Tyrosine Phosphorylation and Activation of JAK Family Tyrosine Kinases by Interleukin-9 in MO7E Cells

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Interleukin-9 (IL-9) is a T-cell-derived multifunctional cytokine that can stimulate the proliferation of a human megakaryocytic leukemia cell line, MO7E. Previous studies suggested that protein tyrosine phosphorylation may be involved in IL-9 signaling pathways. However, tyrosine kinases activated by IL-9 have not been identified. In this report we show that IL-9 induces tyrosine phosphorylation and activation of the JAK family tyrosine kinases including JAK1, JAK3, and Tyk2. The kinetic studies indicate that tyrosine phosphorylation and activation of JAK kinases induced by IL-9 occurred within 1 minute, peaked by 5 to 10 minutes, and persisted at least for 45 minutes. Furthermore, we show that signal transducers and activators of transcription (Stat) 91 or related protein and an 88-kD Stat 91-associated protein are rapidly tyrosine phosphorylated following IL-9 treatment. Gel shift assays confirm that nuclear extracts from MO7E cells stimulated with IL-9 specifically interact with a DNA element termed gamma activated site. These results suggest that actions of IL-9 may, in part, be mediated through JAK kinase-Stat signaling cascades.

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MATERIALS AND METHODS

Reagents. Recombinant human IL-9 (1.2 × 10^6 U/mg protein) was kindly provided by Genetics Institute (Cambridge, MA). Antibodies for JAK1, JAK2, Tyk2, and phosphotyrosine were purchased from Upstate Biotechnology Inc (Lake Placid, NY). Anti–C-terminus of Stat 91 antibodies were produced as described previously and kindly provided by Dr Andrew Larner at the National Institute of Health.

MO7E cell line. The human megakaryocytic leukemia cell line MO7E, which depends on human granulocyte-macrophage colony stimulating factor (GM-CSF) or IL-3 for growth, was obtained from Genetics Institute. The cells were maintained in RPMI 1640 medium supplemented with 20% fetal bovine serum and 100 U/mL recombinant human GM-CSF. For experiments, cells were washed three times with medium and starved for 14 hours in the absence of growth factors. Cells (2 × 10^6/mL) were then stimulated with 100 ng/mL human recombinant IL-9 for the indicated periods of time and lysed with lysis buffer. The detergent soluble proteins were collected for immunoprecipitation.

Immunoprecipitation (IP) and immunoblotting. The detergent soluble proteins from unstimulated or IL-9-stimulated MO7E cells were incubated with anti-JAK1, anti-JAK2, anti-JAK3, or anti-Tyk2 antibodies, respectively, overnight on ice. The protein A-agarose was added and rotated for 2 hours at 4°C. The immunoprecipitates were washed four times with lysis buffer and separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene difluoride (PVDF) membrane and immunoblotted sequentially with antiphosphotyrosine antibodies and anti-JAK1, anti-JAK2, anti-JAK3, or anti-Tyk2 antibodies with enhanced chemiluminescence (ECL) techniques according to manufacturer’s recommendation.

In vitro kinase assay and phosphoamino acid analysis. The immune complex kinase assays were performed as described. The autophosphorylated kinases were separated by 7.5% SDS-PAGE and transferred onto PVDF membrane. The kinases were visualized by exposing the membrane to x-ray film. For phosphoamino acid analysis, the kinase bands were cut and hydrolyzed with 5.7 N HCl at 110°C for 16 hours followed by derivatization and analysis by reversed phase high performance liquid chromatography.

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Fig. 1. Induction of tyrosine phosphorylation of JAK1, JAK3, and Tyk2 kinases by IL-9 in M07E cells. M07E cells were starved for 14 hours and then stimulated with IL-9 or GM-CSF (1,000 U/mL) for 5 minutes (A and B) or for the indicated periods of time (C, D, and E). Cell lysates (2 × 10⁶ cells/sample) were immunoprecipitated with 1 μL of JAK1, JAK2, or Tyk2 antisera or with 5 μg of antihuman JAK3 antibodies as described in Materials and Methods. The immunoprecipitates were separated by 7.5% SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antiphosphotyrosine antibodies as indicated. The membranes were stripped and reimmunoblotted with anti-JAK1, anti-JAK2, anti-JAK3, or anti-Tyk2 antibodies by ECL.

110°C for 60 minutes. Phosphoamino acids were separated by thin layer chromatography (TLC) plate, as described previously. Gel shift assay. M07E cell nuclear extracts were prepared as previously described. Ly-6E gamma activated site (GAS) and high-affinity cis-inducible element (SIE) were prepared by the annealing of oligonucleotides (5'CATGTTATGCATATTCCTGTAAGTG 3' and 5'CATGCCTACAGGAATATGCATAA 3') and oligonucleotides (5'GTCGACATTCCCGTAAATCTTGT 3' and 5'AGATTTACGGAAATGTCGAC 3'), respectively. The probe was labeled by Klenow reaction with [³²P]dCTP in the presence of dATP, dGTP, and dTTP. For gel shift assays, 15 μg of nuclear proteins were incubated with 2 μg of poly d(I-C) poly d(I-C) before the addition of [³²P]-labeled GAS probe (2 × 10⁶ cpm). After incubation at room temperature for 30 minutes, the reactions were analyzed with a 4% native polyacrylamide gel.
ACTIVATION OF JAK KINASES BY IL-9

RESULTS AND DISCUSSION

IL-9 induces tyrosine phosphorylation of JAK1, JAK3, and Tyk2. It has been shown that IL-9 is a mitogenic cytokine for MO7E cells. Although previous studies suggested that tyrosine kinases are involved in the signaling pathways mediated by IL-9 in MO7E,20 such tyrosine kinases have not been identified and characterized. In an attempt to identify tyrosine kinases involved in signaling pathways mediated by IL-9 in MO7E cells, we initially examined whether Src family kinases including Src, Lyn, and Fyn are involved in IL-9 signaling in MO7E cells. Our results showed no evidence that Src, Lyn, and Fyn are activated by IL-9 (our unpublished results). Recently, JAK family nonreceptor tyrosine kinases have been shown to be involved in signaling pathways mediated by cytokines.15-25 To investigate whether IL-9 can induce tyrosine phosphorylation of JAK family tyrosine kinases including JAK1, JAK2, JAK3, and Tyk2 in MO7E cells, we performed immunoprecipitation experiments with antibodies for JAK1, JAK2, JAK3, and Tyk2 followed by immunoblotting with antiphosphotyrosine antibodies. As shown in Fig 1A and B, JAK1, JAK3, and Tyk2, but not JAK2, kinases are tyrosine phosphorylated in MO7E cells after IL-9 stimulation. However, the extent of tyrosine phosphorylation among JAK1, JAK3, and Tyk2 were quite different (JAK1 > JAK3 > Tyk2). The kinetic studies indicated that tyrosine phosphorylation of JAK1, JAK3, and Tyk2 triggered by IL-9 occurred within 1 minute, peaked by 5 to 10 minutes, and persisted at least 45 minutes (Fig 1C through E). Rapid tyrosine phosphorylation of JAK1, JAK3, and Tyk2 induced by IL-9 suggests that JAK kinases are actively involved in IL-9 signaling pathways.

IL-9 activates JAK1, JAK3, and Tyk2 in vitro kinase activity. JAK1, JAK3, and Tyk2 are rapidly tyrosine phosphorylated on stimulation with IL-9 in MO7E cells. We next examined whether tyrosine phosphorylated JAK kinases are activated by performing immune complex kinase assays. As shown in Fig 2, A through C, in vitro kinase activity of JAK1, JAK3, and Tyk2 was significantly increased following IL-9 stimulation. The kinetics of kinase activation corre-
lated with the kinetics of tyrosine phosphorylation seen in Fig 1, C through E. These results indicated that JAK kinases are not only tyrosine phosphorylated, but also activated by IL-9. The rapid tyrosine phosphorylation and activation of JAK family kinases strongly suggest that JAK kinases may directly interact with IL-9 receptor and play an important role in the signaling pathways mediated by IL-9. We also analyzed the phosphoamino acid content of the in vitro auto-phosphorylated JAK kinases. The results in Fig 2D show that JAK1, JAK3, and Tyk2 are phosphorylated on tyrosine, as well as serine/threonine residues. Serine/threonine phosphorylation of JAK kinases has been observed previously in other cytokine systems. The significance of serine/threonine phosphorylation of JAK kinases in the in vitro kinase assay requires further investigation.

Our studies showed that JAK tyrosine kinases, but not Src family tyrosine kinases, are tyrosine phosphorylated and activated by IL-9, extending the list of cytokines that utilize JAK family kinases in signaling. Leonard et al. have recently shown that IL-9 activates JAK1 and JAK3 kinases in T lymphocytes. Unlike GM-CSF, IL-3, or erythropoietin, which has been shown to preferentially activate JAK2 kinase. IL-9 induced tyrosine phosphorylation and activation of three (JAK1, JAK3, and Tyk2) known members of JAK kinases in M07E cells, although the extent of tyrosine phosphorylation (JAK1 > JAK3 > Tyk2) and activation (JAK3 > JAK1 > Tyk2) is different. We have previously shown tyrosine phosphorylation and activation of JAK1, but not JAK2 or Tyk2 kinase, in T lymphocytes after IL-9 stimulation, although the protein level for JAK1, JAK2, and Tyk2 is the same in T cells. These results strongly suggest that cell type specificity can determine which members of JAK tyrosine kinases will be activated in response to the same cytokine.

IL-9 induces tyrosine phosphorylation of Stat 91 and an 88-kD Stat 91-associated protein in M07E cells. Our results clearly indicated that JAK family tyrosine kinases are rapidly tyrosine phosphorylated and activated in M07E cells on stimulation with IL-9. JAK kinase activation may result in tyrosine phosphorylation and activation of Stat family of transcription factors, which were originally described in the interferon system and recently extended to other cytokines. To examine whether Stat 91 is tyrosine phosphorylated and translocated to the nucleus in M07E cells following IL-9 stimulation, the immunoprecipitation experiments with anti-Stat 91 antibodies were performed. The results showed that IL-9 rapidly induced tyrosine phosphorylation and activation of Stat 91 or transcription factors antigenically related to Stat 91 (Fig 3A and B). Our results in Fig 3 clearly indicated that two tyrosine phosphorylated proteins (91 and 88 kD) are immunoprecipitated by anti-Stat 91 antibodies. However, reimmunoblotting of the same membrane with anti-Stat 91 antibodies showed that 91, but not 88 kD protein was recognized by anti-Stat 91 antibodies. Furthermore, the 88 kD protein cannot interact with antibodies against the SH2 domain of Stat 91 protein (data not shown), indicating that the 88-kD protein is not a degradation product of Stat 91. The identity and the role of the Stat 91 associated protein in the signaling pathways mediated by IL-9 in M07E cells requires further investigation.
with 100-fold excess of cold GAS probes but not with 100-fold excess of high-affinity SIE probes (Fig 4B). Furthermore, nuclear extracts from IL-9-stimulated M07E cells did not bind the SIE sequence (data not shown). In comparison with antiphosphotyrosine immunoblotting experiments, the kinetics of GAS binding activity correlated with the extent of tyrosine phosphorylation of the 88-kD Stat 91-associated protein, but not with Stat 91 or proteins antigenically related to Stat 91 (Fig 3B and Fig 4A). These results strongly suggest that the GAS binding proteins in M07E cells are not identical to Stat 91 and, therefore, may represent specific Stats activated by IL-9.

Recent studies with interferons have uncovered a signaling pathway from the cell surface receptor to the nucleus. It is believed that the Stat family of latent cytoplasmic transcription factors, which can be activated by tyrosine phosphorylation, is a key mediator that transduces cell surface signals triggered by interferons to the nucleus. On ligand stimulation, JAK family tyrosine kinases are tyrosine phosphorylated and activated. The Stat family transcription factors are subsequently activated through phosphorylation of tyrosine residues, presumably by activated JAK kinases. The activated Stat proteins are translocated to the nucleus where they bind to specific DNA elements and ultimately stimulate transcriptions of certain genes. The model of interferon signaling pathway from the cell surface receptor to the nucleus has recently been extended to other cytokines and may also be used by IL-9, as we have shown in this report. IL-9 stimulation resulted in rapid tyrosine phosphorylation of Stat 91 or antigenically related Stat and the 88-kD Stat 91-associated protein, which rapidly translocated to the nucleus in M07E cells. Gel shift assays showed specific GAS binding activity in M07E cell nuclear extracts after IL-9 induction, suggesting that Stat 91-related Stat proteins are activated. It should be pointed out that a number of cytokines or growth factors can activate JAK kinases and Stat proteins. However, activation of JAK kinases and Stat proteins is not identical among individual cytokines. The different combination of JAK kinase and Stat protein activation may determine the specificity of each cytokine. Taken together, our results show that IL-9 uses JAK kinase-Stat signaling cascades, and the specificity for IL-9 may be achieved by activation of Stat 91-related proteins and an unidentified 88 kD protein that stably associates with Stat 91-related protein.

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