Malignant Transformation but not Normal Cell Growth Depends on Signal Transducer and Activator of Transcription 3

Karni Schlessinger and David E. Levy

Departments of Pathology and Microbiology and New York University Cancer Institute, New York University School of Medicine, New York, New York

Abstract

Signal transducer and activator of transcription 3 (STAT3) has been indirectly implicated in numerous fundamental cellular processes, including proliferation, survival, and differentiation. We provide genetic evidence from studies of STAT3-null cells that STAT3 is dispensable for normal growth of mouse fibroblasts in culture. STAT3 contributed to the full induction of some (typified by c-myc) immediate early gene expression, but STAT3-independent processes were sufficient to support full cell growth and survival. However, STAT3 was required to manifest a transformed state following expression of v-src, and STAT3-null cells were impaired for anchorage-independent growth as colonies in soft agar and as tumors in mice. The data suggest that STAT3 mediates the maintenance of focal adhesion kinase activity in the absence of cell adhesion by suppressing the action of an inhibitory phosphatase. (Cancer Res 2005; 65(13): 5828-34)

Introduction

Signal transducers and activators of transcription (STAT) are latent transcription factors that are activated by cytokines and growth factors. Tyrosine phosphorylation leads to STAT dimerization, nuclear translocation, and activation of gene expression (1, 2). STAT3 is phosphorylated by various tyrosine kinases such as Janus-activated kinases and src family kinases that are associated with and activated by cytokine and growth factor receptors. STAT3 activation has been implicated in various biological responses, including proliferation, survival, differentiation, and transformation (2). In addition, genetic ablation of STAT3 in mice leads to embryonic lethality, suggesting that STAT3 is essential for basic processes.

An important role for STAT3 in src signaling has been suggested by a variety of experimental data, both as a mediator of proliferation downstream of c-src and as an essential arbiter of cell transformation by the v-src oncogene. For instance, STAT3 is activated by c-src downstream of growth factor receptors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors. Indeed, activated STAT3 was first detected as a DNA binding activity that associated with the c-fos promoter in PDGF-stimulated cells (3). Activation of STAT3 by c-src downstream of the PDGF receptor has also been associated with c-myc transcriptional induction and proliferation of fibroblasts (4), suggesting a direct role for STAT3 in cell growth.

Following the seminal observation that STAT3 is a phosphorylated substrate of v-src (5), increasing evidence has accumulated for a role for STAT3 in the oncogenic action of v-src. Interference with STAT3 signaling by ectopic expression of STAT3 mutants that act as dominant-interfering molecules impairs cell transformation (6, 7). Moreover, a constitutively dimerized form of STAT3 that displays unregulated transactivation potential can promote fibroblast and epithelial cell transformation in the absence of v-src, implying that STAT3 is a potential oncogene on its own (8, 9). This notion is supported by the observation that STAT3 is often abnormally expressed and activated in diverse human tumors, and interference with STAT3 function can impair the viability of transformed cells. Such data provide compelling evidence for a role for STAT3 in tumorigenesis involving src and additional oncogenes (10–12).

Roles for c-src in cell proliferation and malignant transformation remain incompletely understood. During normal cell growth, c-src can be activated by growth factor stimulation and integrin engagement to affect cell proliferation, adhesion, and migration (13). Many of the effects of src on cell adhesion, cell spreading, and migration are thought to be mediated by activation of focal adhesion kinase (FAK), a tyrosine kinase that contributes to the oncogenic action of v-src (14) and is activated by integrins (15). Integrin clustering recruits FAK into focal adhesions where it is activated by autophosphorylation. FAK activation leads not only to proliferation and survival but also regulates cell spreading and migration, most clearly shown by the defects in FAK-null cells (16). Down-regulation of FAK activity can occur through mechanisms involving a naturally occurring dominant-negative form of FAK, proteolytic cleavage, or dephosphorylation by phosphatases (14, 17). FAK down-regulation is important for the turnover of focal adhesions during cell migration and is a major target for v-src in transformed cells, contributing to altered adhesion, migration, and invasion (18).

In this study, we took a genetic approach to studying STAT3 signaling, by conditional gene ablation in primary and immortal mouse fibroblasts. We show that STAT3 is required for some immediate early gene induction downstream of c-src, activated by growth factor receptors such as PDGF receptor, and we identify a novel role for STAT3 in regulation of cell adhesion, through control of FAK phosphorylation in response to v-src. Although we provide definitive evidence for STAT3 involvement in v-src transformation, we found no evidence for an obligatory role for STAT3 in regulation of c-myc expression or for normal fibroblast proliferation.

Materials and Methods

Antibodies, DNA constructs, and chemicals. The following antibodies were obtained from commercial sources: STAT3 (clone 5G7, Zymed, South San Francisco, CA), P-STAT3 (Cell Signaling, Beverly, MA), pp60-src (Upstate, Waltham, MA), tubulin (Sigma, St. Louis, MO), c-Fos, FAK (Santa

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K. Schlessinger is currently at the Medical Research Council Laboratory for Molecular Cellular Biology and Cell Biology Unit, University College London, Gower Street, London WC1E 6BT, United Kingdom.

Requests for reprints: David E. Levy, Departments of Pathology and Microbiology and New York University Cancer Institute, New York University School of Medicine, 550 First Avenue, New York, NY 10016. Phone: 212-263-8192; Fax: 212-263-8211; E-mail: del243@med.nyu.edu.

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Cruz Biotechnology, Santa Cruz, CA), and P-FAR \(^{39}\) (Biosource, Camarillo, CA). PDGF-BB was purchased from Becton Dickinson (Mountain View, CA); SU6656 was purchased from Calbiochem (La Jolla, CA); and staurosporine was purchased from LC Laboratories (Woburn, MA).

Expression construct for v-src (pMv-src) has been described (19). STAT3 constructs were generated by PCR-directed mutagenesis and cloned into the bicistronic green fluorescent protein (GFP) retroviral vector Pallino (20). STAT3\(^{W T}\) carries a mutation of Tyr\(^{705}\) to phe, and STAT3\(^{D BD}\) carries a five- amino-acid substitution in the DNA binding domain (21).

**Cell culture.** Primary mouse embryo fibroblasts (MEF) were prepared by standard techniques (22) from E14-15 STAT3\(^{lox/lox}\) or STAT3\(^{lox/+}\) embryos and used for experiments before passage 5. Immortalized cell lines were generated by the 3T3 protocol (23). The lines used had sustained inactivating mutations of the tumor suppressor p53 (data not shown).

**Protein analysis.** Total cell extracts were prepared in 300 mmol/L NaCl, 50 mmol/L HEPES (pH 7.6), 1.5 mmol/L MgCl\(_2\), 10% glycerol, 1% Triton X-100, 10 mmol/L NaPyriPO\(_4\), 20 mmol/L NaF, 1 mmol/L EGTA, 0.1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L NaVO\(_4\), and protease inhibitor cocktail (Sigma). Proteins were analyzed by SDS-PAGE and immunoblotting, using horseradish peroxidase–labeled secondary antibodies (Pierce, Rockford, IL), as previously described (24).

**Retroviral infection.** Recombinant retroviruses were packaged using Phoenix cells, and infections were carried out using standard protocols (25). In brief, Phoenix cells were transfected in the presence of 25 \(\mu\)g/mL chloroquine using the calcium phosphate method, 48-hour supernatants were used to infect 1 \(\times\) 10\(^5\) cells in the presence of 40 \(\mu\)g/mL polybrene and infected cells were selected by drug resistance. STAT3 constructs also expressed GFP from the same RNA, and GFP expression was used to select infected cells displaying similar expression levels by flow cytometry. Levels of v-src protein in transformed cells were verified by antibody blot.

**RNA quantification.** Abundance of specific RNA species was measured by real-time fluorescent reverse transcription-PCR using Syber green dye (Molecular Probes, Eugene, OR), as previously described (26). Each measurement was done in triplicate, quantified by comparison with a standard dilution series, normalized to the abundance of glyceraldehyde-3-phosphate dehydrogenase or ribosomal protein L32, and presented as the mean and SE. For RNase protection, primary MEF were serum-starved 48 hours in DMEM containing 0.5% serum, stimulated with 40 ng/mL PDGF-BB or 100 ng/mL EGF, and RNA was quantified using the RibOQuant system from BD Pharmingen (San Diego, CA) using multiprobe sets for mFos/Jun and mMyc.

**Colony formation in soft agar.** Cells were seeded in 0.35% low-melting agarose, prepared in growth medium. Colonies that reached 0.2 mm\(^2\) or larger were enumerated after 10 to 14 days (27).

**Focus formation assay.** Confluent cells were incubated in medium containing 0.1% serum for 7 days and foci were enumerated by light microscopy.

**S.c. tumor formation.** BALB/c\(^{nu/nu}\) mice (Taconic Labs, Germantown, NY) were injected with 100 mg/kg cyclophosphamide in PBS 3 days before inoculation with \(1 \times 10^5\) cells. Tumor growth was monitored every 3 to 4 days, and tumor volume was calculated as 4 of 3 of the product of length, height, and depth measured with calipers.

**Cell proliferation.** Cells were plated on 18-mm\(^2\) glass coverslips, serum starved, growth factor stimulated, and labeled with 10\(\mu\)mol/L bromodeoxyuridine (BrdU;rd; Zymed) for 2 hours. Fixed cells were stained with FITC-labeled anti-BrdUrd (Becton Dickinson), counterstained with 0.1 \(\mu\)g/mL propidium iodide, and counted by fluorescence microscopy.

**Adhesion assay.** Nontreated plastic dishes were coated with 25 \(\mu\)g/mL collagen I or 10 \(\mu\)g/mL fibronectin. Cells were detached in 0.5 mmol/L EDTA, washed, and plated in triplicate at \(5 \times 10^4\) cells in DMEM containing 0.5% bovine serum albumin (BSA), 1 mmol/L MgCl\(_2\), and 0.2 mmol/L MnCl\(_2\). When cells exhibited first signs of attachment, but not yet spread, nonattached cells were removed by rinsing with PBS until no cells remained in control wells (BSA coated). Attached cells were stained with 0.4% crystal violet in methanol, extracted in 10% acetic acid and quantified by absorbance at 590 nm.

**Results**

Generation of signal transducer and activator of transcription 3–null fibroblasts. Analysis of STAT3 function in cell proliferation and transformation has largely relied on data generated using dominant-interfering mutants overexpressed in cells. To study the role of STAT3 in a more physiologically relevant environment, STAT3-null fibroblasts were generated from STAT3 conditional knockout mice (28, 29). Primary MEF from STAT3 flox/– or flox/flox embryos (called STAT3\(^{WT}\)) were subsequently infected with a control or a retrovirus encoding Cre recombinase (30) to facilitate deletion of exons 16 to 21 of the STAT3 gene (called STAT3\(^{D BD}\)). STAT3 gene ablation and protein loss were monitored by PCR, electrophoretic mobility shift assay, and Western blotting. Near 100% gene deletion was obtained, accompanied by complete loss of detectable STAT3 protein and DNA binding activity (Supplementary Fig. S1; data not shown). In addition, immortal STAT3\(^{WT}\) cell lines were generated using the 3T3 protocol (23), and STAT3\(^{D BD}\) cells were derived by Cre infection. Primary MEFs were used in basic gene expression and proliferation studies to avoid the potential complication of secondary mutations introduced during immortalization. 3T3 cells were used for transformation studies.

**Proliferation and gene expression in primary signal transducer and activator of transcription 3–null fibroblasts.** Given that STAT3 deletion in mice is embryonic lethal, and that some of the proposed downstream targets for STAT3 are direct regulators of cell proliferation (e.g., c-myc, c-fos, and cyclin D1), deletion of STAT3 in cells could be expected to inhibit proliferation (2). Surprisingly, no effects on proliferation were observed in STAT3\(^{D BD}\) MEF compared with STAT3\(^{WT}\) MEF, as measured by steady-state growth, saturation density, or response to growth factors such as EGF, PDGF, or serum (see below; data not shown). However, because STAT3 has been implicated in induction of gene expression, specifically of c-myc in response to PDGF (4), immediate-early gene induction was compared in STAT3\(^{WT}\) and STAT3\(^{D BD}\) MEF. Interestingly, c-myc RNA levels were not significantly different between STAT3\(^{WT}\) and STAT3\(^{D BD}\) MEF when corrected for levels of the housekeeping RNA, ribosomal protein L32 (Fig. 1A, left). However, c-fos induction was significantly and reproducibly reduced in STAT3\(^{D BD}\) MEF, most notably in serum-stimulated cells, (Fig. 1A, right). STAT3\(^{WT}\) 3T3 fibroblasts expressed a low but

![Figure 1](https://example.com/figure1.png)
measurable level of c-fos protein that was partially reduced in STAT3KO 3T3 fibroblasts (Fig. 2B), reflecting the partial reduction in c-fos mRNA accumulation observed in growth-stimulated primary cells. Levels of c-fos protein were dependent on transcriptionally active STAT3, because reconstitution of STAT3KO cells with recombinant wild type but not transcriptionally inactive STAT3 mutants (STAT3Y705F or STAT3DBD) restored c-fos protein (Fig. 1B) and RNA levels (Supplementary Fig. S2). Interestingly, c-Fos protein levels in cells reconstituted with STAT3 mutants were slightly lower than in STAT3KO cells, suggesting that overexpression of transcriptionally inactive STAT3 may interfere with endogenous transcription of c-fos, perhaps by interfering with upstream signaling pathways or coactivator recruitment to the c-fos promoter. Levels of c-myc were not affected by the presence or absence of STAT3 (data not shown).

Src- and signal transducer and activator of transcription 3–dependent and Src- and signal transducer and activator of transcription 3–independent c-fos induction. STAT3 has been implicated in several signaling events downstream of c-src and v-src. For instance, inhibition of c-src activity by treatment of NIH3T3 cells with the c-src-specific inhibitor SU6656 inhibited STAT3 phosphorylation, c-myc induction, and cell proliferation in response to PDGF (31). To test if STAT3 was required in this response, wild-type and mutant cells were treated with SU6656 and stimulated with PDGF. In STAT3WT cells increasing concentrations of SU6656 abolished STAT3 phosphorylation (Fig. 2A), as previously observed (31). Proliferation under these conditions was also inhibited, confirming that PDGF-induced proliferation was dependent on the activity of c-src (Fig. 2B). Interestingly, proliferation of STAT3KO cells was comparable with that of STAT3WT cells, and this proliferation was equally sensitive to inhibition by SU6656. Therefore, although SU6656 inhibited STAT3 phosphorylation and blocked proliferation, these two processes are not interdependent in that STAT3 is not required for PDGF-induced proliferation.

Because c-fos gene expression was in part dependent on STAT3 (Fig. 1), we tested whether c-fos induction by PDGF also required c-src (Fig. 2C, left). Induction of c-fos mRNA was partially inhibited by SU6656 in STAT3WT cells. However, SU6656 only minimally inhibited the already reduced level of c-fos induction observed in STAT3KO cells, and this inhibition was not statistically significant (Fig. 2C). These results suggest that PDGF stimulates c-fos induction through two parallel pathways, one that requires c-src and STAT3 and a second that is c-src and STAT3 independent, signaling most likely through the Ras/mitogen-activated protein kinase pathway (31–33). Importantly, the partial reduction of c-fos expression in the absence of STAT3 was not reflected by altered growth responses to PDGF (Fig. 2B) or to EGF or insulin-like growth factor-I (data not shown), suggesting that the still substantial STAT3-independent induction of c-fos is sufficient to promote full cell proliferation, making STAT3 dispensable for cell proliferation. In contrast to c-fos, no significant effect was observed on the induction of c-myc RNA following c-src inhibition in cells of either genotype (Fig. 2C, right), supporting our initial finding that c-myc induction by PDGF was independent of STAT3. Absence of STAT3 led to a more profound impairment of c-fos induction than did inhibition of c-src activity in STAT3WT cells, suggesting either that inhibition of c-src by SU6656 was incomplete (note the low levels of phosphorylated STAT3 remaining in SU6656-treated cells; Fig. 2A) or that STAT3 can induce c-fos independent of c-src. In either case, the contribution of c-src toward c-fos induction would seem predominately if not exclusively mediated by STAT3.

Signal transducer and activator of transcription 3 is required for v-src transformation of fibroblasts. Previous studies identified STAT3 as a v-src target, and interference with STAT3 signaling by overexpression of dominant-interfering mutants of STAT3 in v-src transformed fibroblasts significantly reduced transformation (6, 7). To examine the requirement for STAT3 in cell transformation mediated by v-src, STAT3WT and STAT3KO 3T3 fibroblasts were infected with a v-src retrovirus, and transformation was measured by quantifying focus formation on dishes and colony formation in soft agar (Fig. 3A–B). v-src transformation of STAT3KO cells was significantly reduced in both assays, providing direct evidence that STAT3 contributes to v-src transformation. Moreover, the continued presence of STAT3 was required for cell transformation, as deleting STAT3 from cells already transformed by v-src led to a similar reduction in colony formation, showing that STAT3 is required for both initiation and maintenance of the transformed phenotype (Fig. 3B, right). Interestingly, when STAT3 was depleted after v-src introduction, the reduction in colony numbers was less profound, suggesting STAT3 may be preferentially required during early stages of transformation in addition to its role in maintenance of transformation.

To confirm that the defect in transformation was directly due to the absence of STAT3, v-src-expressing STAT3KO cells were...
reconstituted with STAT3 cDNA. In addition to wild type, two transcriptionally inactive mutants of STAT3 were tested, STAT3Y705F or DNA binding mutant STAT3DBD (Fig. 3C). Reconstitution with wild-type STAT3 fully restored transformation by v-src, in a dose-dependent manner. However, reconstitution with either transcriptionally incompetent mutant failed to rescue transformation, reinforcing the notion that gene expression by STAT3 is required for v-src transformation.

The role of STAT3 in v-src transformation was further examined in vitro. Transformed STAT3WT, STAT3KO, and STAT3KO cells reconstituted with wild-type or mutant STAT3 were inoculated s.c. in nude mice (Fig. 3D). Clear impairment of tumor formation was observed for STAT3KO cells, confirming the in vitro results. Tumors from STAT3WT cells developed much more rapidly and reached greater masses compared with tumors derived from STAT3KO cells. Furthermore, cells reconstituted with wild-type STAT3 formed large tumor masses equivalent to those formed by the original STAT3WT cells, whereas cells reconstituted with mutant STAT3 formed minimal tumors similar to those formed by STAT3KO cells.

Signal transducer and activator of transcription 3 is required for reduced adhesion and for focal adhesion kinase activation by v-src. In normal cells, growth and survival are dependent on proliferative and antiapoptotic signals delivered both from growth factor receptors and adhesion receptors, such as integrins. Oncogenes circumvent these requirements, disconnecting cell growth from external signals. The finding that STAT3 is required for v-src transformation but not for growth factor–induced proliferation (Figs. 1–3) suggested a possible role for STAT3 in regulation of adhesion signals. Adhesion of STAT3WT and STAT3KO cells to the integrin substrates collagen and fibronectin was measured (Fig. 4). Whereas nontransformed cells showed no differences in adhesion to the substrates tested, v-src expressing cells exhibited reduced adhesion to collagen I (~60% of nontransformed cells) that was statistically significant. However, oncogene-induced reduction in adhesion was not observed in the absence of STAT3, indicating that loss of adhesion induced by v-src required STAT3. Similar results were observed following different times of adhesion (data not shown). Adhesion to fibronectin was not significantly affected by the absence of STAT3, suggesting that STAT3 is required for signaling of specific integrin subsets and does not affect adhesion nonspecifically.

To confirm the requirement of STAT3 for integrin-mediated adhesion, STAT3KO cells were reconstituted with wild-type or mutant STAT3 and their adhesion to extracellular matrix (ECM) substrates was tested. Adhesion of STAT3KO cells reconstituted with wild-type STAT3 was again reduced by 60%, similar to the effect observed in STAT3WT cells (Fig. 4). In contrast, neither STAT3 mutant mediated the v-Src-dependent decrease in adhesion, suggesting that transcriptionally active STAT3 is required to modulate adhesion.

Impaired cellular adhesion is associated with a decrease in focal adhesion complex turnover, which is dependent on FAK activity. In addition to regulating focal adhesion complexes, FAK has been implicated in cell proliferation and survival (14, 15, 34). Therefore, it was possible that STAT3 regulated FAK, contributing to the reduced cell adhesion and increased survival observed in suspension culture. To characterize FAK activation in STAT3KO cells, v-src-transformed cells were plated in suspension and FAK activity was measured (Fig. 4). Whereas nontransformed cells showed no differences in adhesion to the substrates tested, v-src expressing cells exhibited reduced adhesion, whereas cells reconstituted with mutant STAT3 formed wild-type or mutant STAT3 were infected s.c. into nude mice, and resulting tumor growth is presented.

Figure 3. Transcriptionally competent STAT3 is required for v-src transformation in vitro and in vivo. A. STAT3WT and STAT3KO 3T3 cells, expressing v-src or vector control, were grown until confluent and maintained in low-serum medium (0.5%) until foci were visible. Magnification, ×32. B. 1 × 10^5 cells were plated in 6-cm dishes in complete medium with low-melting agarose (0.35%). After 14 days, colonies larger than 0.2 mm in diameter were counted. Columns, average colony numbers (experiments done in triplicate); bars, ± SE. WT3 was deleted before v-src (left) or after (right) v-src transformation. Stable v-src expression and complete STAT3 deletion were confirmed by immunoblotting and PCR, respectively. C. STAT3 KO cells expressing v-src were infected with a bicistronic retrovirus containing GFP and STAT3 or control. Cells were sorted by fluorescence-activated cell sorting for GFP levels and expanded into “high” and “low” pools. Soft agar colony growth was scored, using 5,000 cells due to higher transformation efficiency of these pure pools. Chart represents two pools isolated for each genotype based on increasing order of GFP (STAT3) expression. E.V., empty vector control (i.e., STAT3 KO cells); WT, Y-F, and DBD, STAT3-null cells reconstituted with wild type STAT3, STAT3Y705F, and STAT3DBD, respectively. D. STAT3WT, STAT3KO, or STAT3KO cells reconstituted with wild-type or mutant STAT3 were injected s.c. into nude mice, and resulting tumor growth is presented.

Figure 4. STAT3 is required for v-src induced loss of adhesion. Adhesion of nontransformed and v-src transformed STAT3WT and STAT3KO cells to collagen I (A) or fibronectin (B). C. adhesion to collagen I of v-src-transformed STAT3KO cells reconstituted with wild-type or mutant STAT3. At least four independent experiments were done, each in triplicate.
autophosphorylation on Tyr397 was measured (Fig. 5A). STAT3WT cells maintained high levels of FAK phosphorylation when grown in suspension, although there was some degree of variability over time. In contrast, STAT3KO cells exhibited a marked reduction in FAK phosphorylation within 24 hours. Loss of FAK phosphorylation correlates with the inability of STAT3KO cells to establish colonies in soft agar, suggesting that in addition to mediating loss of cell adhesion, STAT3 contributes to the enhanced FAK phosphorylation observed in v-src-transformed cells.

To test whether FAK was appropriately activated by integrins, FAK autophosphorylation was measured in starved cells replated on ECM (Fig. 5B). Nontransformed cells placed in suspension lost FAK phosphorylation, which was fully restored by 4 hours of replating in both STAT3WT and STAT3KO cells (Fig. 5B, left), suggesting that FAK is activated in a STAT3 independent manner in the absence of v-src. In transformed cells, the kinetics and extent of FAK activation were different (Fig. 5B, right). First, FAK phosphorylation was not completely abolished in suspension, and second, FAK reactivation was maximal already by 1 hour. Importantly, STAT3WT and STAT3KO cells behaved similarly, suggesting that activation of FAK by integrins occurs normally in both genotypes. Coimmunoprecipitation experiments of FAK with v-src showed no significant differences between STAT3WT and STAT3KO cells, supporting the notion that upstream activation of FAK by either integrin engagement or v-src is not affected by the absence of STAT3 (data not shown).

FAK phosphorylation levels are the consequence of phosphorylation by upstream kinases and dephosphorylation by phosphatases. To measure the rate of FAK dephosphorylation, cells were grown in the presence of the kinase inhibitor staurosporine to block upstream kinase activity. The rate of decay of phosphorylated FAK was subsequently measured over a period of 24 hours (Fig. 5C). When STAT3WT cells were plated in suspension with no inhibitor present FAK phosphorylation was maintained or increased over time. In contrast, STAT3KO cells were unable to maintain FAK phosphorylation, as shown previously (Fig. 5A). In the presence of staurosporine, FAK phosphorylation in STAT3WT cells was still maintained, albeit at somewhat lower levels. However, in STAT3KO cells FAK phosphorylation decayed rapidly and was completely lost by 24 hours, without loss of FAK protein levels. Quantification of two independent experiments showed the half-life of phosphorylated FAK in STAT3KO cells to be ~6 hours, in contrast to a half-life of >24 hours in wild-type cells (Fig. 5D). The same experiment done in the presence of the src inhibitor SU6656 showed a similar although less complete loss of FAK phosphorylation (data not shown), consistent with the Y397 phosphorylation site being an autophosphorylation target and only indirectly dependent on v-src. We conclude that STAT3 contributes to the maintenance of high levels of phosphorylated FAK downstream of v-src through inhibition of FAK dephosphorylation.

Discussion

Numerous reports have suggested a role for STAT3 in cell proliferation, survival, differentiation, and transformation. Much of this previous evidence was derived from studies involving expression of constitutively active or dominant-negative versions of STAT3 (2). In this report, we have directly examined the requirement for STAT3 in fibroblasts by using gene ablation. Given initial gene ablation studies showing a requirement for survival of early embryos (35), it was surprising to find that STAT3 was dispensable for normal growth of fibroblasts in vitro. Wild-type and STAT3-null primary fibroblasts and immortalized 3T3 cells grew at equivalent rates, reached comparable saturation densities, and displayed similar serum dependence. Moreover, no gene product was found to be absolutely dependent on STAT3 for expression, including such previously reported STAT3 target genes as cyclin D, Bcl-X (data not shown), c-fos, or c-myc (Fig. 1).

In spite of no genes being absolutely dependent on STAT3 for expression, induction of c-fos in response to mitogens was substantially impaired. This impairment presumably reflects the recruitment of STAT3 to its cognate binding site (SIE) in the c-fos promoter (3, 36). Although this enhancer element is dispensable in transient transfection assays, it clearly plays a substantial role in the regulation of c-fos gene expression in its normal chromosomal context, as has been also noted in transgenic mice (37). Induction of c-fos by PDGF was partially dependent on c-src activity, as revealed by its impairment by the src inhibitor SU6656 (Fig. 2). A greater inhibition of c-fos expression was observed in the absence of STAT3 than in the absence of c-src activity. Either additional enzymes are involved in STAT3 activity or the SU6656 was unable to completely inhibit c-src activity. In either case, most
if not all of the induction of c-fos by c-src was mediated by STAT3, because inhibition of c-src had an insignificant effect on c-fos expression in STAT3-null cells. c-fos gene expression was also impaired in src-transformed cells in the absence of STAT3, confirming the ability of src to phosphorylate STAT3. This partial loss of c-fos gene expression may have contributed to the impaired transformation of STAT3-null cells, because reconstitution of STAT3-null cells with constitutively expressed c-fos partially rescued the transformation defect (data not shown).

A different pattern was observed for c-myc expression. Although c-myc has been suggested to depend on STAT3 in response to PDGF (4), no significant differences in c-myc mRNA levels were detected in the absence of STAT3. Moreover, inhibition of c-src activity also did not impair c-myc expression, in spite of its ability to block PDGF-induced cell proliferation (Fig. 2). The lack of a requirement for STAT3 for c-myc expression was observed both in primary MEF (Fig 2), as well as in established cell lines (data not shown). It has been postulated that transcription factor requirements for c-myc expression can be affected by the activity of p53 or SV40 T antigen (38). However, this explanation cannot account for lack of a STAT3 requirement observed in these cells, because the primary MEFs were wild type for p53 and neither primary nor immortalized lines expressed SV40 proteins (data not shown).

These results suggest that STAT3 contributes to the full induction of c-fos in response to mitogens, largely through a c-src-dependent pathway. However, residual STAT3-independent induction of c-fos is sufficient to drive full cell proliferation in vitro. On the other hand, STAT3 is dispensable for the induction of c-myc expression, which is driven through a c-src-independent pathway. This model differs from previous conclusions drawn from data using NIH 3T3 cells (39), in which overexpression of c-myc rescued the cell cycle block imposed by inhibition of c-src. This disparity may lie in inherent differences between normal primary fibroblasts and immortalized NIH 3T3 cells that lack p19ARF, a c-myc target gene (40), or may result from the higher levels of myc achieved in those experiments by ectopic expression. In either case, normal primary fibroblasts are capable of maintaining sufficient immediate early gene expression for cell growth in the absence of STAT3.

A different picture emerged for v-src-induced transformation. Here, as previously suggested from experiments involving dominant-negative forms of STAT3 (5, 7), STAT3 played an essential role in the full transforming potential of v-src. STAT3-null cells expressing v-src were impaired in their ability to form foci in culture, to grow as colonies in soft agar, and to develop as tumors in nude mice. This requirement for STAT3 reflected its role as a transcription factor, because only wild-type STAT3 and not transcriptionally compromised mutants was capable of restoring the transforming potential of v-src.

The step in cell transformation compromised by loss of STAT3 involved cell-substratum interactions. Whereas growth of v-src-expressing cells in adherent culture was equivalent in the presence or absence of STAT3 (data not shown), the reduction in cell adhesion to integrin substrates typical of src-transformed cells was abrogated (Fig 4) and a biochemical correlate of this defect was an inability to maintain phosphorylated FAK in the absence of integrin signaling (Fig 5). Direct phosphorylation of FAK did not seem to depend on STAT3, either downstream of c-src activated by integrins during adhesion or following expression of v-src. However, the ability to maintain FAK phosphorylation was impaired, even in the presence of v-src, which seemed to result from more rapid dephosphorylation. Regulation of the rate of FAK dephosphorylation required STAT3 target genes, because only wild type and not transcriptionally compromised STAT3 mediated this process. Although several phosphatases have been proposed to dephosphorylate FAK (e.g., PTP1B and SHP2; refs. 41, 42), we did not detect changes in protein levels of these phosphatases in STAT3KO cells (data not shown). Nevertheless, it is possible that STAT3 regulates the expression of another phosphatase. Alternatively, STAT3-dependent genes might affect phosphatase or regulatory protein recruitment to focal adhesion complexes.

The contrast between a requirement for STAT3 during cell transformation and its dispensable role during cell growth suggests that STAT3 possesses a transformation-specific function, consistent with its postulated role as an oncogene (9). No abnormalities were noted for adherent cell growth in the absence of STAT3, implying normal integrin function is independent of STAT3. However, transformation by dominant oncogenes, such as v-src, overrides the proliferation requirement for integrin engagement through a process at least partially dependent on FAK activation (43), and this process was STAT3 dependent. Therefore, whereas it might have been imagined that what v-src does during transformation is to constitutively activate normal growth signaling pathways, it seems in this case to supplant an integrin-dependent signal by a STAT3-dependent process. Thus, whereas STAT3 is not required for normal cell proliferation, either downstream of growth factor receptors or adhesion receptors, it is necessary for v-src to render cells adhesion-independent. This concept suggests that the activation of STAT3 that is characteristic of many human tumors may contribute to metastatic growth and tumor invasion through a tumor-specific mechanism. It will be important to identify the critical STAT3 target genes that mediate this process.

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Karni Schlessinger and David E. Levy


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