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Leukemia Inhibitory Factor and Interleukin-6 Trigger the Same Immediate Early Response, Including Tyrosine Phosphorylation, upon Induction of Myeloid Leukemia Differentiation

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Leukemia inhibitory factor (LIF) and interleukin-6 (IL-6), two multifunctional cytokines lacking structural homology and binding to distinct receptors, share interesting functional similarities, which include induction of hematopoietic differentiation in normal and myeloid leukemia cells, induction of neuronal cell differentiation, and stimulation of acute-phase protein synthesis in hepatocytes. Structural information on the LIF receptor is not yet available, whereas recent cloning of the IL-6 receptor has shown it to be bipartite, with a signal-transducing subunit that lacks sequence homology to known protein kinases and produces second messengers of unknown nature. The molecular nature of the mechanisms which LIF and IL-6 use to induce cell differentiation is not known. To address this issue, we took advantage of a clone of M1 myeloblastic leukemia cells capable of being induced for terminal differentiation by both LIF and IL-6 and of recently identified myeloid differentiation primary response (MyD) genes. It is shown that LIF and IL-6 directly activate the same set of immediate early response genes upon induction of M1 myeloid differentiation. At least two mechanisms of gene activation, one transcriptional and the other posttranscriptional, are shown to be involved. It is also shown that the LIF and IL-6 immediate early response, at suboptimal cytokine concentrations, is additive. Using a variety of protein kinase activators and inhibitors, we have shown that the intracellular signalling pathways for both LIF and IL-6 are distinct from those of known second messengers and involve protein phosphorylation, notably tyrosine phosphorylation of a 160-kDa protein, as an essential step(s) in the immediate early activation of MyD gene expression. These observations indicate that the functional similarities of LIF and IL-6 as inducers of cell differentiation prevail at the level of the complex differentiation immediate early response and implicate common mechanisms of signal transduction for LIF- and IL-6-induced differentiation.

Since the recent discovery of interleukin-6 (IL-6) as a factor stimulating B-cell differentiation (22, 25), it has become increasingly evident that IL-6 is a multifunctional cytokine which plays important roles in a wide range of biological activities (24, 39, 45), including the acute-phase response to injury and inflammation (2, 14), T-cell activation (43, 44), regulation of neuronal cell differentiation (16, 38), and the differentiation of hematopoietic cells of the myeloid lineage (23, 29, 40). Intriguingly, in the past year it has become evident that another newly discovered cytokine, termed leukemia inhibitory factor (LIF), although lacking structural homology to IL-6 (9, 15) and binding to distinct receptors (21), shares striking functional similarities with IL-6. LIF was first discovered by its ability to inhibit the growth and induce terminal differentiation of M1 myeloid leukemia cells (15), also induced by IL-6 (8, 40). Since then it has been found to share with IL-6 a broad range of other biological activities, including stimulation of synthesis of acute-phase proteins in hepatocytes (4), regulation of normal hematopoietic cell development (35), and induction of neuronal cell differentiation (47).

Recent cloning of the IL-6 receptor has shown it to be a bipartite receptor composed of a ligand-binding subunit that interacts with a signal-transducing subunit (18, 42, 48), lacking sequence homology to any known protein kinases and producing second messengers of yet unknown nature (18, 25). Structural information on the LIF receptor is not yet available. Thus, the molecular nature of the mechanisms which LIF and IL-6 use to exert their common pleiotropic effects on cell development, including the induction of hematopoietic cell differentiation, has not been elucidated. It is not clear to what extent the common functions of LIF and IL-6 as differentiation inducers are due to the activation of one cytokine by the other or, alternatively, are the direct consequence of the ability of each of these cytokines to activate the same or a similar set of differentiation immediate early response genes.

To address these issues, we have taken advantage of a clone of M1 myelobastic leukemia cells capable of being induced for terminal differentiation by both LIF and IL-6 and of a set of myeloid differentiation primary response (MyD) genes, which recently we have shown to be activated in the absence of protein synthesis in M1 cells following induction for terminal differentiation by conditioned media of mouse lungs, a potent physiological source for hemopoietic differentiation inducers, including IL-6 (41), or by recombinant IL-6 (8, 31–33). The set of MyD genes used in this study encompasses those identified as known genes, including *junB*, c-*jun*, and *junD*, encoding leucine zipper transcription factors, and ICAM1, encoding a ligand to a cell surface adhesion receptor, as well as two novel MyD genes, MyD88 and MyD116 (references 31 to 33 and references

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therein). All of these MyD genes also were shown to be expressed in primary cultures of myeloid precursor-enriched bone marrow cells, with expression characteristics similar to what was observed in M1 cells (31–33). The results of the experiments presented in this study show, for the first time, that LIF and IL-6 induce hematopoietic cell differentiation directly via activation of an identical set of immediate early response genes activated transcriptionally and posttranscriptionally, thereby implicating common mechanisms of signal transduction for LIF- and IL-6-induced differentiation. Results of experiments using M1 cells treated with suboptimal dosages of LIF and IL-6 in combination, or with various protein kinase activators and inhibitors, and the finding of common tyrosine phosphorylation of a 160-kDa protein further strengthen this notion.

MATERIALS AND METHODS

Cells and cell culture. The murine M1 and WEHI-3B myeloid leukemia cell lines were obtained from E. R. Stanley (Albert Einstein College of Medicine). They were recloned in soft agar, and clones were tested for differentiation-associated properties, including induction of adherence to the surface of the tissue culture plates, Fc and C3 rosette formation, lysozyme synthesis, changes in proteins synthesized, determined by two-dimensional gel electrophoresis, and morphological maturation (28). M1 differentiation-competent (M1D+) clone 6 and a clone of WEHI-3B (WEHI-3B D− clone 22), not induced for differentiation by these compounds (28, 31, 32), were used in this study. Cells were cultured in Dulbecco's modified Eagle's medium (H-21; GIBCO, Grand Island, N.Y.) and 10% horse serum at 37°C in a humidified atmosphere with 10% CO_2. Cells were seeded at densities as indicated except that for RNA extractions, the cell concentration was adjusted to give a final density of $>0.25 \times 10^6$ cells per ml at the time of extraction. After culture, viable cell number was determined by trypan blue dye exclusion and counting in a hemacytometer.

Cytokines and other reagents. Cytokines used in this study were purified human recombinant IL-6 (0.5 mg/ml), a kind gift from L. Souza Amgen, Thousand Oaks, Calif.; murine IL-6 and murine LIF in Cos cell conditioned medium, prepared via transfection of Cos 7 cells with pcD-mIL6 (27) and pcD-LIF, respectively (gift of F. Lee, DNAX Research Institute, Palo Alto, Calif.); and purified murine LIF (10^6 U/ml), recently available from AMRAD Corp., Victoria, Australia. Cytokines were titrated for differentiation-inducing and growth inhibitory activities with M1D+ clone 6 (28). IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), and granulocyte colony-stimulating factor (G-CSF) were as described before (28). Murine beta interferon (IFN-β; 0.2×10^6 U/ml) and rat IFNγ (10^6 U/ml) were from Lee Biomolecular Research Laboratories Inc. Experiments were conducted with cytokine concentrations which represent the optima of the differentiation or growth inhibition dose-response curves. Optimal concentrations of IL-6 and LIF in Cos cell conditioned media (10 U of differentiation-inducing activity per ml, defined as described in reference 28) and purified IL-6 (50 ng/ml) or LIF (200 U/ml), respectively, gave identical responses with M1D+ clone 6. Conditioned media of mock-transfected Cos cells had no effect on growth, differentiation, or MyD gene expression of M1D+ cells. Other reagents used in this study included 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), *N*-6,2-*O*-dibutyryl cyclic AMP (dBcAMP), 8-bromo-cyclic GMP (8Br-cGMP), cholera toxin, calcium ionophore A23187, and the protein kinase inhibitors 1-(5-isoquinolinesulfonyl)-2-metylpiperazine (H7), *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), and sphingosine (all from Sigma, St. Louis, Mo.). The protein kinase inhibitor tyrphostin (RG50864) was kindly supplied by A. Zilberstein Rorer Central Research (Horsham, Pa.). Monoclonal antibodies (immunoglobulin G1 [IgG1]) against murine IL-6 and control hybridoma supernatant were kindly provided by F. Lee.

Assays for differentiation-associated properties. C3 receptors were assayed with sheep erythrocytes (Lampire Biological Laboratories, Pipersville, Pa.) incubated with antibody to sheep erythrocytes (Diamedix, Miami, Fla.) and mouse serum as a source of complement (Pel-Freez, Rogers, Ark.), as described previously (30). Lysozyme activity in cell extracts and in the growth medium was assayed by the decrease in turbidity at 540 nm of a suspension of *Micrococcus lysoddeikticus* (Sigma), as described previously (28). Cell attachment was determined by counting the number of suspended and attached cells (31), and morphological differentiation was determined on May-Grünwald-Giemsa-stained cytospin smears by counting at least 300 cells and scoring the proportion of immature blast cells, cells at intermediate monocyte stages of differentiation, and mature macrophages (31).

General recombinant DNA techniques and DNA probes. Plasmid preparations, restriction enzyme digestions, DNA fragment preparations, and agarose gel electrophoresis were done as described before (31). Probes for JunB, c-Jun, JunD, ICAM1, H3, MyD88, and MyD116 were prepared with agarose gel-purified *Eco*RI DNA fragments excised from CsCl-banded pBluescript plasmids of the appropriate MyD cDNA clones; the pBluescript plasmids themselves were derived from the corresponding MyD λZAP phage clones isolated from a λZAP M1D+ cDNA library, as described previously (31). The c-Jun and JunD probes were 2.5- and 1.7-kb *Eco*RI DNA fragments excised from MyD c-*jun* and *junD* pBluescript plasmids, respectively, identified by sequencing 300 to 500 nucleotides from both ends of the indicated MyD pBluescript plasmids by using double-stranded DNA dideoxy sequencing (31). The β-actin probe was a 0.7-kb *Eco*RI fragment of the human cDNA clone pHFBA (37). The IL-6 and LIF probes were 1.4-kb *Bam*HI and 0.65-kb *Pst*I-*Eco*RI fragments excised from the pcD-mIL6 (27) and pcD-LIF expression vectors, respectively (kindly provided by F. Lee). DNA for probes was labeled by random priming to a specific activity equal to or greater than 10^9 cpm/μg (31).

RNA extraction, Northern (RNA) blots, and hybridization. RNA was extracted by the method of Chomczynski and Sacchi (10), using guanidinium thiocyanate. Total RNA (5 μg per lane) was electrophoresed on 1% formaldehyde agarose gels. Preparation of Northern blots, hybridization, and washing conditions were as described before (31) except that RNA was blotted on Nylon membrane (GeneScreen Plus; NEN) and UV cross-linked (Stratalinker; Stratagene). For quantitation of individual mRNAs, films were exposed for periods during which band intensity was linear with respect to time, and relative intensities of hybridization signals were measured at 560 nm with the gel scan program of a DU7 Beckman spectrophotometer.

Nuclear run-on transcription assays. For nuclear run-on assays, 40×10^6 cells were washed three times with ice-cold phosphate-buffered saline and lysed on ice in lysis buffer (0.5% Nonidet P-40, 10 mM Tris [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$); after centrifugation twice (4°C, $500 \times g$), nuclei

were resuspended in 100 μl of glycerol storage buffer (40% glycerol, 50 mM Tris [pH 8.3], 5 mM $MgCl_2$, 0.1 mM EDTA). Run-on transcription was performed at 30°C for 30 min in 200 μl of reaction buffer (35% glycerol, 10 mM Tris [pH 7.5], 5 mM $MgCl_2$, 0.1 mM EDTA, 80 mM KCl, 1 mM dithiothreitol, 1 mM nucleoside triphosphates, 150 μCi of [^{32}P]UTP). Transcription was terminated by addition of 2 μl of 100 mM $CaCl_2$ and 300 U of RNase-free DNase (Boehringer Mannheim Biochemicals), incubation at 30°C for 10 min, addition of 25 μl of 10× TES buffer (100 mM Tris [pH 7.5], 50 mM EDTA, 5% sodium dodecyl sulfate [SDS]), 5 μl of tRNA (10 mg/ml), and 2 μl of proteinase K (10 mg/ml), and incubation at 37°C for 45 min. RNA was extracted as described previously (10), and unincorporated nucleotides were removed by passing the probe over Sephadex G-50 (fine) equilibrated with 10 mM Tris (pH 8)–1 mM EDTA–100 mM NaCl. GeneScreen plus strips containing 10 μg of linearized, denatured plasmid DNA per slot, with or without (plasmid) inserts specific for JunB, c-Jun, JunD, ICAM1, MyD88, MyD116 (31), actin (37), and histone H3.3 (31), were hybridized with 2×10^7 cpm of the run-on transcription products in 2 ml of 1 M NaCl–1% SDS–50 mM Tris (pH 7.5) at 65°C for 42 h. Strips were washed as follows: twice for 5 min each time at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), twice for 30 min each time at 65°C in 2× SSC–1% SDS, 30 min at 37°C in 2× SSC–10 μg of RNase A per ml, and twice for 30 min each time at 56°C in 0.1× SSC–0.1% SDS. Relative intensities of hybridization signals were quantitated by densitometry as indicated above.

Immunoblotting of phosphotyrosine-containing proteins. Whole cell lysates were prepared from M1D+ cells, treated as indicated, by solubilization at 4°C in 0.5 ml of RIPA buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM sodium orthovanadate, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 100 μM leupeptin, 10 mM Tris [pH 4]). Following clarification, the lysates were incubated with approximately 5 μg of UP28 affinity-purified polyclonal rabbit antibodies to phosphotyrosine produced in rabbits immunized with v-*abl* expressed in bacteria (46). The immunecomplexes were adsorbed to Pansorbin (Calbiochem, San Diego, Calif.), washed in RIPA buffer, eluted, and fractionated on a 7.5% polyacrylamide gel as described previously (13). The fractionated proteins were then transferred to nitrocellulose and probed with the antiphosphotyrosine antibodies 4G10 (1 μg/ml) (12) and Pγ20 (1 μg/ml) (ICN, Costa Mesa, Calif.) as described previously (13) except that horseradish peroxidase-conjugated protein A (1:1,000) (Cappel, Organon Teknika Corp., Durham, N.C.) or goat anti-mouse immunoglobulins (1:1,000) (Bio-Rad, Richmond, Calif.) were used as secondary antibodies. The antibodies were detected by using the ECL chemiluminescence reagents (Amersham, Arlington, Ill.).

RESULTS

Induction of terminal differentiation in M1 myeloid precursor cells by LIF and IL-6. LIF and IL-6 inhibited the growth of the M1 differentiation-inducible clone used in this study (M1D+ clone 6) to the same degree (Fig. 1a). Both of these cytokines induced, to a similar extent, a spectrum of early to late differentiation markers, including C3 receptors, cell attachment, lysozyme synthesis, and mature macrophage-like cells (Fig. 1). It should be pointed out that the kinetics of growth inhibition and induction of differentiation-associated properties in M1 cells were similar following treatment with either IL-6 or LIF. Cells with C3 receptors could be first detected after 12 h, cell attachment after 1 day, and lysozyme and mature cells after 2 days. These observations lead to the conclusion that LIF and IL-6 exert similar pleiotropic effects, resulting in the induction of terminal myeloid differentiation.

a.

Inducer	No. of cells (10^6/ml)	C_3 Receptors (%)	Cell attachment (%)	Lysozyme (ug.equiv/ $5x10^6$ cells)	cell type (%) Blast	Intermediate	Mature
None	2.3	1.2	0.6	0.12	>99	<1	0
LIF	0.34	59	66.5	7.3	7	58	35
IL6	0.36	56	69.3	8.1	11	57	32

b.

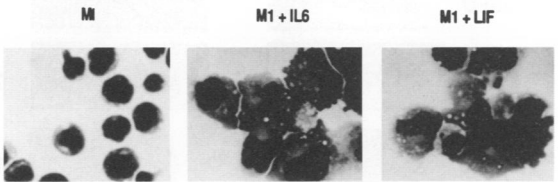


FIG. 1. Induction of terminal differentiation in M1D+ clone 6 by LIF and IL-6. (a) Growth inhibition and differentiation-associated properties; (b) photomicrographs (×488) of May-Grünwald-Giemsa-stained cytospin smears of M1D+ myeloblasts before and 4 days after induction for terminal differentiation. A total of 10^5 cells per ml were seeded, and after 4 days viable cell numbers were determined by trypan blue dye exclusion and counting in a hemacytometer. Differentiation-associated properties were measured 4 days after addition of inducers. Morphological differentiation was determined from May-Grünwald-Giemsa-stained cytospin smears, counting at least 300 cells, and scoring the proportion of immature blast cells, cells at intermediate monocyte stages of differentiation, and mature macrophages. Murine LIF in Cos cell conditioned media and human recombinant IL-6 were used at final concentrations of 10 U/ml and 50 ng/ml, respectively. Values represent means of at least three independent determinations with standard deviations up to ±15%.

Activation of MyD genes by LIF and IL-6. To compare the early genetic response of M1 myeloblasts to LIF and IL-6, expression of MyD genes was examined. As shown in Fig. 2, the steady-state mRNA levels of all MyD genes rapidly accumulated, with identical kinetics and to similar extents, in M1 myeloblasts treated with LIF and with IL-6. Cycloheximide, a potent protein synthesis inhibitor, did not inhibit, and even enhanced, this rapid accumulation of the steady-state levels of MyD mRNAs, indicating that induction of the different MyD genes is an immediate early response to stimulation with LIF as well as with IL-6. Enhanced accumulation of steady-state mRNA levels in the presence of cycloheximide also was commonly observed with many other primary response genes (1, 26) and is believed to reflect decreased mRNA degradation due to the block in translation (6). A variety of other cytokines, including IL-3 (100 U/ml), GM-CSF (100 U/ml), M-CSF (100 U/ml), G-CSF (250 U/ml), IFNβ (1,000 U/ml), and IFNγ (200 U/ml), which did not induce M1 differentiation, did not induce any of the LIF- or IL-6-induced immediate early response. Also, MyD mRNAs were not detected in a clone of WEHI-3B D− myelomonocytic cells treated with LIF or IL-6, which did not induce differentiation (data not shown). Taken together, these observations show that LIF and IL-6 activate an identical set of differentiation immediate early response genes, thereby indicating that they exert similar

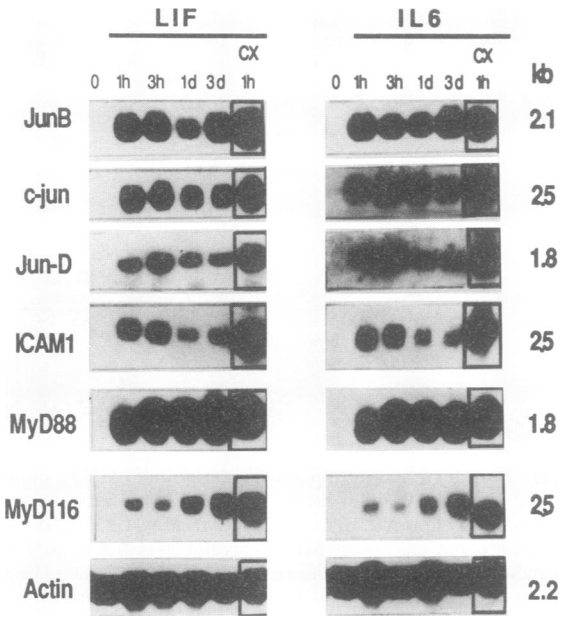
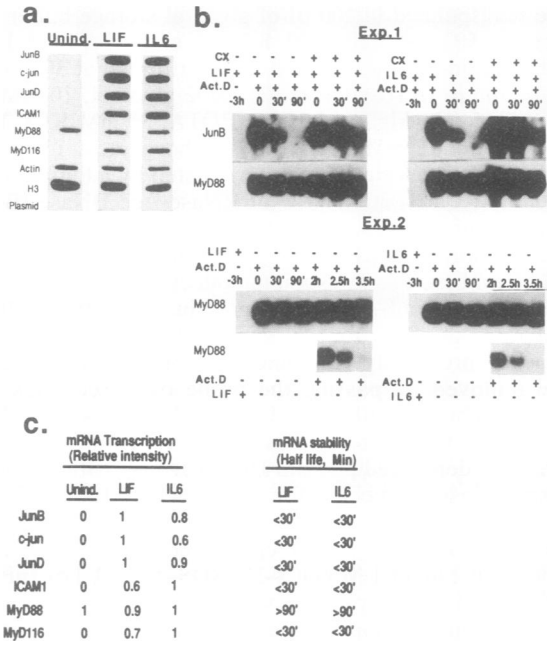


FIG. 2. Immediate early activation of MyD gene expression by LIF or IL-6. A total of 0.2×10^6 cells per ml were seeded in the presence of LIF or IL-6, with or without cycloheximide (CX), and RNA was extracted at the indicated time points. RNA blots were prepared with total RNA (5 μg per lane), and blots were hybridized sequentially with ^{32}P-labeled probes specific to JunB, c-Jun, JunD, ICAM1, MyD88, MyD116, and actin. Resulting autoradiograms are shown. Cycloheximide X was used at a concentration of 10 μg/ml, and concentrations of LIF and IL-6 were as indicated in legend to Fig. 1.



c.

	mRNA Transcription (Relative intensity)			mRNA stability (Half life, Min)	
	Unind.	LIF	IL6	LIF	IL6
JunB	0	1	0.8	<30'	<30'
c-jun	0	1	0.6	<30'	<30'
JunD	0	1	0.9	<30'	<30'
ICAM1	0	0.6	1	<30'	<30'
MyD88	1	0.9	1	>90'	>90'
MyD116	0	0.7	1	<30'	<30'

FIG. 3. MyD gene transcription (a and c) and mRNA stability (b and c) in M1D+ cells stimulated by LIF or IL-6. Nuclear run-on transcription assays were performed as described in Materials and Methods, with nuclei isolated from 40×10^6 M1D+ cells before (Unind.) and 1 h after stimulation with LIF or IL-6. ^{32}P-labeled run-on transcription products were hybridized to nylon membrane filters containing 10 μg of plasmids with or without inserts specific for JunB, c-Jun, JunD, ICAM1, MyD88, MyD116, H3 histone, and β-actin. Resulting autoradiograms are shown in panel a; quantitation of relative intensities of hybridization signals, measured by densitometry with maximum peak areas standardized to 1, is shown in panel c. MyD mRNA stability was determined in M1D+ cells stimulated with LIF or IL-6. In experiment 1 (Exp.1), following 3 h of stimulation, actinomycin D (Act.D; 10 μg/ml) without or with cycloheximide (CX; 10 μg/ml) was added. In experiment 2 (Exp.2), following 3 h of stimulation, cytokines were removed, and actinomycin D was added concomitantly with cytokine removal or after 2 h. For both experiments, at the indicated times, total cellular RNA was isolated from cells and RNA blots were prepared (5 μg per lane). Blots from experiment 1 were hybridized sequentially with probes for JunB, c-Jun, JunD, ICAM1, MyD88, and MyD116. Resulting autoradiograms for JunB and MyD88 are shown. The estimated half-life for each MyD gene, determined by scanning densitometry of the appropriate autoradiograms, is shown in panel c. In experiment 2, blots were hybridized to a MyD88 probe, and resulting autoradiograms are shown. Subsequent hybridization to a JunB probe was used to monitor for efficiency of transcription inhibition by actinomycin D. Data are representative of at least two independent experiments, each performed in duplicate. LIF and IL-6 concentrations were as indicated in the legend to Fig. 1.

pleiotropic effects very early upon induction of M1 terminal differentiation.

To determine and compare the molecular mechanisms that LIF and IL-6 use to activate the immediate early expression of MyD genes, it is necessary to ascertain whether regulation is at the transcriptional or posttranscriptional level. Run-on transcription assays performed in vitro with nuclei isolated from M1D+ cells before and 1 h after stimulation with LIF or IL-6 were used to measure transcription rates of MyD genes (Fig. 3a and c). The inhibitor chase method, using actinomycin D as a potent RNA synthesis inhibitor, was used to assess the stability of specific MyD mRNAs (Fig. 3b and c).

For all MyD genes analyzed except MyD88, transcription was below detection levels in uninduced M1 cells and was highly induced to similar levels by both LIF and IL-6 (Fig. 3a and c). In contrast, MyD88 was transcribed at similar rates in uninduced cells and in cells stimulated with either LIF or IL-6. It should be pointed out that cycloheximide had no effect on the transcription rates for any of the MyD genes. To eliminate the possibility that incomplete MyD88 transcripts are synthesized in uninduced M1 cells, nuclear run-on products of nuclei obtained from M1D+ cells before and after stimulation with LIF or IL-6 were hybridized to cDNA fragments specific to 5′, middle, or 3′ regions of MyD88 mRNA. No differences were seen, indicating that full-length MyD88 transcription is taking place in uninduced M1 cells as well as in M1 cells following stimulation (data not shown).

As shown in Fig. 3b (Exp.1) and c, all MyD mRNAs, with the exception of MyD88 mRNA, had a short half-life (<30 min). The half-life of MyD88 mRNA was markedly longer, evident by essentially no decrease in the steady-state level of MyD88 mRNA even 90 min following treatment with actinomycin D. It also can be seen that cycloheximide, known to decrease mRNA degradation (6), had a considerably greater effect on the stability of the shorter-lived JunB mRNA than on the half-life of the MyD88 mRNA.

The induced steady-state levels of MyD88 transcripts can be accounted for by stabilization of its mRNA in both LIF- and IL-6-treated M1 cells. To gain insights into the mechanism of stabilization of MyD88, its stability was analyzed in M1 cells which, after brief (3-h) treatment with LIF or IL-6,

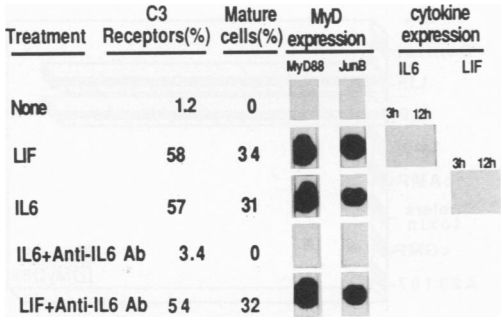


FIG. 4. Direct induction of M1D+ differentiation by LIF and IL-6. To determine the effects of anti-IL-6 antibodies (Ab) on LIF-induced terminal differentiation, 0.1×10^6 cells per ml were seeded with indicated treatments and assayed after 4 days for C3 receptors and morphology, as indicated in the legend to Fig. 1; for MyD expression, 0.5×10^6 cells per ml were seeded, total RNA was extracted 1 h following the indicated treatments, RNA blots were prepared by using 5 μg of RNA per ml, and blots were hybridized sequentially with probes specific to JunB and MyD88. Resulting autoradiograms are shown. Monoclonal antibodies (IgG1) against murine IL-6 were preincubated (1 h, 37°C) with murine IL-6 (10 U/ml) or LIF (10 U/ml) at a 1:100 dilution prior to treatment of cells. Control hybridoma supernatant (IgG1), used under the same experimental conditions, had no effect on M1D+ differentiation-associated properties or MyD gene expression. To determine cytokine expression, M1D+ cells were stimulated with murine IL-6 (10 U/ml) or LIF (10 U/ml), total RNA was extracted at the indicated time points, poly(A)$^+$ mRNA was isolated (31), RNA blots were prepared from poly(A)$^+$ RNA (3 μg per lane), and blots were hybridized with probes specific to murine IL-6 or LIF. Values represent means of at least three independent determinations with standard deviations of up to ±15%. Autoradiograms shown are representative of these experiments.

were washed to remove the inducers, and actinomycin D was added to block further transcription, either immediately or following a lag period of 2 h (Fig. 3b, Exp. 2). It can be seen that upon removal of the inducers and concomitant treatment of the cells with the transcription inhibitor, MyD88 mRNA still exhibited remarkable stability (>3 h). In contrast, removal of inducers and addition of actinomycin D only after 2 h resulted in reduced levels of MyD88 mRNA, which had a half-life shorter than 30 min. Taken together, these observations are consistent with the notion that LIF and IL-6 either prevent the synthesis of a labile MyD88 mRNA nuclease or rapidly inactivate it.

The results of the run-on transcription and MyD mRNA stability analysis indicate that immediate early activation of MyD genes, with the exception of MyD88, can be attributed to regulation primarily at the level of initiation of transcription, whereas activation of MyD88 must be accounted for by posttranscriptional regulation, specifically at the level of mRNA stabilization. Thus, at least two common mechanisms of gene activation are induced by both LIF and IL-6 in the absence of protein synthesis.

LIF and IL-6 induce M1 terminal differentiation directly. The results of the studies described thus far, although highly suggestive of LIF and IL-6 inducing hematopoietic cell differentiation directly, do not completely eliminate the possibility that induction of M1D+ differentiation by LIF or by IL-6 may be conferred, in part, indirectly via activation of one cytokine by the other. Results of experiments addressing this issue are shown in Fig. 4. It can be seen that anti-murine IL-6 antibodies (antibodies against murine LIF are not yet available), at a titer which completely abolished IL-6-induced differentiation, had no effect on LIF-induced terminal differentiation, as indicated by monitoring for the induction of JunB and MyD88 mRNAs, C3 receptors, and macrophagelike cells. Also, no detectable levels of IL-6 mRNA or LIF mRNA were observed at the indicated time points upon induction of M1D+ terminal differentiation by LIF or IL-6, respectively. It should be pointed out that in this M1D+ clone, IL-6 mRNA, but not LIF mRNA, was detected 1 day following induction of differentiation with either IL-6 or LIF (data not shown). That this later expression of IL-6 mRNA has no bearing on LIF-induced myeloid differentiation is indicated by the failure of IL-6 antibodies to inhibit LIF-induced M1 differentiation as well as from the observations that M1 myeloid differentiation induced by LIF is not delayed compared with that induced by IL-6. The expression of IL-6 mRNA in both LIF- and IL-6-treated M1 cells is nevertheless another example of both cytokines activating the same genetic program during M1 differentiation. Thus, taken together, the results of these experiments indicate that LIF and IL-6 induce hematopoietic cell differentiation directly.

LIF and IL-6 utilize common signal transduction pathways to induce M1 myeloid differentiation. The results of the experiments described above, which have indicated that LIF and IL-6 induce myeloid differentiation directly by triggering an identical transcriptional and posttranscriptional immediate early response, raise the possibility that LIF and IL-6 share common signal transduction pathways.

To further investigate this issue, we wanted to determine whether combined treatment of M1 cells with LIF and IL-6 results in an additive rather than a synergistic response, which would be consistent with both cytokines utilizing common signalling pathways. To do this, it is necessary to use suboptimal concentrations of LIF and IL-6, i.e., concentrations at which the response is linear. As shown in Fig. 5, for M1 cells treated concomitantly with suboptimal concentrations of LIF and IL-6, activation of JunB was additive. It can also be seen that following treatment of the cells concomitantly with optimal concentrations of LIF and IL-6, the level of JunB mRNA was not significantly higher than what was observed with each cytokine. Similar results were obtained when we examined the effects of suboptimal and optimal concentrations of LIF and IL-6 on the immediate early activation of MyD88 mRNA accumulation, as well as induction of C3 receptors, tested at 1 and 16 h following cytokine administration, respectively (data not shown).

The possible involvement of known second messengers in LIF- or IL-6-induced immediate early activation of MyD gene expression was tested, using a variety of known protein kinase activators and inhibitors. As shown in Fig. 6a, treatment of M1 cells with the protein kinase activator TPA, dBcAMP, cholera toxin, 8Br-cGMP, or A123187 did not induce significant levels of JunB or MyD88 mRNAs compared with what was observed following treatment of the cells with LIF or IL-6. As expected from the failure of TPA and the calcium ionophore A23187 to induce JunB or MyD88 mRNAs, the protein kinase inhibitor sphingosine, an inhibitor of protein kinase C (17), and the calmodulin antagonist W7 (20) did not affect the immediate early activation of JunB and MyD88 mRNAs induced by LIF or IL-6 (Fig. 6b). However, in contrast to what was observed with sphingosine and W7, the nonspecific protein kinase inhibitor H7 (19) and the tyrosine kinase inhibitor tyrphostin (34) were found to inhibit by at least 90% the LIF- or IL-6-induced immediate early activation of JunB and MyD88 mRNAs. It should be

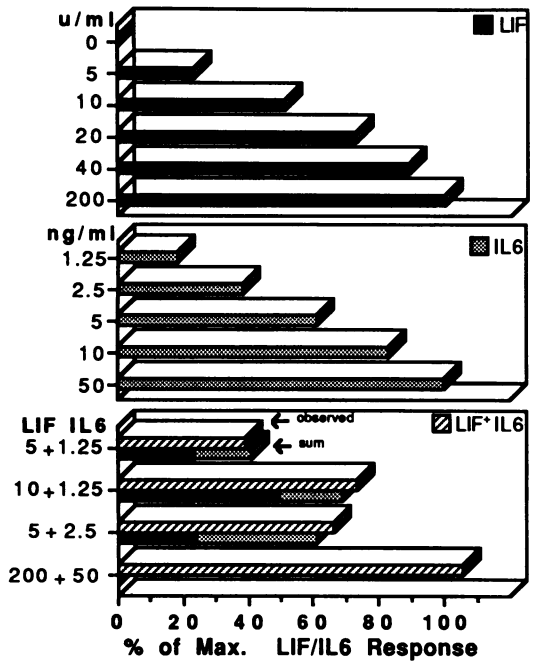


FIG. 5. Analysis of the effects of suboptimal concentrations of LIF and IL-6 on immediate early activation of JunB. A total of 10^6 cells per ml were seeded without or with LIF or IL-6, at the indicated concentrations, and RNA was extracted following 1 h of stimulation. RNA blots were prepared with total RNA (5 μg per lane) and hybridized with ^{32}P-labeled JunB probe. Hybridization signals of resulting autoradiograms were quantitated by densitomery, as indicated in Materials and Methods. Maximum induction with LIF or IL-6 was comparable to what is shown in Fig. 2, and all autoradiograms gave similar hybridization signals with an actin probe. Data are representative of at least three independent experiments.

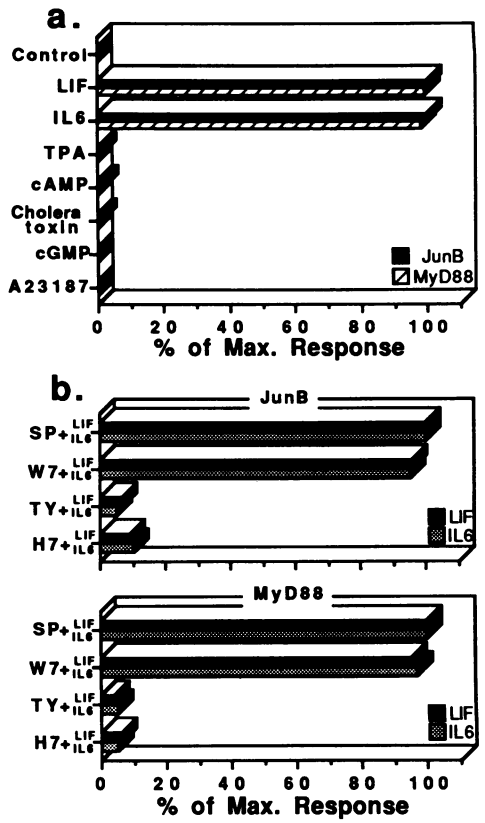


FIG. 6. Analysis of the effects of known protein kinase activators (a) or inhibitors (b) on immediate early activation of JunB and MyD88. For activator studies, cells (10^6/ml) were seeded without or with LIF (200 U/ml), IL-6 (50 ng/ml), TPA (50 ng/ml), dBcAMP (1 mM), cholera toxin (1 μg/ml), 8Br-cGMP (1 mM), or the calcium ionophore A23187 (1 μM), and total RNA was extracted following 1 h of stimulation. For inhibitor studies, cells (10^6/ml) were treated for 1 h with sphingosine (SP; 10 μM), W7 (40 μM), tyrphostin (TY; 150 μg/ml), or H7 (20 μM) prior stimulation (45 min) with IL-6 (50 ng/ml) or LIF (200 U/ml). RNA was extracted, and blots were prepared with total RNA (5 μg per lane) and hybridized sequentially with ^{32}P-labeled probes for JunB and MyD88. Hybridization signals of resulting autoradiograms were quantitated by densitometry, as indicated in Materials and Methods. Maximum induction with LIF or IL-6 was comparable to what was shown in Fig. 2, and all autoradiograms gave similar hybridization signals with an actin probe. Activator and inhibitor concentrations used represent the optima of dose-response curves, i.e., concentrations at which cell viability appeared to be unaffected as indicated by tryphan blue dye exclusion and lack of effect on H3 mRNA expression. Data are representative of at least two independent experiments.

pointed out that the rapid response of these genes to LIF or IL-6 enabled us to limit the exposure time of the cells to the various protein kinase inhibitors, thereby minimizing possible toxic effects, as indicated by the lack of any effect on actin and H3 mRNA expression, as well as on cell viability, determined by trypan blue dye exclusion (see legend to Fig. 6). Similar results with both activators and inhibitors were obtained for the other MyD genes as well (data not shown).

The ability of the protein kinase inhibitors tyrphostin and H7 to block both LIF- and IL-6-induced immediate early activation of MyD genes has implicated protein phosphorylation as an essential step in LIF and IL-6 signal transduction. To further address this issue, we used monoclonal antibodies to phosphotyrosine to directly identify possible changes in tyrosine-phosphorylated proteins following stimulation of M1 cells with LIF or IL-6. As shown in Fig. 7a, rapid tyrosine phosphorylation of a 160-kDa protein (p160), persisting for at least 30 min, was detected following stimulation with either IL-6 or LIF. In different immunoblotting experiments p160 was the only band which consistently and reproducibly showed induced tyrosine phosphorylation following LIF or IL-6 stimulation. As shown in Fig. 6b, the IL-6- or LIF-induced tyrosine phosphorylation of p160 was blocked by tyrphostin but not by H7. Both kinase inhibitors were used at concentrations that blocked the LIF or IL-6 immediate early response (Fig. 6).

Taken together, the additive nature of the LIF and IL-6 immediate early response, the results of the activator and inhibitor studies, and the detection of p160 phosphorylation suggest that LIF and IL-6 share common signalling pathways, which are distinct from the major signal transduction pathways that utilize known mediators, including diacylglycerol, calcium, cAMP, or cGMP, yet include similar protein phosphorylation as an essential step(s).

DISCUSSION

Using recently identified MyD genes and a clone of M1 myeloblastic leukemia cells capable of being induced for terminal differentiation by both LIF and IL-6, it is shown for the first time that LIF and IL-6 induce hematopoietic cell

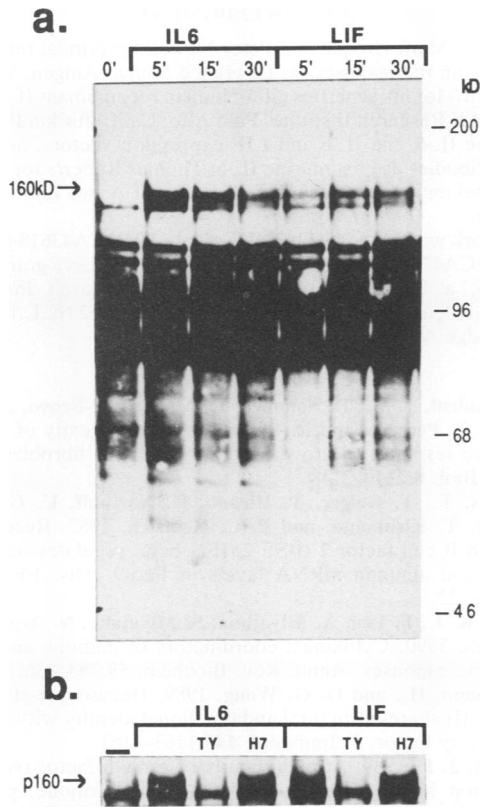


FIG. 7. Tyrosine phosphorylation of a 160-kDa protein following stimulation of M1 cells by IL-6 or LIF (a) and the effects of protein kinase inhibitors on p160 phosphorylation (b). Immunoblots of phosphotyrosine-containing proteins immunoprecipitated from whole cell extracts of M1 cells were probed with monoclonal antibodies to phosphotyrosine as described in Materials and Methods. In panel a, M1D+ cells (10^7/ml) were stimulated for the indicated times (in minutes) with IL-6 (100 ng/ml) or LIF (400 U/ml), and whole cell extracts were prepared. In panel b, cells (0.2×10^7/ml) were treated for 90 min with tyrphostin (TY; 150 ng/ml) or H7 (40 μM/ml) prior to stimulation of cells (10^7/ml) with IL-6 (5 min) or LIF (15 min), and whole cell extracts was prepared. Resulting fluorograms are shown in panel a; only the p160 region is shown in panel b. All results are representative of at least two independent experiments.

differentiation directly, triggering the same differentiation immediate early response. At least two mechanisms of gene activation were shown to be involved, one transcriptional and the other posttranscriptional. In the past year it has become increasingly evident that LIF and IL-6, although structurally distinct and binding to different receptors, are able to induce neuronal cell differentiation (16, 38, 47), stimulate acute-phase protein synthesis in hepatocytes (2, 4, 14), and induce hematopoietic cell differentiation (this work; 23, 29, 35). In light of the present work, it is likely that these biological functions which are common to LIF and IL-6 are directly induced by each of these cytokines, mediated via similar immediate early responses.

As indicated above, LIF and IL-6 induced hematopoietic cell differentiation directly, activating, with similar kinetics and to similar extents, an identical set of myeloid differentiation immediate early response genes, including genes induced primarily at the level of transcription and at least one gene which is induced posttranscriptionally by stabilization of its transcript. Since these mechanisms of gene activation encompass a broad range of biochemical pathways, including modification of preexisting transcription factors and determinants of mRNA stability, a strong similarity in the signal transduction pathways activated by LIF and IL-6 is implied. The additive nature of the LIF and IL-6 immediate early response and the results of the activator and inhibitor studies strengthen this notion, indicating that LIF and IL-6 activate common intracellular signalling pathways which apparently do not utilize any of the known second messengers, including protein kinase C, cAMP-dependent kinase, cGMP-dependent kinase, and Ca^+/calmodulin kinases (this work; for IL-6, see also references 18, 25, and 36).

Structural information on the LIF receptor is not yet available, whereas cloning of the IL-6 receptor and analysis of its possible modes of function have shown that it is a bipartite receptor composed of a ligand-binding subunit that interacts with a signal-transducing subunit (18, 42, 48), which produces second messengers of yet unknown nature (this work; 18, 25). It also has become apparent that the IL-6 receptor is a member of a new superfamily of hematopoietin receptors that includes receptors for most known interleukins and hematopoietic growth and differentiation factors (3, 5, 11). All of these receptors were found to share common amino acid motifs in their extracellular domains, their cytoplasmic domains lacking sequence homology to known protein kinases; also, several lines of evidence suggest that, like the IL-6 receptor, they may couple with other proteins which play a crucial role in signal transduction (reference 3 and references therein). Yet the finding that two protein kinase inhibitors, tyrphostin and H7, efficiently blocked both the LIF- and IL-6-induced immediate early activation of MyD genes, including genes induced at the transcriptional (*junB*) and posttranscriptional (MyD88) levels, suggests that protein phosphorylation is, after all, an essential step in LIF and IL-6 signal transduction. This notion is further strengthened by our finding that both LIF and IL-6 rapidly induced tyrosine phosphorylation of a 160-kDa protein and that this phosphorylation was blocked by tyrphostin. Taking all of these observations together, it is tempting to speculate that the apparent similarity in LIF and IL-6 signal transduction reflects the interaction of the distinct LIF and IL-6 ligand-binding receptors (21) with similar signal-transducing proteins (Fig. 8).

It should be pointed out that recently the immediate early growth response in a murine hybridoma cell line dependent on IL-6 for growth was observed to be blocked by tyrphostin and H7 (36). In this study it was also documented that IL-6 induced tyrosine phosphorylation of 160-kDa protein, blocked by tyrphostin but not by H7. Interestingly, the phosphorylation of p160 is prolonged (at least 30 min) following LIF- or IL-6-induced differentiation of M1 cells compared with its phosphorylation following induction of growth in the hybridoma cell line in which it is transient (36). It is possible, therefore, that persistent phosphorylation of p160 may be necessary for the observed stable expression of MyD genes (Fig. 2) following induction of M1 myeloid differentiation, as opposed to the transient expression of JunB and TIS11 in IL-6-treated murine hybridoma cells in which p160 phosphorylation was also transient. It should also be pointed out that the ability of H7 to block the LIF or IL-6 immediate early response, while not inhibiting the induced phosphorylation of p160, implicates additional protein phosphorylation events in the LIF or IL-6 signal transduction cascade. Additional studies in the context of the

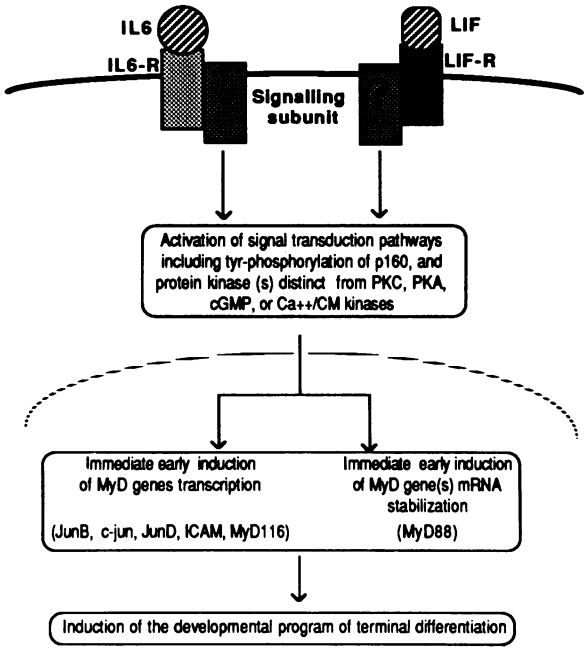


FIG. 8. Schematic summary diagram of molecular events involved in LIF/IL-6-induced differentiation. It is indicated that LIF and IL-6, although lacking structural homology and binding to distinct receptors, utilize common intracellular signalling pathways, including tyrosine phosphorylation of p160 and protein kinase(s) distinct from protein kinase C (PKC), cAMP, cGMP, or Ca^{2+}/calmodulin (Ca++/CM) kinase, to trigger an identical immediate early response upon induction of cellular differentiation. Structural information on the LIF receptor (LIF-R) is not yet available, whereas cloning of the IL-6 receptor (IL6-R) has shown that it is composed of a ligand-binding subunit that associates with a signal-transducing subunit. The identity in the molecular events involved in LIF/IL-6-induced differentiation is postulated to reflect interaction of distinct LIF/IL-6 ligand-binding receptors with similar signal-transducing proteins.

cellular system described in this report will be instrumental in determining the molecular nature and role of protein kinases and phosphorylated proteins which participate in LIF and IL-6 signalling that leads to the induction of hematopoietic cell differentiation.

Summary and concluding remarks. The results of this study have shown, for the first time, that LIF and IL-6 induce hematopoeitic cell differentiation directly, activating an identical transcriptional and posttranscriptional immediate early response. The identity of the LIF and IL-6 immediate early gene response, its additive nature, the results of the studies with a variety of protein kinase activators and inhibitors, and the finding of p160 phosphorylation suggest that LIF and IL-6 share common intracellular signalling pathways that appear not to utilize known second messengers and that include similar protein phosphorylation as an essential step(s) (Fig. 8). The M1 myeloid precursors and the panel of MyD genes used in this study provide a valuable system for further dissecting the molecular mechanisms that are involved in the transduction of LIF and IL-6 signals from the plasma membrane to the nucleus, including the *trans* and *cis* control elements which result in MyD gene activation.

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