Identification of LIL-STAT in monocytic leukemia cells and monocytes after stimulation with interleukin-6 or interferon γ

Henny H. Lemmink, Leonore Tuyt, Gerlinde Knol, Ellen Krikke, and Edo Vellenga

In acute myelogenous leukemia (AML) and adult T-cell leukemia, it has been demonstrated that the transcription factor LIL-STAT is constitutively activated. To identify and characterize this unknown LIL-STAT protein, electrophoretic mobility shift assay (EMSA) and oligoprecipitation assays were performed by using lipopolysaccharide/interleukin-1 (IL-1)–responsive element (LILRE) oligonucleotide probes. EMSA demonstrated a significant increase in LIL-STAT binding to the LILRE oligonucleotides after interferon-γ (IFN-γ) and IL-6 stimulation of THP-1 cells. In unstimulated THP-1 and AML cells, LILRE oligonucleotide probes bound only to STAT1 α and β isoforms. The LILRE element showed a significant increase in binding of both α and β isoforms of STAT1 and STAT3 upon IFN-γ and IL-6 stimulation. Similar results were observed with human monocytes upon IL-6 or IFN-γ stimulation. These studies indicate that LIL-STAT consists of STAT1 and STAT3 proteins that bind to the LILRE DNA consensus site in a stimulus-dependent way.

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Study design

Cell culture, AML cells, cytokines, and antibodies

Human monocytic leukemia cells, namely THP-1 and AML cells, were isolated as described before. Nuclear proteins were extracted from leukemic cells and monocytes after 10 minutes of incubation with 10 ng/mL recombinant human IFN-γ (Endogen, Woburn, MA) or 10 ng/mL IL-6. Peripheral blood cells or bone marrow cells were obtained from AML patients after informed consent. AML blasts were cultured at 37°C in RPMI 1640 medium (Flow, Rockville, MD) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.1% fetal calf serum for 4 hours before cytokine stimulation and subsequent nuclear extract preparation. Mononuclear cells were obtained from healthy blood donors after informed consent, and monocytes were isolated as described earlier. Polyclonal antibodies directed against the N-terminal domain (amino acids 1-194) of STAT1 (G16930) and monoclonal antibodies against the C-terminal domain of STAT1 (sc-346) and STAT3 antibodies (C-20: sc-482; F2: sc-8019) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-STAT3 (Y705) antibodies (no. 9131) were purchased from Cell Signaling.

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RESULTS AND DISCUSSION

BINDING OF STAT1 AND STAT3 TO DIFFERENT GAS ELEMENTS

Nuclear extracts were isolated from THP-1 and B cells after stimulation with IL-6 and IFN-γ. The binding of STAT proteins was monitored by EMSA analysis using LILRE as oligonucleotide probe. IL-6 and IFN-γ stimulation of THP-1 cells increased STAT binding to the radiolabeled LILRE probe (Figure 1A). LIL-STAT binding was completely inhibited by addition of excess LILRE oligonucleotides and partially inhibited by hSIE oligonucleotides (Figure 1B). In contrast, LIL-STAT binding was not inhibited by addition of unlabeled FcγRI oligonucleotides (Figure 1B). By incubating nuclear extracts of unstimulated THP-1 cells with polyclonal anti-STAT1 antibodies, it was shown that the LIL-STAT binding decreased (Figure 1A) as well as upon IL-6 and IFN-γ stimulation (data not shown). To identify STAT oligonucleotide binding proteins as detected in EMSAs, we subsequently performed oligoprecipitations.

IDENTIFICATION OF STAT1 AND STAT3 BINDING TO THE LILRE GAS SITE IN MYELOID LEUKEMIA CELLS AND MONOCYTES

Biotinylated double-stranded LILRE and STAT1-STAT3 (S1-S3) oligonucleotides coupled to streptavidin-coated sepharose beads were incubated with nuclear extracts from unstimulated, IL-6-stimulated, and IFN-γ-stimulated THP-1 cells, AML cells, and monocytes. The oligoprecipitated proteins were separated on SDS-PAGE and analyzed by Western blot using antibodies against STAT1 and STAT3. As shown for unstimulated THP-1 cells, there was binding of STAT1 to LILRE and S1-S3 oligonucleotides (Figure 2A). In the case of IL-6 and IFN-γ stimulation, there was a strong increase of STAT1 binding to both GAS oligonucleotide elements (Figure 2A). In the case of IL-6 and IFN-γ stimulation, there was a strong increase of STAT1 binding to both GAS oligonucleotide elements (Figure 2A). In the case of IL-6 and IFN-γ stimulation, there was a strong increase of STAT1 binding to both GAS oligonucleotide elements (Figure 2A). In the case of IL-6 and IFN-γ stimulation, there was a strong increase of STAT1 binding to both GAS oligonucleotide elements (Figure 2A).
Western blot by using antibodies against phosphotyrosine (pY) STAT3 (Figure 2C). No phospho-STAT3 protein binding was detected for the LILRE or S1-S3 oligoprecipitations using unstimulated THP-1 nuclear extracts (data not shown). However, after IL-6 stimulation, 2 proteins of approximately 90 and 84 kd could be demonstrated. IL-6 stimulation strongly induced binding of both α and β STAT3 isoforms to the LILRE and S1-S3 oligonucleotide-coupled beads (Figure 2C). A minor small band of about 70 kd was detected as well. This may be a proteolytic product of STAT3 α or β that is still capable of DNA binding. STAT3 α and β were precipitated by LILRE on nuclear extracts from IL-6-stimulated THP-1 cells using STAT3 (F2) antibodies (Figure 2C, lane 2), and minor pY-STAT3 α binding was observed in IFN-γ-stimulated THP-1 cells (Figure 2C, lanes 5-6). To further assess the specificity of LILRE oligoprecipitation, we completely abolished the pY-STAT1 and pY-STAT3 binding by unbiotinylated double-stranded LILRE oligonucleotides (Figure 2B,D). In contrast, the addition of nonspecific TRE oligonucleotides did not affect the pY-STAT3 binding (Figure 2D). Similarly, the binding of pY-STAT1 to LILRE was inhibited by unlabeled LILRE (Figure 2B), but no inhibition was obtained in the presence of TRE oligonucleotides (data not shown).

To exclude the binding of tyrosine-phosphorylated proteins other than STAT1-STAT3, we performed additional oligoprecipitation experiments and analyzed them by immunoblot using phosphotyrosine antibodies (PY100). Again, tyrosine-phosphorylated proteins were identified by LILRE and S1-S3 oligoprecipitations in nuclear extracts isolated from IL-6-stimulated and IFN-γ-stimulated THP-1 cells. In the IL-6 sample, 2 tyrosine-phosphorylated proteins of approximately 85 and 90 kd were precipitated by LILRE and S1-S3 oligonucleotides, resembling the molecular weights of STAT3 α and β isoforms. In the IFN-γ sample, a protein band of about 90 kd was detected, which correlates with the molecular weight of STAT1 (Figure 2E). Importantly, no other tyrosine-phosphorylated proteins of different molecular weights could be identified by LILRE and S1-S3 oligoprecipitation.

Finally, oligoprecipitations were performed to identify STAT binding to LILRE in AML cells, which showed a relatively high LILRE binding activity on EMSA (data not shown). As a result, pY-STAT1 was precipitated in the unstimulated AML cells with high LILRE binding (Figure 3A). Upon IFN-γ stimulation, the STAT1 LILRE binding was increased, whereas IL-6 stimulation showed the same amount of STAT1 binding as the unstimulated AML sample (Figure 3A). The binding of STAT1 to LILRE-coupled sepharose beads is LILRE oligonucleotide-mediated because no STAT1 binding was observed in the control precipitations using only streptavidin sepharose beads (Figure 3A, lanes 1, 3, and 5). pY-STAT3 was bound to the LILRE oligonucleotides in IL-6-stimulated AML cells, and was not precipitated in the unstimulated condition or after IFN-γ stimulation (Figure 3A, lanes 8 and 12).

In our previous study, we detected LILRE binding activity in CD34+ cells, which was absent upon differentiation into monocytes and granulocytes. Therefore, nuclear extracts isolated from unstimulated, IL-6-stimulated, and IFN-γ-stimulated monocytes were tested by LILRE oligoprecipitation. In contrast to THP-1 cells, unstimulated monocytes did not show LILRE binding of STAT1 α and STAT3 α, but only a very small amount of the β subunit. IL-6 stimulation clearly showed binding of both STAT1
and STAT3 α and β subunits (Figure 3B). In the case of IFN-γ stimulation, STAT1 bound to the LILRE sequence but in concert with the STAT3 β subunit (Figure 3B). These results are in contrast to previous results demonstrating no LILRE binding activity in IFN-γ-stimulated monocytes as detected by EMSA. The discrepancy might be explained by the fact that LILRE oligoprecipitations were performed in the presence of much more nuclear protein than used in the EMSA.

Based upon our studies, we have identified STAT1 and STAT3 as being the STAT variants that bind to the LILRE DNA element, depending on the stimulus. Unfortunately, because of the low resolution of the LILRE EMSA, we cannot discriminate between STAT1 and STAT3 homodimers or determine whether STAT1-STAT3 heterodimers are involved. However, based upon the relative abundance of STAT3 bound to the LILRE oligonucleotide upon IL-6 stimulation, and of STAT1 upon IFN-γ stimulation, we conclude that the LILRE complex most likely consists of STAT3 or STAT1 homodimers in monocytic leukemia cells and monocytes, depending on the stimulus. Additionally, the involvement of other proteins that interact with STAT1 and STAT3 bound to the LILRE oligonucleotide is possible. Recent reviews have already described the interactions of STATs with other proteins that are also stimulus- and cell-type specific. The binding of STAT1 and STAT3 to the LILRE site present in the IL-1β gene promoter might modulate IL-1β gene expression. Indeed, IL-6 and IFN-γ have been identified as positive modulators of IL-1β production in macrophage cell lines. The finding of STAT1-STAT3 protein association with the LILRE binding site in the IL-1β gene promoter is in line with previous reports showing constitutive activation of STAT1-STAT3 and IL-1β gene expression in AML and adult T-cell leukemia cell lines. Whether constitutive LILRE binding in AML or adult T-cell leukemia cells affects IL-1β gene expression remains to be elucidated.

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