we examined the cross-talk among LIF, CT-1, OSM, CNTF, and IL-6 in adipocytes. We also examined the ability of growth hormone (GH), a potent STAT activator, to modulate gp130 cytokine signaling. Our results demonstrate that a 2-h pretreatment with the gp130 cytokines examined leads to the inhibition of their own subsequent signaling. Interestingly, LIF could also inhibit the signaling of CT-1, CNTF, and OSM, but not IL-6. Similarly, CT-1 could inhibit its own signaling or a cellular response to LIF and OSM. OSM also exhibited similar properties. Inhibition of signaling was measured by three parameters: activation of STATs, activation of ERKs, and the induction of SOCS-3 mRNA. Moreover, we observed that the cross-talk that resulted in an inhibition of signaling correlated with the degradation of the LIFR in a proteasome-independent manner and a loss of the LIFR at the plasma membrane. In summary, our findings strongly suggest that inhibitory cross-talk among specific gp130 cytokines in fat cells is mediated by the ligand-dependent lysosome-mediated degradation of the LIFR.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium was purchased from Invitrogen. Bovine and fetal bovine sera were purchased from Sigma and Invitrogen, respectively. Rat recombinant CNTF and human recombinant CT-1 were purchased from Calbiochem. Recombinant mouse IL-6 and recombinant human OSM were purchased from Bio Source International. Mouse recombinant LIF was purchased from Chemicon International. Insulin, human recombinant GH, leupeptin, chloroquine, and cycloheximide were all purchased from Sigma. Epoxomicin and MG132 were purchased from Boston Biochem. TRIzol was purchased from Invitrogen. All STAT antibodies were monoclonal IgGs purchased from Transduction Laboratories or polyclonal IgGs purchased from Santa Cruz. The highly phospho-specific polyclonal antibodies for STAT 1 (Tyr^{701}), STAT 3 (Tyr^{705}), and STAT 5 (Tyr^{694}) were IgGs purchased from BD Transduction Laboratories and Upstate Biotechnology, Inc. LIFR and ERK1/ERK2 antibodies were rabbit polyclonal IgGs purchased from Santa Cruz. Active ERK antibody was a rabbit polyclonal IgG purchased from Cell Signaling Technology. Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson Immunoresearch. An Enhanced Chemiluminescence (ECL) kit was purchased from Pierce. Nitrocellulose and Zeta Probe-GT membranes were purchased from Bio-Rad.

Preparation of Whole Cell Extracts—Monolayers of 3T3-L1 preadipocytes or adipocytes were rinsed with phosphate-buffered saline and then harvested in a nondenaturing buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 1 μM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μM leupeptin, and 2 mM sodium vanadate. Samples were extracted for 30 min on ice and centrifuged at 15,000 rpm at 4 °C for 15 min. Supernatants containing whole cell extracts were analyzed for protein content using a BCA kit (Pierce) according to the manufacturer's instructions.

Gel Electrophoresis and Western Blot Analysis—Proteins were separated in 5, 7.5, 10, or 12% polyacrylamide (acrylamide from National Diagnostics) gels containing SDS according to Laemmli (58) and transferred to nitrocellulose membrane in 25 mM Tris, 192 mM glycine, and 20% methanol. After transfer, the membrane was blocked in 4% fat-free milk for 1 h at room temperature. Results were visualized with horseradish peroxidase-conjugated secondary antibodies and ECL.

RNA Analysis—Total RNA was isolated from cell monolayers with TRIzol according to the manufacturer's instructions with minor modifications. For Northern blot analysis, 20 μg of total RNA was denatured in formamide and electrophoresed through a formaldehyde/agarose gel. The RNA was transferred to Zeta Probe-GT, cross-linked, hybridized, and washed as described previously (59). Probes were labeled by random priming using the Klenow fragment and [α-^{32}P]dATP.

Rodent Adipose Tissue Isolation—Animals were euthanized by cervical dislocation, and tissues were immediately removed and frozen in liquid nitrogen. Frozen tissues were homogenized in a buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 1 μM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, and 10 μM leupeptin, and 2 mM sodium vanadate. Homogenates were centrifuged for 10 min at 5,000 rpm to remove any debris and insoluble material and then analyzed for protein content. All C57BL/6J mice were obtained from a colony at the Pennington Biomedical Research Center. All animal studies were carried out with protocols that were reviewed and approved by institutional IACUCs.

3T3-L1 Cell Membrane Fractionation—Untreated and LIF-treated serum-deprived 3T3-L1 adipocytes were rinsed with buffer A (250 mM sucrose, 20 mM HEPES, 1 mM EDTA, pH 7.4) at 37 °C and then harvested at 4 °C in buffer A and homogenized with a Teflon pestle. Total membranes were pelleted at 250,000 × *g* for 90 min and resuspended in buffer B (20 mM HEPES, 1 mM EDTA, pH 7.4). For some experiments, membranes were fractionated into plasma membrane, intracellular membranes (low density microsomes, LDM), and a nuclear/mitochondrial fraction as we have described previously (59). Membrane and cytosolic fractions were divided and immediately stored at −70 °C. The protein content for all fractions was determined with a BCA kit according to the manufacturer's instructions.

RESULTS

One of the unifying features of all gp130 cytokines is their ability to activate JAK/STAT and MAPK (ERK1/2) signaling pathways. We therefore wanted to determine whether gp130 cytokines, which affect these pathways in fat cells, can work synergistically to initiate these signals, or whether their actions antagonize each other. To address this question, we pretreated fully differentiated 3T3-L1 adipocytes with several different gp130 cytokines (IL-6, LIF, OSM, CNTF, and CT-1), as well as with GH for 2 h. Next, we treated the cells for 15 min with either CT-1, CNTF, LIF, or GH. As shown in Fig. 1, gp130 cytokines interacted primarily in an antagonistic fashion. The results in Fig. 1*A* represent an experiment in which 3T3-L1 adipocytes were pretreated with CT-1, which is known to activate STAT 1 and 3 in these cells potently (60). We observed that CT-1 signaling was impaired by a 2-h pretreatment with

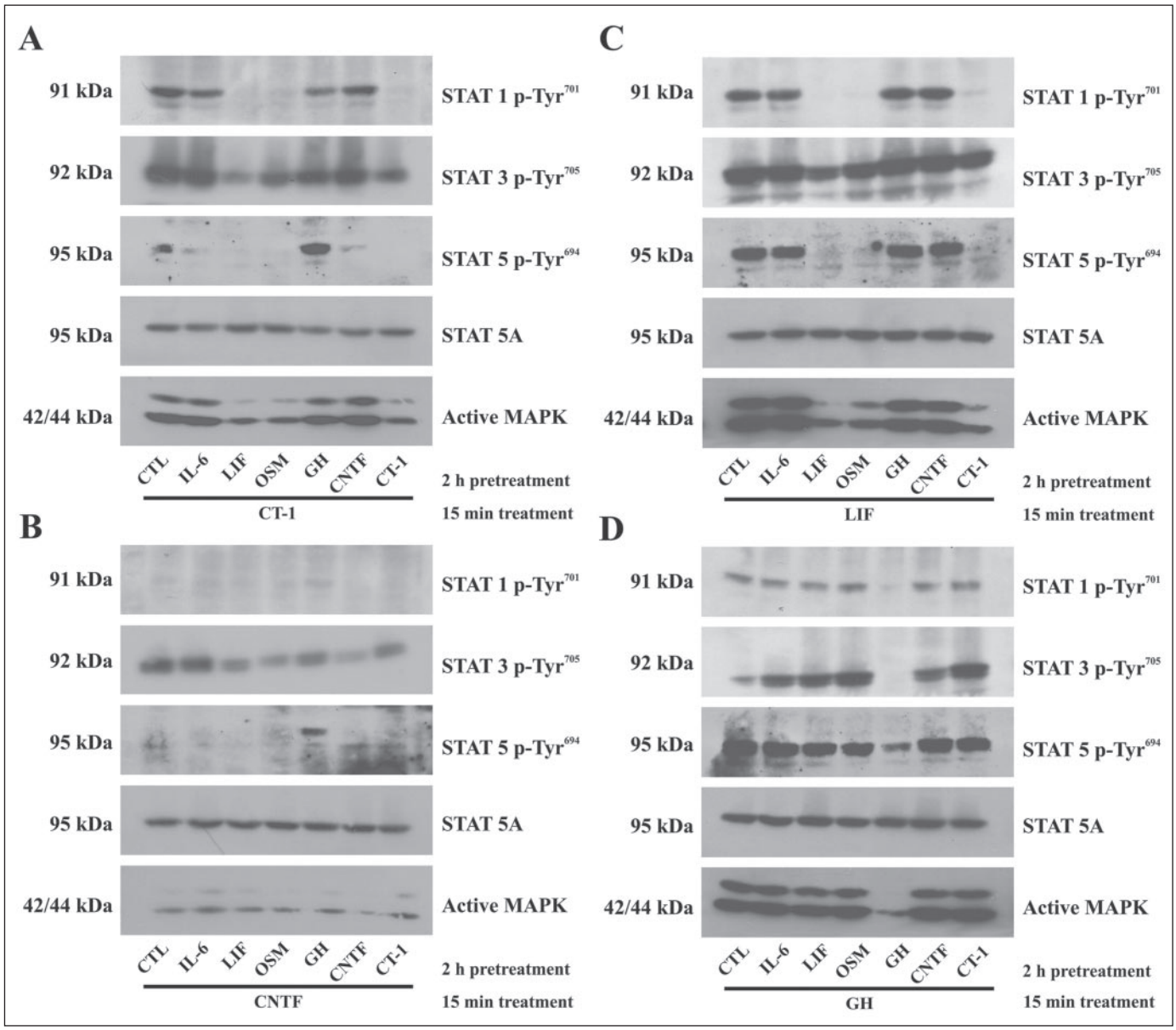

FIGURE 1. **gp130 cytokine administration inhibits cell signaling of other gp130 cytokines.** Fully differentiated 3T3-L1 adipocytes were preincubated with 2 ng/ml IL-6, 0.1 nM LIF, 0.5 ng/ml OSM, 125 ng/ml GH, 0.45 nM CNTF, or 0.2 nM CT-1 for 2 h. Next, the cells were treated for 15 min with CT-1 (*A*), CNTF (*B*), LIF (*C*), or GH (*D*). After the treatment, whole cell extracts were prepared, and 150 μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment performed independently two times.

several gp130 cytokines, including CT-1. Our results demonstrate that a 2-h pretreatment with LIF, OSM, or CT-1 completely blocked the STAT 1 activation induced by a 15-min CT-1 treatment. These same cytokines also interfered with the activation of STAT 3 by CT-1. In this case, LIF acted as the most potent inhibitor, whereas CT-1 and OSM pretreatments consistently resulted in a partial, but significant, inhibition of STAT activation. No substantial STAT 5 activation occurred with CT-1 treatment, whereas GH pretreatment caused a robust STAT 5 activation that was detectable even after the 2-h pretreatment period. The ability of LIF, OSM, and CT-1 to block further actions of CT-1 was also evident through their effects on MAPK (ERK1/2) signaling because the activation of this pathway by acute CT-1 was attenuated through pretreatment with these cytokines.

Even though CNTF is not a potent activator of JAK/STAT or MAPK signaling in adipocytes (54, 60), its ability to activate STAT 3 (*first lane*, Fig. 1*B*) was affected by the pretreatment with all other gp130 cytokines examined, except for IL-6. As was the case with the acute CT-1 treatment, a 2-h pretreatment of adipocytes with LIF, OSM, or CT-1 decreased the magnitude of STAT 3 activation by CNTF. This pattern of regulation was not observed with active MAPK, which we attribute to the weak activation of this kinase by CNTF.

The data in Fig. 1*C* verify that LIF acts as a potent activator of JAK/STAT and MAPK signaling in 3T3-L1 adipocytes. However, the ability of LIF to activate STAT 1 and 5 was completely abolished by a 2-h pretreatment with LIF, OSM, or CT-1. An identical pattern of inhibition was also observed with the activation of MAPK (ERK1/2) by a 15-min LIF treatment. Acute LIF administration resulted in a robust STAT 3 activation, and only the 2-h pretreatment with LIF resulted in an attenuation of STAT 3 activation. Hence, the gp130 cytokines LIF, OSM, and CT-1 pretreatment inhibited the LIF-induced activation of STAT 1 and 5, but not of STAT 3.

Acute treatment with GH, shown in Fig. 1*D*, also leads to a robust

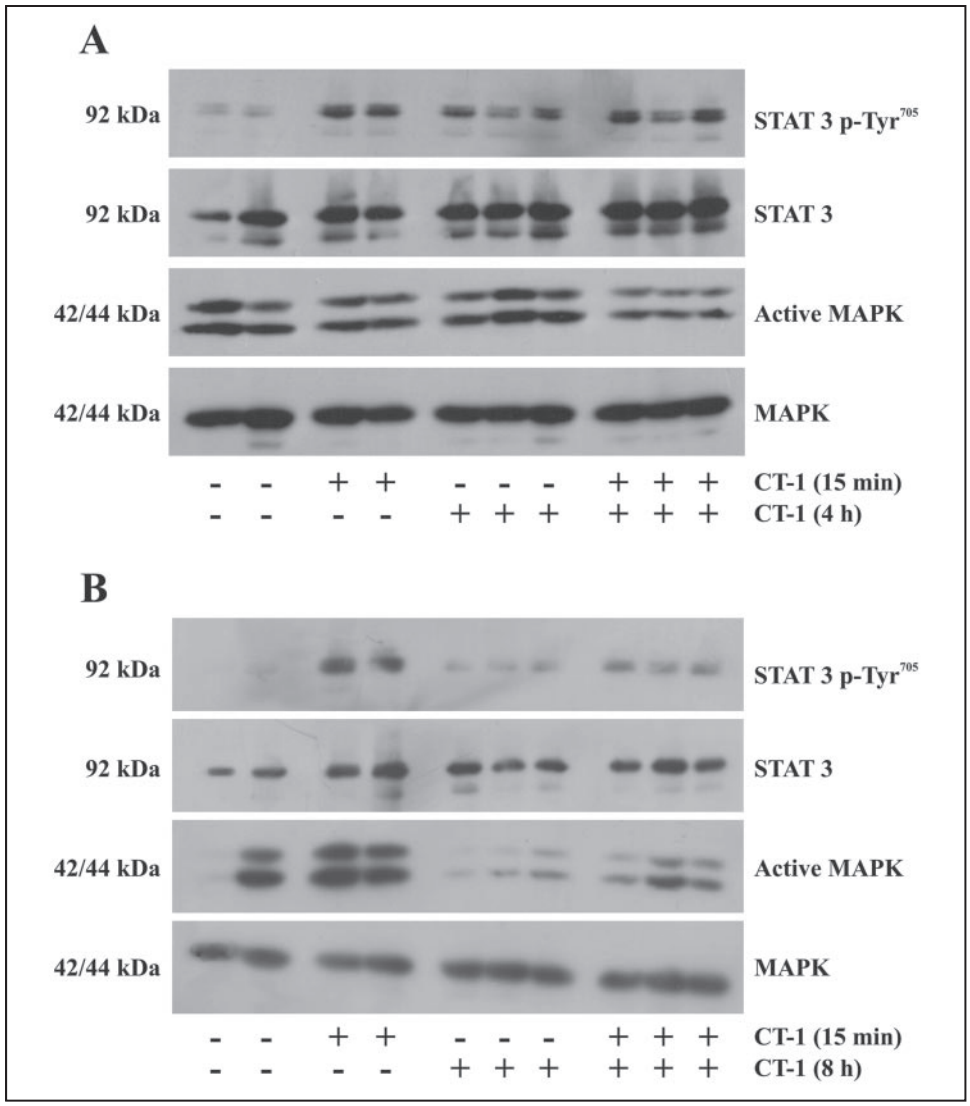

FIGURE 2. **CT-1 treatment *in vivo* blocks subsequent CT-1 signaling in rodent fat pads.** Six-week-old male C57BL/6J mice were given an intraperitoneal injection of 0.05 nM CT-1 (0.25 μg/animal) or vehicle (saline) control. Two animals were used for each condition. After 4 (*A*) or 8 (*B*) h the animals were injected again with CT-1 or vehicle. In this set of experiments, three animals were used for each condition. 15 min after the second injection the mice were sacrificed, and epididymal fat pads were immediately removed and frozen in liquid nitrogen. 150 μg of each tissue extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment performed independently two times.

induction of JAK/STAT and MAPK signaling, as evident by the activation of STAT 1, 3, and 5 and ERK1/2. However, unlike gp130 cytokines, the ability of GH to activate these pathways was not affected by a 2-h preincubation with any of the gp130 cytokines examined. In fact, LIF, OSM, and CT-1 mildly enhanced the GH-induced activation of STAT 3, but not of STAT 1 and 5. The only inhibitory effect observed for GH singling was the inhibition of STAT and MAPK activation after a 2-h pretreatment with GH itself. STAT5A levels are shown in all panels of Fig. 1 to demonstrate equivalent loading of whole cell extracts. The expression of STAT 3 and total level of ERK1/2 (MAPK) also remained unchanged by all of the treatments shown in Fig. 1 (data not shown).

We utilized a similar experimental approach in an *in vivo* model to examine further the nature of interactions among gp130 cytokines. C57BL/6J mice were injected with either 0.05 nM CT-1 or the appropriate vehicle (saline) control, then injected again either four (Fig. 2*A*) or eight (Fig. 2*B*) h later, and then sacrificed 15 min after the second round of injections. As shown in Fig. 2, acute intraperitoneal administration of CT-1 lead to the activation of STAT 3 in mouse epididymal fat pads. Interestingly, MAPK (ERK1/2) was active even in animals injected with vehicle (saline) control. As expected, phosphorylated STAT 3 proteins were present in the adipose tissue extracts from mice after a 15-min injection with CT-1. We still observed active STAT 3 at 4 h after CT-1 administration (Fig. 2*A*) at levels comparable with that following a 15-min treatment. In an independent experiment, six animals were pretreated with CT-1 after an intraperitoneal injection with CT-1 or vehicle, and epididymal fat pads were harvested for analysis. We observed that the active forms of both STAT 3 and MAPK were absent from the fat pads 8 h after a CT-1 injection (Fig. 2*B*). The animals injected with CT-1 for 4 h, and then again for 15 min (Fig. 2*A*), showed no additional increase in STAT 3 or MAPK activation compared with animals injected with CT-1 for 15 min or 4 h only. Yet, in the fat pads of mice injected with CT-1 for 8 h, and then again for 15 min (Fig. 2*B*), we observed no increase in STAT 3 or MAPK activation compared with animals injected with CT-1 for 8 h only. Total MAPK levels are shown to demonstrate equivalent levels of protein loading. In summary, these results clearly demonstrate that pretreatment of mice with CT-1 blocks the acute STAT 3 activation by CT-1 in adipose tissue.

We have shown previously that the activation of the JAK/STAT pathway in 3T3-L1 adipocytes by gp130 cytokines is associated with the induction of SOCS-3 mRNA (60). Therefore, we also examined the effects of gp130 cytokine interaction on the induction of SOCS-3 mRNA. As shown in Fig. 3*A*, the ability of a 40-min LIF treatment to induce SOCS-3 mRNA expression was impaired by a 2-h pretreatment with LIF, CT-1, and, to a lesser degree, by OSM. Conversely, IL-6,

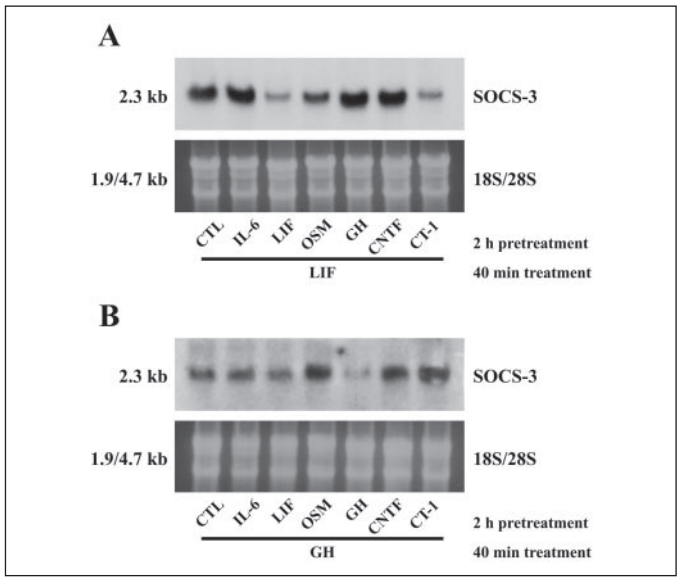

FIGURE 3. **Cross-talk among gp130 cytokines inhibits the induction of SOCS-3 mRNA.** Fully differentiated 3T3-L1 adipocytes were preincubated with 2 ng/ml IL-6, 0.1 nM LIF, 0.5 ng/ml OSM, 125 ng/ml GH, 0.45 nM CNTF, or 0.2 nM CT-1 for 2 h. The cells were then treated for 45 min with either LIF (*A*) LIF or GH (*B*). After the treatment total RNA was collected from the cells, and 20 μg of each total RNA was electrophoresed, transferred to nylon, and subjected to Northern blot analysis. This is a representative experiment performed independently three times.

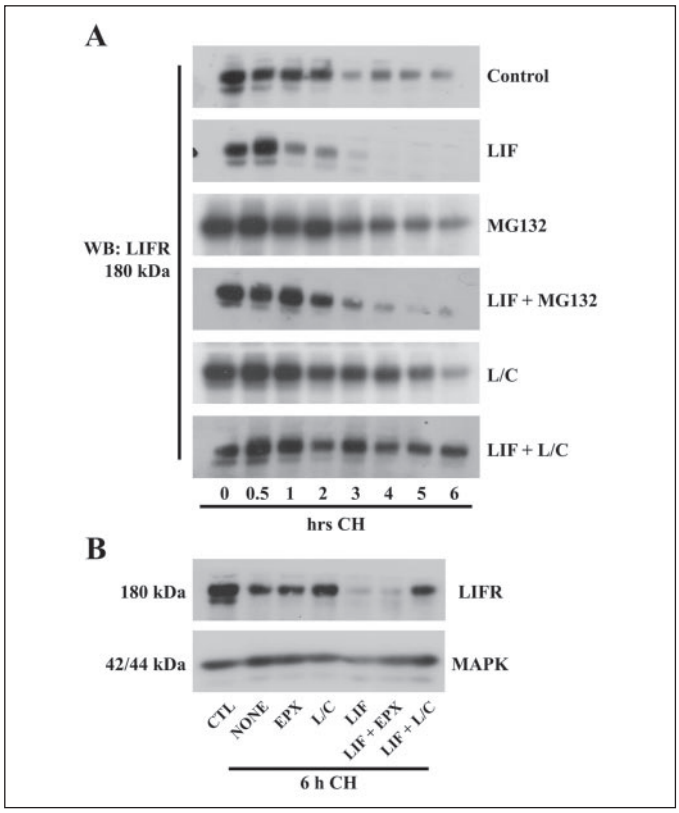

FIGURE 4. **Administration of LIF alters the half-life of LIFR protein in adipocytes.** *A*, fully differentiated 3T3-L1 adipocytes were treated with 0.1 nM LIF, 20 μM MG132, a mixture of 200 μM chloroquine and 10 μM leupeptin (*L/C*), or a combination of LIF and the inhibitors. The treatments were done in the presence of 5 μM cycloheximide (*CH*). Whole cell extracts were prepared from untreated (*0*) or adipocytes treated at the times indicated. *B*, whole cell extracts were prepared from fully differentiated 3T3-L1 adipocytes that were untreated (*CTL*) or treated for 6 h with 0.1 nM LIF, 100 nM epoxomicin (*EPX*), a mixture of 200 μM chloroquine and 10 μM leupeptin, or a combination of LIF and the inhibitors, in the presence of 5 μM cycloheximide. In each panel, 150 μg of extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot (*WB*) analysis. This is a representative experiment performed independently two times.

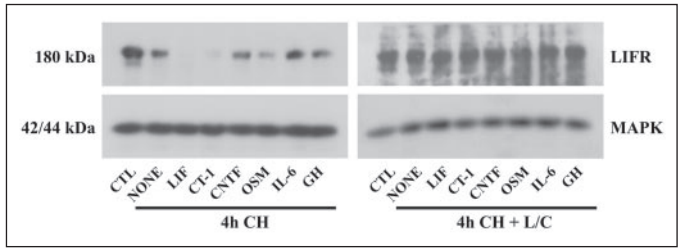

FIGURE 5. **Half-life of LIFR protein in adipocytes is decreased by gp130 cytokine administration in a proteasome-independent manner.** Whole cell extracts were prepared from 3T3-L1 adipocytes treated with 0.1 nM LIF, 0.2 nM CT-1, 0.45 nM CNTF, 0.5 ng/ml OSM, 2 ng/ml IL-6, or 125 ng/ml GH for 4 h in the presence of 5 μM cycloheximide (*CH*), or a mixture of 5 μM cycloheximide and 200 μM chloroquine and 10 μM leupeptin (*L/C*). 100 μg of each sample was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment performed independently three times.

CNTF, or GH pretreatment had no effect on the LIF-induced increase of SOCS-3 mRNA. Pretreatments with LIF, OSM, or CT-1, but not IL-6, CNTF, or GH, also attenuated the induction of SCOS-3 mRNA by OSM and CT-1 (data not shown). Consistent with the data shown in Fig. 1*D*, pretreatment with gp130 cytokines did not inhibit the ability of GH to induce SOCS-3 mRNA. In fact, LIF, OSM, and CT-1 seemingly enhanced the GH-induced increase in SOCS-3 mRNA expression, much like their synergistic effect on STAT 3 activation observed in Fig. 1*D*. In addition, the data shown in Fig. 3*B* clearly indicate that the increase in SOCS-3 mRNA expression induced by GH was only inhibited by pretreatment with GH, but not by any of the gp130 cytokines examined. In these figures, 18 and 28 S ribosomal RNAs are shown to demonstrate even loading of total RNA.

Another common feature of gp130 cytokines is their shared use of gp130 and LIFR proteins as signal transducers that comprise their receptor complexes. Therefore, we examined the effect of gp130 cytokine signaling on the expression and decay of these two receptor components. As shown in Fig. 4*A*, the expression levels of LIFR in fully differentiated 3T3-L1 adipocytes dramatically decreased within 6 h in the absence of *de novo* protein synthesis (achieved through incubation with cycloheximide). Addition of LIF, a ligand for LIFR, further accelerated this degradation process. In the presence of ligand, we observed a significant decrease in LIFR levels within 1 h of the treatment, and no protein was detectable after 4 h. Addition of a proteasome inhibitor, MG132, failed substantially to prevent the loss of LIFR protein under basal conditions. Moreover, this inhibitor did not have a marked effect on the increased decay of LIFR after LIF treatment. However, the addition of lysosome inhibitors leupeptin and chloroquine prevented the degradation of LIFR under both basal and LIF-treated conditions. These findings were confirmed by the experiment shown in Fig. 4*B*. In this case, we used a highly specific proteasome inhibitor, epoxomicin, in lieu of MG132. The data shown clearly indicate that the loss of LIFR after a 6-h treatment with cycloheximide was not affected by epoxomicin but was prevented with a combination of leupeptin and chloroquine. Furthermore, the addition of LIF alone, or in the presence of epoxomicin, resulted in a complete loss of LIFR protein, whereas the combination of leupeptin and chloroquine completely prevented this loss. Consistent with previously reported findings (61, 62), the levels of gp130 protein remained unchanged throughout all of the treatments (data not shown and see Fig. 7). Total MAPK (ERK1/2) levels are also shown to demonstrate even loading of protein samples.

To elucidate why LIF, CT-1, and OSM seemed to have prominent inhibitory effects on gp130 cytokine signaling, we examined the ability of all gp130 cytokines to affect the expression levels of LIFR protein in

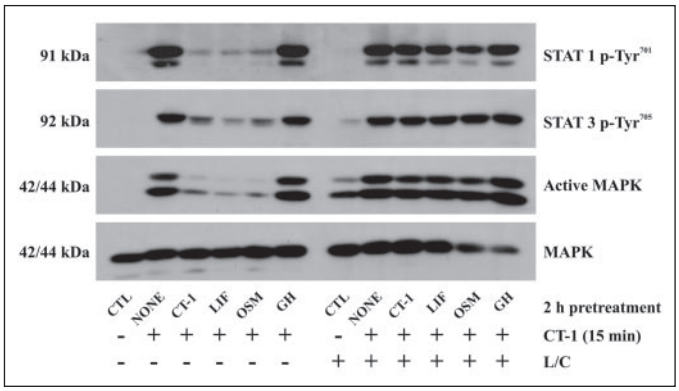

FIGURE 6. **Cross-talk among gp130 cytokines is abolished by inhibiting LIFR degradation in a lysosome-dependent manner.** Fully differentiated 3T3-L1 adipocytes were preincubated with 0.2 nM CT-1 , 0.1 nM LIF, 0.5 ng/ml OSM, or 125 ng/ml GH for 2 h in the presence of 200 μM chloroquine and 10 μM leupeptin (*L/C*). The cells were then treated for 15 min with CT-1. After the treatment, whole cell extracts were prepared, and 150 μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment performed independently three times.

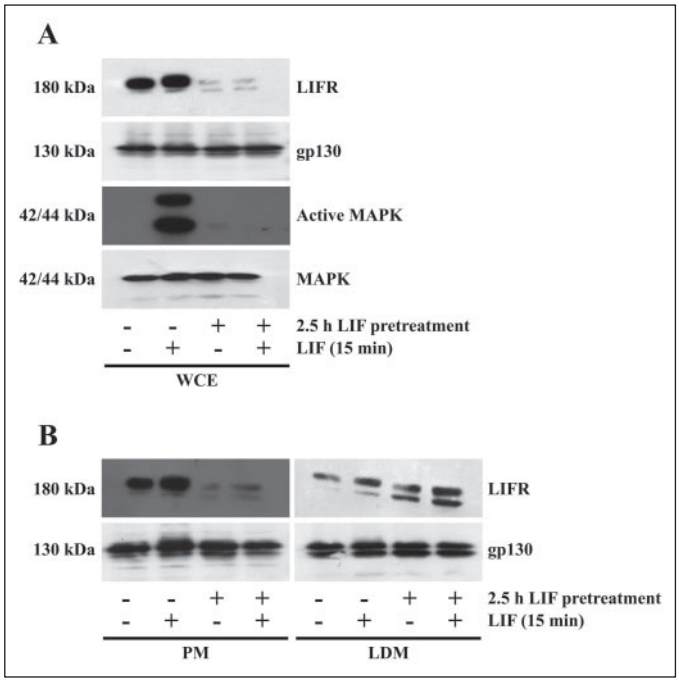

FIGURE 7. **LIF pretreatment results in deceased levels of LIFR on the plasma membrane.** Fully differentiated 3T3-L1 adipocytes were preincubated with 0.1 nM LIF for 2.5 h, followed by a 15-min LIF treatment. The whole cell extracts (*WCE*) (*A*) and the plasma membrane (*PM*) and low density microsome fractions (*LDM*) (*B*) were isolated from the treated cells. An equal amount of each type of extract (100 μg of WCE, 40 μg of PM, and 20 μg of LDM) was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment performed independently two times.

3T3-L1 adipocytes. As shown in Fig. 5, a 4-h incubation with cycloheximide induced a notable decrease in LIFR levels. However, addition of LIF, CT-1, or OSM to these cells resulted in a complete or, in the case of OSM, a nearly complete loss of LIFR expression. Interestingly enough, the addition of CNTF, IL-6, or GH did not induce a decrease in LIFR levels greater than the decrease induced by cycloheximide. Consistent with the data from Fig. 4, the loss of LIFR expression through the action of cycloheximide alone, or with LIF, CT-1, and OSM, was blocked by the addition of leupeptin and chloroquine to the cell media. Total MAPK (ERK1/2) levels are shown as controls for even loading.

Because the inhibitory effects of LIF, CT-1, and OSM correlated with their ability to induce a decrease in LIFR protein levels, and the loss of LIFR could be reversed by leupeptin and chloroquine, we tested whether the addition of leupeptin and chloroquine could reverse the inhibitory effects of LIF, CT-1, and OSM on JAK/STAT and MAPK signaling. Consistent with Fig. 1*A*, the data in Fig. 6 demonstrate that CT-1, LIF, or OSM administration significantly impairs the ability of CT-1 to activate STATs 1 and 3, as well as MAPK (ERK1/2) in adipocytes. Once again, GH demonstrated no such ability. However, the addition of leupeptin and chloroquine to adipocytes prior to cytokine administration reversed the inhibitory effects of CT-1, LIF, and OSM, as evidenced by the ability of CT-1 to activate STATs 1 and 3, and MAPK (ERK1/2) in these cells. Total MAPK (ERK1/2) levels are shown as even loading controls in this experiment.

To determine whether the inhibition of LIF signaling was mediated by a decrease in the levels of LIFR on the cell surface, we isolated membrane fractions from LIF-treated cells that had been previously untreated or pretreated with LIF. As shown in Fig. 7*A*, an examination of whole extracts confirmed that a 2-h LIF pretreatment resulted in a decrease of LIFR expression and the ability of LIF to induce signaling, evident by the activation of MAPK (ERK1/2) in the cells. However, we did not observe any decrease in gp130 levels. These whole cell extracts were also used for membrane fractionation to isolate plasma membrane and LDM fractions. After an acute LIF stimulation (Fig. 7*B*), high levels of LIFR were still present at the plasma membrane. Yet, we observed a decrease in LIFR at the plasma membrane in cells pretreated with LIF, without significant change in the levels of LIFR in LDM. There was no change in the levels of gp130 in either membrane compartment with any of the treatment conditions.

DISCUSSION

Synchronous functional redundancy and specificity may be the most interesting characteristic of gp130 cytokine biology (10, 11). Although the specific mechanisms governing the actions of these cytokines are still unknown, there is a general consensus regarding several aspects of gp130 cytokine signaling. Foremost, all members of the gp130 cytokine family use gp130 protein as a trans-membrane signal transducer within their functional receptor complexes (22, 24). Also, gp130 is crucial for the recruitment of other receptor complex components (63) and for the propagation of signal to the downstream targets, such as the components of the JAK/STAT or MAPK pathways (47, 50). Several gp130 cytokines (LIF, OSM, CNTF, CT-1) use LIFR as a part of their functional receptor complexes (25–29). Both gp130 and LIFR are expressed *in vivo* as well as in cultured adipocytes (54, 64), and many studies have shown that various gp130 cytokines have the ability to induce intracellular signaling in these cell types (54, 60, 65, 66).

In our studies, we examined whether gp130 cytokines can act synergistically to activate their downstream targets or whether their actions antagonize the ability of the individual cytokine to induce signaling. The results from our studies support the latter scenario. CT-1, LIF, and OSM could attenuate or, in some cases, completely abolish, subsequent signaling by their family members. CNTF or IL-6 did not have the same capabilities. There was no the inhibitory cross-talk between gp130 cytokines and GH. GH was only able to interfere with its own signaling. In fact, our data in Figs. 1 and 3 suggest that CT-1, LIF, and OSM may have synergistic effects on GH-induced STAT 3 activation and the induction of SOCS-3 mRNA. These results suggest that the attenuation of gp130 cytokine signaling does not occur primarily at the level of intracellular signal transducers. If this were the case, then GH signaling would have been affected as well because it propagates its signal through the JAK/

STAT and MAPK pathways, in a manner similar to gp130 cytokines (67). Rather, it is indicative of a signaling inhibition at the receptor level because GH does not share receptor components with the members of gp130 cytokine family. Our observations that LIFR is decreased at the plasma membrane support these observations (Fig. 7).

Interestingly, the results from our *in vivo* study were also consistent with our findings. Unlike in 3T3-L1 adipocytes, STAT 3 and MAPK activation by CT-1 was sustained throughout a 4-h pretreatment period in live animals. However, no additional STAT 3 or MAPK activation occurred after a subsequent 15-min CT-1 treatment, demonstrating the inhibitory effect of the pretreatment. The inhibitory effect of CT-1 pretreatment was particularly evident after an 8-h pretreatment period. This extended time frame allowed the activated STAT 3 and MAPK levels to return to basal. Because of the limited scope of our study, we have not yet examined whether the inhibitory cross-talk *in vivo* occurs, but, based on our *in vitro* results, we predict that LIF and OSM pretreatment would also attenuate CT-1 signaling *in vivo*.

We also demonstrated that the ability of gp130 cytokines to inhibit the initiation of signaling by their family members results in marked changes in target gene expression. In these studies, we observed a loss in the induction in SOCS-3 mRNA coincided with an impairment of signaling by gp130 cytokines. Similar findings were recently reported by a group studying OSM-mediated induction of SOCS-3 mRNA in cultured human umbilical vein endothelial cells (68). The results of our experiment are very important because they clearly demonstrate that the inhibition of signal induction is also observed in the nucleus at the level of gene transcription. Furthermore, our results suggest that there are no compensatory mechanisms by which the cells can maintain a particular level of signaling by some cytokines when faced with reduction in signaling because of the inhibitory cross-talk of gp130 cytokines.

Because our experiments suggested a possible inhibition of signaling at the level of the receptor, we examined the effects of gp130 cytokines on the expression and stability of LIFR. Previous studies have shown that gp130 cytokine signaling affects the turnover and degradation of LIFR while having no effect on gp130 (61, 62). Consistent with these findings, we have shown that the half-life of LIFR is dramatically shortened by LIF administration in 3T3-L1 adipocytes. We have also demonstrated that basal or ligand-induced degradation of LIFR can be prevented by the addition of lysosomal inhibitors. However, proteasome inhibitors did not alter the half-life of LIFR under basal conditions or after LIF administration. In addition to LIF, both CT-1 and OSM had profound effects on LIFR degradation. All of these cytokines use LIFR as a component of their receptor complexes and therefore induce its endocytosis and degradation (62). The effect of CNTF was marginal, whereas IL-6 and GH had no effect on LIFR levels. IL-6 signals via the complex of gp130 and IL-6Rα and therefore does not affect LIFR, whereas the receptor for GH shares no common components with gp130 cytokine receptor complexes. CNTF signals through the receptor complex of gp130, LIFR, and CNTFRα. However, differentiated 3T3-L1 adipocytes do not express CNTFRα (54), so the ability of this cytokine to form functional receptor complexes, and therefore induce LIFR degradation, is severely impaired. Consistent with our previous experiment, inhibition of the lysosome with leupeptin and chloroquine completely prevented the loss of LIFR following gp130 cytokine treatment.

However, one of our most interesting findings was the fact that the inhibition of the lysosome prior to cytokine administration completely blocks the ability of gp130 cytokines to impair signaling of their family members. Together with the subcellular fractionation data, these results strongly suggest that the loss of LIFR protein during the pretreatment period prevents the initiation of subsequent signaling events by cytokine administration and that the inhibitory cross-talk is not the result of a direct effect on STATs, but rather the decreased levels of LIFR on the plasma membrane.

In summary, a striking parallel has emerged during this study, linking the ability of a specific gp130 cytokine to block its own signaling or the signaling of other gp130 cytokines in a manner that correlates with the degradation of LIFR. Specifically, CT-1, LIF, and OSM, the cytokines with the strongest inhibitory effects on the signaling of their family members, were also the most potent inducers of lysosome-mediated LIFR degradation. We hypothesized that their ability to inhibit, or at least attenuate, the signaling of gp130 cytokines is associated with their ability to induce LIFR degradation and limit the availability of LIFR for the formation of receptor complexes required for further stimulation of signaling by gp130 cytokines. Conversely, CNTF, IL-6, and GH did not affect the turnover of the LIFR and therefore did not inhibit the signaling abilities of other gp130 cytokines. Further studies will be necessary to elucidate the exact fate of LIFR after cytokine treatment and to determine whether lysosomal inhibition prevents endocytosis of LIFR, as well as its degradation. However, our data clearly demonstrate that lysosomal inhibitors reverse the ability of CT-1, LIF, and OSM pretreatment to inhibit gp130 cytokine-induced STAT activation, suggesting that the cross-talk among these cytokines occurs because of LIFR endocytosis, which makes this protein unavailable for further signaling events. These findings provide a strong basis for future studies on cytokine interactions that could be used to mimic various disease states marked by simultaneous expression of several cytokines, or to create novel approaches in inducing the differentiation of various progenitor cell lines (69, 70). This system may also be used to understand the signaling events in adipocytes under the influence of macrophages. Two recent studies (55, 71) have demonstrated that macrophage accumulation in adipose tissue, and subsequent secretion of cytokines by these macrophages, could possibly be some of the initial events leading to the onset of obesity and type 2 diabetes.

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