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Activation of the Jun N-Terminal Kinase Pathway by Friend Spleen Focus-Forming Virus and Its Role in the Growth and Survival of Friend Virus-Induced Erythroleukemia Cells

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Members of the mitogen-activated protein kinase (MAPK) family, including Jun amino-terminal kinase (JNK) and extracellular signal-related kinase (ERK), play an important role in the proliferation of erythroid cells in response to erythropoietin (Epo). Erythroid cells infected with the Friend spleen focus-forming virus (SFFV) proliferate in the absence of Epo and show constitutive activation of Epo signal transduction pathways. We previously demonstrated that the ERK pathway was constitutively activated in Friend SFFV-infected erythroid cells, and in this study JNK is also shown to be constitutively activated. Pharmacological inhibitors of both the ERK and JNK pathways stopped the proliferation of primary erythroleukemic cells from Friend SFFV-infected mice, with little induction of apoptosis, and furthermore blocked their ability to form Epo-independent colonies. However, only the JNK inhibitor blocked the proliferation of erythroleukemia cell lines derived from these mice. The JNK inhibitor caused significant apoptosis in these cell lines as well as an increase in the fraction of cells in G_2/M and undergoing endoreduplication. In contrast, the growth of erythroleukemia cell lines derived from Friend murine leukemia virus (MuLV)-infected mice was inhibited by both the MEK and JNK inhibitors. JNK is important for AP1 activity, and we found that JNK inhibitor treatment reduced AP1 DNA-binding activity in primary erythroleukemic splenocytes from Friend SFFV-infected mice and in erythroleukemia cell lines from Friend MuLV-infected mice but did not alter AP1 DNA binding in erythroleukemia cell lines from Friend SFFV-infected mice. These data suggest that JNK plays an important role in cell proliferation and/or the survival of erythroleukemia cells.

Friend spleen focus-forming virus (SFFV), in conjunction with its natural helper virus Friend murine leukemia virus (MuLV), causes a rapid erythroleukemia when injected into susceptible adult or newborn mice (for a review, see reference 48). Friend SFFV, a replication-defective retrovirus, carries a unique *env* gene encoding a 55-kDa glycoprotein, which is responsible for its pathogenicity. The first stage of Friend SFFV-induced disease is characterized by splenomegaly and polycythemia, which is due to the polyclonal expansion and differentiation of erythroid cells in the absence of the erythroid hormone erythropoietin (Epo). This Epo-independent erythroblastosis is due to the interaction at the cell surface of SFFV gp55 with the erythropoietin receptor (EpoR) and a short form of the receptor tyrosine kinase Stk (sf-Stk) (6, 13, 25, 39). This interaction results in the constitutive activation of Epo and/or Stk signal transduction pathways, including the Ras/Raf-1/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase, and Jak/STAT pathways (32, 33, 38, 40). The second stage of the disease consists of the outgrowth of Friend SFFV-infected erythroid cells that have become transformed due to integration of the virus into the *Sfpi-1* locus (31, 43, 44). This leads to inappropriate expression of the *Sfpi-1* gene product, PU.1, in erythroid cells, causing a block in their differentiation and the outgrowth of transformed erythroleukemia cells (50).

Friend MuLV, the natural helper virus for Friend SFFV, can also cause erythroleukemia, characterized by splenomegaly and severe anemia, if injected alone into newborn mice (55). Unlike Friend SFFV, Friend MuLV is replication competent and does not carry any unique genes that are required for its pathogenicity. Rather, Friend MuLV interacts with specific endogenous retroviral envelope gene sequences in the mouse, generating a new virus, Friend mink cell focus-inducing virus, which is responsible for the first stage of the disease (47). The erythroid hyperplasia induced by Friend MuLV, in contrast to that induced by Friend SFFV, still requires Epo. Friend MuLV-induced erythroleukemia also has a transformation stage, which can be detected after several passages of primary erythroleukemic cells in mice. These cells have become transformed primarily due to virus integration at the *Fli-1* locus, resulting in up-regulation of the Fli-1 protein in erythroid cells (2, 3). Both PU.1 and Fli-1 belong to the *ets* oncogene family and have the ability to bind to specific DNA sequences. This allows them to alter the expression of distinct downstream target genes, consistent with their nonoverlapping involvement in the induction of erythroleukemias by Friend SFFV or Friend MuLV. Overexpression of PU.1 and Fli-1 blocks the differentiation of erythroid cells (50, 54), perhaps through modulating the Epo/EpoR or sf-Stk signal transduction pathways.

The MAP kinases constitute an important group of serine/threonine signaling kinases that modulate the phosphorylation, and therefore the activation status, of transcription factors and

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link transmembrane signaling with gene induction events in the nucleus (37). It has been shown that Epo can activate components of the MAP kinase pathway, including extracellular signal-related kinase (ERK), p38 MAPK, and Jun N-terminal kinase (JNK) (20, 30, 34, 35). Withdrawal of Epo has also been shown to activate p38 MAPK (56). JNKs were first described biochemically following exposure of cells to environmental stresses (23, 24) and are now recognized as being activated in a variety of cells by cytokines and growth factors (17). The JNKs have also been characterized by their ability to associate with and phosphorylate regulatory sites in the N terminus of the transcription factor c-Jun, a member of the activator protein 1 (AP1) complex (9) and to phosphorylate transcription factors such as ATF-2, ELK-1, NFAT, and p53 (8, 14, 28, 63). Although it has been shown that Friend SFFV, like Epo, can activate the MAP kinase ERK (32), little is known about the ability of Friend SFFV or Friend MuLV to activate JNK or the role of any of the MAP kinases in the proliferation and survival of Friend SFFV and Friend MuLV-induced erythroleukemia cells. The purpose of this study, therefore, was to investigate the role of MEK/ERK and JNK, using specific pharmacological inhibitors for these kinases, in the proliferation and survival of murine erythroleukemia cells.

MATERIALS AND METHODS

Cell lines and primary erythroleukemic cells. The erythroleukemia cell lines NP1, NP4, NP5, NP7, and NP13 (60) were established from mice infected with helper-free Friend SFFV polycythemia (SFFV-P) (59). These cell lines were maintained in Dulbecco minimal essential medium supplemented with 10% fetal calf serum (FCS). HCD-57 cells, Epo-dependent erythroleukemia cells derived from an NIH Swiss mouse infected with Friend MuLV (49), and HCD-57 cells in which Epo dependence had been abrogated by infection with Friend SFFV-P (49) were maintained in Iscove's modified Dulbecco minimal essential medium supplemented with 30% FCS and 5×10^{-5} M 2-mercaptoethanol, with or without Epo (2 U/ml). The Friend MuLV-transformed cell lines TP1, TP3 (41), CB7 (53), and HB22.2 (2) were maintained in Dulbecco minimal essential medium supplemented with 10% FCS. The Friend MuLV-transformed cell line HB9.2ED (16) was maintained in the same medium but with the addition of 2 U/ml of Epo. CB7, HB22.2, and HB9.2ED cell lines were kindly provided by Yaacov Ben-David (University of Toronto, Toronto, ON, Canada). Primary erythroleukemia cells were prepared by injection of Friend MuLV/Friend SFFV-P into adult NIH Swiss mice, as described previously (46).

Inhibitors. The protein kinase inhibitors PD98059 (12) and SP600125 (4) were purchased from Calbiochem (La Jolla, CA). The inhibitors were reconstituted in dimethyl sulfoxide (DMSO) to make a 5-mg/ml stock of PD98059 and a 10 mM stock of SP600125.

Cell proliferation assays. Cell proliferation was assayed by using the WST-1 reagent (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions and as previously described (38). Cells were plated in 96-well microtiter plates and incubated for 24 or 48 h with the concentrations of the MEK inhibitor PD98059 or JNK inhibitor SP600125 indicated in the figures before the addition of the cell proliferation reagent WST-1. After incubation of the samples in a humidified atmosphere, the absorbances of the samples were measured at 450 nm against those of background controls using an enzyme-linked immunosorbent assay reader.

Erythroid colony formation assays. Spleen cells from Friend SFFV-P-infected NIH Swiss mice were assayed for colony formation in methylcellulose medium as previously described (46). Briefly, spleen cells (10^6) were plated in 24-well dishes with 1% methylcellulose, 1% dialyzed bovine serum albumin (BSA), 30% FCS, L-glutamine, penicillin-streptomycin solution, 0.1 mM 2-mercaptoethanol, and DMSO or the inhibitors indicated above. After 72 h of growth at 37°C in a humidified atmosphere containing 5% CO_2, 100 μl of benzidine (2 mg/ml) was added to each well and 15 different square areas (2 by 2 mm/area) within a well under the microscope were saved as pictures by the Photoshop software in a computer. Numbers of hemoglobin-positive (blue) erythroid SFFV CFU were determined as small colonies of less than 10 cells or large colonies of more than 10 cells. The average was calculated for three wells per sample.

Cell cycle and flow cytometry. The cell cycle was analyzed by using flow cytometry of propidium iodide (PI)-stained cells. Cells (1×10^6) were fixed in 70% ethanol overnight at 4°C. The cells were washed in phosphate-buffered saline with 0.1% BSA. Cells were incubated with 1 U/ml of RNase A (DNase free) and 10 μg/ml of PI (Sigma Chemical Corp., St. Louis, MO) overnight at room temperature in the dark. Cells were analyzed by using a FACScan flow cytometer (Becton Dickenson, Rutherford, N.J.). Amounts of cells containing sub-G_1 DNA, indicating apoptosis, were determined as a percentage of the total number of cells. For annexin V staining, live cells were washed in phosphate-buffered saline and then incubated with annexin V-fluorescein isothiocyanate (R&D Systems, Minneapolis, MN) and PI for 15 minutes. Cells were analyzed using the FACScan flow cytometer.

Protein analysis. Cell lysates were prepared by resuspending cells in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 μg/ml each of aprotinin and leupeptin), followed by incubation on ice for 20 min. Insoluble components were removed by centrifugation, and protein concentrations were determined with a protein assay kit (Bio-Rad Laboratories, Hercules, Calif.). Proteins were separated by electrophoresis on Tris-glycine or NuPAGE Bis-Tris minigels (Invitrogen, Carlsbad, CA) and then transferred electrophoretically to nitrocellulose filters for Western blotting with anti-JNK, anti-phospho JNK, anti-MEK, and anti-phospho-MEK antibodies (Cell Signaling Technology, Beverly, MA, or Upstate Biotechnology, Lake Placid, NY), followed by visualization using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, N.J.).

Electrophoretic mobility shift assays. Nuclear extracts were prepared as previously described (40) and used in electrophoretic mobility shift assays. Ten to 30 μg of nuclear extract was incubated with a [^{32}P]ATP-labeled double-stranded DNA fragment corresponding to the consensus binding site for AP1 (sense strand, 5′CGCTTGATGACTCAGCCGGAA3′) or an AP1 mutant oligonucleotide (sense strand, 5′CGCTTGATGACTTGGCCGGAA3′) at room temperature for 30 min in the presence of binding buffer (13 mM HEPES [pH 7.9], 80 to 120 mM NaCl, 0.15 mM EDTA, 8% glycerol, and 1 mM dithiothreitol), 1 μg BSA, 0.25× protease inhibitor cocktail, 0.05% NP-40, and 3 μg of poly(dI-dC). A mutant AP1 oligonucleotide with a substitution of TG for CA in the AP1 binding motif was used as a negative control. Samples were then subjected to electrophoresis using a 6% polyacrylamide gel containing 5% glycerol with 0.5× Tris-borate-EDTA buffer. Gels were dried and complexes visualized by autoradiography. The integrity of the nuclear extracts obtained from drug-treated cells was demonstrated using a specific probe for another transcription factor (STAT5).

Statistical analysis. Statistical methods used in this study included Leven's test, one-way analysis of variance, Welch's modified one-way analysis of variance, and the Dunnett test for post hoc comparisons.

RESULTS

MEK and JNK are constitutively phosphorylated in primary leukemic spleens and erythroleukemia cell lines derived from Friend SFFV-infected mice. We previously demonstrated that Epo activates the Ras-Raf-MEK-ERK pathway in the Epo-dependent erythroleukemia cell line HCD-57 and that infection of these cells with Friend SFFV results in constitutive activation of this pathway (32). To determine if Friend SFFV infection of erythroid cells also results in the constitutive activation of JNK, we examined cells grown in the absence of Epo for the expression of phosphorylated JNK. As shown in Fig. 1A, JNK is phosphorylated in uninfected HCD-57 cells after stimulation with Epo but is constitutively phosphorylated in Friend SFFV-infected HCD-57 cells. JNK is also highly phosphorylated in primary leukemic spleens from Friend SFFV-infected mice (Fig. 1B), in five independently derived erythroleukemia cell lines from mice infected with helper-free Friend SFFV (Fig. 1C) and in five independent erythroleukemia cell lines from Friend MuLV-infected mice (Fig. 1D). Differences in the amounts of pJNK1 and pJNK2 were observed between the SFFV MEL lines and the F-MuLV MEL lines and may be due to differences in the stages of erythroid cell differentiation

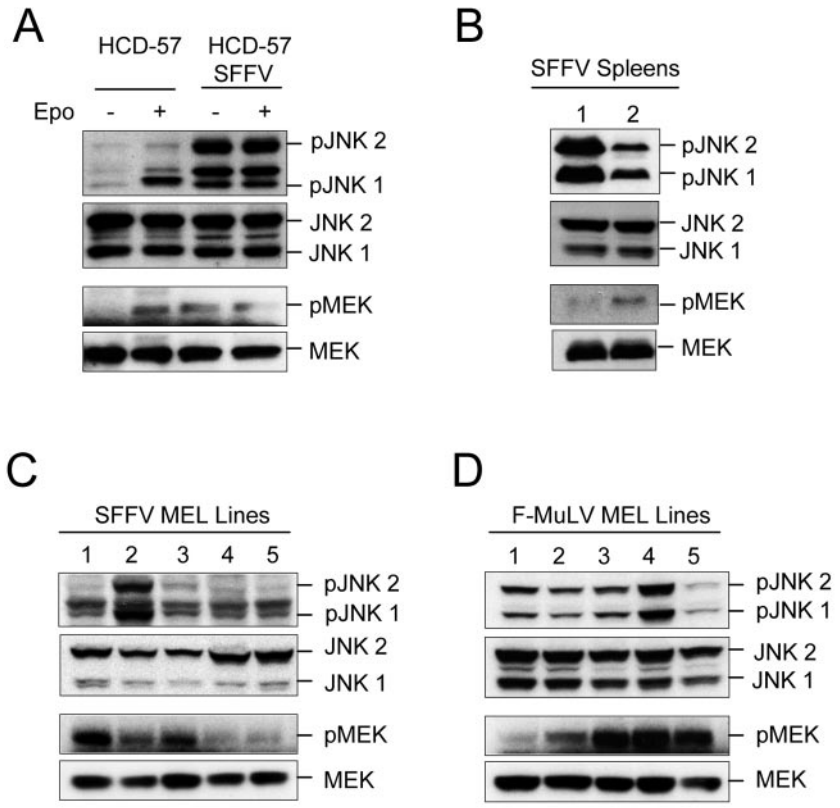

FIG. 1. Expression of phosphorylated MEK and JNK in erythroleukemia cells from Friend SFFV- and Friend MuLV-infected mice. JNK and MEK phosphorylation was determined in uninfected and Friend SFFV-infected HCD-57 cells grown in the presence or absence of Epo (A), primary erythroleukemia cells derived from the spleens of two separate Friend SFFV-infected mice (B), erythroleukemia cell lines (lane 1, NP1; lane 2, NP4; lane 3, NP5; lane 4, NP7; lane 5, NP13) derived from mice infected with helper-free Friend SFFV (C), and erythroleukemia cells lines derived from mice infected with Friend MuLV (lane 1, TP1; lane 2, TP3; lane 3, CB7; lane 4, HB22.2; lane 5, HB9.2ED) (D). Western blot analysis of total cell lysates was carried out using anti-phospho-JNK and anti-phospho-MEK antibodies. The filters were stripped and then incubated with anti-JNK or anti-MEK antibodies to determine total kinase levels.

between the cell lines. MEK is phosphorylated in all of these erythroleukemia cells (Fig. 1A to D).

Both MEK and JNK inhibitors block the proliferation of uninfected and Friend SFFV-infected HCD-57 cells. Previous studies have shown that the Epo-dependent proliferation of erythroid cells is dependent upon MEK/ERK and JNK (18, 27, 33). To determine whether Friend SFFV infection of erythroid cells alters their requirement for these kinases, we compared the Epo-dependent cell line HCD-57 with its Friend SFFV-infected, Epo-independent counterpart for their sensitivity to the MEK inhibitor PD98059 as well as to the JNK inhibitor SP600125, which blocks both JNK1 and JNK2. After treatment of these cells with PD98059 or SP600125 for 48 h, the amount of ERK or JNK kinase activity, respectively, was negligible (data not shown). To determine the effect of each inhibitor on the proliferation of these cells, the cells were grown in the presence of various concentrations of the inhibitor for 48 h and cell proliferation was determined using the WST-1 reagent. As shown in Fig. 2, blocking of either MEK (panel A) or JNK (panel B) inhibited cell proliferation in both HCD-57 and Friend SFFV-infected HCD-57 cells in a dose-dependent fashion. The kinetics of cell growth inhibition between Friend SFFV-infected and uninfected HCD-57 cells were similar, with 50% inhibitory concentrations of between 50 and 100 μM for PD98059 and approximately 5 μM for SP600125. Thus, activation of both MEK and JNK by both Epo and Friend SFFV appear to be critical for the proliferation of erythroid cells.

We also examined the percentages of apoptotic cells in cultures treated with the MEK and JNK inhibitors by flow cytometry analysis of PI-stained cells (Fig. 2C). Cells containing sub-G_1 DNA, indicative of apoptosis, were gated and shown as a percentage of the total number of cells. After 24 h, only SP600125 induced apoptosis (data not shown). After 48 h, both drugs caused an increase in the percentage of apoptotic cells, although a higher percentage of Friend SFFV-infected cells became apoptotic, especially after treatment with the JNK inhibitor (49% of the SP600125-treated HCD-57/SFFV cells versus 19% of the uninfected HCD-57 cells) (Fig. 2C). Treatment of uninfected and Friend SFFV-infected HCD-57 cells with SP600125, but not PD98059, increased the number of cells in the G_2/M phase fraction, and a few multinucleated cells could be seen in the cultures after 3 to 4 days (data not shown).

The proliferation of both primary and immortal erythroleukemia cells from Friend SFFV-infected mice is inhibited by treatment with a JNK inhibitor, but only primary erythroleukemia cells are inhibited by treatment with a MEK inhibitor.

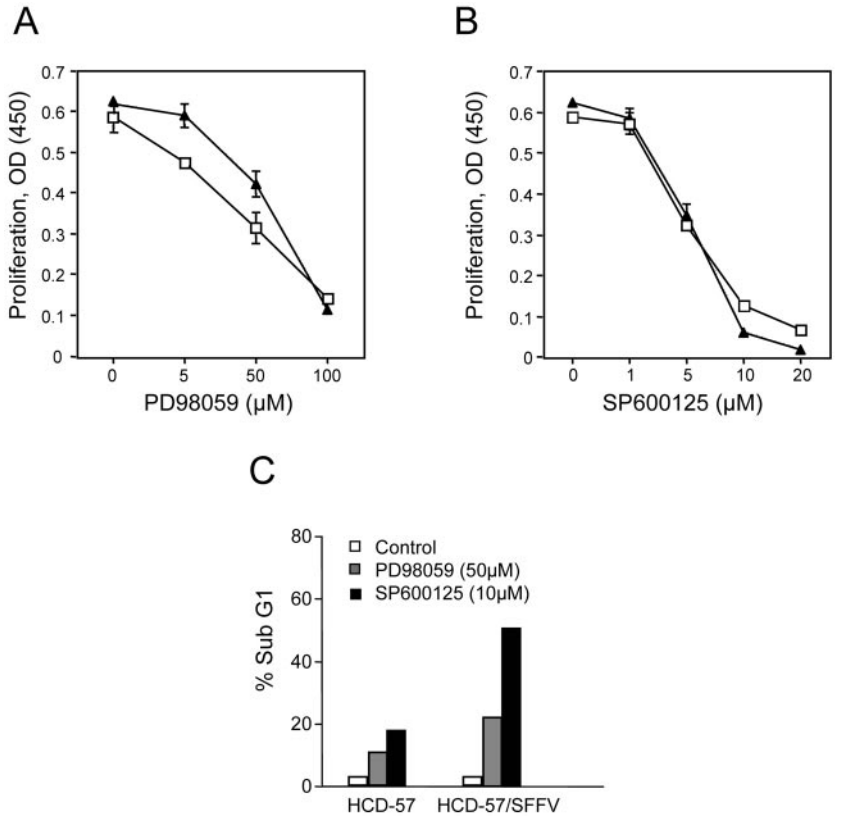

FIG. 2. Effect of MEK and JNK inhibitors on the proliferation of uninfected and Friend SFFV-infected HCD-57 cells. Epo-dependent HCD-57 cells (□) and Epo-independent HCD-57/SFFV cells (▲) were cultured with and without Epo, respectively, for 48 h in the presence of various concentrations of the MEK inhibitor PD98059 (A) or the JNK inhibitor SP600125 (B), and proliferation was measured using the WST-1 reagent. Graphs represent results with triplicate samples, with bars showing standard errors. Cells were also analyzed using flow cytometry for PI-stained cells 48 h after treatment with the DMSO control, PD98059 (50 μM), or SP600125 (10 μM) (C). The sub-G_1 fraction was assessed as apoptotic cells, and the percentages of the total were determined. OD, optical density.

Friend SFFV-induced erythroleukemia occurs in two stages (for a review, see reference 48). In the first stage, Friend SFFV causes Epo-independent erythroid proliferation and differentiation in the animal. Primary erythroleukemic splenocytes from these animals can grow in liquid culture for only a short time but can both proliferate and differentiate in semisolid medium, forming hemoglobin-positive erythroid colonies. In the second stage, Friend SFFV-infected erythroid cells become blocked in differentiation, resulting in the outgrowth of transformed erythroleukemia cells that can be grown as cell lines. As shown in Fig. 1B and C, cells from both stages show constitutive activation of MEK and JNK. To determine what role these constitutively activated kinases play in cells derived from both stages of SFFV disease, we used the inhibitors PD98059 and SP600125 to inhibit MEK and JNK, respectively.

As shown in Fig. 3, both PD98059 (panel A) and SP600125 (panel B) inhibited the liquid growth of primary erythroleukemic splenocytes from Friend SFFV-infected mice in a dose-dependent fashion, with kinetics similar to that seen using Friend SFFV-infected HCD-57 cells. In contrast to HCD-57 cells (Fig. 2C), neither the MEK nor the JNK inhibitor caused an increase in the percentage of primary erythroleukemia cells undergoing apoptosis (data not shown), and no multinucleated cells were observed. Since a previous study showed that antisense JNK oligonucleotides induced apoptosis only in p53-deficient human tumor lines (45), it is possible that the failure of primary erythroleukemia cells from SFFV-infected mice to undergo apoptosis in response to JNK inhibition may reflect their wild-type-p53 status.

When primary erythroleukemia cells were plated in the SFFV CFU assay with or without inhibitors, the formation of large, hemoglobin-positive colonies, which represent primarily proliferating cells, was significantly reduced in the presence of both the MEK inhibitor PD98059 (P was 0.004 at 50 μM and <0.001 at 100 μM) and the JNK inhibitor SP600125 (P was 0.001 at 10 μM) (Fig. 3C). In contrast, the formation of small hemoglobin-positive colonies, which represent differentiating cells, was not significantly reduced after treatment with either inhibitor ($P > 0.1$). This suggests that MEK and JNK inhibitors may predominantly block a proliferation pathway in Friend SFFV-infected erythroid cells rather than a differentiation pathway.

We next examined five independently derived immortal erythroleukemia cell lines from Friend SFFV-infected mice. As shown in Fig. 4, the MEK inhibitor PD98059 had little effect on the growth of any of these cells, even after 48 h (panel A). In contrast, the JNK inhibitor SP600125 dramatically inhibited the growth of all of these cell lines as early as 24 h

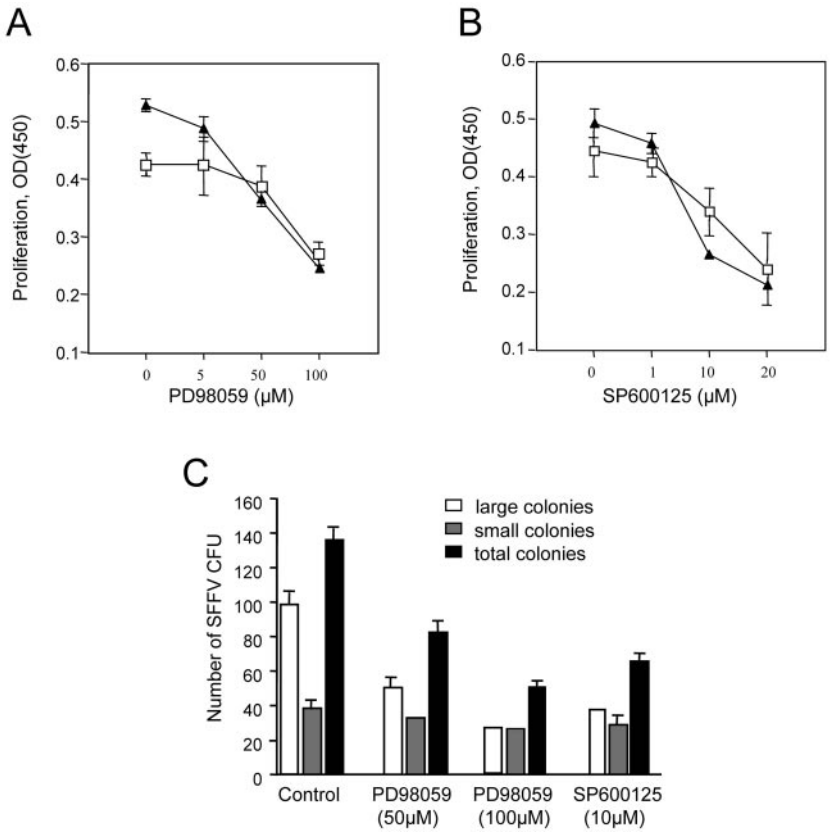

FIG. 3. Effect of MEK and JNK inhibitors on the proliferation of primary erythroleukemia cells from Friend SFFV-infected mice. Primary erythroleukemic cells from the spleens of Friend SFFV-infected mice were cultured in Epo-free medium with various concentrations of the MEK inhibitor PD98059 (A) or the JNK inhibitor SP600125 (B) for 24 (□) or 48 (▲) hours, and proliferation was measured using the WST-1 reagent. Cells were also plated in methylcellulose in the absence of Epo, and benzidine-positive erythroid cells (numbers of SFFV CFU) were determined after 72 h (C). Graphs represent mean results from triplicate samples, with bars showing standard errors. OD, optical density.

(panel B), with similar dose-dependent kinetics among cell lines. When we examined the percentage of apoptotic cells in cultures treated with the MEK and JNK inhibitors using annexin V staining (see Fig. 6A), we could detect little if any increase in the percentage of apoptotic cells after treatment with the MEK inhibitor, but all five of the Friend SFFV-induced cell lines showed a high percentage of apoptotic cells (38 to 92%; $P < 0.001$) after treatment with the JNK inhibitor for 48 h. All of these MEL cells are either mutant or null for p53 (data not shown).

The proliferation of immortal erythroleukemia cells from Friend MuLV-infected mice is inhibited by treatment with both MEK and JNK inhibitors. To determine if immortal erythroleukemia cell lines in general were resistant to inhibition by MEK, we examined five independently derived erythroleukemia cell lines from mice infected with Friend MuLV. As shown in Fig. 5, both the MEK inhibitor PD98059 (panel A) and the JNK inhibitor SP600125 (panel B) inhibited the proliferation of Friend MuLV erythroleukemia cell lines as early as 24 h in a dose-dependent fashion. When we examined the percentage of apoptotic cells in cultures treated with the MEK and JNK inhibitors using annexin V staining (Fig. 6B), we could detect a modest increase in the percentage of apoptotic cells in four out of five of the cultures treated with the MEK inhibitor (15 to 27%; P was 0.005 to <0.001). In contrast to the Friend SFFV-induced erythroleukemia cell lines, the Friend MuLV-induced cell lines were less susceptible to apoptosis induced by the JNK inhibitor, with all lines showing a modest increase in the percentage of apoptotic cells (P was 0.015 to <0.001) but only two of five lines showing greater than 30% apoptosis after drug treatment for 48 h. Differences in sensitivity to apoptosis after JNK inhibition are not due to the p53 status of the cells because all of the F-MuLV MEL lines were mutant or null for p53 (data not shown).

Cell cycle analysis in erythroleukemia cell lines treated with the JNK inhibitor. In all of the erythroleukemia cell cultures grown in the presence of the JNK inhibitor SP600125 for 3 to 5 days, we saw evidence of multinucleated (polyploid) cells. This prompted us to test the effect of SP600125 treatment on the cell cycle. All of the Friend SFFV-transformed erythroleukemia cell lines, including NP4 and NP7 (Fig. 7A) and NP1, NP5, and NP13 (data not shown) showed similar patterns of the cell cycle after treatment with SP600125. After 12 h of exposure to the drug, 62% of NP4 cells and 84% of NP7 cells stained for DNA content in a manner consistent with the G_2/M phase of the cell cycle, in comparison with 23% and 16%, respectively, after treatment with DMSO. At 24 h after drug treatment, the cells entered the endoreduplication cycle, with 32% of NP4 and 19% of NP7 cells showing a DNA content of 8N (polyploid). Twenty-four hours after drug treatment, 56%

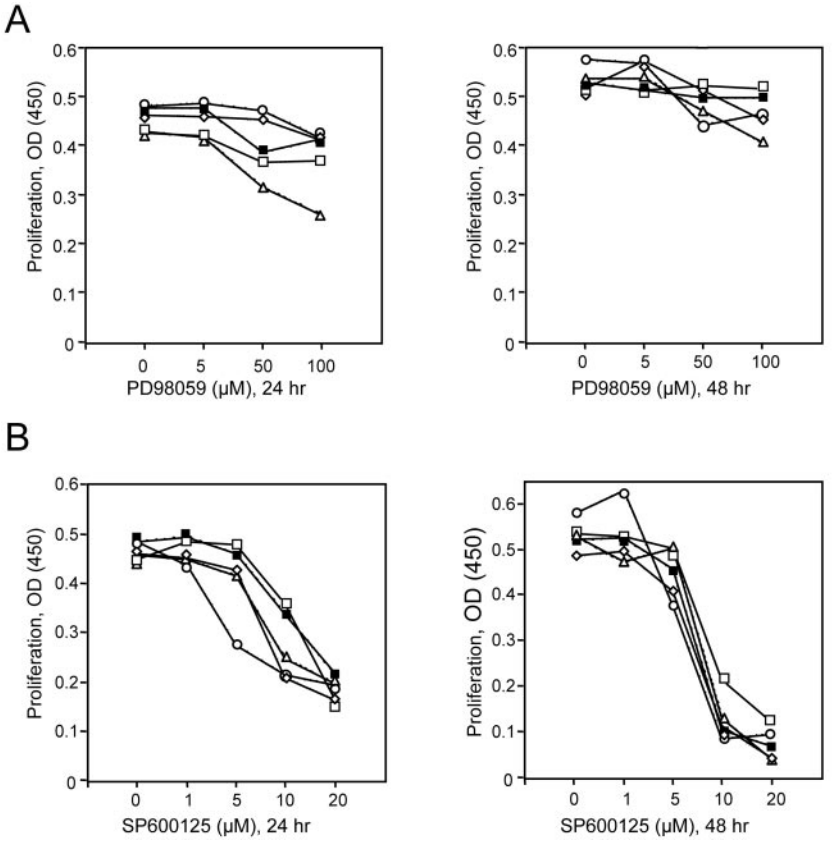

FIG. 4. Effects of MEK and JNK inhibitors on the proliferation of erythroleukemia cell lines from Friend SFFV-infected mice. Erythroleukemia cell lines from Friend SFFV-infected mice were cultured with different concentrations of the MEK inhibitor PD98059 (A) or the JNK inhibitor SP600125 (B) for 24 or 48 h. Proliferation was then measured using the WST-1 reagent. Cell lines analyzed were NP1 (□), NP4 (◇), NP5 (○), NP7 (Δ), and NP13 (■). Graphs represent mean results from triplicate samples. The standard error was less than 0.06. OD, optical density.

of NP4 and 29% of NP7 cells stained for DNA in a manner consistent with the G_0/G_1 phase, which includes apoptotic cells. The Friend MuLV-induced erythroleukemia cell lines TP3 and CB7 also showed a large increase in the percentage of cells in the G_2/M phase after treatment with SP600125 for 12 h, with 76% of TP3 cells and 58% of CB7 cells staining for G_2/M DNA (Fig. 7B) in comparison with DMSO-treated cells (21% for TP3 and 22% for CB7). Similar results were obtained with TP1 and HB22.2 (data not shown). Differences were noted, however, after 24 h of treatment with SP600125, and the results directly correlated with the sensitivity of the cells to the induction of apoptosis by the JNK inhibitor. Fifteen percent of TP3 cells (Fig. 7B) and 35% of TP1 cells (data not shown), both of which are sensitive to apoptosis induction by SP600125 (Fig. 6B), showed a DNA content of 8N (polyploid) 24 h after SP600125 treatment, and 43% of TP-3 (Fig. 7B) and 35% of TP1 (data not shown) cells were in the sub-G_1 phase. In contrast, CB7 cells (Fig. 7B) and HB22.2 cells (data not shown), neither of which are very sensitive to apoptosis induction by SP600125, continued to accumulate in the G_2/M phase and showed no evidence of apoptosis. As noted above, these differences were not related to the p53 status of the cell lines. Treatment of any of the erythroleukemia cell lines with the MEK inhibitor PD98059 did not induce an accumulation of cells in the G_2/M phase or polyploidy (data not shown). These results suggest that in the majority of erythroleukemia cell lines, whether they are induced with Friend SFFV or Friend MuLV, the JNK inhibitor SP600125 induces an accumulation of cells in the G_2/M phase of the cell cycle, with some of the erythroleukemia cells going into apoptosis and others into an endoreduplication cycle.

Regulation of AP1 activity through JNK in erythroleukemia cells. AP1 is one of the downstream molecules that is regulated by JNK and MEK (21). We, therefore, investigated whether mouse erythroleukemia cells express AP1 DNA-binding activity and whether or not it is regulated by JNK. It has previously been demonstrated that Epo induces AP1 DNA binding in Epo-dependent cells (5, 19, 42). Using AP1 consensus and mutant probes in electrophoretic mobility shift assays, we could detect constitutive AP1 DNA-binding activity in all of the virus-infected erythroleukemia cells examined, including primary splenocytes from Friend SFFV-infected mice (Fig. 8, lanes 2). In contrast, erythroblasts from the spleens of uninfected mice or primary leukemic splenocytes from mice infected with Friend MuLV showed AP1 DNA-binding activity only in the presence of Epo (data not shown). In HCD-57 cells and Friend SFFV-infected HCD-57 cells examined 24 h after treatment with SP600125, AP1 DNA-binding activity decreased compared to that in control cells (Fig. 8A, lanes 4). Treatment of primary erythroleukemic splenocytes from

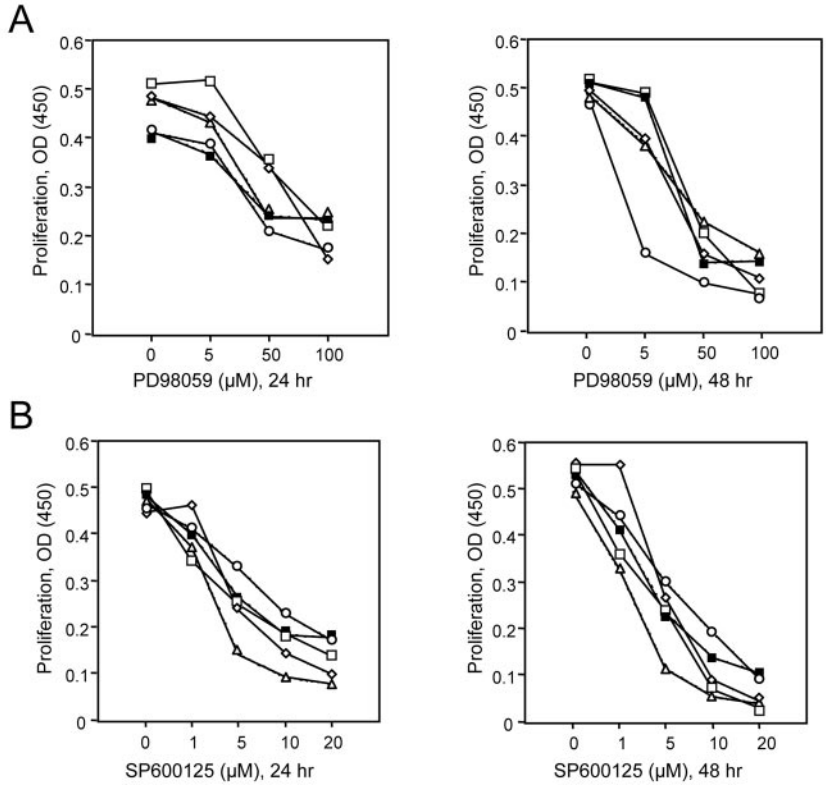

FIG. 5. Effects of MEK and JNK inhibitors on the proliferation of erythroleukemia cell lines from Friend MuLV-infected mice. Erythroleukemia cell lines from Friend MuLV-infected mice were cultured with different concentrations of the MEK inhibitor PD98059 (A) or the JNK inhibitor SP600125 (B) for 24 or 48 h. Proliferation was then measured using the WST-1 reagent. Cell lines analyzed were TP1 (□), TP3 (◇), CB7 (○), HB22.2 (Δ), and HB9.2ED (■). Graphs represent mean results from triplicate samples. The standard error was less than 0.06. OD, optical density.

Friend SFFV-infected mice (Fig. 8B, lane 4) or erythroleukemia cell lines from Friend MuLV-infected mice (Fig. 8D, lanes 4) with SP600125 also caused a reduction in AP1 DNA-binding activity. However, AP1 DNA binding in erythroleukemia cell lines from mice infected with Friend SFFV was generally unaffected by the JNK inhibitor SP600125 (Fig. 8C, lanes 4). In Friend SFFV-induced erythroleukemia cell lines, AP1 DNA-binding activity levels remained unchanged at several time

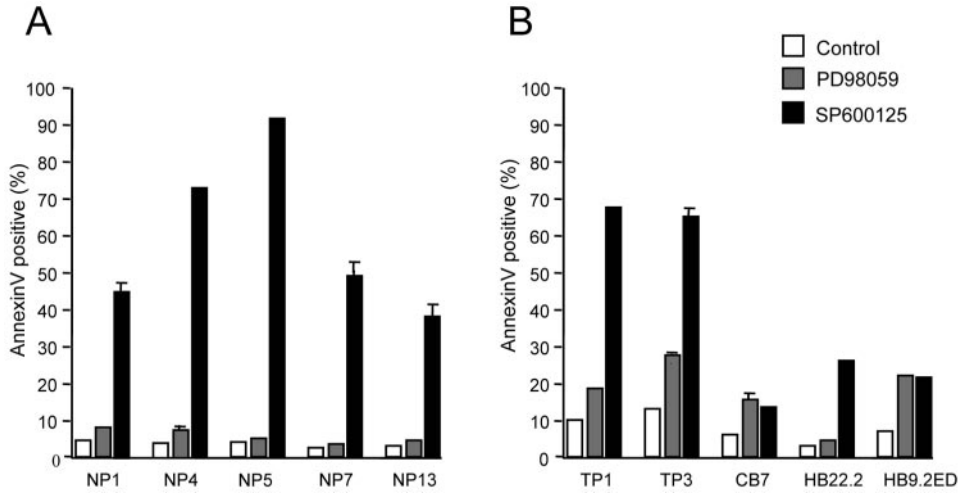

FIG. 6. Effects of MEK and JNK inhibitors on the induction of apoptosis in erythroleukemia cell lines from Friend SFFV- and Friend MuLV-infected mice. Erythroleukemia cell lines from Friend SFFV-infected mice (A) and Friend MuLV-infected mice (B) were cultured for 48 h with the DMSO control, the MEK inhibitor PD98059 (50 μM), or the JNK inhibitor SP600125 (10 μM). Numbers of apoptotic cells were then determined by flow cytometry analysis using annexin V-fluorescein isothiocyanate staining. Graphs represent mean results from triplicate samples, with bars showing standard errors.

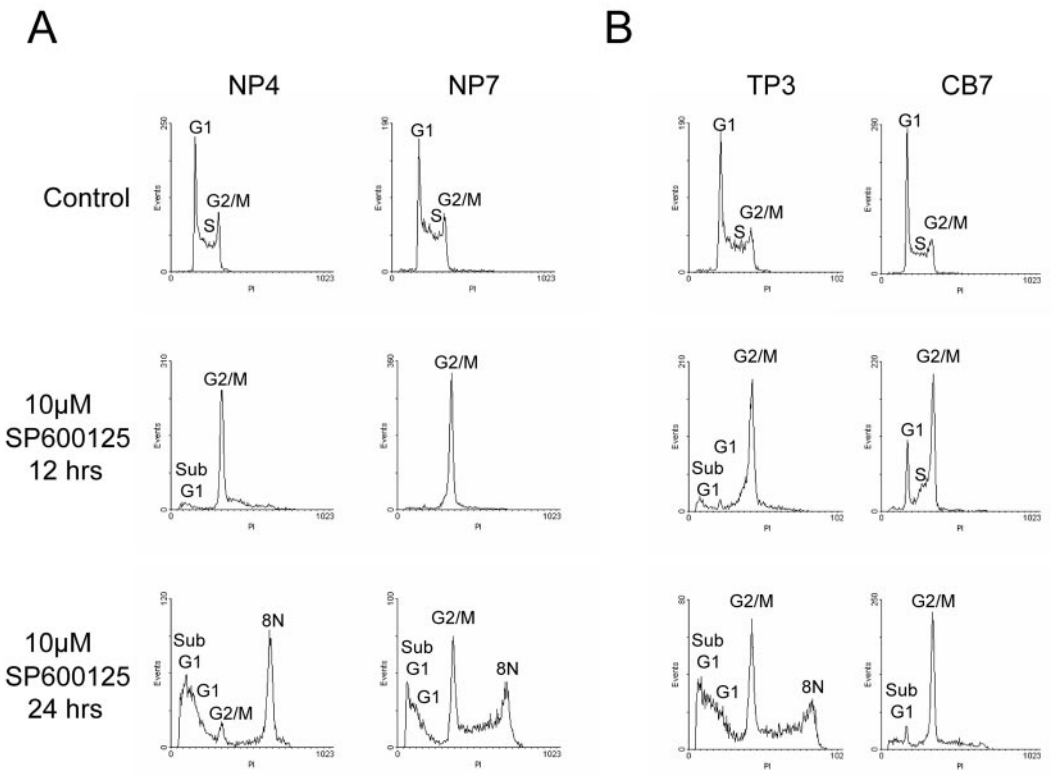

FIG. 7. Effect of a JNK inhibitor on the cell cycle of transformed erythroleukemia cell lines from Friend SFFV- and Friend MuLV-infected mice. Erythroleukemia cells from Friend SFFV-infected mice (A) or Friend MuLV-infected mice (B) were cultured for 12 or 24 h in the presence of DMSO (control) or 10 μM SP600125. Cells were analyzed by flow cytometry of PI-stained cells. Results shown are representative of two separate experiments.

points in the first 24 h of treatment with SP600125, even though SP600125 inhibited cell growth and induced apoptosis (data not shown). In contrast, PD98059 treatment of all erythroleukemia cells examined resulted in a decrease in AP1 DNA-binding activity (Fig. 8A to D, lanes 3), even though proliferation of Friend SFFV-transformed erythroleukemia cells was not inhibited by the MEK inhibitor (Fig. 4A).

These results suggest that in all of the Friend MuLV-transformed erythroleukemia cell lines examined and in primary erythroleukemia cells, AP1 DNA-binding activity is regulated, at least in part, by both the MEK and JNK pathways, whereas in Friend SFFV-transformed erythroid cells, the MEK pathway, but not the JNK pathway, has a role in AP1 DNA binding.

DISCUSSION

In Epo-dependent erythroid cells, the MAP kinases ERK and JNK have been shown to promote proliferation (20). Infection of Epo-dependent erythroid cells with Friend SFFV renders them Epo independent, and this is associated with the constitutive activation of various Epo signal transduction pathways, including the ERK pathway (32). In this study, we show that the JNK pathway is also constitutively activated in erythroid cells infected with Friend SFFV. To determine whether constitutive activation of the ERK and/or JNK pathways promotes the proliferation of Friend SFFV-infected erythroid cells, we took advantage of specific pharmacological inhibitors of these pathways. PD98059, a potent inhibitor of MEK, which activates ERK 1 and ERK 2, blocked the growth of Friend SFFV-infected HCD-57 cells and primary erythroleukemic spleens from Friend SFFV-infected mice but surprisingly had no effect on the growth of immortal erythroleukemia cell lines derived from these mice. PD98059, however, was a potent inhibitor of immortal erythroleukemia cell lines derived from mice infected with Friend MuLV. In contrast to the MEK inhibitor, SP600125, a specific inhibitor of JNK1 and JNK2, was a potent inhibitor of the growth of all erythroleukemia cell lines examined. The SP600125-treated erythroleukemia cell lines all showed significant apoptosis as determined either by analyzing the number of cells in the sub-G_1 fraction or by annexin V staining. Interestingly, when we compared HCD-57 cells and Friend SFFV-infected HCD-57 cells after treatment with SP600125, we found similar kinetics of cell growth inhibition but observed that Friend SFFV-infected HCD-57 cells were more sensitive to the induction of apoptosis after drug treatment than uninfected HCD-57 cells, suggesting that JNK may play an important role for antiapoptosis in Friend SFFV-infected erythroid cell lines. In contrast to what occurred with erythroleukemia cell lines, inhibition of JNK did not induce apoptosis in primary erythroleukemia cells from Friend SFFV-infected mice. This may reflect the wild-type-p53 status of these primary cells or may suggest that JNK does not play an antiapoptotic role in primary erythroleukemic spleens from Friend SFFV-infected mice. We conclude from our data that

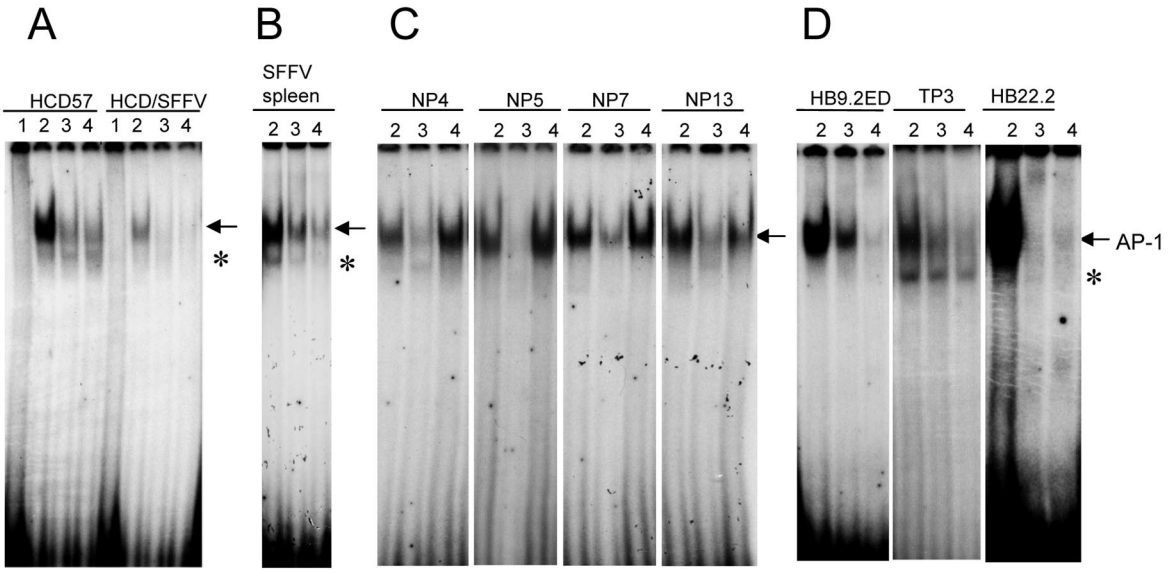

FIG. 8. Effect of MEK and JNK inhibitors on AP1 binding in primary and transformed erythroleukemia cells from Friend SFFV- and Friend MuLV-infected mice. Cells were treated with the DMSO control (lanes 1 and 2), the MEK inhibitor PD98059 (50 μM) (lanes 3), or the JNK inhibitor SP600125 (10 μM) (lanes 4) for 48 h, and nuclear extracts were prepared. Extracts were then subjected to electrophoretic mobility shift assays using an AP1 consensus probe (lanes 2 to 4) or an AP1 mutant probe (lanes 1). Cells examined were (A) HCD-57 cells grown in Epo or Friend SFFV-infected HCD-57 cells grown in the absence of Epo, (B) a primary leukemic spleen from a Friend SFFV-infected mouse, (C) erythroleukemia cell lines from mice infected with Friend SFFV, and (D) erythroleukemia cell lines from mice infected with Friend MuLV. Arrows indicate the AP1 band. The asterisk indicates a nonspecific band.

the JNK pathway promotes the growth and/or survival of erythroleukemia cells from Friend SFFV-infected mice. We have initiated studies to determine if SP600125 will also block the growth of these erythroleukemia cells in vivo.

It is curious that erythroleukemia cell lines derived from Friend SFFV-infected and Friend MuLV-infected mice differ in their sensitivities to the drug PD98059. The Friend SFFV-derived erythroleukemia cell lines are thought to represent a later stage of erythroid cell differentiation than the Friend MuLV-derived erythroleukemia cell lines (41, 52), and the cells may, therefore, have different requirements for the ERK pathway. However, primary leukemic splenocytes from Friend SFFV-infected mice were sensitive to growth inhibition by PD98059, and they should represent the same stage of erythroid cell differentiation as in the cell lines derived from them. The Friend SFFV-induced erythroleukemia cell lines, however, differ from the primary erythroleukemia cells as well as the Friend MuLV-induced erythroleukemia cell lines in their expression of the *ets*-related transcription factor PU.1 (1, 31). F-MuLV MEL cells express the *ets*-related transcription factor Fli-1 (1), and treatment with the MEK inhibitor did not alter the expression of Fli-1 in these cells or PU.1 in the SFFV MEL cells (data not shown). Perhaps expression of PU.1 activates pathways in Friend SFFV-induced erythroleukemia cells that can substitute for the ERK pathway, allowing the cells to grow in the presence of the MEK inhibitor.

Although our data indicate that the JNK pathway is important for the growth and survival of Epo-independent erythroleukemia cells, the role that the pathway plays in these cells is unclear. Mice with homozygous deletions of either JNK1 (10) or JNK2 (62) fail to show any abnormalities in erythropoiesis, and deletion of both JNK1 and JNK2 (22), while lethal to the embryo, does not affect the development of the fetal liver, the site of erythropoiesis in the fetus. JNKs do play a role in tetradecanoylphorbol-13-acetate-induced papilloma progression, where JNK1 acts as a suppressor of skin tumor development (51) and JNK2 is critical in the tumor promotion process (7). It will be interesting to test if the progressions of erythroleukemia induced by Friend SFFV and Friend MuLV are different in JNK1- and JNK2-deficient mice. In all of the erythroleukemia cell lines treated with SP600125, we observed an accumulation of cells in the G_2/M phase of the cell cycle, consequent apoptosis, and endomitosis. Previous studies have suggested a role for JNK in the cell cycle (61). Active JNK is at the centrosome from S phase to anaphase (26). Also, the JNK inhibitor SP600125 has been reported to cause G_2/M arrest or endoreduplication in a variety of human cancer cell lines, including multiple myeloma, breast cancer, prostate cancer, and erythroleukemia (11, 15, 18, 29, 58). In Friend virus-induced SKT6 cells, which unlike the cells used in this study can differentiate in response to Epo, antisense oligonucleotides of JNK1 and JNK2 inhibited Epo-induced differentiation (36), although cell cycle analysis was not shown. In another study, expression of a dominant negative form of JNK1 inhibited the Epo-dependent proliferation of HCD-57 erythroleukemia cells but did not increase the fraction of cells in G_2/M or the number undergoing endoreduplication (18). In our studies, treatment of erythroleukemia cell lines with the JNK inhibitor SP600125 did not induce their differentiation (data not shown).

Because the JNK pathway can regulate AP1 activity, we investigated AP1 DNA binding in the erythroleukemia cells used in this study. Although it has previously been shown that Epo induces AP1 DNA-binding activity in Epo-dependent cells (5, 19, 42), our data are the first to demonstrate that AP1

DNA binding is constitutive in Friend SFFV-infected HCD-57 cells, in primary splenocytes from Friend SFFV-infected mice, and in erythroleukemia cell lines from Friend SFFV and Friend MuLV-infected mice. By examining AP1 DNA binding in these cells before and after treatment with the JNK inhibitor, we were able to evaluate whether the JNK inhibitor may block the proliferation of these cells by inhibiting AP1 activity. Our data showed that AP1 activity in Friend SFFV-infected HCD-57 cells, in primary splenocytes from Friend SFFV-infected mice, and in erythroleukemia cell lines from Friend MuLV-infected mice was reduced after treatment of the cells with the JNK inhibitor SP600125. However, treatment with SP600125 had no effect on AP1 DNA binding in erythroleukemia cell lines from mice infected with Friend SFFV, even though the drug inhibits cell proliferation. Thus, AP1 DNA binding appears to be regulated by the JNK pathway and correlates with cell proliferation in some erythroleukemia cells but not others. Although loss of AP1 DNA binding after treatment of F-MuLV MEL lines with the JNK inhibitor correlates with inhibition of cell proliferation, it will be necessary to specifically block AP1 DNA-binding activity in these cells in order to determine if AP1 is essential for their growth. A previous study showed that the components in the AP1 complex activated after Epo stimulation of HCD-57 cells differed from the components in the AP1 complex activated after Epo withdrawal from HCD-57 cells (19). Also, it was recently shown that fibroblasts from mice deficient for JNK1 and JNK2 showed decreased expression of some but not all AP1 components (57). Perhaps the components in the AP1 complex activated in the Friend SFFV-induced erythroleukemia cell lines are different from those in the other erythroleukemia cells examined in this study and that only the components in the latter cells are under JNK regulation. Interestingly, the MEK inhibitor was able to reduce AP1 DNA binding in the Friend SFFV-induced erythroleukemia cell lines, even though it did not alter the growth of these cells. This suggests that the components of the AP1 complex activated in the Friend SFFV-induced erythroleukemia cell lines may be regulated by the MEK pathway but not by JNK. We could not detect any differences, either before or after drug treatment, in the expression of AP1 components (c-Jun, JunB, JunD, and c-Fos) in MEL cells from Friend SFFV- versus F-MuLV-infected mice (data not shown). Since JNK also regulates other transcription factors in addition to AP1, it will be interesting to determine if the activity of any of these is altered after treatment of the various erythroleukemia cell lines with the JNK inhibitor.

In conclusion, our study highlights the importance of the JNK pathway in maintaining the growth and survival of a diverse group of mouse erythroleukemia cells. In light of our data, inhibition of JNK using the drug SP600125 might be a useful strategy for treating erythroleukemia in humans.

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