Src Family Kinase-independent Signal Transduction and Gene Induction by Leukemia Inhibitory Factor*

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Members of the interleukin-6 (IL-6) family of cytokines exert their biological effects via binding to their cognate ligand-binding receptor subunit on a target cell. The subsequent recruitment of the common signal transducer glycoprotein 130 and activation of the JAK/STAT and SHP-2/Ras/mitogen-activated protein kinase (MAPK) pathways are responsible for the majority of cellular responses elicited by IL-6 cytokines. Several types of experiments suggest that the Src family of kinases (SFK) also participates in IL-6 family cytokine-mediated signaling events. SYF cells, which lack expression of SFKs Src, Yes, and Fyn, were used to determine the role of SFKs in IL-6 family cytokine signaling and gene induction. SYF and wild type (WT) control fibroblasts displayed similar activation of signaling intermediates following stimulation with leukemia inhibitory factor (LIF). LIF-stimulated tyrosine phosphorylation of SHP-2 and subsequent activation of MAPK in SYF cells were identical to that seen in LIF-stimulated WT cells. Both LIF-stimulated tyrosine phosphorylation of STAT1 and STAT3, as well as LIF-stimulated DNA binding activity of STAT-containing nuclear complexes were indistinguishable when compared in SYF and WT cells. In addition, the phosphatidylinositol 3-kinase-sensitive Akt kinase and p38 MAPK were activated by LIF in both SYF and WT cells. Furthermore, LIF-stimulated expression of c-fos, egr-1, and suppressor of cytokine signaling-3 was retained in SYF cells. The IL-6 family cytokine oncostatin M was also capable of activating MAPK, STAT3, STAT1, Akt, and p38 in both WT and SYF cells. These results demonstrate that IL-6 family cytokines can activate a full repertoire of signaling pathways and induce gene expression independent of SFKs.

The interleukin-6 (IL-6)† family of cytokines, which includes leukemia inhibitory factor (LIF), ciliary neurotrophic factor, oncostatin M (OSM), cardiotoxin-1, cardiotoxin-like cytokine (1), IL-6, and IL-11, produces a wide variety of responses in a number of cell types (2). For example, LIF has been shown to maintain embryonic stem (ES) cell pluripotency (3) and cause increased lipolysis in adipocytes (4), whereas IL-6 can cause proliferation of myeloma cell lines. In vitro these cytokines frequently produce redundant responses, such as the ability of ciliary neurotrophic factor, IL-11, LIF, OSM, or IL-6 to all cause myeloma cell line proliferation (5). This redundancy is usually attributed to the fact that following binding of an IL-6 family cytokine to its cognate cell surface receptor, the common signal transducing receptor subunit gp130 is recruited, along with constitutively associated JAK tyrosine kinases (6). Subsequently, a common set of signal transduction molecules is recruited to activated receptor complexes, including STAT1 and STAT3, which are activated by phosphorylation and ultimately translocate to the nucleus where they induce transcription of target genes (7). One such STAT-induced target gene is suppressor of cytokine signaling-3 (SOCS-3) (8, 9), which negatively regulates cytokine signaling (10).

In addition to the JAK/STAT pathway, the SHP-2/Ras/MAPK (ERK1/2) (11), phosphatidylinositol 3-kinase (PI3K)/Akt (12), and p38 MAPK (13) pathways are also stimulated by IL-6 family cytokines. SHP-2 serves as a LIF-sensitive adaptor molecule for recruitment of downstream signaling molecules via an SH2-mediated interaction with gp130 (14). SHP-2 is thought to couple the IL-6 family cytokines to the MAPK (ERK1/2) cascade (15). The SHP-2 binding site on gp130 is required for both IL-6-mediated ERK1/2 activation and induction of immediate early genes egr-1 and c-fos (16, 17). SHP-2 is also thought to couple IL-6 cytokines to the PI3K/Akt pathway (14, 18). The PI3K/Akt pathway was linked recently to LIF- or IL-6-mediated phosphorylation of Akt/protein kinase B and subsequent protection against apoptosis. The p38 MAPK has only recently been implicated in IL-6 family cytokine signaling (13, 21, 22). In addition to these pathways, members of the Src family kinases (SFKs) display increased catalytic activity following stimulation of cells with LIF, IL-6, or IL-11 (23–25).

The SFKs are non-receptor tyrosine kinases and include eight murine members: Src, Yes, and Fyn, which are expressed ubiquitously, as well as Lyn, Fgr, Lck, Blk, and Hck, which display a more restricted expression (26). LIF-mediated self-renewal of ES cells is accompanied by both the activation of Hck and the recruitment of Hck to gp130. Furthermore, ES cells expressing a constitutively active form of Hck require 15-fold less LIF to maintain pluripotency compared with LIF-stimulated parental ES cells (24). In multiple myeloma (MM) cell lines, IL-6 stimulation increases the activity of Fyn, Hck, and Lyn (23). Recent functional studies in MM cell lines suggest that a novel region of gp130 is involved in both Hck and MAPK activation (27). In 3T3-L1 preadipocytes, IL-11 activates both Src and Yes (25). Although these results suggest a
role for SFKs in IL-6 family cytokine signaling, there is no direct evidence that endogenous SFKs either are required for or modulate IL-6 family cytokine-mediated stimulation of signal transduction and subsequent gene induction. In this paper we test the role of endogenous SFKs in IL-6 family cytokine signaling.

Because of the ability of SFK family members to compensate for the loss of another SFK (28, 29), and because of the activation of multiple SFKs by IL-6 family cytokines, we used a system where SFKs are not expressed. SYF cells are a mutant cell line derived from src/fl;fyn triple-knock-out mouse embryonic stem cells and have also been shown to lack expression of Lyn (30). Because the expression of Hck, Lck, Fgr, and Blk is thought to be restricted to cells of hematopoietic lineage, SYF represent a cell line lacking all known murine SFKs (30). We used SYF cells to determine whether the lack of SFKs has an effect on LIF-mediated signaling pathways or LIF-mediated gene induction.

We show here that a loss of expression of all SFKs does not affect canonical LIF-mediated signal transduction pathways. STAT1 and STAT3 display similar cytokine-stimulated responses in SYF and a wild type (WT) control cell line. Activation of the MAPK cascade by LIF appears unchanged by the loss of SFKs. Both the PI3K-sensitive Akt kinase and p85 MAPK are phosphorylated following LIF-stimulation of either SYF or WT cells. In addition, LIF stimulates the expression of the LIF-sensitive genes c-fos, egr-1, and SOCS-3 in both SYF and WT cells. Furthermore, OSM-mediated activation of STAT3, STAT1, MAPK, p38, and Akt is also unaffected in SYF and WT cells. Furthermore, OSM-mediated activation of the LIF-sensitive genes c-fos, egr-1, and SOCS-3 in both SYF and WT cells. Furthermore, OSM-mediated activation of STAT1 and STAT3 display similar cytokine-stimulated responses in SYF and a wild type (WT) control cell line. ACTIVATION OF THE MAPK CASCADE BY LIF APPEARS UNCHANGED BY THE LOSS OF SFKs.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Recombinant human LIF was purchased from Alomone (Jerusalem, Israel). Recombinant human PDGF (B chain homodimer) and human OSM were purchased from Peprotech (Rocky Hill, NJ). The polyclonal antibodies against p85, Akt 1/2, c-fos, egr-1, and SOCS-3, monoclonal antibody against STAT1, and the SIE oligonucleotide were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal antibodies against phospho-p42/p44 MAPK, phospho-STAT3 (Tyr-705), STAT3, phospho-STAT1 (Tyr-701), phospho-p38, and phospho-Akt (Ser-473) were purchased from New England Biolabs (Beverly, MA). The monoclonal antibody against MAPK (ERK1/2) was purchased from Zymed Laboratories Inc. (South San Francisco, CA). The monoclonal anti-phosphotyrosine antibodies PY20 and 4G10 were purchased from BD Biosciences. Protein G-agarose, poly(dI-dC)-oligonucleotides, polyacrylamide 0.5% Tris borate-EDTA gels pre-run at 150 V for 2 h, autoradiographic film, and Kodak X-omat film were purchased from ICN (Costa Mesa, CA) and Upstate Biotechnology (Lake Placid, NY), respectively. This monoclonal anti-SHP-2 antibody was purchased from Beckman Coulter. Protein G-agarose, poly(dI-dC)-poly(dI-dC)-poly(dI-dC), and G-25 Sephadex Quick Spin columns were purchased from Roche Applied Science. T4 polynucleotide kinase was purchased from New England Biolabs (Beverly, MA). γ-32P/ATP was purchased from PerkinElmer Life Sciences. Dulbecco’s modified Eagle’s medium and fetal bovine serum were obtained from Invitrogen. SYF cells were obtained from ATCC (CRL-2459). The WT mouse embryonic fibroblast cell line was derived from hybrid C57Bl6J/129Sv E13.5 embryos and has also been shown to lack expression of Lyn (30). Because the expression of Hck, Lck, Fgr, and Blk is thought to be restricted to cells of hematopoietic lineage, SYF represent a cell line lacking all known murine SFKs (30). We used SYF cells to determine whether the lack of SFKs has an effect on LIF-mediated signaling pathways or LIF-mediated gene induction.

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Western analysis—Cells were plated on 10-cm dishes, serum-starved overnight, and stimulated the next day at ~80% confluence with varying concentrations of PDGF, LIF, OSM, or PBS (vehicle). Following stimulation, cells were aspirated, and cells were rinsed three times in 4 ml of ice-cold PBS. Cells were then lysed in 100 µl of 4X SDS sample buffer, scraped, sonicated, cooled, boiled for 5 min, and centrifuged as described previously (31). Supernatants were removed, and ~50 µg of total cellular proteins were loaded onto SDS/10% PAGE. Electrophoresis, transfer, probing of membranes with phosphotyrosine antibodies, visualization with enhanced chemiluminescence, and stripping of blots were performed as described previously (15, 31). All other blots were blocked and probed using 5% non-fat milk in Tris-buffered saline/0.1% Tween. Film was analyzed as described (31) except that ratios of phosphorylated to total protein are represented as either a percentage of the corresponding PDGFR-stimulated ratio or as a percentage of the maximal LIF-stimulated ratio. Student's t test for independent samples was used, and only samples with a p value <0.05 were considered statistically significant.

Immunoprecipitation—Cells were stimulated with 100 ng/ml LIF for various amounts of time or PBS (vehicle), rinsed three times in ice-cold PBS, and lysed in 1 ml of elution buffer (1% Triton X-100, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 2 mM NaOH, 2 mM NaVO4, 1% aprotinin, 10 µg/ml Tris, pH 7.4, 1 mM phenylmethylsulfonyl fluoride), solubilized on ice for 60 min, and centrifuged for 15 min at 4 °C. Supernatants were then immunoprecipitated overnight with 3 µl of anti-SHP-2 antibodies and 30 µl of protein G-agarose beads. After recovery by brief centrifugation, immunocomplexes were washed twice with elution buffer (without Triton-X), and beads were then resuspended in SDS sample buffer and boiled, and supernatants were loaded onto SDS/PAGE and analyzed as described above.

Preparation of Nuclear Extracts—Following stimulation with LIF or PBS (vehicle), nuclear lysates were obtained as described previously (32). Briefly, cells were rinsed, scraped, and pelleted by centrifugation at 3000 rpm in a tabletop variable-speed microfuge. Next, cells were resuspended in 400 µl of Buffer A (100 mM HEPES, pH 7.8, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, 100 µM NaVO4) and incubated on ice for 10 min. Cells were lysed in 0.25% Nonidet P-40, vortexed, and spun at 6000 rpm to pellet the nuclei. The pellet was re-suspended in 50 µl of Buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 0.2 µg/ml aprotinin, 100 µM NaVO4) and incubated on ice for 15 min with frequent vortexing to elute nuclear proteins. Samples were then spun at top speed in a tabletop microfuge at 4 °C for 5 min, and supernatants were snap-frozen and stored at −70 °C. Protein concentration was determined by Bradford assay (33).

Electrophoretic Mobility Shift Assay (EMSA)—EMSAs were performed as described previously (34) with minor modifications. Briefly, 40 ng of a double-stranded 27-mer corresponding to an optimized high affinity STAT1/3 DNA binding site of the c-fos promoter (SIE-DNA, 5′-GTG-CATTCCCCTGAACTTTGCTTACA-3′) was end-labeled using 20 units of T4 polynucleotide kinase and 50 µCi of [γ-32P]ATP. Radiolabeled oligonucleotides were separated from unincorporated nucleotides using a G-25 Sephadex Quick Spin column. Binding reactions were incubated in binding buffer (25 mM HEPES, pH 7.9, 50 mM KCl, 0.5 mM EDTA, and 10% glycerol) with 20 fmol of radiolabeled SIE, 10 µg of total nuclear lysate, and 2 µg of poly(dI-dC)-poly(dI-dC). For supershift analysis, 3 µl of either STAT1 or STAT3 antibody was added to samples. Binding reaction components were added as follows: 1) 10 µg of nuclear protein, 2) 200 fold excess of cold competitor DNA, 3) cold competitor DNA, 4) probe. Components 1–3 were preincubated at room temperature for 30 min before addition of probe. Binding reactions were incubated for 20 min at room temperature and separated on a 6% polyacrylamide 0.5× Tris borate-EDTA gel pre-run at 150 V for 2 h at 4 °C. Dried gels were exposed to Eastman Kodak Co. X-omatic film at −70 °C, sandwiched between two DuPont lighting intensifying screens. Autoradiographic film was scanned and analyzed for statistical significance as described above.

RESULTS

LIF Stimulates Phosphorylation of Both MAPK and SHP-2 in WT and SYF Cells—Dual phosphorylation of MAPK (ERK1/ERK2) on Thr-202 and Tyr-204 is used frequently to quantify MAPK activation in both cultured cells and intact tissues (35–37). We performed SDS-PAGE and subsequent immunoblot assays on lysates from either SYF or WT cells. Blots were first probed with antibodies against activated MAPK and then stripped and re-probed with an antibody that recognizes total ERK1/2 to control for slight variations in protein loading. The time course for activation of MAPK by LIF in both WT and SYF whole cell lysates was determined (Fig. 1A). A robust LIF-stimulated activation of MAPK can be seen at both 10 and 30 min.
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Fig. 1. Immunoblot analysis and quantitative analysis of LIF-stimulated MAPK and SHP-2 phosphorylation in WT and SYF lysates. A, quiescent WT or SYF cells were stimulated with LIF (100 ng/ml) for various times or PDGF (100 ng/ml) for 5 min. Whole cell lysates were analyzed by SDS-PAGE, using antibodies specific for phosphorylated MAPK (P-MAPK). Membranes were then stripped and re-probed for total MAPK. Quantitative analysis using densitometry shows LIF phosphorylation of ERK1 (B) or ERK2 (C) as a percentage of maximal PDGF-stimulated MAPK phosphorylation. D, quiescent WT or SYF cells were stimulated with varying concentrations of LIF for 15 min or PDGF (100 ng/ml) for 5 min. E, SHP-2 immunoprecipitants were analyzed using antibodies specific for phosphotyrosine (P-Tyr), followed by stimulation of cells with LIF (100 ng/ml) for various times. Membranes were then stripped and re-probed for total SHP-2. F, quantitative analysis using densitometry shows LIF phosphorylation of SHP-2 as a percentage of maximal LIF-stimulation (5 min). Data from WT (□) or SYF (○) cells are represented as the mean ± S.D. of three separate experiments (B, C, and F). Variations in intensity of bands because of protein loading were corrected for by normalizing to total MAPK. Representative blots from three separate experiments are shown (A, D, and E).

15 min of agonist stimulation in both cell lines. MAPK activation by LIF returns to basal levels by 30 min of agonist treatment. PDGF signaling to MAPK is unchanged in SYF cells (30), and thus we treated cells with 100 ng/ml PDGF (5 min) as a control for activated MAPK. LIF stimulated ERK1/p44 (Fig. 1B) and ERK2/p42 (Fig. 1C) activation to a similar level, relative to PDGF, in both cell lines. Next we examined whether there was any difference in coupling of LIF to MAPK activation at submaximal doses when comparing WT and SYF cells. There was a similar dose dependence for stimulation of MAPK phosphorylation in both cell lines; maximal phosphorylation of MAPK was attained at 30 ng/ml LIF in both WT and SYF cells. SHP-2 tyrosine phosphorylation is often used to determine activation of SHP-2 by IL-6 family cytokines (14, 38). To confirm that the SHP-2/Ras/MAPK pathway was unperturbed by the loss of expression of SFKs, we also measured SHP-2 tyrosine phosphorylation (Fig. 1E). SHP-2 was immunoprecipitated from either WT or SYF lysates following LIF stimulation for various times. Immunoprecipitants were subject to SDS-PAGE and subsequent immunoblot assays with phosphotyrosine-specific antibodies. Blots were then stripped and probed for total immunoprecipitated SHP-2. A large increase in SHP-2 tyrosine phosphorylation over basal was seen at 5 min after LIF stimulation in WT and SYF cells. SHP-2 tyrosine phosphorylation returned to basal levels 30 min after addition of agonist in both cell lines. Quantitative analysis of LIF-stimulated SHP-2 activation is represented as a percentage of maximal (5 min) LIF-stimulated SHP-2 tyrosine phosphorylation in Fig. 1F (n = 3). These data suggest that the SHP-2/MAPK pathway is unaltered by the loss of SFKs.

LIF-stimulated Activation of STAT1 and STAT3 Is Unperturbed in SYF Cells—Next we examined whether a loss of expression of SFKs affected LIF-mediated activation of the JAK/STAT pathway. Phosphorylation of Tyr-705 or Tyr-701 of STAT3 or STAT1, respectively, results in SH2-mediated hetero- or homodimerization of STAT1/3, followed by rapid translocation to the nucleus (39, 40). Whole cell lysates were collected from either WT or SYF cells, separated using SDS-PAGE, and analyzed first with antibodies specific for either Tyr-705-phosphorylated STAT3 or Tyr-701-phosphorylated STAT1. Blots were then stripped and re-probed with antibodies specific for either total STAT3 or STAT1. As shown in Fig. 2A, LIF stimulation of either WT or SYF cells resulted in phosphorylation of STAT3 within 5 min. STAT3 phosphorylation remained elevated for 30 min and returned to basal levels 1 h after addition of LIF in both WT and SYF cell lysates. Quantitative analysis of the data (Fig. 2B) is represented as a percentage of maximal LIF-stimulated STAT3 phosphorylation. STAT1 was also transiently phosphorylated following treatment of WT or SYF cells with LIF (Fig. 2C). Although there was a statistically significant difference between LIF-stimulated phosphorylation of STAT1 in WT and SYF cells at 5 min (Fig. 2D), this difference was not seen at later time points. To ensure that there were in fact no minor differences in the ability of LIF to stimulate STAT phosphorylation in SYF cells, we stimulated cells with varying concentrations of LIF (Fig. 2, E and F) and measured STAT activation as described above. LIF-stimulated STAT3 phosphorylation was observed at a concentration of 10 ng/ml in both WT and SYF cells (Fig. 2E). Fig. 2F reveals that WT and SYF cells have a similar sensitivity to LIF as measured by STAT1 phosphorylation in both WT and SYF cells. These data suggest that the activation of the JAK/STAT pathway, in terms of both temporal response and sensitivity to ligand, is unaffected by the loss of SFKs.

DNA Binding Activity of Nuclear STAT-containing Complexes Is Unaltered by the Loss of SFKs in Immortalized Mouse Embryonic Fibroblasts—Although it appeared as though there were no changes in the ability of LIF to stimulate phosphorylation of STATs on tyrosine residues in SYF cells relative to WT cells, there is evidence that DNA binding activity and subsequent transcriptional activation by STATs can be both positively (41) and negatively (42) regulated by serine phosphorylation. To determine whether there were any differences in the ability of STATs to transactivate target genes, we performed EMSA analysis using a double-stranded 27-mer corresponding to a STAT consensus binding site within the c-fos promoter (43), known as the SIE. SIE was radiolabeled and incubated with nuclear extracts from LIF-stimulated SYF or WT cells (Fig. 3, A and C). Fig. 3A shows a time course for STAT binding to SIE. Three complexes, designated previously as SIF-A, SIF-B, and SIF-C, have been shown to bind SIE and represent the STAT3 homodimer, STAT1/3 heterodimer, and STAT1 homodimer, respectively (44). Binding reactions using LIF-stim-
SYF (f) cells are represented as the mean ± H18554 percentage of maximal LIF stimulation (10 min). Data from WT analysis using densitometry shows LIF phosphorylation of STATs as a phosphorylated STAT3 (P-STAT3; lysates where analyzed by SDS-PAGE using antibodies specific for total STAT3 (A and B) and a STAT3 homodimer, and that the intermediate complex con-plex is a STAT1 homodimer, the slowest migrating complex is stimulated extracts. Supershift analyses with anti-STAT1 and the addition of a 100-fold molar excess of unlabeled SIE to of binding to SIE is shown by a loss of complex formation with three distinct nuclear complexes bound to SIE. The specificityulated nuclear extracts from either WT or SYF cells reveals (A and B) or STAT1 (C and D) or phosphorylated STAT1 (E and F). Variations in intensity of bands because of protein loading were corrected for by normalizing to total STAT3 or protein loading. 2 These data show that multiple members of STAT3, MAPK, p38, and Akt in responses in both cell lines (Fig. 7). LIF and OSM stimulated respective responses to OSM are identical to LIF-stimulated gp130 receptor complex. We found that not only do LIF stimulates the formation of a LIF receptor ligand-binding receptor subunits are products of distinct genes. Although LIF and OSM both utilize the gp130 signal transducer, their ligand-binding receptor subunits are products of distinct genes. LIF stimulates the formation of a LIF receptor-gp130 complex, whereas stimulation of cells with OSM results in an OSM receptor-gp130 receptor complex. We found that not only do both WT and SYF cells respond to OSM but also that their respective responses to OSM are identical to LIF-stimulated responses in both cell lines (Fig. 7). LIF and OSM stimulated phosphorylation of STAT3, STAT1, MAPK, p38, and Akt in both WT and SYF cells. PDGF-stimulated lysates were used as a control. Blots were stripped and re-probed to confirm equal protein loading. LIF-stimulated p38 phosphorylation can be seen in both cell lines following 15 min of treatment with LIF. PDGF is used as a control for p38 phosphorylation. Quantitative analysis of LIF-mediated p38 activation is represented as a percentage of PDGF-stimulated p38 phosphorylation (Fig. 5B). No difference is seen in the ability of LIF to activate p38 MAPK in SYF or WT cells. These data suggest that LIF sig-naling to the p38 MAPK pathway is unaffected by the loss of SFKs.

The LIF-sensitive Genes c-fos, egr-1, and SOCS-3 Are Induced by LIF in SYF Cells—Changes in protein expression of LIF-sensitive genes were analyzed to measure the effect of LIF stimulation of signaling pathways in SYF and WT cells. The early immediate genes c-fos, egr-1, and SOCS-3 have all been shown previously (13, 16) to be regulated by LIF. SDS-PAGE was used to assess the protein expression of LIF-sensitive gene products in cell lysates following stimulation of cells with LIF. Blots were re-probed for STAT3 to correct for minor differences in protein loading. LIF-stimulated expression of c-fos, egr-1, and SOCS-3 peaks at 1 h and returns to basal levels by 4 h post-stimulation in both WT and SYF cells (Fig. 6A). In con-trast, no changes were seen in the expression of SOCS-1, c-jun, or junB in either cell line following LIF-stimulation. Cells were stimulated with varying concentrations of LIF for 1 h to see whether there were minor differences between expression of LIF-sensitive proteins in WT and SYF cells. A dose-dependent increase in expression of c-fos (Fig. 6B) and egr-1 (Fig. 6C) is seen following stimulation of WT or SYF cells with LIF. These data suggest that the loss of SFK gene expression has no overt effect on the ability of LIF to stimulate gene expression.

The IL-6 Cytokine Family Member Oncostatin M Also Retains Normal Signal Transduction in SYF Cells—Although LIF and OSM both utilize the gp130 signal transducer, their ligand-binding receptor subunits are products of distinct genes. SYF and WT cells. As a control for Akt Ser-473 phosphorylation, we stimulated cells with PDGF (Fig. 4B). Quantitative analysis of LIF-mediated Akt activation represented as a percentage of PDGF-stimulated activity shows no difference between LIF coupling to the activation of Akt in SYF or WT cells.

The p38 MAPK Is Activated by LIF in Both SYF and WT Fibroblasts—p38 MAPK was determined to be LIF-sensitive in both WT and SYF cells (Fig. 5A), using SDS-PAGE and a dual-specificity phospho-specific p38 antibody (Thr-180/Tyr-182). Blots were stripped and re-probed for total p38 to control for protein loading. LIF-stimulated p38 phosphorylation can be seen in both cell lines following 15 min of treatment with LIF. PDGF is used as a control for p38 phosphorylation. Quantitative analysis of LIF-mediated p38 activation is represented as a percentage of PDGF-stimulated p38 phosphorylation (Fig. 5B). No difference is seen in the ability of LIF to activate p38 MAPK in SYF or WT cells. These data suggest that LIF sig-naling to the p38 MAPK pathway is unaffected by the loss of SFKs.

The P38-sensitive Akt Kinase Is Phosphorylated in Response to LIF, Independent of SFKs—To rule out the possibility that SFKs couple IL-6 family cytokines to the P38K pathway, we measured phosphorylation of Akt on Ser-473 in response to treatment of cells with LIF. Maximal activation of Akt by LIF occurred at 15 min after addition of agonist (Fig. 4A) in both WT and SYF cells. As a control for Akt Ser-473 phosphorylation, we stimulated cells with PDGF (Fig. 4B). Quantitative analysis of LIF-mediated Akt activation represented as a percentage of PDGF-stimulated activity shows no difference between LIF coupling to the activation of Akt in SYF or WT cells.

DISCUSSION

The results shown here suggest that LIF-mediated signaling and gene induction can occur independently of SFKs. There are numerous examples of increases in SFK catalytic activity follow-ing stimulation of cells with IL-6 family cytokines; however, there is little evidence linking this SFK catalytic activation to subsequent signaling or gene induction. Our results demon-strate that LIF-mediated activation of the canonical JAK/STAT and SHP-2/Ras/MAPK pathways is unperturbed by a loss of

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G. S. L. and N. M. N., unpublished observations.
expression of SFKs. In addition, the activation of the PI3K-sensitive Akt pathway, as well as the activation of the p38 MAPK pathway by LIF, are unaffected by a lack of SFKs. Furthermore, induction of the LIF-sensitive genes c-fos, egr-1, and SOCS-3 occurs in both the presence and absence of SFKs, suggesting that SFKs are not required for a cell to respond to LIF. The ability of OSM to signal normally in SYF cells suggests that multiple members of the IL-6 family of cytokines can induce a response in cells without SFKs.

The SFKs were first implicated in IL-6 cytokine signal transduction in human endothelial cells in which OSM stimulation in vitro leads to increased activity of Yes and Fyn (45). Next it was discovered that LIF-stimulated ES cells display both increased Hck tyrosine kinase activity in vitro and a stimulation-dependent biochemical association between gp130 and Hck (24). Furthermore, ES cells expressing a constitutively active Hck required 15-fold less LIF to maintain pluripotency compared with LIF-stimulated parental ES cells. These data suggested that increased Hck activity might amplify the endogenous response to LIF. Using chimeric granulocyte colony-stimulating factor receptor/gp130 receptors expressed in transiently transfected ES cells, Ernst et al. (46) were able to implicate a region of gp130 in granulocyte colony-stimulating factor-stimulated activation of Hck. Subsequent studies revealed that a dominant negative STAT3 could shunt ES cells onto a path toward differentiation (47), whereas suppression of SHP-2 or ERK activation resulted in maintenance of ES cell pluripotency (48). Collectively, these results suggested that Hck might lie either upstream of LIF-stimulated STAT3 activation or upstream of MAPK inhibition. Multiple myeloma cell lines display an analogous increased catalytic activation of SFKs and a biochemical association of SFKs with gp130 following stimulation with IL-6 (23). Most recently, using a erythropoietin receptor/gp130 chimeric receptor and overexpressed Hck, Schaeffer et al. (27) implicated a region of gp130 as a positive regulator of Hck, as well as MAPK. However, this region of gp130 was unable to account for all gp130-mediated activation of MAPK, consistent with SHP-2 acting as a regulator of LIF-mediated MAPK activation. As the chimeric receptor mutation of Schaeffer et al. (27) created agp130 deletion of substantial size (41 amino acids), it is not clear how the subsequent loss of activation of MAPK and Hck are related. Interestingly, we were unable to find any effect of a loss of SFK expression on MAPK or STAT3 signaling in SYF cells, suggesting that the loss of MAPK activation in MM cells transfected with the gp130 deletion mutant chimeric receptor may be because of uncoupling of agonist-induced MAPK activation that is independent of SFKs. Alternatively, mechanisms of coupling to

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**Fig. 3.** EMSA analysis of STAT DNA binding activity in LIF-stimulated WT or SYF nuclear extracts. A, time course for DNA binding activity is shown following stimulation of cells with LIF (100 ng/ml) for various times, using a radiolabeled SIE oligonucleotide and nuclear extracts, resolved on 0.5× Tris-buffered EDTA/6% PAGE. The first lane shows SIE probe without any nuclear extracts. The 100× SIE lane shows binding of WT LIF-stimulated nuclear extracts to SIE in the presence of 100-fold molar excess of unlabeled competitor SIE. The lane labeled STAT1 shows binding of WT LIF-stimulated nuclear extracts to SIE after preincubation with an anti-STAT1-specific antibody. The last lane shows binding of WT LIF-stimulated nuclear extracts to SIE after preincubation with an anti-STAT3-specific antibody. B, quantitative analysis of data represented as percent-maximal LIF-stimulated STAT/SIE binding activity. Data from WT (open bars) or SYF (filled bars) cells are represented as the mean ± S.D. of three separate experiments. C, dose-response for LIF-stimulated STAT DNA binding activity. Representative autoradiographs from three separate experiments are shown (A and C).
MAPK activation by IL-6 cytokines may differ in a cell type-specific fashion.

In 3T3-L1 mouse preadipocytes Fyn has been shown to co-purify with Grb2 and JAK2 in an IL-11-dependent fashion. Furthermore, Fyn is tyrosine-phosphorylated in response to IL-11 stimulation (49). Yes and Src also appear to be activated in 3T3-L1 cells following IL-11 stimulation (25), and Yes appears to co-precipitate with JAK2 in a stimulation-dependent fashion (50). These data argue for both physical association of SFKs with gp130 and activation of SFKs following treatment of 3T3-L1 cells with IL-11. Although ES cells, MM cell lines, and 3T3-L1 preadipocytes display increased activity of SFKs following stimulation with LIF, IL-6, or IL-11, respectively, the consequences of IL-6 family cytokine-stimulated increases in SFK activity remains unclear. There is no direct evidence linking IL-6 family cytokine-mediated stimulation of endogenous SFKs with downstream effectors and no direct evidence of cellular consequences specific to endogenous SFKs on IL-6 cytokine-mediated gene induction. Our findings show that the absence of SFKs has no effect on known IL-6 cytokine family signaling pathways or gene induction.

Although most cellular responses mediated by SFKs are dependent upon the catalytic activity of SFKs, both the SH2

![Fig. 4. LIF-stimulated phosphorylation of Akt kinase in WT and SYF cells.](http://example.com/fig4)

WT or SYF cells were stimulated with 100 ng/ml of either LIF (15 min) or PDGF (5 min). Whole cell lysates were analyzed by SDS-PAGE, using antibodies specific for phosphorylated Akt (P-Akt). Representative blots are shown from at least three separate experiments.Quantitative analysis using densitometry shows LIF phosphorylation of Akt as a percentage of maximal PDGF-stimulated MAPK phosphorylation (5 min). Variations in intensity of bands because of protein loading were corrected for by normalizing to total Akt. Data from WT (open bars) or SYF (filled bars) cells are represented as the mean ± S.D. of four (WT) or five (SYF) experiments.
and SH3 domains found in SFKs have been implicated in catalytic region-independent functioning of SFKs as a scaffolding protein (65–67). Furthermore, Hck-mediated activation of STAT3 has been shown recently (51) to require the SH3 domain of Hck. Thus, although SFK inhibitors that abrogate kinase activity are highly useful in the study of SFK function, they may not be able to inhibit all functions of SFKs. Unlike the use of inhibitors of SFK catalytic activity, which only addresses the role of catalytic activity of SFKs, SYF cells should also elucidate any scaffolding functions of SFKs. Thus, the lack of an observable deficit in LIF-mediated signaling and gene induction in SYF cells suggests that both SFK scaffolding and catalytic functions are not required for cellular responses to LIF.

There is evidence that both overexpression of SFKs or expression of constitutively active v-Src can lead to activation of both STAT3 (51–53) and STAT1 (54, 55). The fact that no deficit is seen in STAT3 or STAT1 phosphorylation in LIF-stimulated SYF cells suggests that SFK-mediated activation of STATs is not required for IL-6 family cytokine-driven STAT activation and subsequent gene induction. The regulation of STAT activation by c-Src may be specific to other signaling systems, such as G protein-coupled-receptors and receptor tyrosine kinases, whereas the activation of STAT by v-Src may be specific to cellular transformation such as that seen in oncogenesis (56). The G protein-coupled-receptor family member angiotensin II receptor couple to STAT1 via Fyn (57) and STAT3 via Src (58). Upon binding of epidermal growth factor to the receptor tyrosine kinase ErbB, the resultant receptor-mediated STAT activation is Src-dependent (59), whereas STAT activation by basic fibroblast growth factor signaling through the receptor tyrosine kinase ErbB, the resultant receptor-mediated STAT activation is Src-dependent (59), whereas STAT activation by basic fibroblast growth factor signaling through the receptor tyrosine kinase fibroblast growth factor receptor is inhibited by the SFK-selective inhibitor PP1 (60). Our results suggest that the effects on STATs downstream from cytokine stimulation seen with viral forms of SFKs are not relevant to normal cytokine signaling, a view that is supported by findings in v-Src-transformed NIH3T3 cells (61). The JAK inhibitor SOCS-1 is capable of inhibiting cytokine-induced JAK/STAT activation; however, SOCS-1 cannot inhibit v-Src-induced JAK/STAT activation. v-Src has been shown to phosphorylate a chimeric granulocyte colony-stimulating factor/ gp130 receptor on Tyr-759, the SHP-2 docking site on gp130 (62); however, we saw no effect on tyrosine phosphorylation of SHP-2 or subsequent activation of MAPK in SYF cells following LIF stimulation. v-Src has also been shown to induce c-fos expression via the SIE of the c-fos promoter (63). We did not detect a deficit in LIF-mediated c-fos induction or SIE binding in SYF cells, supporting the conclusion that the effects of overexpression of v-Src are unrelated to normal IL-6-cytokine-mediated signaling and gene induction.

Experiments in white blood cells have elucidated a requirement for SFKs in p38 activation by cell surface receptors. SFKs have been implicated in bacterial tripeptide fMet-Leu-Phe-induced p38 MAPK-dependent activation of chemoattractant-induced degranulation of neutrophils (64), whereas pretreatment of monocytes with the SFK inhibitor PP2 completely inhibits soluble E-selectin-induced chemotaxis (65). These observations, along with our observation that p38 MAPK is phosphorylated in WT fibroblasts following stimulation with LIF, led us to investigate whether there was any effect of a loss of SFK expression on LIF-mediated p38 MAPK activation in SYF cells. We found no evidence that SFKs are involved in LIF- or OSM-stimulated p38 activation.

Inhibitors of SFKs PP1 and PP2 have been shown to inhibit prolactin-stimulated Akt phosphorylation on Ser-473 in W53 cells (66). We saw no deficit in the ability of LIF to stimulate Akt phosphorylation in SYF cells compared with WT cells. Recent evidence suggests that LIF (67) and IL-6 can activate p70S6K (68); however, we did not see any activation of p70S6K by LIF in WT or SYF cells using a phospho-Ser-411-specific antibody. Based on our results, we would not expect to see a difference, because IL-6-mediated induction of p70S6K is PI3K- and MAPK-sensitive, and we saw no effects on either signaling pathway in SYF cells. Shc has been shown recently (69) to be a direct effector molecule of SFKs; however, as Shc is primarily thought to couple IL-6 family cytokine receptors to MAPK (70), and we did not observe a MAPK deficit, it does not appear that this is a mechanism utilized by OSM or LIF in WT or SYF cells.

Because of the redundant nature of SFKs and the activation of multiple SFKs by multiple IL-6 family cytokines, we chose to study a system where SFKs are not expressed. This allowed us to ask whether any SFKs are required for LIF-mediated signaling and gene induction. Although the SYF cells are an immortalized cell line, simian virus 40 large T antigen is thought to immortalize cells via changes to the cyclin D1-dependent cell cycle regulatory pathway (71) and does not effect STAT activation (72). It is possible that there may be a redundancy in IL-6 cytokine signaling that would prevent observable effects of a loss of SFKs on the actions of LIF. For example, there is evidence that LIF activation of MAPK in 3T3-L1 cells occurs by protein kinase C-dependent and -independent pathways (67). However, our data show that SFKs are not required for known LIF-mediated signaling mechanisms or gene induction. The ability of OSM to signal in SYF cells suggests that multiple members of the IL-6 family can elicit a response in cells without SFKs.

In conclusion, we have shown that the absence of SFKs does not affect canonical LIF-mediated signal transduction and gene induction. Previous work implicating SFKs in IL-6 family cytokine signaling has utilized the expression of viral forms of SFKs or has shown increased catalytic activity of SFKs following stimulation of cells with IL-6 family cytokines; however, no previous study has revealed any requirement of SFKs for signaling or gene induction. This work is the first to test the effect of removing all expressed members of the Src family of kinases on IL-6 family cytokines in a cellular context. Our results show that SFKs are not required for signal transduction or gene induction by IL-6 family cytokines.

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REFERENCES

1. Elson, G. C., Lelièvre, E., Guillet, C., Chevalier, S., Plun-Favreau, H., Froger, J., Suard, I., de Cognac, A. B., Delhete, Y., Bonnefoy, J. Y., Gauchat, J. F., and Gascon, H. (2000) Nat. Neurosci. 3, 867–872.
2. Heinrich, P. C., Behrmann, I., Muller-Newen, G., Schaper, F., and Graeve, L. (1998) Biochem. J. 334, 297–314.
3. Williams, R. L., Hilton, D. J., Pease, S., Wilson, T. A., Stewart, C. L., Gearing, D. P., Wagner, E. F., Metcalf, D., Nicola, N. A., and Gough, N. M. (1988) Nature 336, 684–687.
4. Marshall, M. K., Doerr, W., Feingold, K. R., and Grunfeld, C. (1994) Endocrinology 135, 141–147.
5. Zhang, X. G., Gu, J. J., Lu, Z. Y., Yasukawa, K., Yancopoulos, G. D., Turner, K., Shoyab, M., Taga, T., Kishimoto, T., and Bataille, R., et al. (1994) J. Exp. Med. 179, 1357–1342.
6. Stahl, N., and Yancopoulos, G. D. (1994) J. Neurobiol. 25, 1454–1466.
7. Darnell, J. E., Jr., Kerr, I. M., and Stark, G. R. (1994) Science 264, 1415–1421.
8. Adams, T. E., Hansen, J. A., Starr, R., Nicola, N. A., Hilton, D. J., and Billestrup, N. (1998) J. Biol. Chem. 273, 1285–1287.
9. Auernhammer, C. J., Boussquet, C., and Melmed, S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6964–6969.
10. Starr, R., Wilson, T. A., Viney, E. M., Murray, I. J., Rayner, J. R., Jenkins, B. J., Gonda, T. J., Alexander, W. S., Metcalf, D., Nicola, N. A., and Hilton, D. J. (1997) Nature 387, 917–921.
11. Boulton, T. G., Stahl, N., and Yancopoulos, G. D. (1994) J. Biol. Chem. 269, 11648–11655.
12. Oh, H., Fujio, Y., Kuniyoda, K., Hirota, H., Matsui, H., Kishimoto, T., and Yamashiki-Takahara, K. (1998) J. Biol. Chem. 273, 9703–9710.
13. Duval, D., Reinhardt, B., Keding, C., and Boeuf, H. (2000) FASEB J. 14, 1577–1584.
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