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Association of Stat3-Dependent Transcriptional Activation of p19^{INK4D} with IL-6-Induced Growth Arrest

Masahiro Narimatsu, Koichi Nakajima, Makoto Ichiba, and Toshio Hirano[1]

Division of Molecular Oncology, Department of Oncology, Biomedical Research Center, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka, 565, Japan

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Signal transducer and activator of transcription 3 (Stat3) is the major mediator of the IL-6-induced signals regulating growth and differentiation. In the M1 myeloleukemic cell line, Stat3 is a critical transcription factor causing repression of c-*myc* and c-*myb* genes, expression of *junB* and IRF1, growth arrest at G1, and subsequent macrophage differentiation. To understand the mechanisms by which Stat3 causes such effects, we searched for other Stat3-regulated genes possibly involved in growth arrest. We identified this inducible molecule as p19^{INK4D} using a specific antibody. Both p19^{INK4D} mRNA and protein were rapidly induced by IL-6 treatment without requiring *de novo* protein synthesis and the induction was fully suppressed by dominant-negative forms of Stat3. Thus both Stat3-regulated events, repressions of c-*myc* and c-*myb* and induction of p19^{INK4D}, are likely to be involved in IL-6-induced growth arrest in M1 cells.

The extracellular signals, such as cytokines, growth factors and hormones, control cellular fate by regulating cell growth, differentiation and cell death. Interleukin 6 (IL-6), one of cytokines showing pleiotrophic function, determines cell fate through activating a variety of gene expression programs in various tissues including lymphoid tissue, hematopoietic lineage cells, liver and bone (1,2,3,4). Among the signal transduction pathways known to be activated by IL-6, Stat3-mediated pathway and other pathways including SHP-2/Ras/MAP kinase pathway have been shown to be critical for IL-6-induced growth and differentiation (5,6,7). Especially, Stat3 is critical in the IL-6-induced growth arrest and differentiation of M1 leukemic cells (5). Stat3 is also critical in gp130(a common signal-transducing subunit of the IL-6 family cytokine receptors)-mediated cell survival and cell growth in a BaF/B03 proB cell line (7). M1 cells have been shown to be induced growth arrest and macrophage differentiation by IL-6 and LIF (8,9,10,11,12). Among the genes regulated by IL-6 signals, the repression of c-*myc* and c-*myb* expression is likely to be responsible for IL-6-induced growth arrest and the subsequent terminal differentiation, since deregulated expression of exogenous c-*myb* or c-*myc* inhibits the IL-6-induced growth arrest and terminal differentiation (13,14). IL-6 and LIF, however, partially inhibit the growth of M1 cells expressing exogenous c-*myc* (13). Therefore it is conceivable that in conjunction with the repressed c-*myc* and c-*myb*, other unknown mechanism(s) initiated by IL-6 signals are involved in the IL-6-induced growth arrest of M1 cells. In this study, we showed that IL-6 induced a CDK4-associated molecule with a size of around 20 kDa and identified it as p19^{INK4D}. Furthermore, Stat3 was found to be essential for the induction of p19^{INK4D}.

MATERIALS AND METHODS

Cell lines. Murine myeloid leukemia cell line M1 cells were grown in Dulbecco's Modified Eagles Medium (DMEM; GIBCO) supplemented with 10% horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2. The M1 transformant expressing dominant-negative Stat3 (M1-Stat3D clone 3) was previously described (5).

Immunoprecipitation and immunoblotting. Whole-cell extracts (WCE) were prepared by lysing cells in ice-cold lysis buffer as previously described(5). For immunoblotting, immunoprecipitates of whole-cell extracts were separated on SDS-polyacrylamide gels, transferred to Immobilon P (Millipore) membranes, probed with the following specific antibodies and detected for signals with the chemiluminescence system (Renaissance, DuPont NEN). The antibodies used are as follows; antibodies against CDK2, CDK4, CDK6, cyclin D1, D2, D3, E, A, CDK inhibitors p21, p27, p57, p15, p16, and p18 (purchased from Santa Cruz Biotechnology) and anti-p19 (a gift from C. Sherr) (20).

[1] To whom correspondence should be addressed. Fax: 81-6-879-3889. E-mail: hirano@molonc.med.osaka-u.ac.jp.

Abbreviations used: Stat, signal transducer and activator of transcription; IL, interleukin; INK4, inhibitor of CDK4; CDK, cyclin-dependent kinase.

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Northern blot analysis. Total RNA was prepared from treated cultures by using TRIzol (GIBCO BRL). 20 μg of total RNA per sample was separated by electrophoresis in 1% agarose formaldehyde gels and transferred to Hybond N^+ (Amersham) nylon filters. Filters were hybridized with ^{32}P-labeled cDNA fragments overnight and washed three times with 0.2 × SSC, 0.1 % SDS at 56 °C for 20 min and subjected to autoradiography. The amount of loaded RNA was tested by the expression level of CHO-B mRNA or by the ethidium bromide staining of RNA. The probes used are as follows; the 1.3-kb *Pst*I-*Xho*I fragment of human cyclin A cDNA (a gift from H. Nojima), the 2.5-kb *Eco*RI fragment of human cyclin E cDNA, the 1.3-kb *Bst*XI fragment containing the coding region of mouse CDK4 cDNA (gifts from T. Akiyama), the 0.5-kb *Bam*HI-*Eco*RI containing the entire coding sequence of mouse p19 (a gift from C. Sherr) and the 0.6-kb *Eco*RI-*Bam*HI fragment of CHO-B cDNA (a gift from J. Darnell, Jr.).

Metabolic labeling. M1 cells were washed twice with DMEM without l-methionine or l-cysteine, containing 10% dialyzed horse serum and incubated with the same medium containing ^{35}S-methionine and ^{35}S-cysteine for 16 hr. M1 cells were unstimulated or stimulated with IL-6 at 50 ng/ml for the last 8 hr of the incubation period. Anti-CDK4 precipitates were separated on a SDS-polyacrylamide gel, and subjected to autoradiography. Normal rabbit IgG was also used for immunoprecipitation as a control.

RESULTS

Induction of a 19kDa CDK4-associated molecule by IL-6 precedes the increase in the amount of the hypophosphorylated form of Rb in M1 cells. M1 cells have been shown to gradually accumulate at G1 phase in response to IL-6 (5,15). This accumulation of G1 cells accompanies the gradual increase in the amount of hypophosphorylated retinoblastoma protein (Rb) relative to hyperphosphorylated form of Rb, consistent with the results reported by Resnitzky et al (15). (Fig. 1A). Hypophosphorylated form of RB was apparent at 24 hr after IL-6 stimulation and increased thereafter, suggesting that the kinase activity phosphorylating Rb, cyclin-dependent kinases CDK4/6 and CDK2 with cor-

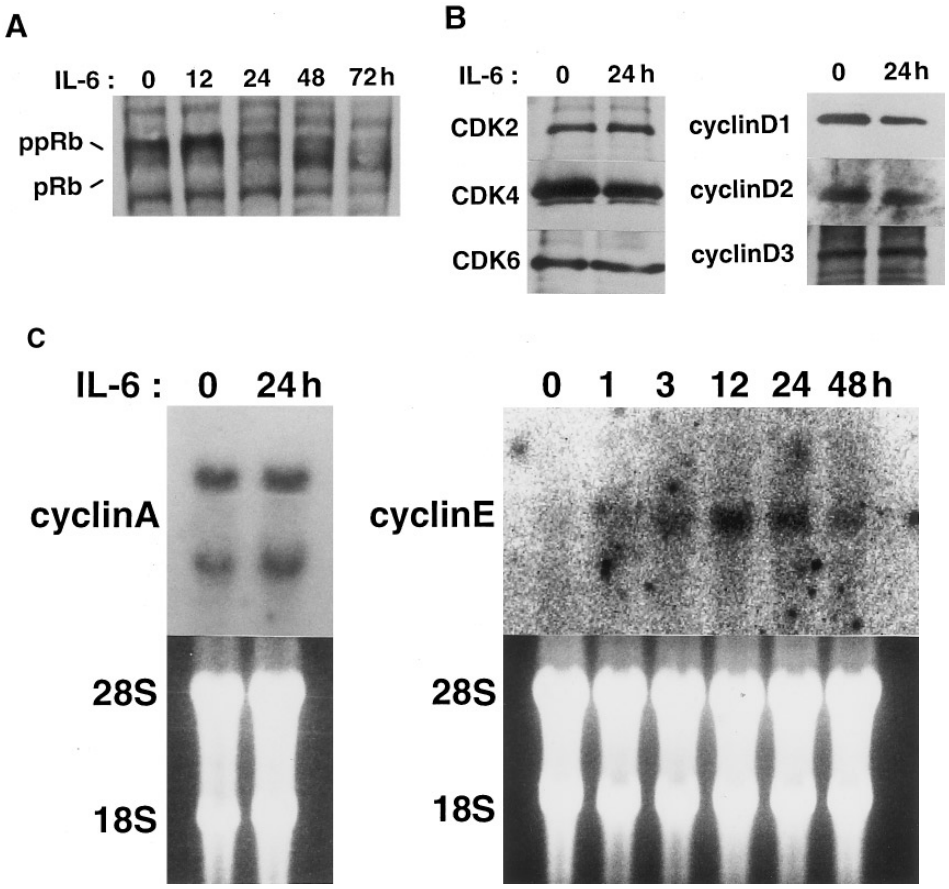

FIG. 1. IL-6-induced appearance of hypophosphorylated Rb without decreasing the amounts of CDKs/cyclins acting at the G1/S transition. (A) The whole-cell extracts (WCE) (30 μg) from M1 cells stimulated with IL-6 at 50 ng/ml for the indicated period were analyzed for the migration pattern of Rb by blotting with anti-Rb antibody. Hyperphosphorylated form of Rb (ppRb) and hypophosphorylated form of Rb (pRb) are indicated at left. (B) The WCE from M1 cells unstimulated or stimulated with IL-6 for 24 hr were analyzed for the amounts of CDK2, CDK4, CDK6 (left panel) and D-type cyclins, D1, D2, D3 (right panel) by immuno-precipitation and immunoblotting using specific antibodies. (C) Total RNAs from M1 cells unstimulated or stimulated with IL-6 at 50 ng/ml for the indicated period were analyzed for the steady state mRNA expression of cyclin A (left panel) and cyclin E (right panel).

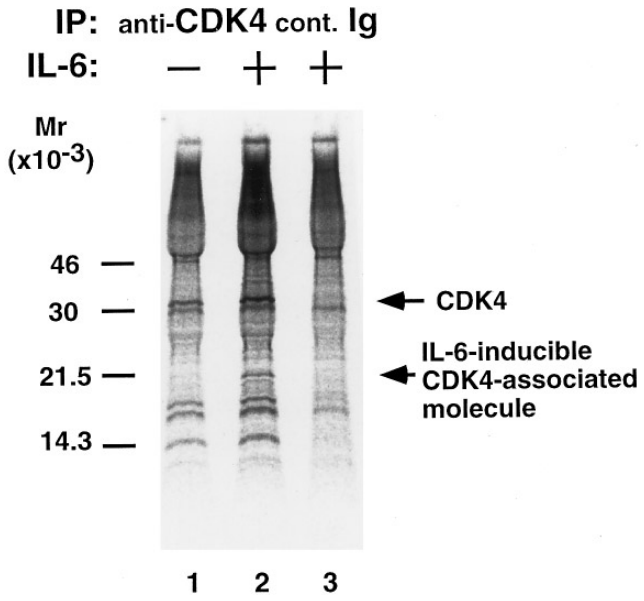

FIG. 2. Induction of a CDK4-associated protein with a size of around 20 kDa by IL-6. CDK4-immunoprecpitates (lane1 and 2) or control Ig-immuno-precipitates (lane 3) of WCE from unstimulated (lane 1) or IL-6-stimulated (lane 2 and 3) M1 cells, which had been metabolically labeled with ^{35}S-methionine and ^{35}S-cysteine, were separated on a SDS-polyacrylamide gel and subjected to autoradigraphy.

responding regulatory cyclins (16) are negatively regulated by IL-6 signals. To understand the mechanisms by which IL-6 increases hypophosphorylated Rb, we have first examined whether IL-6 caused changes in the amount of the constituents of CDK/cyclins acting at the G1/S boundary in M1 cells after 24 hr stimulation with IL-6. The protein levels of CDK2, CDK4 and CDK6, and D-type cyclins (cyclin D1, D2 and D3) did not change at 24 hr after IL-6 stimulation (Fig. 1B). Since we could not detect the protein levels of cyclin A and E, we tested the amount of mRNA for cyclin A and E. Cyclin A mRNA level did not decrease at least for 24 hr after IL-6 stimulation but cyclin E mRNA level increased several fold in response to IL-6 with being at a plateau level through 12 hr and 24 hr and declined at 48 hr (Fig. 1C)

The constant amounts of the CDKs and cyclins except for cyclin E during the initial course of growth arrest in M1 cells prompted us to examine whether IL-6 induces CDK-associated CDK inhibitors or other unknown CDK-associated proteins. First we examined whether IL-6 induced CDK4-associated molecules by using whole-cell extracts from untreated or IL-6-treated M1 cells which had been metabolically labeled with ^{35}S-methionine and ^{35}S-cysteine. Fig. 2 shows that IL-6 stimulation induced a CDK4-associated molecule with the apparent size of 19-20 kDa (lane 2). This molecule was not detected in the precipitates using normal rabbit immunoglobulin, confirming the specific association between CDK4 and the IL-6-induced 19-20 kDa molecule. It is of note that the induction of CDK4-associated molecule by IL-6 precedes to the appearance of hypophosphorylated form of Rb and growth arrest. This CDK4-associated molecule may be functionally relevant to the IL-6-induced growth arrest.

Identification of the IL-6-induced CDK4-associated protein as p19^{INK4D} and its Stat3-dependent induction. We tried to identify the CDK4-associated molecule. Specific antibodies to known CDK inhibitors (see 17 for a review) were used for immunoblotting the CDK4 immunoprecipitates. As shown in Fig. 3A, the amount of p27 in the CDK4 immunoprecipitates did not change over 24 hr IL-6-stimulation. Other members of the p21 family (p21 and p57) were not detected in the CDK4 immunoprecipitates (data not shown). We failed to detect the proteins of both p21 and p57 by using immunoprecipitation and immunoblotting with specific antibodies, indicating that the expression levels of these inhibitors were very low in the M1 cell line we used. Specific antibodies to members of the INK4 family (p16, p15, p18 and p19) were also used. Among them, neither p15, p16 nor p18 was detected in the CDK4 immunoprecipitates even after stimulation with IL-6 up to 24 hr (data not shown). On the other hand, p19^{INK4D} CDK inhibitor appeared first at 2 hr IL-6 stimulation in the CDK4-immunoprecipitates and the protein levels gradually increased up to 24 hr stimulation (Fig. 3A). From the identical size and the induction pattern of p19 and other CDK inhibitors, it is reasonable to conclude that the 19-20 kDa CDK4-associated molecule shown in Fig. 2 is p19^{INK4D} itself.

Since we showed that IL-6-induced growth arrest of M1 cells is dependent on Stat3 activation (5), we examined wether Stat3 activity is essential for IL-6-induction of p19^{INK4D}. As shown in Fig. 3A, the dominant-negative Stat3 inhibited the IL-6-induced increase of p19^{INK4D} protein in the CDK4-immunoprecipitates. The protein levels of p19^{INK4D} in IL-6-stimulated M1 parental cells and dominant negative Stat3-expressing M1 cells were well correlated with the levels of p19^{INK4D} in the CDK4-immunoprecipitates (data not shown). Northern blot analysis of the p19 mRNA in M1 parental cells and the M1 transformant expressing the dominant-negative Stat3 (M1-Stat3D) shows that IL-6 rapidly induced p19^{INK4D} at the mRNA level, with the level being first detected at 1 hr after stimulation and increasing thereafter, in M1 parental cells but not in M1-Stat3D cells (Fig. 3B). Together with the failure of protein synthesis inhibitor cyclohexamide to inhibit p19^{INK4D} mRNA induction by IL-6 in M1 cells (data not shown), these results suggest that the p19^{INK4D} gene is an immediate target of Stat3 in M1 cells.

DISCUSSION

We have provided an evidence that IL-6 rapidly induces p19^{INK4D} mRNA and protein in a manner depen-

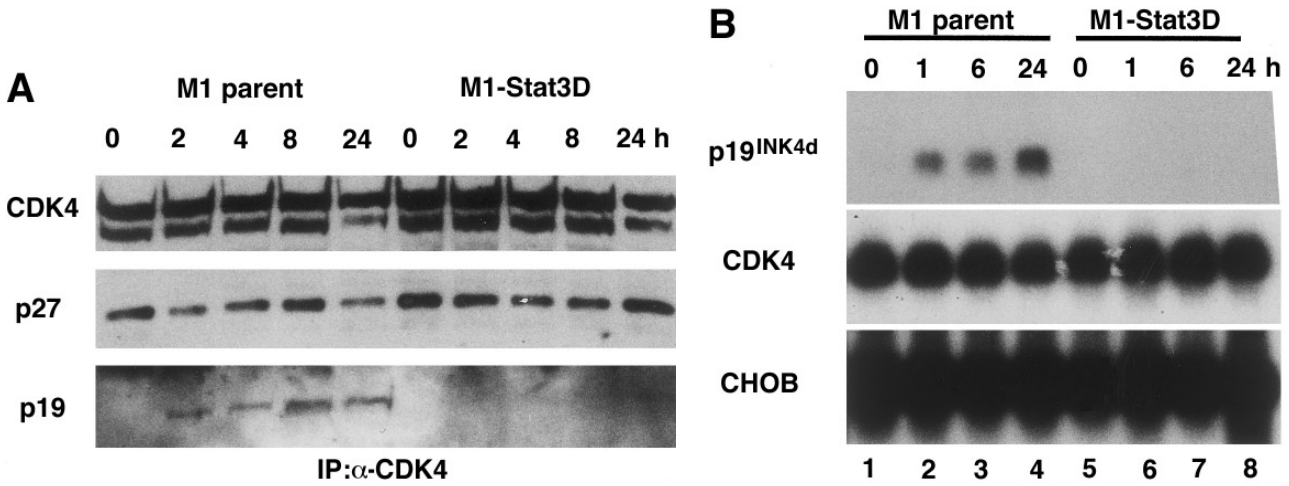

FIG. 3. Induction of $p19^{INK4D}$ at both protein and mRNA levels by IL-6 in a manner dependent on Stat3. (A) CDK4-immnouprecipitates from M1 parental cells and M1 cells expressing dominant-negative Stat3 (M1-Stat3D) stimulated for the indicated periods were immunoblotted with anti-CDK4, anti-p27 and anti-p19 antibody. (B) Total RNAs from M1 parental cells and M1 cells expressing dominant-negative Stat3 (M1-Stat3D) stimulated with 50 ng/ml IL-6 for the indicated period were analyzed for the expression levels of $p19^{INK4D}$, CDK4 and CHOB mRNAs.

dent on Stat3 in M1 leukemic cells. Interestingly, the IL-6-induction of $p19^{INK4D}$ occurs within 1 hr without requiring de novo protein synthesis and persists for more than 24 hr, consistent with the expected role of $p19^{INK4D}$ in the decrease in CDK4/6 activity and eventual accumulation of M1 cells at G1 phase, which is apparent later after two days. The sustained induction of $p19^{INK4D}$ is well correlated with the sustained activity of Stat3 (Fig. 3A, 5). This sustained induction of the Stat3 target genes is also observed for the *junB* and *Stat3* genes (unpublished observation). IL-6 did not induce other known CDK inhibitors including p21, p57, p27, p15, p16 or p18 in this cell line, although Steinman et. al. reported that IL-6 induces p21 in a subclone of M1 cells (18) and Morse et. al. reported p18 induction in a B cell line by IL-6 (19). The activation of some CDK inhibitor genes may require cell-type specific transcription factor(s) in addition to Stat3 or other IL-6-activated transcription factors.

Although it has been shown that CSF-1 induces expression of $p19^{INK4D}$ in a cell-cycle regulated manner and the overexpression of $P19^{INK4D}$ alone in NIH3T3 causes growth arrest of the cell (20), this is the first report showing the induction of $P19^{INK4D}$ by a factor causing growth arrest in a manner dependent on Stat3. Induction of other CDK inhibitors, including p21, p27 and p15, have been shown to be associated with growth arrest and differentiation of a variety of cells in response to a number of extracellular signals, including the cases for TGFβ induction of p15 (21), and vitamin D3 induction of p21 (22). In some cases, Stat proteins have been claimed to be involved in causing growth arrest partly by inducing CDK inhibitors. For instance, IFNγ-induced growth arrest of several cell lines accompany with the induction of p21 through Stat1 (23). Growth arrest and megakaryocyte differentiation of a cell line by thrombopoietin accompanies the induction of p21 most likely through Stat5 (24). Thus some of CDK inhibitors, such as p21 and $p19^{INK4D}$, are target genes for the Stat family proteins.

The results shown here, together with our previous report showing the critical role of Stat3 in the IL-6-induced repression of c-*myc* and c-*myb*, growth arrest and subsequent macrophage differentiation of M1 cells, suggest that Stat3-regulated gene regulation program leading to the repression of c-*myc* and c-*myb* and induction of $p19^{INK4D}$ is responsible for the IL-6-induced growth arrest in M1 cells.

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