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IL-6 Induces Expression of the *Fli-1* Proto-oncogene via STAT3[1]

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Induction of gene expression by IL-6 has become an area of intense interest due to the role this cytokine plays in mediating aspects of inflammation, cellular differentiation, and proliferation. The ETS family of transcription factors represents a group of positive and negative regulators of transcription, that are differentially expressed in a cell and tissue specific manner. The ETS protein Fli-1 is known to induce differentiation in the erythroblastic leukemia cell line K562 along megakaryocytic developmental pathways. Here we show that IL-6 treatment of K562 induces the expression of *Fli-1* via the STAT3 transcription factor. Upregulation of *Fli-1* expression can be abrogated by the addition of AG490, a chemical inhibitor of JAK kinases, and by transfecting the cells with a dominant negative STAT3 expression construct.

The *Fli-1* proto-oncogene, a member of the ETS family, was first discovered in erythroleukemias induced as a result of retroviral integration by the Friend Leukemia Virus, into the upstream region of the *Fli-1* gene (1). The *Fli-1* transcription factor is expressed in many hematopoietic cell types, and it's expression in endothelial cells also suggests a role in angiogenesis (2). K562 erythroleukemia cells express low levels of both erythrocytic and megakaryocytic specific genetic markers, and can be induced to differentiate along one of these two major pathways depending upon the external stimuli applied to the cells (3). The ETS family of hematopoietic transcription factors, of which *Fli-1* is a member, plays an important role in this differentiation process (4). *Fli-1,* along with other factors, induces K562 cells to enter the megakaryocytic differentiation pathway by transactivating important megakaryocytic marker genes, including the thrombopoietin receptor (MPL) (5), glycoprotein IX, and glycoprotein IIb (GpIIb) (6). Conversely, erythroleukemic cells induced to progress along erythroid differentiation pathways express greatly reduced levels of *Fli-1,* and ectopic overexpression of *Fli-1* in these cells serves to reduce the levels of erythroid markers present (7). Not surprisingly, the homozygous knock-out of the *Fli-1* gene in mice produces neuro-vascular hemorrhaging due to lack of platelets, resulting in fetal death on day 11 (8). Hematopoietic development is also affected with a reduction in the numbers of pronormoblasts, basophilic normoblasts, and colony-forming cells (8).

Interleukin-6 is a known inducer of thrombocyte differentiation in K562 cells, driving the cells towards megakaryocytic development, and repressing γ-globin gene mRNA production (9). The *s*ignal *t*ransducers and *a*ctivators of *t*ranscription (STAT) are activated in response to cytokine receptor stimulation, via tyrosine phosphorylation (10, 11). Therefore, it is of interest to determine the molecular signaling pathways involved in the induction of *Fli-1* expression, and whether IL-6 is part of this mechanism. Here we show that IL-6 regulates *Fli-1* gene expression via STAT3, thus identifying an important transcriptional pathway for the effects of IL-6 on megakaryocyte development.

MATERIALS AND METHODS

Cell culture. 293 cells were obtained from the American Type Culture Collection (CRL-1573) and maintained in DMEM high glucose supplemented with 10% FBS, Glutamine and penicillin-streptomycin solutions. Human erythroleukemia K562 cells were also obtained from the ATCC (CCL-243), and were maintained in

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RPMI-1640 media supplemented with 10% FBS. Recombinant interleukin-6 (Cat. 200-06) was purchased from Pepro-Tech Inc. (Rocky Hill, NJ, USA).

RT-PCR assays. To examine the effects of IL-6 on *Fli-1* expression, K562 cells were rinsed twice in PBS pH 7.4, re-suspended in RPMI-1640 media supplemented with 0.05% FBS for 48 hours, then stimulated with 100 ng/mL IL-6. Cells were collected and RNA was harvested for RT-PCR assays to determine the relative amounts of *Fli-1* expression from each experimental condition described. Kinase inhibitors AG-490, and PD98059 were added 60 minutes prior to IL-6 stimulation, and used at a concentration of 50 μM as described in the text. All cDNAs were prepared from TriZol (Invitrogen, Inc., Gaithersburg, MD) extracted total RNAs, and RT reactions were run on 2 μg total RNA. Following RT reactions, PCR reactions were run to the mid-point of each PCR fragment's linear synthesis curve. GAPDH bands on agarose gels were scanned on a *TYPHOON* Scanner (Molecular Dynamics) to ensure equalization of expression levels at each time point. Primers specific for the *Fli-1* gene (GenBank Accession Number M98833) were 5′-CGCCACCACCCTCTACAACACGGAA-3′ nt. 703-728 (sense), and 5′-CGGGCCCAGGATCTGATACGGATCT-3′ nt. 952-977 (anti-sense) which yielded a 274 bp. fragment. Amplification primers specific for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene 5′-AGGTGAAGGTCGGAGTCAACGG-3′ (sense), and 5′-CCCAGCCTTCTCCATGGTGGTG-3′) (anti-sense) were utilized to amplify a constitutively expressed internal control fragment of 319 bp.

Luciferase assays. The pGL3Promoter plasmid (Promega) contains the SV40 promoter positioned downstream of a multiple cloning site followed by the luciferase reporter gene. We transfected a pGL3-Promoter vector construct containing a portion of the *Fli-1* promoter (GenBank Accession Number L-47416, nts. 2165-2299) into either 293HEK, or human erythroleukemia K562 cells. Expression plasmids encoding a known JAK2 inhibitor protein, JAB, a dominant-negative STAT3, and a constitutively-activated STAT3, were used in co-transfection experiments.

An empty expression plasmid was co-transfected along with the reporter vector to maintain consistent amounts of DNA for a reporter baseline activation level in response to IL-6. Transfected cells were incubated for 36 hours then harvested, and lysed in 1× luciferase lysis buffer (20 mM Tris-Cl pH 7.8; 1% Triton X-100 (V/V); 0.1 mM EDTA; 1 mM DTT added just prior to use); 200 μL of medium was removed for normalization assays using the CLONTECH pSEAP positive control vector and CSPD chemiluminescent substrate kit (CSPD disodium 3-(4-methoxyspiro [1,2-dioxetane-3, 2′-(5chloro)tricyclo[3.3.1.1^{3,7}]-decan]-4-yl) phenyl phosphate Cat. K-2041-1) prior to lysis. Experiments to determine luciferase activity for each condition were run in triplicate and normalized against CSPD substrate assay values per μg of protein to ensure consistent transfection levels between experiments. Transactivation results are shown as the fold-increase ratios between unstimulated cells and those treated with 100 ng/mL IL-6.

RESULTS AND DISCUSSION

IL-6 upregulates Fli-1 mRNA expression. K-562 cells were quieted in 0.05% FBS for 48 hours, treated with IL-6 at a concentration of 100 ng/mL, and collected at various time-points. The total RNA from each time-point was harvested, and cDNA synthesized to determine if *Fli-1* expression is affected by treatment with IL-6. Gel electrophoresis of each PCR reaction time-point shows that expression of the 274 bp *Fli-1* band first appears at 4 hours post IL-6 treatment (Fig. 1), and continues to increase throughout the collection period, while GAPDH expression remains relatively constant throughout the collection period. Based on RT-PCR analysis, expression levels of several common

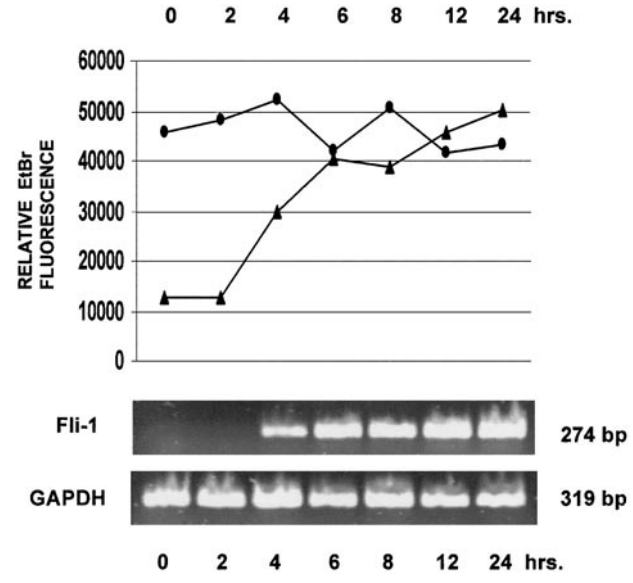

FIG. 1. IL-6 stimulation of K-562 cells results in upregulation of *Fli-1* expression. RT-PCR of total RNA from K-562 cells treated with 100 ng/mL IL-6. Induction of *Fli-1* expression (upper gel panel) is evident beginning at 4 hours post-IL-6 treatment. GAPDH levels (lower gel panel) remain relatively constant throughout the sampling period. Shown above each *Fli-1* and GAPDH time-point PCR band is the corresponding relative EtBr fluorescence intensity value as determined by scanning with Typhoon Digital Scanner, Molecular Dynamics, Inc. (▲) *Fli-1* levels; (●) GAPDH levels.

ETS family transcription factors (ETS 1 and 2, PU-1, and PEA-3) were not affected by treatment with IL-6 (data not shown).

Use of inhibitors to determine IL-6 stimulation pathway. Next, K-562 cells were rested in low serum as described previously, pre-treated for one hour with 50 μM of the MEK-1 Kinase inhibitor PD-98059, or the JAK Kinase inhibitor AG-490, followed by IL-6 stimulation to determine which pathways were involved in the induction of *Fli-1* expression. After 8 hours exposure to 100 ng/mL IL-6, total cellular RNA was harvested and RT-PCR was performed to assess levels of gene expression. As shown in the upper panel of Fig. 2, lanes 1 and 2, which received no inhibitor pretreatment, expression of *Fli-1* is induced only in cells treated with IL-6 (lane 2), while the unstimulated control (lane 1) shows no induction. Treatment of cells with the MEK-1 inhibitor PD-98059 does not inhibit IL-6 mediated induction of *Fli-1* expression (lane 4), and once again the unstimulated control cells (lane 3) show no induction of expression for *Fli-1*, indicating that the p42/44 MAP kinase pathway does not contribute significantly to the observed IL-6 mediated increase of *Fli-1* expression. However, treatment of K-562 cells with the JAK Kinase inhibitor AG-490, completely inhibits expression of *Fli-1* (lane 6). As expected, Lane 5, the unstimulated control, shows no expression of *Fli-1*. IL-6 alone, PD-98059, and AG-490

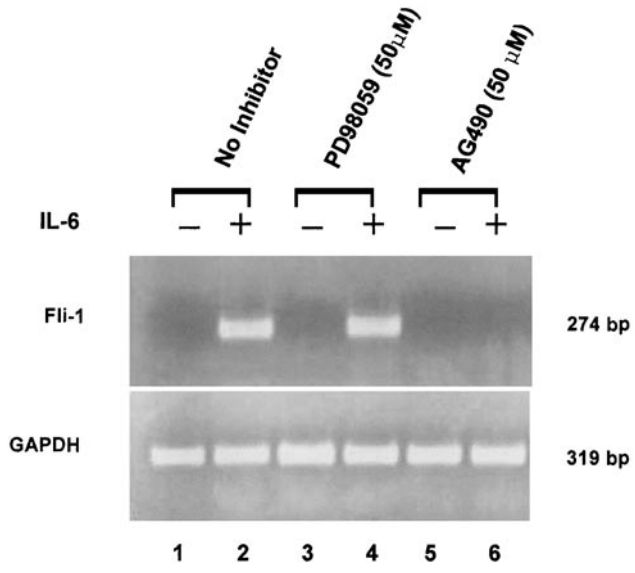

FIG. 2. Use of MAPK and JAK inhibitors to determine IL-6 stimulation pathways in K562 by RT-PCR analysis. Rested K562 cells were treated with MAPK and JAK inhibitors AG-490, and PD98059 respectively, at a concentration of 50 μM to determine their effect on IL-6 stimulation pathways in K562. K562 cells were quieted for 48 hours in RPMI-1640 w/0.1% FBS, then 5×10^6 cells were treated with inhibitor (or carrier) 60 minutes prior to IL-6 addition as indicated. IL-6 treatment was terminated at 8 hours and total RNA isolated for RT-PCR. Upper Panel shows *Fli-1* expression, and Lower Panel shows GAPDH expression with and without IL-6 treatment for each condition as indicated at the top of the figure.

treatment does not appear to influence the levels of GAPDH expression, as shown in the lower panel of Fig. 2. This experiment shows that the stimulation of *Fli-1* expression by IL-6 is mediated by a JAK kinase, and that blocking the p42/44 MAP Kinase pathway does not adversely affect *Fli-1* expression induced by IL-6 treatment.

STAT3 induces Fli-1 expression. Since treatment of K562 cells with IL-6 induces expression of the *Fli-1* transcription factor, we wanted to determine by what mechanism IL-6 produced this effect. Computer assisted searches of potential STAT binding sites within the entire *Fli-1* promoter region revealed only one potential site, containing three putative STAT DNA binding elements (Fig. 3). This region of the *Fli-1* promoter is located approximately 250 base pairs upstream of the initiation codon for *Fli-1*, and contains three potential STAT3 binding sites in close proximity to each other. The consensus sequence for STAT3, 5′-TTMXXXDMA-3′, where D is A, G, or T and M is A or C, based on several previously identified binding sites, is shown for comparison (12). These three putative STAT3 binding sites reside within a previously characterized enhancer region, which was shown to bind GATA and other ETS transcription factors (13, 14). Having already established that IL-6 increases *Fli-1* mRNA levels *in vivo* (Fig. 1), the 126 bp Sac1-Hpa1 fragment containing the potential STAT3 binding elements was excised from its original vector (a gift from B. Barbeau, Ref. 14), sub-cloned into the pGL3-Promoter vector, and analyzed for it's ability to respond to IL-6 stimulation. First, non-hematopoietic 293HEK cells, which are RT-PCR negative for *Fli-1* expression but responsive to IL-6, were transiently transfected with pGL3-Prom-*Fli-1* reporter vector and empty expression plasmid (NULL), then with expression plasmids for JAB, Dominant-Negative STAT3 (STAT3DN), and constitutively activated STAT3 (STAT3C). The cells were rested in low serum media overnight, treated with IL-6 at 100 ng/mL, and later harvested for luciferase assays. Figure 4, panel A, lane 1 shows a five-fold activation (compared with the IL-6 untreated control) resulting from IL-6 stimulation of the pGL3-Prom-*Fli-1* reporter vector, and empty expression vector. Co-transfection of the known JAK-2 kinase inhibitory protein JAB, along with the pGL3-Prom-*Fli-1* reporter plasmid (panel A, lane 2), reduces luciferase activity to approximately two-fold. Similarly, inclusion of the dominant negative STAT3DN expression construct (Fig. 4, panel A, lane 3) greatly reduces luciferase activity to approximately background levels as compared to the IL-6 untreated control. Co-transfection of a plasmid expressing constitutively activated STAT3C (panel A, lane 4) produces an approximately fourteen-fold increase in luciferase activity. Repeating these same experiments in K562 cells produced similar results as shown in panel B of Figure 4. The ability of JAB (Fig. 4, panel B, lane 2) and the dominant negative STAT3DN (panel B, lane 3) construct to down-regulate expression from the pGL3-Prom-*Fli-1* reporter supports the conclusion that the JAK2 activated STAT3 transcription factor appears to be a potent inducer of *Fli-1* expression following treatment of cells with IL-6. As expected, the null vector and

5'-TTMXXXDMA-3'

5'-TCCTCCCCGA**TTCGCAAA**GTGAAGTCAC**TTCCCAAA**A**TTAGCTGAA**AAAAAAGTTTCAT-3'
AGGAGGGGCTAAGCGTTTCACTTCAGTGAAGGGTTTTAATCGACTTTTTTTTCAAAGTA

FIG. 3. Putative STAT3 binding sites within the 126 bp Sac1-Hpa1 *Fli-1* promoter fragment (GenBank Accession Number L-47416, nts. 2165-2299) in pGL3PROM, STAT sites are bold and underlined. Consensus sequence of known Stat3 binding sites is shown for comparison, where D is A, G, or T and M is A or C.

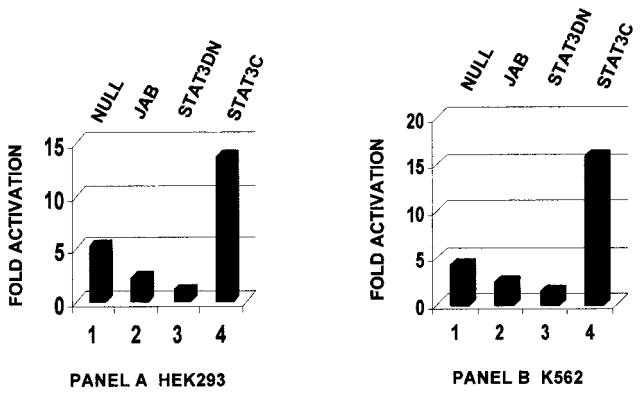

FIG. 4. Response of putative STAT DNA binding elements in the *Fli-1* promoter to IL-6 stimulation. The pGL3-Prom-*Fli-1*-STAT3 Luciferase Reporter Vector was co-transfected into either 293 (panel A) or K-562 (panel B) cells, along with Empty or Null expression vector (lane 1), the JAK2 inhibitor JAB (lane 2), the STAT3DN dominant negative (lane 3), or the STAT3C constitutively activated (lane 4) expression plasmids. Transactivation results are shown as the fold-increase ratios between unstimulated cells and those treated with 100 ng/mL IL-6.

pGL3-Prom-*Fli-1* reporter gives approximately four-fold activation, and the activated STAT3C construct greatly amplifies the activation of the reporter vector (Fig. 4, panel B, lane 4), about fifteen-fold. The ability of the dominant-negative STAT3 construct, STAT3DN, to significantly and specifically block activation of the pGL3-Prom-*Fli-1* luciferase reporter strongly suggests that wild-type STAT3 transcription is responsible for the observed IL-6 mediated increase in *Fli-1* mRNA levels *in vivo.* Since these putative STAT3 binding domains reside within a region already known to exhibit enhancer activity, our conclusions are consistent with previous observations regarding the importance of this promoter region in the regulation of *Fli-1* expression (14).

In this report, we have demonstrated the relationship between the inflammatory cytokine IL-6 and the proto-oncogene *Fli-1.* Stimulation of cells with IL-6 causes the activation of the STAT-3 transcription factor, shown previously to increase cell proliferation and mediate anti-apoptotic signaling pathways (15). The ability of *Fli-1* to induce expression of the anti-apoptotic protein Bcl-2, along with evidence which shows that *Fli-1* can inhibit expression of the cell cycle regulating Retinoblastoma protein (Rb), suggests an important role for *Fli-1* in tumor development, as well as hematopoietic differentiation, and may represent an important IL-6 mediated pathway (16, 17). Furthermore, it has been shown that the strong anti-apoptotic properties of IL-6 serve to increase the resistance of multiple myeloma cells to chemotherapeutic drugs, effectively blocking dexamethasone-induced apoptosis in human myeloma cells (18). This effect, previously shown to be mediated by AKT activation, may also involve the effects of *Fli-1* directed activity as well, given the data we have presented (18).

The induction of *Fli-1* expression by IL-6 mediated STAT3 transactivation may also affect other important cell survival and tumor promotion pathways. Interestingly, the ability of *Fli-1* to recognize and bind to the same or similar sites within many promoters as the prototypic ETS proteins Ets-1, and Ets-2, increases the likelihood that IL-6 mediated *Fli-1* expression could exert wide ranging effects on cellular function (19, 20). For example, the Flt-1 tyrosine kinase serves as a high-affinity receptor for the vascular endothelial growth factor (VEGF), and contains within it's promoter at least five known ETS family DNA binding sites (21). Treatment of stromal and microvascular endothelial cells with recombinant human (rh) VEGF increases their expression of IL-6, which can then increase VEGF expression and secretion from multiple myeloma cell lines (22). This cyclical relationship demonstrates how amplification of important tumor promoting pathways are cooperative in their effects, i.e., the potential upregulation of VEGF receptor Flt-1 by Ets factors such as *Fli-1* in response to IL-6 stimulation.

The ability of *Fli-1* to bind a wide array of similar ETS binding sites increases the range of potential targets to genes involved in tumor metastasis (23, 24, 25). The expression of the urokinase plasminogen activator (uPA) in prostate and other cancers, correlates with metastasis and serves as a prognostic indicator. The uPA promoter is transactivated by several ETS family proteins, and immuno-histological examination of prostate adenocarcinoma shows high levels of *Fli-1* and another ETS gene, Elf-1 (26). Another gene family involved in metastasis are the matrix metalloproteinases (MMP). Several promoter regions of these proteases have been examined, and all contain EBS sites that allow ETS family proteins to regulate their transcriptional activities (27).

Consistent with these observations, IL-6 mediated stimulation of *Fli-1* expression, a known activator of important tumor- and metastatic-promoting genes in numerous hematopoietic and vascular tumors, suggests that strategies directed towards ameliorating the effects of IL-6 might be warranted to block or reduce the activation of *Fli-1.* Furthermore, these findings lend support to the use of anti-inflammatory drugs during chemotherapy protocols as an adjunct to cytotoxic drugs.

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