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Immunology

STAT3 activation is a critical step in gp130-mediated terminal differentiation and growth arrest of a myeloid cell line

(cytokine/signal transduction/macrophage)

MASASHI MINAMI*, MASAHIRO INOUE*, SHI WEI*, KIYOSHI TAKEDA*, MAKOTO MATSUMOTO*, TADAMITSU KISHIMOTO†, AND SHIZUO AKIRA*‡

*Institute for Molecular and Cellular Biology, Osaka University 1-3 Yamada-oka, Suita, Osaka 565, Japan; and †Osaka University Medical School, Department of Medicine III, 2-2 Yamada-oka, Suita, Osaka 565, Japan

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ABSTRACT **Myeloid leukemia M1 cells can be induced for growth arrest and terminal differentiation into macrophages in response to interleukin 6 (IL-6) or leukemia inhibitory factor (LIF). Recently, a large number of cytokines and growth factors have been shown to activate the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) signaling pathway. In the case of IL-6 and LIF, which share a signal transducing receptor gp130, STAT3 is specifically tyrosine-phosphorylated and activated by stimulation with each cytokine in various cell types. To know the role of JAK–STAT pathway in M1 differentiation, we have constructed dominant negative forms of STAT3 and established M1 cell lines that constitutively express them. These M1 cells that overexpressed dominant negative forms showed no induction of differentiation-associated markers including Fcγ receptors, ferritin light chain, and lysozyme after treatment with IL-6. Expression of either c-myb or c-myc was not downregulated. Furthermore, IL-6- and LIF-mediated growth arrest and apoptosis were completely blocked. Thus these findings demonstrate that STAT3 activation is the critical step in a cascade of events that leads to terminal differentiation of M1 cells.**

The Janus kinase–signal transducer and activator of transcription (JAK–STAT) signaling pathway, which was first discovered from studies of interferon signaling, has turned out to be widely used by a number of cytokines and growth factors (1, 2). The JAK family (Tyk2, JAK1, JAK2, and JAK3) are non-receptor-type protein tyrosine kinases that phosphorylate STATs on one particular tyrosine residue. Upon tyrosine phosphorylation, STATs form homo- and heterodimeric complexes through the SH2 domain of the molecules and translocate to the nucleus, where they regulate transcription from the promoters containing interferon γ activation site-related DNA sequences. To date, six members of the STAT family, STAT1 through STAT6, have been identified. Different combinations of one or more STAT family members are activated in response to different cytokines, which is assumed to contribute to the diversity of cytokine responses. In the case of the interleukin 6 (IL-6) signaling pathway, the functional IL-6 receptor complex is composed of two chains, an IL-6 receptor α chain that binds IL-6 and another signal-transducing receptor component, gp130 (3). IL-6 initiates signaling via homodimerization of gp130, which then leads to both tyrosine phosphorylation of the cytoplasmic domains of gp130 and activation of a family of JAKs that are constitutively associated with the intracellular domains of gp130. After recruited to gp130, STAT3 (and also STAT1α, albeit to much lesser extent) becomes tyrosine-phosphorylated by JAK kinases, dissociates from the receptor, dimerizes, and translocates to the nucleus. STAT3 is also phosphorylated in response to the IL-6-related cytokines, such as leukemia inhibitory factor (LIF), ciliary neurotrophic factor, and oncostatin M, that share gp130 and in response to granulocyte colony-stimulating factor, which utilizes a receptor highly related to gp130 (4, 5). Epidermal growth factor induces tyrosine phosphorylation of STAT3 and STAT1α (6). Recently, it has been shown that selectivity of STAT activation is determined by a specific docking interaction between a STAT SH2 domain and a phosphorylated tyrosine-containing sequence in the signal-transducing receptor component (7). A consensus sequence YXXQ present in gp130 and LIF receptor cytoplasmic domains is necessary and sufficient for tyrosine phosphorylation of STAT3. In addition to the obligatory requirement of tyrosine phosphorylation for dimer formation and DNA binding by STATs, serine phosphorylation, probably mediated by mitogen-activated protein (MAP) kinases, was shown to be required for maximal transcriptional activation of STATs (8, 9). In spite of the extensive studies on STAT activation mechanisms, the biological function of STATs, particularly in the cell proliferation and differentiation has not yet been assessed although there are some reports about the involvement of STAT proteins in certain specific gene expressions.

In this study we examined the functional role of STAT by overexpressing the dominant negative forms of STAT3 in a myeloid cell line, M1, that undergoes growth arrest and macrophage differentiation in response to IL-6. The results provide evidence that the IL-6-mediated growth arrest and differentiation of M1 cells are absolutely dependent on the activation of STAT3 proteins.

MATERIALS AND METHODS

Cells. Myeloid leukemia M1 cells were cultured in Eagle's minimal essential medium supplemented with twice the normal concentrations of amino acids and vitamins and 10% (vol/vol) fetal calf serum. M1 cells were treated with recombinant human IL-6 at 100 ng/ml and LIF at 1000 units/ml, respectively. Viable cell numbers were determined by trypan blue exclusion and counted in a hemocytometer.

Plasmid Construction and DNA Transfection. Wild-type and mutant STAT3 constructs were generated by polymerase chain reaction (PCR) and site-directed mutagenesis. A point mutation converting Tyr-705 of STAT3 to Phe in STAT3(Y705F) was introduced by using the Transformer site-directed mutagenesis kit (Clontech). STAT3(Δ715) and STAT3(Δ579) were generated by point mutation converting Thr-716 and Trp-580 to stop codons (TGA), respectively. All

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Abbreviations: JAK, Janus kinase; STAT, signal transducer and activator of transcription; IL-6, interleukin 6; LIF, leukemia inhibitory factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Neo^r, neomycin resistance.

‡To whom reprint requests should be addressed.

constructs were epitope-tagged with FLAG(DYKDDDDK) (Kodak) at their N termini. FLAG-tagged STAT3 constructs were cloned into a mammalian expression vector, pEF-Bos, and expressed under the control of the elongation factor gene promoter. M1 cells were transfected via electroporation with the STAT3 expression vector and pSV2neo at a 20:1 ratio, and neomycin-resistant clones were selected in growth medium containing Geneticin (GIBCO) at 750 μg/ml.

Immunoprecipitation and Immunoblot Analysis. M1 tranfectants were stimulated with IL-6 for 15 min and solubilized with ice-cold lysis buffer containing 0.5% Nonidet P-40, 10 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM Na_2VO_3, and aprotinin (5 μg/ml). Whole cell lysates were incubated with anti-STAT3 antibody and protein A-Sepharose (Pharmacia) for 4 hr at 4°C. Immune complexes were analyzed by SDS/PAGE, transferred to a nitrocellulose membrane, and incubated with anti-phosphotyrosine monoclonal antibody (4G10, Upstate Biotechnology). Bound antibody was visualized with an enhanced chemiluminescence system (Amersham).

Northern Blot Analysis. Cytoplasmic RNA was extracted by using IsoGen (Nippon Gene). Total RNA (20 μg per lane) was subjected to agarose gel electrophoresis and transferred to a nylon membrane (Hybond N+, Amersham). The membrane was hybridized with radiolabeled cDNA probes for M1 differentiation-associated markers. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used as an internal control.

Flow Cytometry Analysis. Transfectants (5×10^5 cells) were cultured with IL-6 for 1 day. After harvesting, cells were incubated with 1 μg of mouse IgG2a for 20 min on ice. After washing with PBS, cells were incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG antibody (DAKO) for 20 min on ice. Cells were analyzed in a fluorescence-activated cell sorter (Becton Dickinson FACS).

RESULTS

Establishment and Analysis of M1 Cell Lines Constitutively Expressing Several Mutant STAT3 Proteins. Two mutant STAT3 proteins [STAT3(Y705F) and STAT3(Δ715)] were expected to work as dominant negative forms of STAT3. STAT3(Y705F) is a mutant in which the tyrosine residue phosphorylated by JAK kinases is mutated to phenylalanine. STAT(Δ715) is a mutant that lacks the C-terminal transcriptional activation domain. STAT(Δ579), which lacks the SH2 domain as well as the C-terminal region, was used as negative control. Wild-type STAT3, STAT3(Y705F), STAT3(Δ715), and STAT(Δ579) were N-terminal-FLAG-epitope-tagged to distinguish them from endogenous STAT3 proteins and expressed under the control of the elongation factor gene promoter to get high expression of the exogenous gene (Fig. 1*A*). These constructs were transfected with the neomycin-resistance (Neo^r) selection marker and permanent transformants were obtained. The level of mutant STAT3 proteins were comparable among these transformants as assessed by immunoblot analysis of the cell lysates with anti-FLAG monoclonal antibody (data not shown). Transfectants that contained only the Neo^r vector were also isolated as controls.

We first examined the activation of endogenous STAT3 in these transformants (Fig. 1*B*). The cells were stimulated with IL-6 and the cell lysates were immunoprecipitated with STAT3 antibody, followed by immunoblot analysis with anti-phosphotyrosine antibody. Surprisingly, the endogenous STAT3 protein was not tyrosine-phosphorylated in the case of M1 cells overexpressing STAT3(Y705F). In contrast, in the case of M1 cells overexpressing STAT3(Δ715), the endogenous STAT3 protein was still tyrosine-phosphorylated, although much more of the exogenous mutant STAT3 protein was tyrosine-phosphorylated than was observed with endogenous

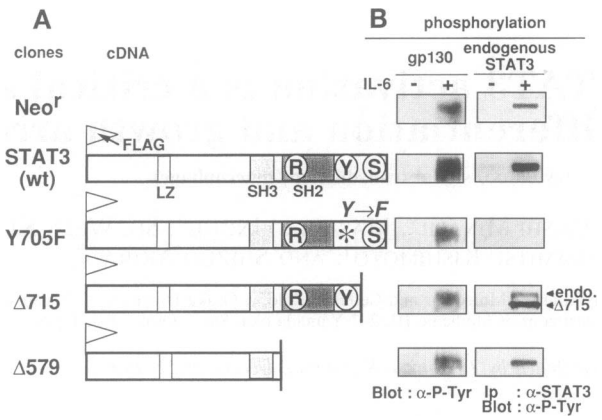

FIG. 1. Effects of overexpression of various STAT3 constructs on the endogenous STAT3 phosphorylation after IL-6 treatment. (*A*) Schematic representation of various STAT3 constructs. LZ, leucine zipper domain; R, arginine; Y, tyrosine; S, serine; F, phenylalanine. (*B*) M1 cells expressing the indicated STAT3 constructs were stimulated with IL-6 for 15 min. Immunoprecipitation obtained with STAT3 antibody or total cell lysates were examined by Western blot analysis with anti-phosphotyrosine antibody 4G10. α-, Anti-; P-Tyr, phosphotyrosine.

STAT3 protein. To exclude the possibility of any defects in the IL-6 receptor signaling pathway in these transformants, gp130 phosphorylation was examined. gp130 was tyrosine-phosphorylated to similar extents among these transformants after IL-6 stimulation. Thus, these results indicate that gp130 was tyrosine-phosphorylated but endogenous STAT3 protein was not phosphorylated in M1 cells overexpressing STAT3(Y705F) in response to IL-6.

Blockade of IL-6-Mediated Growth Arrest and Apoptosis of M1 Cells Overexpressing the Dominant Negative STAT3 Proteins. We next examined the effect of ectopic expression of mutant STAT3 proteins on the IL-6-mediated growth arrest and cell death of M1 cells. As shown in Fig. 2, the Neo^r and STAT3(Δ579) clones underwent growth arrest and cell death after IL-6 stimulation as demonstrated in parent M1 cells. The dead cells showed characteristics of apoptotic cells, such as chromatin condensation, cytoplasmic blebbing, and DNA fragmentation (data not shown). In the case of wild-type STAT3 clones, the growth arrest and cell death were accelerated. In contrast, STAT3(Y705F) clones did not show any growth arrest and cell death after IL-6 stimulation. STAT3(Δ715) clones showed marginal growth arrest and cell death only after 3 days. These results show that IL-6-mediated growth arrest and apoptosis were blocked in the M1 cells overexpressing the dominant negative STAT3 proteins and that STAT3(Y705F) was a more potent dominant negative form compared to STAT3(Δ715).

Effects of Constitutive Expression of Dominant Negative STAT3 Proteins on the Expression of Protooncogenes and Differentiation-Associated Properties. We examined the effect of dominant negative STAT3 proteins on the expression of differentiation-associated markers (Fig. 3). Expression of Fcγ receptor, ferritin light chain, and lysozyme was not induced in M1 cells overexpressing the dominant negative STAT3 proteins, STAT3(Y705F) and STAT3(Δ715). Expression of c-myb and c-myc was not downregulated in these cell lines. Interestingly, lysozyme mRNA was not induced in M1 cells overexpressing wild-type STAT3. Consistent with this finding, the wild-type STAT3-overexpressing M1 cells did not adhere to the plastic dish, unlike parent M1 cells (data not shown). These indicate that wild-type STAT3-expressing M1 cells did not undergo terminal differentiation although they expressed Fcγ receptor.

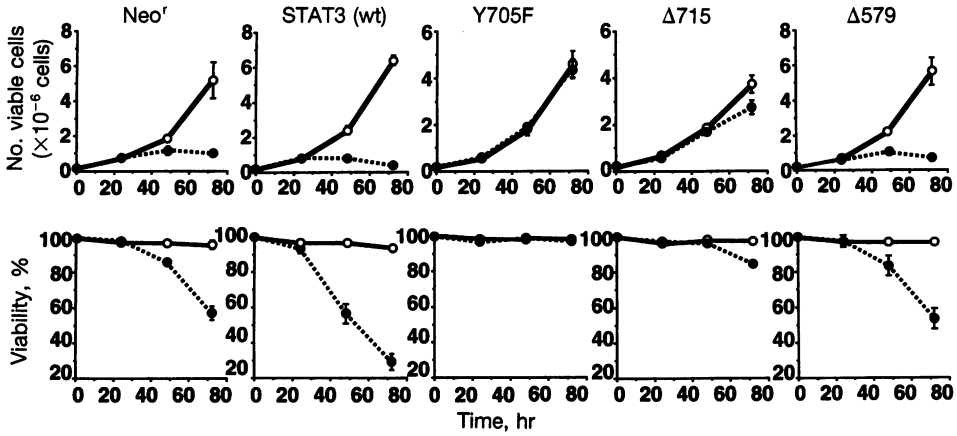

FIG. 2. Growth of M1 clones expressing STAT3 constructs after treatment with IL-6. Cells were seeded (0.2 × 10^6 cells) with (●) or without (○) IL-6 at day 0, and viable cell counts were determined on days 1, 2, and 3. Each point represents the mean ± SD of three experiments.

Dominant-Negative STAT3 Proteins also Block LIF-Mediated Differentiation and Growth Arrest of M1 Cells. LIF also induces terminal differentiation and growth arrest in M1 cells. Receptor signaling for both IL-6 and LIF occurs via a common signal transducing component, gp130. Therefore, we examined the effect of dominant negative STAT3 proteins on LIF-mediated growth arrest and apoptosis. As shown in Fig. 4, LIF-mediated growth arrest and apoptosis of M1 cells were also blocked by constitutive expression of STAT3(Y705F).

DISCUSSION

In this report we have shown that terminal myeloid differentiation and growth arrest induced by IL-6 or LIF are completely blocked by overexpression of dominant negative STAT3 proteins, indicating that STAT3 activation is essential for gp130-mediated growth arrest and differentiation into macrophages in M1 cells. Macrophage differentiation is a highly ordered and tightly regulated cascade that involves multiple changes in gene expression. In the murine M1 myeloid leukemia cell line, treatment with IL-6 or LIF induces rapid induction of immediate early response genes such as junB, c-jun, junD, MyD88, MyD116, MyD118, intercellular adhesion molecule 1, and interferon regulatory factor 1, and suppression of c-myb, which is followed by induction of Fcγ and C3 receptors, as well as downregulation of c-myc (10, 11). Ferritin light chain and lysozyme are induced late during differentiation, accompanied by morphologic differentiation to macrophages, and suppression of proliferative capacity. In the case of M1 cells with dominant negative STAT3 proteins, there was no downregulation of c-myb and c-myc and no induction of differentiation-associated markers such as Fcγ receptor, ferritin light chain, and lysozyme. These findings show that STAT3 activation is the initial step in a cascade of events that lead to terminal differentiation of M1 cells. In fact, it has been demonstrated that STAT proteins bind to the functional regulatory sequences identified in the promoter regions of several immediate early response genes such as interferon regulatory factor 1, junB, c-fos, intracellular adhesion molecule 1, and MyD88, as well as the Fcγ receptor (12–14). Some of the immediate early response genes that are regulated by STAT3 may be associated with growth arrest and differentiation directly and/or indirectly by induction of genes with such activities.

Noteworthy is the finding that M1 cells overexpressing wild-type STAT3 undergo apoptosis without terminal differentiation. In these cells, IL-6 treatment induced Fcγ receptor but not late activation markers such as lysozyme and ferritin. Cytokine signaling usually requires at least two pathways including the Ras–Raf–MEK–MAP kinase cascade, the JAK–STAT pathway, and a pathway for *bcl-2* gene induction (15). Apopotosis may result from an imbalance of signal transduction, in this case, excess activation of JAK–STAT pathway without concomitant activation of other signal pathways.

The structure–function analyses, carried out by expressing wild-type or mutant receptors, have shown that STAT activa-

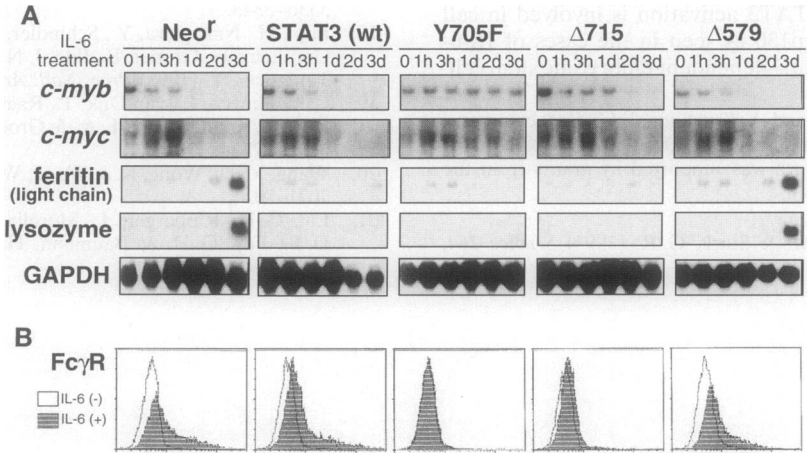

FIG. 3. Analysis of expression of c-myb, c-myc, ferritin light chain, lysozyme, and Fcγ receptors after stimulation with IL-6. (*A*) Total RNA was extracted from cells at the indicated times and analyzed by Northern blots. d, Day(s). (*B*) M1 cells were seeded (0.5 × 10^6 cells) with (hatched area) or without (open area) IL-6 for 1 day. Expression of Fcγ receptors was determined by flow cytometry analysis.

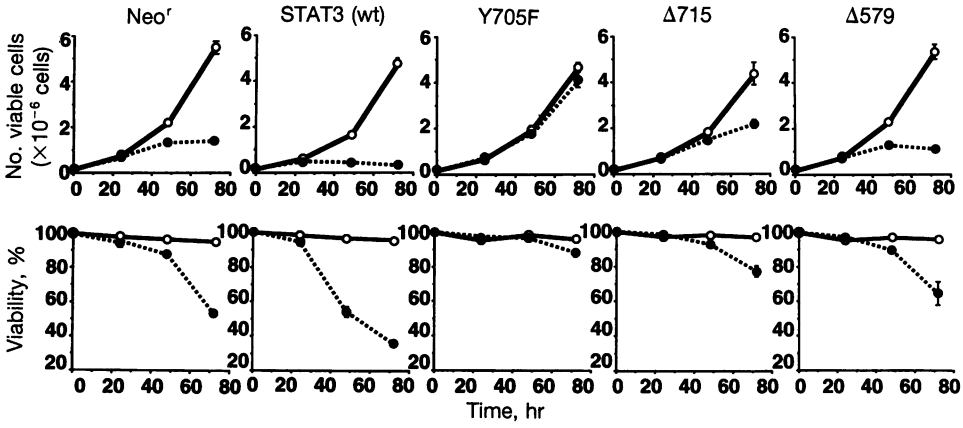

FIG. 4. Growth of M1 cells expressing STAT3 constructs after treatment with LIF. Cells were seeded (0.2×10^6 cells) with (●) or without (○) LIF at day 0, and viable cell counts were determined on days 1, 2, and 3. Each time point represents the mean ± SD of three experiments.

tion is not required for induction of mitogenesis after stimulation of several cytokines. In the case of IL-6 signaling, the membrane-proximal portion of gp130 containing so-called box1 and box2 is indispensable for mediating IL-6-mediated DNA synthesis, and the JAK family associated with box1 (16). However, box3 essential for the IL-6-mediated tyrosine phosphorylation of STAT3 is not required for mitogenesis (3). Deletional analysis of the IL-4 receptor α chain also showed that the cytoplasmic domain of the human IL-4 receptor required for a mitogenic response contains no tyrosine residues (17). IL-4-induced tyrosine phosphorylation of STAT6 was lost with such truncate receptor. A similar result was provided in IL-2-induced STAT5 activation (18). On the other hand, some evidence indicates activation of STATs in the absence of receptor phosphorylation. For example, experiments with C-terminal truncation of the IL-5 receptor demonstrate that a βc receptor mutant lacking intracellular tyrosine residues was still able to mediate STAT activation (19). The same observation was made for STAT1α activation via the growth hormone receptor (20). Also in a STAT–JAK coexpression experiment, a box3-independent activation of STAT proteins was achieved by overexpression of JAK2 or Tyk2 (21). In these cases, it is possible that STATs associate with phosphotyrosines on activated JAKs via their SH2 domain and/or that additional STAT domains interact with JAK–receptor complexes. Therefore, activation of STATs might be facilitated but not absolutely dependent on the presence of a box3 motif. Further studies are required to conclude that the JAK–STAT pathway is not involved in cell proliferation. The dominant negative forms of STAT3 will be useful for such experiments that determine whether STAT3 activation is involved in cell proliferation induced by gp130 as seen in the cases of IL-6-dependent myelomas and LIF-dependent embryonic stem cell.

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