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Abrogation of Interleukin-3 Dependence of Myeloid Cells by the v-*src* Oncogene Requires SH2 and SH3 Domains Which Specify Activation of STATs

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The v-*src* oncogene encodes a nonreceptor tyrosine kinase. When this gene was expressed in the myeloblastic cell line 32Dcl3, it was found to abrogate interleukin-3 (IL-3) dependence of this cell line and to block its ability to terminally differentiate into granulocytes in response to granulocyte colony-stimulating factor (GCSF). In contrast, a highly related tyrosine kinase gene, v-*fgr*, fails to render this cell line IL-3 independent for growth or to block its ability to undergo terminal differentiation in the presence of GCSF. The active structural domains of v-*src* that are responsible for the abrogation of IL-3 dependence of myeloid cells and the mechanisms by which v-*src* transforms these cells are at present unclear. To identify the domains in v-*src* which are responsible for this activity, we constructed several chimeric recombinants between the v-*src* and the related Src family member v-*fgr* by replacing portions of v-*src* with corresponding domains of v-*fgr*. These chimeric DNAs were transfected into 32Dcl3 cells and examined for their abilities to render this cell line IL-3 independent. Our results show that only chimeras containing both the SH3 and the SH2 domains of v-*src* were capable of rendering the 32Dcl3 cell line IL-3 independent. To understand the possible mechanisms underlying the IL-3-independent growth of v-*src*-transformed 32Dcl3 cells, we examined the phosphorylation status of JAK-1, JAK-2, and JAK-3 kinases in the v-*src*- and v-*fgr*-transformed 32Dcl3 cells. Our results show that none of the JAK kinases are constitutively phosphorylated by v-*src* or v-*fgr*. We then examined the phosphorylation status of the STAT (signal transducers and activators of transcription) family of transcription factors. Our results show that STAT1, STAT3, and STAT5 exist in a constitutively phosphorylated state in v-*src*-transformed 32Dcl3 cells, while such constitutive phosphorylation is not seen in v-*fgr*-transformed cell lines. Our results also show that STAT3 coimmunoprecipitates with v-Src, suggesting that the activation of STAT3 occurs due to direct association with v-Src. However, STAT1 and STAT5, which also exist in a constitutively phosphorylated state in v-*src*-transformed 32Dcl3 cells, do not coimmunoprecipitate with v-Src, suggesting that these proteins either interact weakly with v-Src or are phosphorylated by a mechanism distinctive from that of STAT3.

It is becoming increasingly clear that hematopoietic cell growth and differentiation are mediated by a group of soluble factors known as cytokines (14, 17, 35). These molecules bind to their cognate receptors and mediate intracellular signal transduction events which result in the modulation of gene expression (13). During the past few years, new evidence has emerged to indicate that most cytokines transmit their signals via a new family of tyrosine kinases termed JAK kinases (7, 31, 35, 38). To date, this family consists of four members, i.e., JAK-1, JAK-2, JAK-3, and TYK-2. These kinases, either alone or in conjunction with each other, appear to be responsible for effects mediated by several cytokines and neurokines (2, 12, 17, 22, 30, 31, 35). Current models suggest that interaction of cytokines with their receptors induces receptor dimerization, which increases the affinity of the cytoplasmic domain of the receptor for JAK kinases, resulting in a ligand-dependent increase of a complex that contains the receptors and JAK kinases. This results in activation of the JAK kinases through an event associated with tyrosine phosphorylation. The activated kinases appear to subsequently phosphorylate the receptors as well as cellular substrates, the most important of which are the STATs (signal transducers and activators of transcription). These transcription factors were originally described by Darnell et al. (7) as transcription factors associated with an interferon-mediated signaling mechanism. To date, six different STATs have been discovered, all of which seem to participate in cytokine- or growth hormone-mediated signal transduction (13, 23).

It is now well-established that several of the oncogenic tyrosine kinases belonging to the Src family have a profound effect on the cytokine dependence of hematopoietic cell lines (1, 26). Most notably, it has been demonstrated that constitutive expression of v-*src* and v-*abl* oncogenes in interleukin-3 (IL-3)-dependent myeloid cell lines renders them cytokine independent for growth (1, 26, 28, 29). This alteration in growth factor dependence had suggested that these oncogenes might interfere with signal transduction pathways associated with cytokines. One intriguing observation that was made by us and others was that several of the activated Src family tyrosine kinases (such as v-Fgr and the activated form of c-Fgr, which show extensive homology to v-Src and transform NIH 3T3 cells in vitro as efficiently as v-Src, fail to induce cytokine-independent growth in IL-3-dependent myeloid cell lines. To understand the molecular basis of this phenomenon, we carried out a detailed analysis of the domains of the v-*src* oncogene that are required for induction of IL-3-independent growth in my-

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eloid cell lines and the mechanisms by which v-Src brings about this effect.

MATERIALS AND METHODS

Cells. The murine hematopoietic cell line 32Dcl3 (32D) is a diploid cell line derived from normal mouse bone marrow and strictly requires IL-3 for growth (37). It was maintained in Iscove's modified Dulbecco medium (IMDM), which was supplemented with 10% fetal bovine serum (complete medium) and 10% WEHI3B-conditioned medium as a source of crude IL-3 (39). The v-*src*- and chimera C-transformed 32D cells were maintained in the same medium but lacking WEHI3B supernatant. JAK-3-overexpressing 32D cells were generated by transfection of an expression vector such that the JAK-3 cDNA encoding the p120 form of JAK-3 was placed under the control of the cytomegalovirus (CMV) promoter (pcDNA3JAK3). This DNA was electroporated into 32Dcl3 cells, and single cell clones expressing high levels of JAK-3 were derived (26a).

Antibodies. The antibodies against STAT1 (E-23), STAT3 (C-20), STAT5 (C-17), and Src (SRC-2 and N-10) were purchased from Santa Cruz Biotechnology. The anti-rabbit c-Fgr antibody was raised against the glutathione *S*-transferase–c-Fgr protein (125 to 570 amino acids). The JAK antibodies and antiphosphotyrosine antibody 4G10 were purchased from UBI (Lake Placid, N.Y.). Rabbit anti-mouse antibody (secondary antibody for anti-STAT and anti-JAK) and mouse monoclonal antibody (secondary antibody for antiphosphotyrosine) were purchased from Amersham.

Construction of the chimeras. The v-*src* and v-*fgr* recombinants were constructed by employing the fusion PCR technique (40). This technique allowed the joining between two different genes, A (*src*) and B (*fgr*). The primers included two 20-nucleotide flanking primers, a sense primer corresponding to the 5′ untranslated region of *src* or *fgr*, and an antisense primer corresponding to the 3′ untranslated region of *src* or *fgr*. One sense and antisense, 42-nucleotide-long base primer (A/B hybrid primer corresponding to the last seven codons of the A molecule up to the joining point and the first seven codons of the B molecule beyond the joining point) were used as joining molecules. PCR was carried out in two steps. In the first step, the 20-nucleotide end primers along with the 42-nucleotide middle primers were used to obtain the portions of each gene required for fusion by using the full length v-*src* and v-*fgr* DNAs as the template. The PCR product of the first step was purified, extracted with phenol-chloroform, and ethanol precipitated. In the second round of PCR, this DNA was used as the template with the 42-nucleotide hybrid primer and the end primers in such a proportion that fusion between the two segments of the gene was promoted. PCR was carried out with reagents purchased from Perkin-Elmer Cetus (Norwalk, Conn.).

DNA transfection. DNA was transfected into the 32D cells by application of high voltage (240 V, 500 μF; Bio-Rad Gene Pulser). A 10-μg amount of linearized DNA in the PMV-7 vector was transfected into 10^7 cells per ml. Two days after the electroporation, G418 was added at a concentration of 150 to 250 μg/ml for selection. After about 8 days of selection, drug-resistant colonies were screened by Northern and Southern blot analyses for integration and expression of the transgene. Western blot assays were carried out to confirm the synthesis of appropriately sized proteins by the expression vectors.

In vitro transcription-translation assay. Transcription-translation assays were performed with the DNA template of all of the *src-fgr* chimeric constructs and with wild-type v-*src* and v-*fgr* genes in pGEM-3Z vector to confirm the synthesis of appropriately sized proteins. Approximately 3 μg of plasmid DNA was linearized by using appropriate restriction enzymes to generate linear DNA. Subsequent to linearization, the template DNA was extracted with a phenol-chloroform-isoamyl alcohol mixture (75:24:1) and precipitated with ethanol. A 0.5-μg amount of template DNA was used in each reaction. The DNA was transcribed and translated in one step by using the TNT lysate-coupled transcription-translation reaction kit purchased from Promega, according to the manufacturer's instructions. The ^{35}S-labeled translation products were resolved by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (20).

Tyrosine kinase assay. The cells were washed with phosphate-buffered saline (PBS) and lysed at 4°C for 20 min in lysis buffer containing 25 mM HEPES (pH 7.6), 0.1% Triton X-100, 300 mM NaCl, 20 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 2.0 μM dithiothreitol (DTT), 0.2 mM Na_3VO_4, 2-μg/ml leupeptin, and 4-μg/ml aprotinin. Lysates were first precleared by incubation with preimmune serum and protein A-Sepharose for 5 min at 4°C. After preclearing, the lysates were incubated with respective antibodies (1:250 dilution) for 1 h followed by incubation with protein A-Sepharose for 45 min at 4°C. The immunoprecipitates were washed thrice with lysis buffer and once with kinase buffer containing 20 mM HEPES (pH 7.6), 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenylphosphate, 0.1 mM Na_3VO_4, and 2 mM DTT. Kinase reactions in vitro were performed with 20 μM rATP, 5 μCi of [γ-^{32}P]ATP in 100 μl of kinase buffer for 20 min at 30°C (11). The samples were analyzed by SDS–12% PAGE after boiling in Laemmli buffer (20).

Electrophoretic mobility shift assay. To prepare nuclear extracts from 32D cells, exponentially growing normal cells were washed three times with PBS and were replated in complete medium without IL-3 and incubated for 18 h before making the extracts. The 32D/v-Src and 32D/chimera C cells were maintained in complete medium lacking IL-3. For each preparation, 5×10^6 cells were harvested in PBS and centrifuged for 5 min at 12,000 × *g* to obtain a pellet. The pellet was then suspended in 400 to 800 μl of buffer containing 10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2-μg/ml aprotinin, 2-μg/ml leupeptin, 2-μg/ml pepstatin, and 2-μg/ml antipain by gentle pipetting, and the suspension was incubated on ice for 15 min. To this suspension, 25 to 50 μl of Nonidet P-40 (NP-40) was added, vortexed, and centrifuged at 12,000 × *g* in an Eppendorf centrifuge. Both the supernatant and the pellet were saved. The supernatant constituted the cytoplasmic extract, while the nuclear pellet was further resuspended in 50 to 100 μl of cold buffer containing 20 mM HEPES-KOH (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA 1 mM DTT, 1 mM PMSF, 2-μg/ml aprotinin, 2-μg/ml leupeptin, 2-μg/ml pepstatin, and 2-μg/ml antipain by mixing vigorously at 4°C for 15 to 20 min with a pipet tip. This suspension was then centrifuged at 12,000 × *g* for 15 min, and the supernatant which constituted the nuclear extract was collected. STAT-specific oligonucleotides and their mutant counterparts were purchased from Santa Cruz Biotechnology. The binding sequence for STAT3 was described by Yu et al. (42), and those for STAT1 and STAT5 were described by Mui et al. (24). The sequences of the probes used in the assays are as follows: STAT1, 5′ CATGTTATGCATATTCCTGTAAGTG 3′; STAT1* (mutant), 5′ CATGTTATGCATATTG*G*A*GTAAGTG 3′; STAT3, 5′ GATCCTTCTGGGAATTCCTAGATC 3′; STAT3* (mutant), 5′ GATCCTTCTGGGC*C*G*TCCTAGATC 3′; STAT5, 5′ AGATTTCTAGGA ATTCAATCC 3′; and STAT5* (mutant), 5′ AGATTTA*G*T*T*T*AATTCA ATCC 3′.

For STAT mobility shift assays, 5 μg of nuclear extract was incubated with 1 ng of ^{32}P-labeled acute-phase response element (APRE) probe or with mutant oligonucleotide in 10 μl of binding buffer [50 mM Tris (pH 7.5), 5 mM $MgCl_2$, 250 mM NaCl, 5 mM EDTA, 2.5 mM DTT, 20% glycerol, 50-μg/ml single-stranded DNA, 0.25-μg/ml poly(dI-dC)] for 30 min at room temperature and electrophoresed on a nondenaturing 5% polyacrylamide gel in 0.5% TBE buffer (25 mM Tris, 25 mM boric acid, 10 mM EDTA) as described by Shreiber et al. (33). One microgram of poly(dI-dC) was used as nonspecific DNA competitor in each reaction. The probe was a double-stranded oligonucleotide that was end labeled with [γ-^{32}P]ATP by using T4 nucleotide kinase. For antibody competition, antisera diluted to 1:250 were added at the end of the binding reaction, and the mixtures were incubated at 4°C for an additional 2 h before addition of the probe.

Western blot analysis. A 60-μg amount of nuclear extract or 120 μg of total cell cytoplasmic extracts was used for Western blot analysis of STAT proteins. Protein samples were separated by SDS–10% PAGE on polyacrylamide gels and then transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% milk for 30 min at room temperature and then incubated with primary antibody (4 μg per sample) for 60 min at room temperature with constant agitation, washed thrice with T-TBST (0.05% Tween 20, 20 mM Tris [pH 7.5], 150 mM NaCl), and incubated with secondary antibody (goat antirabbit immunoglobulin G linked to horseradish peroxidase) (1:10,000 dilution) for 30 min at room temperature and detected with enhanced chemiluminescence (ECL; Amersham). For Western blot analysis of JAK-3 proteins and phosphotyrosine proteins, cell extracts or immunoprecipitates were separated on an 8% polyacrylamide gel and transferred onto nitrocellulose membranes. The filters were then blocked in 1% bovine serum albumin for 1 h at room temperature and incubated with 10 μl of JAK-3 antibody or 1-μg/ml 4G10 monoclonal antiphosphotyrosine antibody in 10 ml of T-TBST containing 3% bovine serum albumin at 4°C overnight. The immunocomplexes were then visualized as described for STAT Western blots. For JAK-1 and JAK-2 Western blot analysis, the protein extracts were separated on 8% polyacrylamide gels, transferred onto nitrocellulose membranes, and blocked in PBS containing 3% nonfat dry milk for 20 min at room temperature. JAK antibody diluted (1:1,000) in PBS–3% milk was then incubated overnight at 4°C. The nitrocellulose filter was washed twice with water and then incubated with 1:10,000 diluted secondary antibody for 30 min at room temperature. The membrane was then washed with PBS–0.05% Tween 20 for 3 to 5 min, followed by four to five rinsings in water. ECL (Amersham) was used for visualization of immune complexes.

Immunoprecipitation. The immunoprecipitation studies were performed with total cell lysates. For STAT immunoprecipitation, protein was extracted by sonicating 5×10^8 cells in a buffer containing 150 mM NaCl, 50 mM Tris (pH 7.5), 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 4-μg/ml aprotinin, 4-μg/ml pepstatin A, and 4-μg/ml leupeptin. After sonication, the lysate was incubated on ice for 30 min and then sedimented at 12,000 × *g* for 15 min to remove insoluble material. For each immunoprecipitation, 1 mg of total protein for each sample was used. The cell extract was first precleared by incubating the extract with preimmune serum and protein A-Sepharose (50 μl of slurry) for 5 min at 4°C. After preclearing, the lysates were then incubated with the respective antibodies (1:250 dilution) for 1 h, followed by incubation with protein A-Sepharose for 45 min at 4°C. The immunoprecipitates were then collected by centrifugation, washed thrice with the lysis buffer, dissolved by boiling in Laemmli's buffer (20), and subjected to SDS-PAGE.

For JAK immunoprecipitations, normal 32D cells were starved of IL-3 for 18 h and stimulated with IL-3 for 15 min before being harvested for lysis. As negative controls for phosphorylation, 32Dcl3 cells incubated in the absence of IL-3 for 15 to 30 min were used for the preparation of cell lysates. Under these conditions,

32D cells are known to contain detectable levels of JAK-1 and JAK-2 in an unphosphorylated state. The lysis buffer consisted of 10 mM Tris-HCl (pH 7.6), 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 200 mM sodium orthovanadate, 1% Triton X-100, 1 mM PMSF, 4-μg/ml aprotinin, 4-μg/ml pepstatin A, and 4-μg/ml leupeptin. The lysate was incubated overnight with the JAK-specific antibody. The precipitates were washed and analyzed by SDS-PAGE as described above for STATs.

The lysis buffer for v-Src and STAT coimmunoprecipitation consisted of 1% NP-40 in PBS, 1 mM PMSF, 4-μg/ml aprotinin, 4-μg/ml pepstatin A, and 4-μg/ml leupeptin. The conditions for immunoprecipitation and SDS-PAGE were identical to those used for STAT and JAK immunoprecipitations.

RESULTS

Construction of chimeric molecules containing portions of v-*src* and v-*fgr* genes and introduction into 32Dcl3 cells. To study the mechanisms associated with v-*src*-mediated transformation of myeloid cells, we used the 32Dcl3 cell line, which is derived from normal mouse bone marrow, strictly requires IL-3 for growth, and is nontumorigenic (37). This cell line, when transformed with expression vectors containing v-*abl*, *bcr-abl*, or v-*src*, was found to grow indefinitely in the absence of IL-3 and to form tumors in nude mouse assays (19, 21, 29). However, when v-*fgr* or an activated form of c-*fgr* which lacks the C-terminal 12 amino acids (c-*fgr*-Δ) was expressed in this cell line, they failed to render these cells IL-3 independent for growth (our unpublished data). Since the *fgr* and *src* genes share extensive homology in their SH2, SH3, and tyrosine kinase domains (15, 25), this observation offered us a very convenient method to test the structural domains required for v-*src*-mediated effects on 32Dcl3 cells. Figure 1a shows the various chimeric molecules that were generated by swapping SH2, SH3, and tyrosine kinase domains between v-*src* and c-*fgr* or v-*fgr* genes. Following the construction of these chimeras in pGEM3Z vector, sequence analysis was performed on all of the constructs to verify their sequences. Then, in vitro transcription-translation reactions were performed to determine that all of the chimeras encoded proteins with appropriate sizes. The results presented in Fig. 1b show that v-*fgr* and chimera A gene encode proteins of approximately 70 kDa, while v-*src* and chimera B, C, and D genes produce proteins of approximately 60 kDa. The larger size of v-Fgr and chimera A is due to the presence of a Gag-actin region at the amino termini of these proteins.

Effect of *src/fgr* chimeras on IL-3 dependence of 32Dcl3 cells. The v-*src*, v-*fgr*, and c-*fgr* DNAs were then cloned into the retrovirus vector pMV7 and used for electroporation into 32Dcl3 cells. This vector contains the G418 resistance gene under the control of the herpes simplex virus-thymidine kinase promoter (16). Following electroporation, cell lines were selected by culturing cells in a medium containing IL-3 and G418 (500 μg/ml). Similar single cell clones were isolated from 32Dcl3 cells transfected with pMV7 vector alone, which were used as negative controls. Both mass cultures and single cell clones were isolated from individual electroporation and used for the analysis of RNA and protein synthesis. These studies showed that all mass cultures and single cell clones contained copies of viral DNA stably integrated into the genome, which was efficiently transcribed, as determined by Northern blot analysis (data not shown). To ascertain that all of the DNA constructs efficiently produced the appropriate proteins in vivo, Western blot analysis of the total cell lysates was performed with polyclonal antibodies raised against the C-terminal and N-terminal ends of v-Src protein. The results presented in Fig. 1c show that cells transfected with chimeras B, C, and D (which contain the SH3 domain of v-*src*) produced proteins of approximately 60 kDa that cross-reacted with the antibody raised against the N-terminal end of v-Src (N10). Cells transfected with chimeras A and D (which contain the tyrosine kinase domain of *src*) cross-reacted with the antibody raised against the C-terminal end of v-Src (SRC-2). These results show that all of the cell lines used in this study produce appropriately sized proteins of expected immunoreactivities with antibodies raised against v-Src. To ascertain that the 32D/v-*src*, 32D/v-*fgr*, and *src/fgr* chimeras were kinase active, in vitro kinase assays were performed with immunoprecipitates derived from cell lysates made from the transfected cells. The results presented in Fig. 1d show that proteins encoded by v-Src and v-Fgr as well as the chimeras A, B, C, and D showed an associated kinase activity, while the immunoprecipitates derived from control 32D cells did not exhibit such an activity.

To determine the effects of Src/Fgr chimeric proteins on IL-3-independent proliferation of 32D cells, normal and DNA-transfected cells were cultured in IMDM containing 10% FBS but lacking IL-3 and were analyzed for cell viability and growth for 10 days. The results presented in Fig. 1e show that 100% of the normal 32Dcl3 cells lost their viability in a period of 48 to 72 h following the removal of IL-3. DNA analysis from these cells showed the appearance of fragmented DNA, suggesting the induction of apoptosis in these cells (data not shown). Cells transfected with v-*fgr* and c-*fgr*-Δ expression vectors lost viability in a similar fashion. In contrast, the v-*src*-transformed cells continued to proliferate in the absence of IL-3 and maintained 90 to 100% viability over a period of several months. Of all of the cell lines transfected with *src/fgr* chimeras, only cells transfected with chimera C showed IL-3-independent growth. Cells expressing chimeric proteins A, B, and D behaved very similarly to v-*fgr*- or c-*fgr*-Δ-transformed cells. Examination of the structures of these chimeric molecules showed that the exchange of tyrosine kinase domains between *src* and *fgr* did not make any difference with respect to the biological phenotype obtained. The kinase domains of either v-*src* or v-*fgr* in conjunction with the SH2 and SH3 domains of v-*src* were adequate to induce IL-3-independent growth in the transfected cells. However, exchange of the SH2 domain of v-*src* with that of v-*fgr* (chimera D) was adequate to render this chimeric molecule inactive with respect to its ability to induce IL-3 independence in 32Dcl3 cells. Similarly, replacement of the SH3 domain of v-*src* with the Gag-actin region of v-*fgr* or the SH3 domain of c-*fgr* resulted in disruption of the ability of v-*src* to induce IL-3 independence in these cells. In addition, a chimera that contained the SH3 and SH2 domains of c-*fgr* and the tyrosine kinase domain of v-*src* also failed to render 32Dcl3 cells IL-3 independent (data not shown), further supporting the above conclusion. These results strongly suggest that both the SH3 and the SH2 domains of v-Src are essential for this oncoprotein to induce IL-3-independent proliferation in 32Dcl3 cells.

Activation of STATs by v-*src*, v-*fgr*, and *src/fgr* chimeras. Current models suggest that interaction of cytokines with their receptors results in the activation of the JAK kinases through an event associated with tyrosine phosphorylation. The activated kinases appear to then phosphorylate the receptors as well as STATs (13), which in turn mediate phenotypic changes associated with different cytokines. Since the effects of v-Src appear to mimic the effects of IL-3, and since it has been recently shown that v-Src can activate STAT3 in NIH 3T3 cells (4, 42), we examined the subcellular localization and phosphorylation statuses of various STATs in 32Dcl3 cells expressing v-*src*, v-*fgr*, and *src/fgr* chimeras (Fig. 2). STATs, in their inactive form, are known to be localized in the cytoplasm and upon phosphorylation by JAK kinases translocate into the nucleus, where they initiate transcription of target genes (7, 8, 10, 12–14). Because of this phenomenon, nuclear localization of

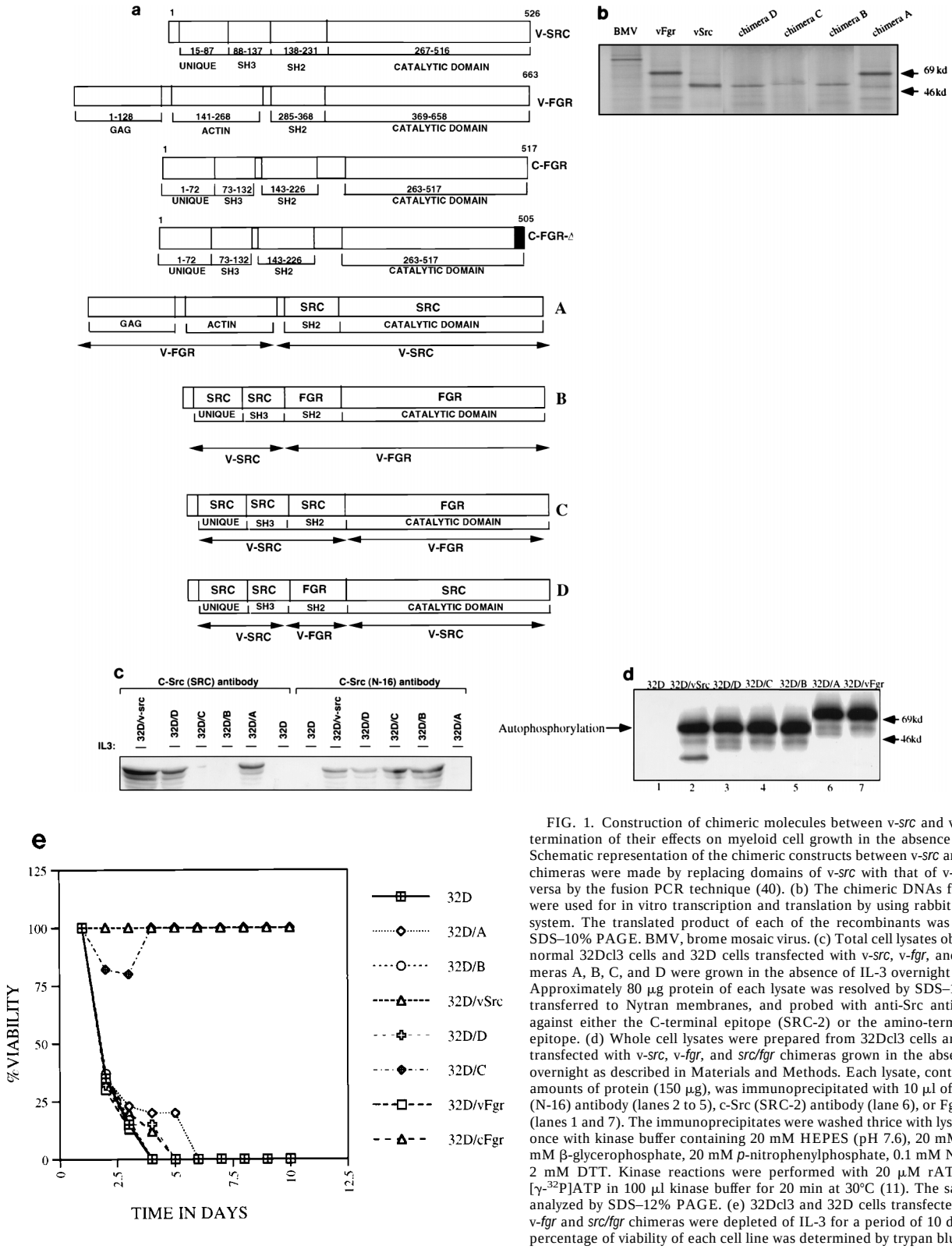

FIG. 1. Construction of chimeric molecules between v-*src* and v-*fgr* and determination of their effects on myeloid cell growth in the absence of IL-3. (a) Schematic representation of the chimeric constructs between v-*src* and v-*fgr*. The chimeras were made by replacing domains of v-*src* with that of v-*fgr* and vice versa by the fusion PCR technique (40). (b) The chimeric DNAs from panel a were used for in vitro transcription and translation by using rabbit reticulocyte system. The translated product of each of the recombinants was resolved by SDS–10% PAGE. BMV, brome mosaic virus. (c) Total cell lysates obtained from normal 32Dcl3 cells and 32D cells transfected with v-*src*, v-*fgr*, and *src/fgr* chimeras A, B, C, and D were grown in the absence of IL-3 overnight before lysis. Approximately 80 μg protein of each lysate was resolved by SDS–10% PAGE, transferred to Nytran membranes, and probed with anti-Src antibody raised against either the C-terminal epitope (SRC-2) or the amino-terminal (N-10) epitope. (d) Whole cell lysates were prepared from 32Dcl3 cells and 32D cells transfected with v-*src*, v-*fgr*, and *src/fgr* chimeras grown in the absence of IL-3 overnight as described in Materials and Methods. Each lysate, containing equal amounts of protein (150 μg), was immunoprecipitated with 10 μl of either c-Src (N-16) antibody (lanes 2 to 5), c-Src (SRC-2) antibody (lane 6), or Fgr antibodies (lanes 1 and 7). The immunoprecipitates were washed thrice with lysis buffer and once with kinase buffer containing 20 mM HEPES (pH 7.6), 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenylphosphate, 0.1 mM Na_3VO_4, and 2 mM DTT. Kinase reactions were performed with 20 μM rATP–5 μCi of [γ-^{32}P]ATP in 100 μl kinase buffer for 20 min at 30°C (11). The samples were analyzed by SDS–12% PAGE. (e) 32Dcl3 and 32D cells transfected with v-*src*, v-*fgr* and *src/fgr* chimeras were depleted of IL-3 for a period of 10 days, and the percentage of viability of each cell line was determined by trypan blue exclusion.

any given STAT indicates its activated status. When Western blots were performed for the detection of STAT1, STAT3, and STAT5 in 32Dcl3 cells grown overnight in the absence of IL-3, all three STATs were found to be localized in the cytoplasm, while none of these transcription factors were seen in the nuclear extracts. Figure 2 shows that STAT1 antibody (E-23) recognizes two bands of 91 and 84 kDa, which represent STAT1a and STAT1b (8), respectively. Similarly, STAT5 an-

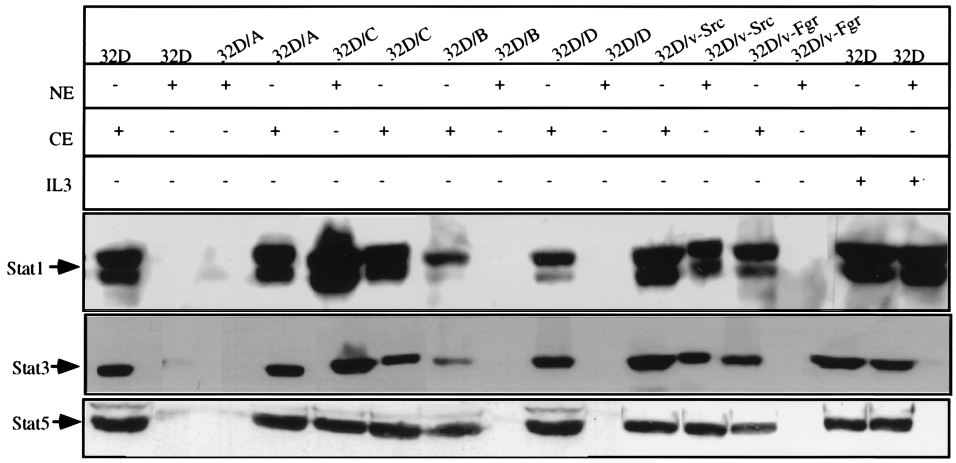

FIG. 2. Constitutive activation of STATs in 32D cells transformed by v-*src* and chimera C. Normal 32Dcl3 and 32D cells transfected with v-*src*, v-*fgr*, and *src/fgr* chimeras A, B, C, and D were grown in the presence or absence of IL-3, and cytoplasmic extracts (CE) and nuclear extracts (NE) were prepared. Following fractionation by SDS–10% PAGE, the proteins were transferred to Nytran membranes and probed with anti-STAT1, anti-STAT3, and anti-STAT5 antibodies.

tibody (C-17) was found to detect a doublet of 94 and 96 kDa, which represents STAT5 (3, 41). STAT3 antibody (C-20) recognized a single band of 92 kDa, which is characteristic of STAT3 (43). On the other hand, when these cells were incubated overnight in IL-3 and then analyzed for the subcellular localization status of these three STATs, they were all found to be localized in the nucleus, indicating their activated status. In v-*src*-transformed cells, each of the STATs was found to be present in the nucleus even in the absence of IL-3 in the medium. An identical profile was found in cells transformed with *src/fgr* chimera C. Interestingly, when we examined 32D cells transfected with *src/fgr* chimeras A, B, and D grown overnight in the absence of IL-3, all three STATs were found to be localized exclusively in the cytoplasm, suggesting their inactivated state. These results demonstrate that the activation of STATs is exclusively dependent on the presence of the SH2 and SH3 domains in *src/fgr* chimeras and that the kinase domain of either v-*src* or v-*fgr* can phosphorylate each of the STATs studied here. Therefore, it appears that substrate specificity lies in the combined SH2 and SH3 domains of v-*src*.

Tyrosyl phosphorylation of STAT proteins by v-*src* and *src/fgr* chimeras. To analyze the relationship between the expression of v-*src*, v-*fgr*, and *src/fgr* chimeras and STAT protein tyrosyl phosphorylation, we examined the tyrosine phosphorylation statuses of STATs in 32D cells transfected with various DNA constructs. Total cell extracts were prepared from various cell lines, and the cell lysates were used for immunoprecipitation by STAT-specific antibodies. These immunoprecipitates were then subjected to SDS-PAGE followed by Western blot analysis. The Western blots were probed with STAT-specific antibodies to visualize the different STAT proteins (Fig. 3A, C, and E), which showed that all of the cell lines examined here contained detectable levels of STAT1, STAT3, and STAT5. Probing of the same extract with monoclonal antibody 4G10, which specifically recognizes the phosphotyrosine moiety, showed that this antibody does not recognize any of the STATs that are immunoprecipitated from normal 32D cells grown in the absence of IL-3 or cells transfected with chimeras A, B, and D (Fig. 3B, D, and E). However, this antibody could readily detect STAT1, STAT3, and STAT5 immunoprecipitated from normal 32D cells grown in the presence of IL-3 as well as from 32D/v-Src and 32D/chimera C cells grown in the absence of IL-3. These results provide direct evidence that indeed v-Src and chimera C activate STAT1, -3, and -5 by phosphorylating them on tyrosine.

Mobility shift assays. Following cytokine-induced tyrosyl phosphorylation, STAT proteins translocate to the nucleus and modify gene expression by binding to STAT-specific sequences present in the promoter-enhancer regions of target genes. To examine whether the STATs that are activated in 32D cells transfected with v-*src*, v-*fgr*, and *src/fgr* chimeras can bind to their target sequences, nuclear extracts were prepared from normal 32Dcl3 cells and from 32D/v-Src and 32D/chimera C cell lines grown in the absence of IL-3. These extracts were examined for their abilities to bind ^{32}P-labeled STAT1-, -3, and -5-specific oligonucleotides. As negative controls, mutant oligonucleotides in which the STAT-binding sequence was disrupted were used.

The probe used for STAT-1 was a gamma interferon-activated site, Ly6ε, which was described earlier (24). This probe has been shown not to bind to STAT3 or STAT5 and specifically to interact with STAT1. In our assays, this probe gave two bands, whose composition is not fully characterized. However, it is known that the STAT1 gene codes for two proteins of 91 kDa (STAT1α) and 84 kDa (STAT1β), due to alternative splicing of mRNAs (7, 23). In cells in which both forms of the protein are expressed, multiple bands representing homodimers of STAT1α and heterodimers containing both STAT1α and STAT1β have been observed. Mutant oligonucleotides in which the STAT1 binding site has been disrupted failed to show similar binding, suggesting that the observed shift is dependent on the presence of the STAT1-binding motif in the oligonucleotide probe (Fig. 4 lane 4). To further determine the specificity of this binding, we examined the abilities of antibodies specific to STAT1 to interfere with the formation of DNA-protein complexes. The antibodies chosen for these studies were raised against a peptide corresponding to amino acids 688 to 710 derived from the C-terminal domain of the protein. Since this region is essential for the dimerization activity of STAT1 (13), the antibodies are expected to abrogate the DNA binding activity of STAT1. The results presented in Fig. 4 (lane 1) show that incubation of nuclear extracts with these antibodies completely abrogated the STAT1-binding activity of the extracts.

Gel shift assays performed with STAT3- and STAT5-specific probes gave essentially similar results. The STAT3 probe was

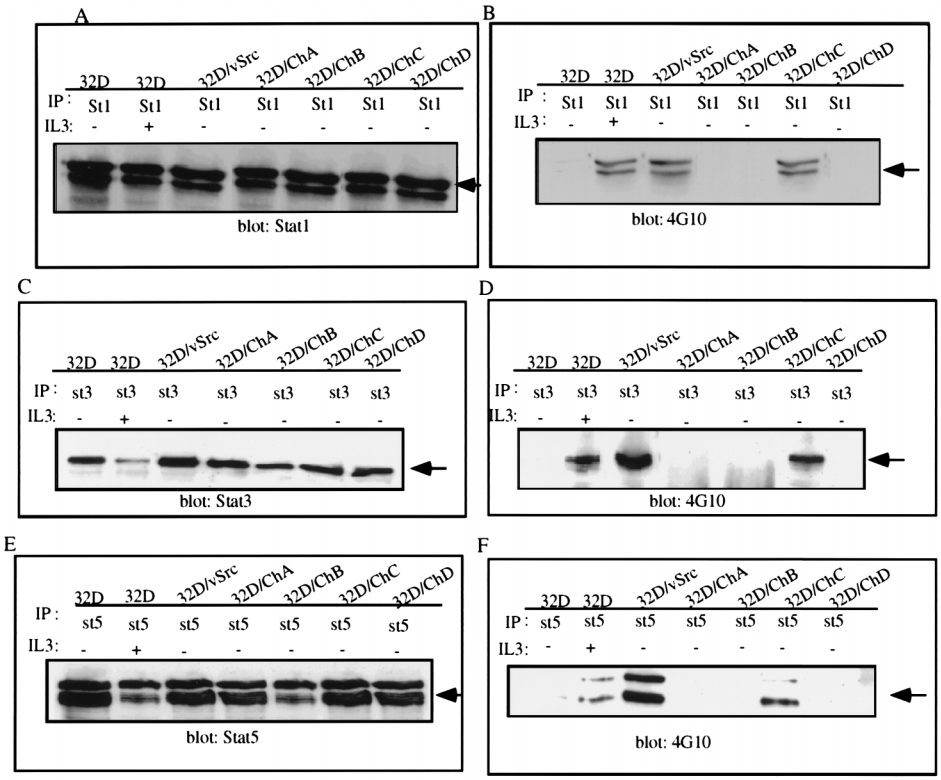

FIG. 3. Tyrosine phosphorylation of STATs in 32D cells transformed by v-*src* and *src/fgr* chimeras. Control 32D and 32D/chimera A (ChA), 32D/chimera B (ChB), and 32D/chimera D (ChD) cell lines were maintained in IMDM containing 10% IL-3, while 32D/v-Src and 32D/chimera C cells were maintained in IMDM without IL-3. When indicated, the cells were maintained in IL-3-free medium overnight before preparation of cell lysates. (A and B) Cell lysates were immunoprecipitated with anti-STAT1 and subjected to SDS-PAGE and Western blotting. The Western blots were then probed with anti-STAT1 antibody (A) or with antiphosphotyrosine antibody 4G10 (B). (C and D) Cell lysates were immunoprecipitated with STAT3 and probed with anti-STAT3 antibody (C) or with 4G10 antibody (D) on Western blots. (E and F) Cell lysates were immunoprecipitated with anti-STAT5 and probed with anti-STAT5 antibody (E) or with 4G10 antibody (F) on Western blots. IP, immunoprecipitate; St, STAT.

the APRE high-affinity Sis-inducible element (SIE) probe described earlier (43). Homodimers of STAT3 have been shown to bind to this probe specifically and yield a single band (42). In our assays, this probe gave a single band with extracts derived from v-*src* and chimera C-transformed cells (Fig. 4, lanes 11 and 12), while such interaction was not seen with extracts derived from normal 32D cells incubated in the absence of IL-3 (lanes 13 and 14). Mutant oligonucleotides in which the STAT3-binding site was disrupted failed to form this complex (lane 12). In addition, an antibody raised against a peptide corresponding to amino acids 750 to 769 derived from the C-terminal domain of STAT3 effectively blocked complex formation (Fig. 4, lane 9), further suggesting that this complex contains activated STAT3. To determine STAT5-binding activity, a probe derived from β-casein was used (24). This probe has been shown to bind specifically to STAT5 with high affinity, and the nuclear extracts derived from 32D/v-Src and 32D/chimera C cells (lanes 6 and 7) readily formed complexes with this oligonucleotide. A mutant form of this oligonucleotide in which the binding sequence was altered failed to form a specific DNA-protein complex (lane 8). In addition, an antibody raised against a peptide corresponding to amino acids 711 to 727 derived from the C-terminal domain of STAT5 effectively blocked complex formation (Fig. 4, lane 5), further suggesting that this complex contains activated STAT5. Similar assays with nuclear extracts derived from 32D cells transfected with v-*fgr* and *src/fgr* chimeras A, B, and D were performed. The nuclear extracts derived from these cell lines failed to form

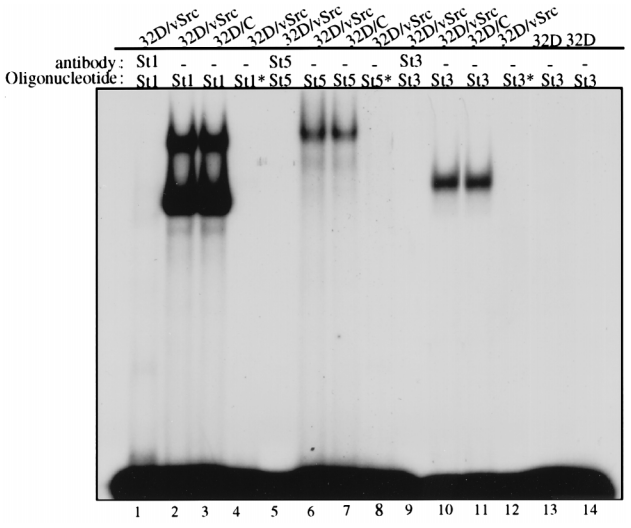

FIG. 4. Induction of STAT-binding activity in 32D cells transformed by v-*src* and chimera C. Nuclear extracts were prepared from normal 32D cells that were starved for IL-3 overnight and 32D cells transformed with v-*src* and chimera C were continuously maintained in IL-3-deficient medium and used for the preparation of nuclear extracts. Nuclear extracts containing 5 μg of total protein were used to carry out electrophoretic mobility shift assays with 10,000 cpm of ^{32}P-labeled STAT-specific (St) and mutant oligonucleotides (indicated by asterisks). A 5-μl volume of respective antibodies against STAT1, STAT3, or STAT5 was included in the binding reaction mixture for abrogation of the specific complex. The nuclear extracts prepared from 32D/chimera A, 32D/chimera B, and 32D/chimera D did not show any detectable binding activity (data not shown).

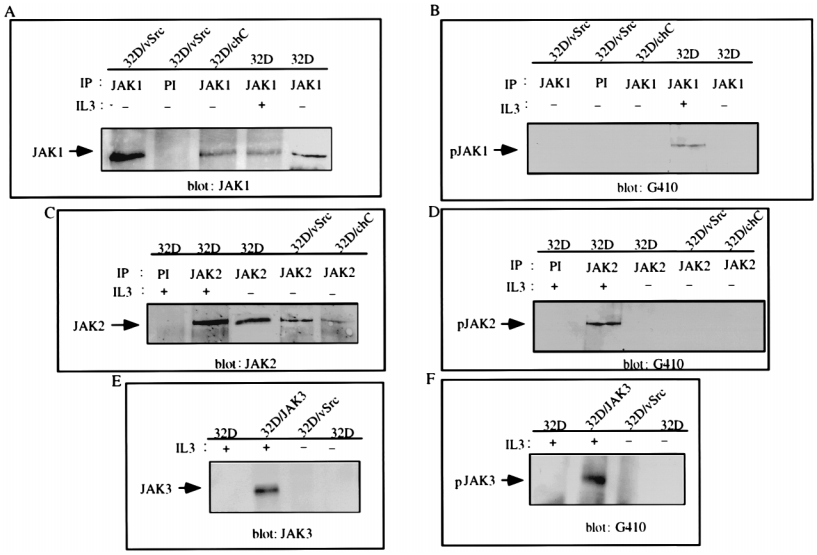

FIG. 5. v-Src does not phosphorylate JAK kinases. 32D cells starved for IL-3 (−) or stimulated with IL-3 for 15 min (+) were used as positive controls for JAK-1 and JAK-2 activation. JAK-3-overproducing cells (32D/JAK3) were maintained in IMDM containing 10% IL-3, while other 32D/Src and 32D/chimera C cells were maintained in IMDM without IL-3 for 18 h before lysis. As negative controls for phosphorylation, 32D cells incubated in the absence of IL-3 for 15 to 30 min were used for the preparation of cell lysates. Whole-cell lysates from the indicated cells containing equal amounts of protein (1 mg) were immunoprecipitated (IP) with 5 μl each of anti-JAK-1 (A), anti-JAK-2 (B), anti-JAK-3 (C), or preimmune serum (PI). The immunoprecipitates were dissolved in sample buffer as described by Laemmli (20) and were separated on an SDS–8% PAGE gel, and Western blot analysis was performed with anti-JAK antibodies or 4G10 monoclonal antibody, which recognizes phosphotyrosine. (A) Cell lysates were immunoprecipitated with anti-JAK-1 antibody or preimmune serum, and the Western blot was probed with anti-JAK-1 antibody. (B) The blot from panel A was stripped and reprobed with 4G10 antibody. (C) Cell lysates were immunoprecipitated with anti-JAK-2 antibody or preimmune serum, and the Western blot was probed with anti-JAK-2 antibody. (D) The blot from panel C was stripped and reprobed with 4G10 antibody. (E) Cell lysates were immunoprecipitated with anti-JAK-3 antibody or preimmune serum, and the Western blot was probed with anti-JAK-3 antibody. (E) The blot from panel D was stripped and reprobed with 4G10 antibody. ch, chimera.

STAT-specific DNA-protein complexes with all of the oligonucleotides under identical conditions (data not shown).

JAK kinases are not constitutively activated by v-*src* or chimera C. It is now well established that interaction of IL-3 with its receptor induces receptor dimerization, which increases the affinity of the cytoplasmic domain of the receptor for JAK-1 and JAK-2 kinases and results in a ligand-dependent increase in a complex that contains the receptors and JAK kinases (5, 41). This results in activation of JAK-1 and JAK-2 through an event associated with tyrosine phosphorylation. It is possible that v-*src* mediates its effect through constitutive activation of one or more of these JAK kinases. To test this possibility, we immunoprecipitated cell extracts from normal 32D cells and 32D/v-Src cells with JAK-specific antibodies. These immunoprecipitates were subjected to SDS-PAGE, and the Western blots were probed with respective JAK antibodies and monoclonal antibody 4G10. The results presented in Fig. 5A and C show that while JAK-1 and JAK-2 were present in the 32D cell line, they were phosphorylated only in IL-3-treated 32D cells (Fig. 5B and D). Both JAK-1 and JAK-2 appeared to be present in detectable quantities in v-*src*- and chimera C-transformed cells, but in an unphosphorylated state. Also, the presence and phosphorylation of JAK-3 were readily seen in 32D/JAK-3 cells (Fig. 5 E and F) that were transfected with a JAK-3 expression vector which was absent in v-*src*- or chimera C-transformed cells. These results indicate that the v-Src protein does not activate the JAK kinases.

Coimmunoprecipitation of STATs with v-Src. It has been recently demonstrated that STAT3 can be coimmunoprecipitated with anti-Src antibodies from cells that are transformed with v-*src* (4). To examine whether all of the STATs that are activated by v-*src* can be coimmunoprecipitated with anti-Src antibodies, we carried out coimmunoprecipitation analyses. In the first set, cell extracts were immunoprecipitated with anti-Src antibodies and then subjected to PAGE. Following the transfer of proteins onto a Nytran membrane, Western blotting was carried out with different anti-STAT antibodies. For all experiments, total cell extracts derived from 32D/v-Src cells were directly loaded onto the gel as positive controls (lanes 1 of Fig. 6A to C). Western blotting with anti-STAT antibodies showed that these antibodies readily detect STAT1, -3, and -5 in total cell extracts derived from 32D/v-Src cells. Immunoprecipitation of these extracts with preimmune serum (Fig. 6A, B, and C, lanes 3) failed to precipitate any protein that cross-reacted with anti-STAT antibodies on Western blots. However, immunoprecipitation of extracts with anti-Src antibodies followed by Western blot analysis with anti-STAT antibodies revealed that STAT3 can be readily coimmunoprecipitated with anti-Src antibodies (Fig. 6A, lane 2). On the other hand, STAT1 and STAT5 failed to coimmunoprecipitate with anti-Src antibodies (Fig. 6B and C, lanes 2). However, these proteins could be readily immunoprecipitated by STAT-specific antibodies (lanes 4 of Fig. 6B and C). When cell extracts of control 32D cells grown in the presence of IL-3 were used in these coimmunoprecipitation assays, we observed that anti-Src antibodies coimmunoprecipitated STAT3. This appears to be due to the presence of c-Src protein in actively proliferating 32D cells, since the same result could be obtained with two different antibodies (SRC-2 and N-16) that immunoprecipitate c-Src (data not shown). This band was not seen in 32D cells grown in the absence of IL-3 (lane 4 of Fig. 6B), in which c-Src levels become undetectable, further suggesting that the band seen in lane 5 of Fig. 6A is STAT3 that coimmunoprecipitated with c-Src. This observation is consistent with those made by others with c-Src (42). These results suggest that while STAT3 stably associates with v-Src as well as c-Src, STAT1 and

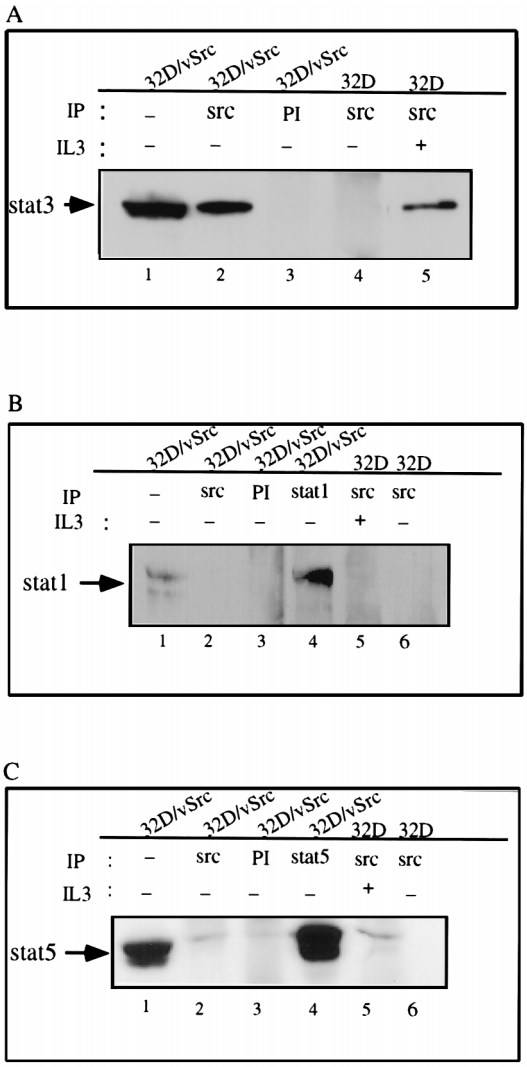

FIG. 6. Coimmunoprecipitation of STAT3 with v-Src in 32D/v-Src cells. 32D/v-Src cells were maintained in the absence of IL-3. Normal 32D cells were maintained in the presence of IL-3 and, when indicated (−), cells were maintained for 18 h in the absence of IL-3 prior to the preparation of cell extracts. Whole-cell lysates from the indicated cells were immunoprecipitated (IP) with 5 μl of anti-Src antibody (SRC-2) or preimmune serum (PI). Immune complexes were resolved by SDS–10% PAGE, transferred to Nytran membranes, and probed with anti-STAT3 (A)-, anti-STAT1 (B)-, or anti-STAT5 (C)-specific antibodies.

STAT5, which are activated by this oncogene, do not coimmunoprecipitate with v-Src even though they are phosphorylated and activated in v-*src*-transformed cells.

DISCUSSION

In this study, we have examined the molecular mechanisms associated with v-*src*-mediated transformation of IL-3-dependent myeloid cells, which results in the IL-3 independent proliferation of transformed cells (1). It has been observed in the past by several investigators that several activated tyrosine kinases that belong to the Src oncogene family transform NIH 3T3 cells in vitro. However, only a few of these genes seem to be capable of transforming myeloid cells such that they become IL-3 independent for growth. Several highly oncogenic tyrosine kinases that are closely related to v-Src (such as v-Fgr) and that can readily transform NIH 3T3 cells fail to bring about this phenotypic effect in IL-3-dependent cell lines (4, 42). The molecular basis for this difference is unknown. To address this specific issue, we have taken advantage of the close structural relationship between the v-*src* and v-*fgr* genes and constructed chimeras, in which the tyrosine kinase domain- and SH2 or SH3 domain-encoding regions of these two genes have been interchanged. We have examined the abilities of these chimeras to transform the 32Dcl3 cell line, which is derived from normal mouse bone marrow and strictly requires IL-3 for growth. This cell line has been shown to become IL-3 independent for growth when transformed by v-*src* but not by v-*fgr* (our unpublished data). Our results show that while the tyrosine kinase domain of v-*src* can be replaced by the tyrosine kinase domain of v-*fgr*, neither the SH2 nor the SH3 domain of v-*src* can be replaced by those of *fgr*. Thus, while chimera C, which contained the SH2 and SH3 domains of v-*src* and the tyrosine kinase domain of v-*fgr*, could render 32D cells IL-3 independent for growth, chimeras A, B, and D failed to do so. Of these, chimera A lacked the SH3 domain of v-*src*, which was replaced by the Gag-actin domain of v-*fgr*. In chimera B, the SH2 and catalytic domains of v-*src* were replaced by the SH2 and catalytic domains of v-*fgr*, while in chimera D, only the SH2 domain of v-*src* was replaced by that of v-*fgr*. Similarly, c-*fgr*-Δ, which contained both the SH2 and the SH3 domains of c-*fgr*, also failed to render 32D cells IL-3 independent. Replacing the kinase domain of c-*fgr*-Δ with that of v-*src* failed to produce a difference in the phenotype seen with c-*fgr*-Δ (data not shown). Since only v-*src* and chimera C can render the 32Dcl3 cells IL-3 independent, we conclude that both the SH2 and the SH3 domains of v-*src* are necessary to bring about this biological change in 32D cells. It is now well established that the SH2 domains of the Src family of kinases play an important role in binding to the phosphotyrosine moiety on cellular substrates and mediate their binding to specific signaling molecules present within the cell (18, 32). On the other hand, the SH3 domain has been shown to play an important role in protein-protein interactions and appears to mediate binding of the Src family of tyrosine kinases to proline-rich sequences present in other cellular proteins that are specifically recognized by different SH3 domains (18). The present report is the first which demonstrates that these two domains act in concert in the recognition of specific cellular substrates such as STATs, which themselves contain an SH3 domain.

Current models suggest that the binding of cytokines to their cognate receptors brings about their dimerization, which in turn increases the affinity of the receptors for JAK kinases and results in the activation of JAK kinases through an event associated with tyrosine phosphorylation. The activated kinases appear to then phosphorylate cellular substrates, the most important of which are the STATs (31). STATs seem to belong to a growing family of transcription factors, of which six members (STAT1 to STAT6) have been identified so far. All of the STAT proteins are highly related and are characterized by the presence of functional C-terminal SH2 and SH3 domains (10). STATs in their inactive state are localized to the cytoplasm and, following phosphorylation, appear to migrate to the nucleus and activate transcription of target genes. Since IL-3-mediated growth of myeloid precursor cells results in activation of the JAK-STAT pathway (3, 34), it could be hypothesized that abrogation of IL-3 dependence by v-*src* is brought about via the activation of this pathway. The results presented in this study show that the phosphorylation status of JAKs is unaffected in v-*src*-transformed cells. However, when the phosphorylation status of STATs was examined, several members of this family were found to be constitutively activated in v-*src*-transformed cells, even in the absence of any

cytokines in the medium. Thus, we find that STAT1, STAT3, and STAT5 are constitutively phosphorylated on tyrosine and stay localized in the nuclei of v-*src*-transformed cells. These results suggest that these transcription factors are direct targets of v-*src*, which phosphorylates these proteins, resulting in their constitutive localization in the nucleus. In control cells or cells transfected with v-*fgr*, phosphorylation of these proteins was observed only in the presence of IL-3. This led us to examine whether v-*src* physically associates with STATs via its SH2 and SH3 domains. Our coimmunoprecipitation studies provide strong support to the idea that STAT3 and v-Src physically associate with each other, which, in turn, seems to result in the phosphorylation of STAT3. Thus, we could precipitate STAT3 with anti-Src antibodies. However, under identical conditions, we could not precipitate STAT1 and STAT5 proteins with anti-Src antibodies. This could be due to the possibility that STAT1 and STAT5 interact with v-Src with a lower affinity than that of STAT3, and the rigorous immunoprecipitation protocols used in this study do not allow coimmunoprecipitation of these two proteins with v-Src. Alternatively, STAT1 and STAT5 could be activated by v-Src by an indirect mechanism which involves intermediary kinases that are activated by v-Src. The interaction between STAT3 and v-Src seems to require both the SH2 and the SH3 domains of v-*src*, since only chimera C could activate STAT3. We did not find coimmunoprecipitation of STAT3 with anti-Src antibodies in 32D cells transformed by chimeras A, B, and D (data not shown). This is consistent with the data presented in Fig. 2, 3, and 4, in which we did not observe their activation as measured by phosphorylation on tyrosine, migration to the nucleus, and an ability to bind STAT-specific DNA sequences.

These results suggest that v-*src*-mediated abrogation of IL-3 dependence in 32Dcl3 cells is achieved via constitutive phosphorylation of STAT1, STAT3, and STAT5, in the absence of any detectable JAK activation. These results are consistent with recent reports, in which other investigators have shown that v-Src phosphorylates STAT3 in NIH 3T3 and rat embryo fibroblast cells (4, 42). However, the mechanisms associated with NIH 3T3 cell transformation by v-*src* appear to differ from that seen with 32D cells, in that STAT1 and STAT5 are not activated by v-*src* in NIH 3T3 cells. The mechanisms associated with v-*src*-mediated transformation of myeloid cells seem to be also different from that of v-*abl*, since in v-*abl*-transformed cells, activation of JAK-1 and JAK-3 was observed (6). This is not surprising, since v-*abl* lacks the SH3 domain (28) and might be incapable of interacting directly with STATs. Similarly, recent reports indicate phosphorylation of STAT1 and STAT5 in *bcr-abl*-transformed cells (5). However, the mechanism by which Bcr-Abl mediates this activation appears to be different from that of v-Src, since Bcr-Abl coimmunoprecipitates with and constitutively phosphorylates the β_c subunit of the IL-3 receptor (27). This interaction appears to lead to the tyrosine phosphorylation of JAK kinases, which in turn seem to mediate STAT phosphorylation (9, 27).

The requirement of the SH2 and SH3 domains of Src for substrate interaction has also been observed with one other substrate, p68, which becomes tyrosine phosphorylated and physically associates with Src during mitosis in mouse fibroblasts (36). Interaction of p68 with Src involves the SH2 and SH3 domains of Src. In the case of Src-p68 interaction, it has been proposed that the SH2 domain binds phosphotyrosine-containing sequences within p68, whereas the SH3 domain may provide an additional p68-binding site or may mediate transient p68-Src binding before tyrosine phosphorylation and SH2 binding. Since both v-Src and STATs that are phosphorylated by Src contain SH2 and SH3 domains as well as phosphotyrosine, it is possible that phosphotyrosine of v-Src binds to the SH2 domain of STAT3 and the phosphotyrosine moiety of STAT3 binds to the SH2 domain of Src, allowing a very stable interaction between the proteins. Determination of the domains of STATs that mediate binding to v-Src should further aid in understanding the molecular mechanisms that contribute to the signal transduction pathways associated with v-*src*-mediated transformation.

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