Constitutive Activation of JAK1 in Src-transformed Cells*

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We have previously found that the signal transducer and activator of transcription (Stat) 3 is constitutively activated in cells stably transformed by the v-Src oncoprotein. While activation of Stat proteins has also been observed following epidermal growth factor or platelet-derived growth factor stimulation, Stat3 activation is more commonly associated with signaling through cytokine receptors and activation of the Janus family tyrosine kinases JAK1 or JAK2. We therefore investigated whether JAK1 or JAK2 were activated in Src-transformed cells. In three v-Src-transformed fibroblast cell lines (NIH3T3, Balb/c, and 3Y1), JAK1 displayed increased tyrosyl phosphorylation compared to non-transformed cells. The level of tyrosyl phosphorylation of JAK1 was significantly greater in NIH3T3 cells transformed by expression of v-Src or high levels of a constitutively active mutant of c-Src (Y527F) than in cells overexpressing the less transforming normal c-Src. Enzymatic activity of JAK1 was assessed using autophosphorylation assays. In anti-JAK1 immunoprecipitates from v-Src-transformed NIH3T3 cells, a protein with the same migration as JAK1 showed substantially increased levels of 32P incorporation compared to immunoprecipitates from non-transformed cells. Similar results were obtained using anti-JAK2 immunoprecipitates; however, the level of JAK2 tyrosyl phosphorylation and 32P incorporation in anti-JAK2 immunoprecipitates were markedly lower than in anti-JAK1 immunoprecipitates. We conclude that JAK1, and possibly JAK2, are constitutively activated in Src-transformed cells, raising the possibility that Janus family kinases contribute to the constitutive activation of Stat3 previously observed in these cells and/or other properties of Src-transformed cells.

The product of the v-src oncogene, v-Src, is a constitutively activated protein-tyrosine kinase that is thought to induce cell transformation through unregulated tyrosyl phosphorylation and activation of normal cellular pathways involved in the control of cellular growth (1, 2). Although transformation by oncogenic forms of Src is one of the most thoroughly character-
EXPERIMENTAL PROCEDURES

Cells and Reagents—3Y1, NIH3T3, Balb/c, and their Src-transformed counterparts have been described earlier (11-13). Anti-JAK2 serum (αJAK2), raised against a synthetic peptide corresponding to amino acids 755-764, was prepared in our laboratory in conjunction with Pels Freese Laboratories as described previously (9). Anti-JAK1 serum (αJAK1), raised against a synthetic peptide corresponding to amino acids 785-804 of murine JAK1, was kindly provided by J. Ihle, St. Jude Children's Research Hospital, Memphis, TN. Anti-Src monoclonal N2-17 was obtained from the NCI repository (Quality Biotech.). Mouse monoclonal anti-phosphotyrosine antibody 4G10 (αPY) was purchased from Upstate Biotechnology Inc. TritonX-100 came from Pierce, recombinant protein A-agarose from Repligen, and enhanced chemiluminescence (ECL) detection system from Amersham Corp. All other reagents were of reagent grade or better.

Immunoprecipitation and Western Blotting—Exponentially growing cultures were rinsed three times with PBSV (10 mM sodium phosphate, pH 7.4, 137 mM NaCl, 1 mM sodium orthovanadate) and scraped in lysis buffer (50 mM Tris, pH 7.5, 0.1% Triton X-100, 137 mM NaCl, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin) on ice. Cell lysates were centrifuged at 12,000 × g for 10 min and the resulting supernatants were incubated on ice for 2 h with the indicated antibody. Immune complexes were collected on protein A-agarose during a 60-min incubation at 8°C, washed three times with 50 mM Tris, pH 7.5, 0.1% Triton X-100, 137 mM NaCl and boiled for 5 min in a mixture (80:20) of lysis buffer and 5 × SDS-PAGE sample buffer (95 mM Tris (pH 6.8), 10% SDS, 10% β-mercaptoethanol, and 40% glycerol). The supernatant was subjected to SDS-PAGE followed by Western blot analysis with the indicated antibody using the ECL detection system (14).

Kinase Assays—Exponentially growing cultures were rinsed three times with PBSV and scraped on ice in 25 mM HEPES, pH 7.4, 0.1% Triton X-100, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. Cell lysates were centrifuged at 12,000 × g for 10 min and the resulting supernatants were incubated on ice for 2 h with the indicated antibody. Immune complexes were collected on protein A-agarose during a 60-min incubation at 8°C, washed three times with 50 mM Tris, pH 7.5, 0.1% Triton X-100, 137 mM NaCl and once with phosphorylation buffer (50 mM HEPES, pH 7.6, 0.1% Triton X-100, 0.5 mM dithiothreitol, 150 mM NaCl) and once with phosphorylation buffer (50 mM HEPES, pH 7.6, 0.1% Triton X-100, 0.5 mM dithiothreitol, 6.25 mM manganese chloride, 100 mM NaCl). For autophosphorylation assays, 100 µl of phosphorylation buffer containing ~100 µCi of γ-32P-ATP and 100 µg/ml each of aprotinin and leupeptin was added directly to the immune complexes on ice. Reactions were incubated at 30°C for 10 min (a time at which 32P incorporation was still in the linear range) then stopped by addition of stop buffer (wash buffer containing 10 mM EDTA). The immune complexes were washed once with stop buffer, twice with wash buffer, and boiled for 5 min in SDS-PAGE sample buffer. Solubilized proteins were resolved by SDS-PAGE on 3-10% polyacrylamide gradient gels. Phosphorylated proteins were visualized by autoradiography.

RESULTS

JAK1 and JAK2 Are Tyrosyl-phosphorylated in v-Src-transformed Cells—Since the Janus kinases are tyrosyl-phosphorylated when activated, we first examined whether αJAK1 or αJAK2 displays increased tyrosyl phosphorylation in v-Src-transformed cells. JAK1 and JAK2 were immunoprecipitated from 3Y1, murine NIH3T3, or murine Balb/c cells and their v-Src-transformed counterparts SR1, NIH-VS, and Balb/c-VS, respectively. The immunoprecipitated proteins were analyzed by Western blotting using an antibody to phosphotyrosine (αPY) (Fig. 1). Both JAK1 and JAK2 showed increased levels of tyrosyl phosphorylation in all three v-Src-transformed lines when compared to their non-transformed counterparts. However, the apparent level of tyrosyl phosphorylation of the αJAK1 immunoprecipitates was substantially greater than that observed in αJAK2 immunoprecipitates. Additionally, while an elevated level of JAK1 tyrosyl phosphorylation was detectable in all experiments, tyrosyl phosphorylation of JAK2 was more variable, being undetectable in some experiments.

The increased levels of tyrosyl-phosphorylated JAK1 and JAK2 reflect increased tyrosyl phosphorylation of the proteins rather than increased levels of expression. Expression of v-Src did not increase expression levels of JAK1 or JAK2 in any of the three cell lines as judged by Western blotting (data not shown). In fact, NIH3T3 cells expressing v-Src or other Src variants (see below) had slightly decreased (by 30-40%) JAK1 expression compared to untransformed NIH3T3 cells.

Interestingly, αJAK1 immunoprecipitates from v-Src-transformed cells contained a number of other tyrosyl-phosphorylated proteins in addition to JAK1 (Fig. 1, lanes A-F, and F). Additional tyrosyl-phosphorylated proteins were also apparent in the αJAK2 immunoprecipitates (Fig. 1, lanes H, J, and L) but, like the tyrosyl phosphorylation of JAK2 itself, their number, intensity, and reproducibility were less than observed in αJAK1 immunoprecipitates. One of these associated proteins appears to be Src (see below). While we do not know the identity of any of the other proteins at the present time, they may represent potential JAK substrates.

The Level of Tyrosyl Phosphorylation of JAK1 and -2 Parallels the Transforming Potency of Src—We examined the level of tyrosyl phosphorylation of JAK1 and JAK2 in cells overexpressing forms of Src with different transforming potency (Fig. 2). JAK1 (Fig. 2, lanes A-D) and JAK2 (Fig. 2, lanes E-H) were immunoprecipitated from control NIH3T3 cells (Fig. 2, lanes A and E) and from NIH3T3 cells transformed by high levels of expression of normal c-Src (Fig. 2, lanes B and F), the Y527F constitutively active mutant of c-Src (Fig. 2, lanes C and G) or v-Src (Fig. 2, lanes D and H), and analyzed by immunoblotting with αPY. The level of tyrosyl-phosphorylated JAK1 and JAK2 was significantly higher in immunoprecipitates from cells transformed by the more highly transforming c-Src Y527F and v-Src than in cells partially transformed by overexpression of the more weakly transforming normal c-Src.

Src Co-precipitates with JAK1—The αJAK Immunoprecipitates from Src expressing NIH3T3 cells contain tyrosyl-phosphorylated proteins migrating at a position appropriate for Src (Fig. 2). To test if Src was indeed present, we used an anti-Src antibody to probe αJAK1 immunoprecipitates from control, c-Src overexpressing, and v-Src expressing NIH3T3 cells (Fig. 3, lanes A-C). αJAK recognized a band of the appropriate size (~60 kDa) for Src in immunoprecipitates from cells either expressing v-Src or overexpressing c-Src. No Src was detected in immunoprecipitates from control NIH3T3 cells or where non-immune serum was used in place of αJAK1 (data not shown). Significantly more Src was detected in the immuno-
Fig. 2. JAK1 and JAK2 show greater tyrosyl phosphorylation in cells transformed by more oncogenic variants of Src. aJAK1 (lanes A–D) and aJAK2 (lanes E–H) immunoprecipitates were prepared from actively growing subconfluent cultures of NIH3T3 cells (lanes A and E) and NIH3T3 cells transformed by overexpression of normal c-Src (lanes B and F), expression of c-Src Y527F (lanes C and G), or v-Src (lanes D and H). The immunoprecipitated proteins were subjected to Western blot analysis using αPY. Immunoblot analysis of equivalent immunoprecipitates with the immunoprecipitating antibody (αJAK1 or αJAK2) showed that equal amounts of JAK1 and JAK2 were present in the immunoprecipitates from each of the four different cell lines (data not shown). The unlabeled arrow (↑) marks the estimated migration of Src proteins.

Fig. 3. Src co-precipitates with JAK1. Lysates of NIH3T3 cells (lanes A and D) and NIH3T3 cells transformed by v-Src (lanes C and F) or overexpression of c-Src (lanes B and E) were immunoprecipitated with αJAK1 (lanes A–C) or αSrc (lanes D–F). The immunoprecipitated proteins were subjected to immunoblot analysis using αSrc. Equivalent amounts of cellular protein were used in each immunoprecipitate, and the two panels shown are from the same experiment. The exposure time for the autoradiograph shown for lanes A–C was 6 times longer than that for lanes D–F.

Precipitates from cells overexpressing c-Src than in those from v-Src-transformed cells. Western blotting of αSrc immunoprecipitates with αSrc (Fig. 3, lanes D–F) indicated that this difference most likely reflects the different levels of Src protein expressed in these two cell lines.

JAK1 and JAK2 Immunoprecipitates from v-Src-transformed Cells Have Increased Kinase Activity—We sought to determine if JAKs 1 and 2 isolated from Src-transformed cells possess increased tyrosyl phosphorylation. JAK1 and JAK2 immunoprecipitates from normal and Src-transformed cells were subjected to immune-complex kinase assays to measure autophosphorylation. In αJAK1 immunoprecipitates assayed for kinase activity, a protein with the same migration as JAK1 displays increased incorporation of 32P in immunoprecipitates from v-Src-transformed NIH3T3 cells compared to untransformed cells (Fig. 4, lane B). The amount of JAK1 present in each immunoprecipitate was the same as judged by Western blotting with αJAK1 (data not shown). In addition to JAK1, several other proteins had increased 32P incorporation in immunoprecipitates from v-Src-transformed cells and may represent JAK1-associated proteins. Preincubation of αJAK1 with its antigenic peptide (Fig. 4, lane C), but not with an analogous peptide derived from JAK2 (Fig. 4, lane D), prevented the appearance of all bands showing increased phosphorylation in immunoprecipitates from v-Src-transformed cells. These bands were also absent when non-immune rabbit serum was used in place of αJAK1

DISCUSSION

Our results show that the Janus kinases JAK1 and JAK2 are constitutively tyrosyl-phosphorylated in Src-transformed cells and suggest that JAK1, and possibly JAK2, is in an activated state in these cells. These findings raise the possibility that JAK1 and/or JAK2 might be at least partly responsible for the constitutive activation of Stat3 previously described (11) in Src-transformed cells. In this regard, it is interesting to note that both JAK1 and JAK2 contain putative binding sites (YXXQ motif, Ref. 15) for Stat3, while neither v-Src nor c-Src contains these sites. It is also interesting to speculate that the apparent greater activation of JAK1 compared to JAK2 might account for the observed targeting of Stat3 rather than Stat1 for activation in Src-transformed cells. While JAK2 contains a motif similar to the putative Stat1 binding site (16) in the interferon-γ receptor, JAK1, v-Src and c-Src do not. Although both JAK1 and JAK2 have been shown to phosphorylate Stat1 in vitro (see Fig. 5 in Ref. 17), thus, under conditions of normal levels of expression of the JAK and Stat proteins, JAK1 activation alone may not be sufficient for Stat1 activation. On the other hand, when both Src and Stat3
are expressed in yeast, Src can phosphorylate Stat3 without the assistance of JAK1. However, these results in yeast do not preclude the possibility that JAK1 could be required for Stat3 activation by Src in mammalian cells. Further work will be required to resolve the role of JAK1 in the constitutive activation of Stat3 observed in Src-transformed cells.

These findings also raise the question of whether activation of JAK1 and Stat3 might be important in the normal function of c-Src. It has been established that one of the functions of c-Src is to participate in intracellular signaling initiated by the binding of a number of growth factors, such as PDGF, colony-stimulating factor 1, and EGF, to their cell surface receptors. Src binds directly to these activated receptors and becomes itself activated (18–21). Microinjection of either neutralizing anti-Src family kinase antibodies or a dominant-negative mutant of c-Src into cells prior to stimulation blocks PDGF, colony-stimulating factor 1, or EGF-induced entry into S-phase (22, 23), suggesting that activation of Src family kinases is necessary for these factors to induce mitogenesis. Each of these factors has been shown to promote the tyrosyl phosphorylation and DNA binding activity of proteins recognized by an antibody directed against the C-terminal portion of Stat3 (10). Activation of JAK1 has also been reported in EGF-stimulated cells. It is possible that c-Src has a role in activation of Stat3 and JAK1 by these growth factor receptors.

What role the constitutive activation of the JAKs might play in Src-dependent cell transformation is not clear. Unlike the growth factor receptor tyrosine kinases, such as the receptors for EGF and PDGF, that have been directly linked to mitogenesis, signaling through the JAK-Stat pathway(s) has been primarily associated with the maintenance, differentiation, and activation of cells of the hematopoietic and lymphocytic lineages. Yet, cellular differentiation often involves proliferation, and several lines of evidence suggest that increased activity of JAK2 may be responsible for increased proliferation of EPO-dependent hematopoietic progenitor cells. In most systems examined thus far, JAK and Stat activation is highly transient, even in the continued presence of the activating cytokine. The tight regulation of JAK-Stat signaling is thought to be accomplished through the activity of cellular phosphatases. In CHO cells expressing a mutant form of the EPO receptor that lacks the ability to bind SHP-1, EPO-induced JAK2 activation is greatly prolonged compared to that observed in 32D cells expressing the wild type EPO receptor (24). In mice bearing the mutations “Motheaten” or “Motheaten viable,” the hematopoietic progenitor cells lack functional SHP-1 (25). These mice are expressed in yeast, Src can phosphorylate Stat3 without the assistance of JAK1. However, these results in yeast do not preclude the possibility that JAK1 could be required for Stat3 activation by Src in mammalian cells. Further work will be required to resolve the role of JAK1 in the constitutive activation of Stat3 observed in Src-transformed cells.

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