Full Oncogenic Activities of v-Src Are Mediated by Multiple Signaling Pathways

Ras AS AN ESSENTIAL MEDIATOR FOR CELL SURVIVAL*

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Junko Odajima‡, Itaru Matsumura§§, Junko Sonoyma‡, Hanako Daino‡, Akira Kawasaki‡‡, Hirokazu Tanaka‡, Naohiro Inohara†, Toshio Kitamura‡, Julian Downward†**, Koichi Nakajima‡‡‡, Toshio Hirano§§, and Yuzuru Kanakura‡

From the ‡Department of Hematology/Oncology and §§Molecular Oncology, Biomedical Research Center, Osaka University Medical School, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan, the ¶Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan 48109, the ‡Department of Hematopoietic Factors, The Institute of Medical Science, The University of Tokyo, Minato, Tokyo 108-0071, Japan, the ††Imperial Cancer Research Fund, 44 Lincoln’s Inn Fields, London WC2A 3PX, United Kingdom, and the ‡‡Department of Immunology, Osaka City University Medical School, 1-4-3 Asahi-machi, Abeno, Osaka 545-0051, Japan

Tyrosine kinase oncoproteins cause simultaneous activation of multiple intracellular signaling pathways. However, the precise mechanisms by which individual pathways induce oncogenesis are not well understood. We have investigated the roles of individual signaling pathways in v-Src-dependent cell growth and survival by inhibiting one particular pathway. v-Src induced constitutive activation of signal transducers and activators of transcription 3 (STAT3), phosphatidylinositol 3-kinase, and Ras in murine Ba/F3 cells and led to factor-independent proliferation. Dominant-negative mutants of STAT3 (STAT3D) and phosphatidylinositol 3-kinase (Ap85) inhibited v-Src-dependent growth by ~60 and ~40%, respectively. Moreover, dominant-negative Ras (N17) induced severe apoptosis, which was accompanied by down-regulation of Bcl-2 and activation of caspase-3. Although cells overexpressing Bcl-2 or caspase-3 inhibitors remained viable even when N17 was expressed, the growth was reduced by ~85%. During N17- and STAT3D-induced growth suppression, expression of cyclin D2, cyclin D3, c-myc, and c-fos was suppressed by N17, whereas that of cyclin D2, cyclin E, and c-myc was suppressed by STAT3D. Thus, v-Src-activated Ras and STAT3 are involved in distinct but partly overlapping transcriptional regulation of cell cycle regulatory molecules. These results suggest that the full oncogenic activity of v-Src requires simultaneous activation of multiple signalings, in which Ras is particularly required for survival.

Cell growth and survival are regulated by a wide variety of external stimuli propagated from growth factors, extracellular matrices, cell-to-cell interactions, and others. These stimuli are transmitted to intracellular signal transducers and trigger activation of signaling cascades including Ras/mitogen-activated protein kinase (MAPK), Janus family of protein-tyrosine kinase

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To whom correspondence should be addressed: Dept. of Hematology/Oncology, Osaka University Medical School, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. Tel.: 81-6-6879-3871; Fax: 81-6-6879-3879; E-mail: matumura@bldon.med.osaka-u.ac.jp.

1 The abbreviations used are: MAPK, mitogen-activated protein kinase; STAT, signal transducers and activators of transcription; PI3-K, phosphatidylinositol 3-kinase; IL, interleukin; Ab, antibody; PY, phospho-tyrosine; IPTG, isopropyl-β-D-thiogalactopyranoside; TUNEL, terminal deoxynucleotidyl-transferase-mediated biotin-dUTP nick end labeling; LacR, Lac repressor.

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activate Ras/Raf/MAPK pathway, cannot prevent cell death in response to granulocyte-macrophage colony-stimulating factor in murine interleukin-3 (IL-3)-dependent Ba/F3 cells (19). It has also been reported that Ras is capable of protecting cells from apoptosis through the induction of Bcl-2 and Bcl-XL expression (20).

The STAT family of transcription factors, another set of signal transducers activated by v-Src, has been demonstrated to play a pivotal role in growth factor- and hormone-induced signal transduction (for reviews see Refs. 21 and 22). It has been shown that STAT3 is involved in cell growth control through the transcriptional regulation of c-myc and cyclin D1 genes (23, 24) and also in IL-6 receptor-mediated anti-apoptotic signals via the induction of Bcl-2 in Ba/F3 cells (25). In addition, many lines of evidence suggest a close association between deregulated activation of STATs and oncogenesis in various types of tumors. Constitutive activation of STATs has been found in breast carcinoma and hematologic malignancies (26–28). STAT3, particularly, has been found to be constitutively activated in the bone marrow mononuclear cells from patients with multiple myeloma and in a human myeloma cell line U266, which is inherently resistant to Fas-induced apoptosis (29). Furthermore, a recent study has demonstrated that point-mutated, constitutively active STAT3 (STAT3-C) confers anchorage-independent growth on a fibroblastic cell line 3Y1, and injection of STAT3-C-expressing 3Y1 cells into nude mice led to tumor formation (23).

Although a growing number of studies elucidate the function of various signal transducers, it is hard to assign a specific contribution of an individual signaling pathway to the induction of each aspect of the transformed phenotype, because many different signaling pathways are activated simultaneously by a single oncogene product. To investigate the role of individual signaling pathways, we utilized dominant-negative forms of signaling molecules to inhibit activation of one particular signal transduction pathway. In this study, we inducibly expressed dominant-negative forms of Ras (N17), STAT3 (STAT3D), and PI3-K (p85) in v-Src-transformed Ba/F3 cells and examined their effects on v-Src-induced cell growth and survival in association with the expression and function of cell cycle and apoptosis regulatory molecules.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies—**Highly purified recombinant murine IL-3 was provided by Kirin Brewery (Tokyo, Japan). The antibodies (Abs) against STAT1, STAT3, STAT5b, and PI3-K p110α were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-pan-Ras Ab was from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-phosphotyrosine (PY) Ab was from Medical & Biological Laboratories (Nagoya, Japan), anti-Bcl-2 Ab from Transduction Laboratories (Lexington, KY), and anti-MAPK and anti-phospho-MAPK Abs from New England BioLabs (Beverly, MA). The antibodies (Abs) against cyclin D1, cyclin D2, and cyclin D3 from Dr. H. Matsuzawa (Nippon Roche Research Institute, Kanagawa, Japan); human cyclin A, cyclin B, cyclin E,cdc2, and cdk4 from Dr. H. Kiyokawa (University of Illinois, Cancer Center, Chicago, IL); and human cdc2 from Dr. E. Harlow (Massachusetts General Hospital, Boston, MA). To construct expression vectors of v-src, bel-2, cIAP1, and cIAP2, these cDNAs were subcloned into multicloning site of pcDNA3 (Invitrogen, Diemel Schöpf, The Netherlands). To generate an expression vector of v-srcASH2, a Smal fragment coding to amino acids 169–264 was excised from the plasmid pcDNA3-v-src and self-ligated. To construct a Lac-inducible expression vector of Δp85, its cDNA was subcloned into EcoRV site of pOPRSVI (Stratagene, La Jolla, CA). Δp85 is a mutant 85-kDa subunit of PI3-K that cannot bind the 110-kDa catalytic subunit (30). The construction of Lac-inducible expression vectors of STAT3ΔD and N17 (Ha-RasΔN17), these were described previously (33). STAT3ΔD bears mutations in the DNA-binding domain and inhibits binding of endogenous STAT3 with the response element on target DNA (34). N17 is the Asn-17 mutant of Ha-Ras, which blocks multiple downstream signalings including the activation of c-Raf and subsequent phosphorylation of MAPK (35). Expression vectors for constitutively active STAT5 (pcDNA3-1’-6’-STAT5A), Ha-RasΔN17 (pOPRSVI-Ha-RasΔN17), Ha-RasΔN17 (pSG5-Ha-RasΔN17), Ha-RasΔN17 (pSG5-Ha-RasΔN17; pSG5-Ha-RasΔN17 was described in previous papers (36–39).

**Lac-inducible System—**To express a target cDNA, we used a LacStitch™II inducible expression system (Stratagene, La Jolla, CA). In short, Ba/F3 cells were initially transfected with expression vectors of Lac repressor and CMV-αβ-gal, by electroporation. The transfected cells were screened by culturing with 0.5 mg/ml hygromycin (Sigma). Out of several hygromycin-resistant clones, one clone in which LacR was most intensely expressed was further transfected with pOPRSVI containing STAT3ΔD, Δp85, or N17. The expression vector of pOPRSVI contains Rous sarcoma virus promoter linked to the Escherichia coli lactose operon, and expression of target cDNA is suppressed by LacR through the lactose operon. When isopropyl-β-thiogalactopyranoside (IPTG) is added to the culture medium, LacR is released from lactose operon, and transcription of the target cDNA is initiated. After selection with 1.5 mg/ml G418 (Life Technologies, Inc.), the induction levels of the target proteins in each clone were examined by Western blot analyses and after the treatment with 0.5 mg/ml of IPTG. To further transfected the plasmid (pcDNA3-Bcl-2, pcDNA3-cIAP1, or pcDNA3-cIAP2) into hygromycin- and G418-resistant Ba/F3 cells, an expression vector of baculovirus d stained nano (Pakam Pharma, Tokyo, Japan) was cotransfected. After the selection with 30 μg/ml of baculovirus and subsequent phosphorylation of MAPK (35). Expression vectors for constitutively active STAT5 (pcDNA3-1’-6’-STAT5A), Ha-RasΔN17 (pOPRSVI-Ha-RasΔN17), Ha-RasΔN17 (pSG5-Ha-RasΔN17), Ha-RasΔN17 (pSG5-Ha-RasΔN17; pSG5-Ha-RasΔN17 was described in previous papers (36–39).

**Luciferase Assay—**Four tandem repeats of APRE sequence (40) and three tandem repeats of AP-1-binding sequence (41) were linked to the murine interleukin-3-dependent pro-B cell line 5×10⁶ cells. These 5×10⁶ cells were solubilized in lysis buffer, and lysates were performed by using Dual-Luciferase Reporter System (Promega), in which transfection efficiency was monitored by cotransfected pRL-CMV-RLuc, an expression vector of Renilla reniformis luciferase. In short, cultured cells (1 × 10⁶ cells/sample) were electroporated (300 V, 220 μF, 1000 Ω) with 10 μg of an appropriately purified plasmid together with 2 μg of pRL-CMV-RLuc. After a 12-h recovery period in the culture medium, the cells were serum- and IL-3-starved for 24 h and subjected to luciferase assays. To analyze the effects of IL-3 on AP-1 activities, the serum- and IL-3-deprived cells were stimulated with IL-3 (1 ng/ml) for 6 h and subjected to luciferase assays. To examine the effects of dominant-negative mutants, the cells were pretreated with 0.5 μM of IPTG for 24 h before electroporation and cultured with IPTG during the assay. The cells were lysed in lysis buffer supplied by the manufacturer, followed by the measurement of firefly (Photinus pyralis) and Renilla luciferase activities on luminometer LB96P (Berthold Japan, Tokyo, Japan). The relative firefly luciferase activities were calculated by normalizing transfection efficiency according to the Renilla luciferase activities.

**Northern Blot Analysis—**The isolation of total cellular RNA and the method for Northern blot were described previously (42).

**Immunoprecipitation and Immunoblotting—**Preparation of cell lysates, immunoprecipitation, gel electrophoresis, and immunoblotting were performed according to the methods described previously (42). Immunoreactive proteins were visualized with the enhanced chemiluminescence detection system (Telok Life Science Products).

**PI3-K Assay—**Cells (5 × 10⁶) were solubilized in lysis buffer, and lysates were immunoprecipitated with an anti-PY (2 μg) at 4 °C for 2 h and subsequently with protein G-Sepharose beads at 4 °C for 2 h (Amersham Pharmacia Biotech). The immunoprecipitates were washed twice with lysis buffer, twice with buffer containing 0.5 μM LiCl and 0.2% Nonidet P-40, and finally with 10 mM HEPES, pH 7.4, and 0.15 mM NaCl. Then 30 μl of 10 μM...
phenyl phosphate (dissolved in 20 mM HEPES, pH 7.4) was added. After a 10-min incubation at 37 °C with 100 μl of kinase buffer containing 0.2 mg/ml phosphatidylinositol, 40 μM ATP, 30 mM MgCl₂, and 10 μCi of [γ-³²P]ATP, the reaction was stopped by adding 100 μl of 1 N HCl, and the lipid layer was extracted with 200 μl of chloroform/methanol (1:1 v/v). The extracts were washed with methanol/1 N HCl (1:1 v/v), and the lipids were separated by TLC using silica gel G60 (250 mm; Merck). The TLC was developed in chloroform/methanol/H₂O/NH₄OH (43:38:7:5 v/v) and visualized by autoradiography.

DNA Content Analysis—The DNA content of cultured cells was examined by staining with propidium iodide and analyzed on FACSort (Becton Dickinson, Oxnard, CA) with a program ModFit LT2.0 (Becton Dickinson) as described previously (42).

TUNEL Assays—TUNEL assays were performed with In Situ Cell Death Detection Kit (Roche Molecular Biochemicals). Briefly, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline for 30 min, transferred into permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate), and incubated on ice for 2 min. After washing with phosphate-buffered saline, the cells were resuspended in TUNEL reaction mixture containing TdT enzyme and digoxigenin-nucleotide. Incorporation of nucleotides into 3'-DNA fragmented ends was detected by flow cytometry.

Annexin-V Staining—Cells were washed with RPMI 1640 twice and resuspended in 100 μl of labeling solution containing avdin-annexin-V conjugates at room temperature for 30 min. The cells were rinsed and developed with fluorescein-conjugated avidin (Becton Dickinson) at 4 °C for 30 min. The stained cells were analyzed by flow cytometry.

Assays for Caspase-3 Activities—Caspase-3 activities were measured with PhiPhiLux-G1D2 kit (OncoImmunin, College Park, MD). Briefly, cells were washed with phosphate-buffered saline and resuspended in 50 μl of substrate solution supplied by the manufacturer, which contains the caspase-3-specific substrate. After a 60-min incubation in a 5% CO₂ incubator at 37 °C, the cells were suspended in 500 μl of dilution buffer supplied by the manufacturer and subjected to flow cytometry. In this system, caspase-3 activities are measured by fluorescence that is derived from the cleaved substrate specific for caspase-3.

RESULTS

Overexpression of v-Src but Not v-SrcΔSH2 Leads to IL-3-independent Growth of Ba/F3 Cells—To analyze the signaling events induced by v-src oncogene, we initially introduced an expression vector of v-src, v-srcΔSH2, which lacked the SH2 domain or an empty control vector into a murine IL-3-dependent cell line Ba/F3. After the selection with G418, several clones were isolated and subjected to Northern blot analysis with 32P-labeled v-src probe. The lower panel shows CHO-B mRNA as a loading control. B, parental Ba/F3 cells, v-src-transfected cells, and v-srcΔSH2-transfected cells were seeded at a cell density of 100,000/culture with or without IL-3 (IL-3 concentration is as indicated), and the total number of viable cells was counted by trypan blue dye exclusion method. The results are shown as the means ± S.D. of triplicated cultures.

v-SrcΔSH2 expressing cells. Next, we examined the activation state of specific signaling molecules in these clones. STAT1, STAT3, STAT5, and PI3-K p85α were immunoprecipitated from the total cellular lysates and subjected to Western blot analysis with an anti-PY Ab. MAPK activation was assessed by Western blot analysis on the whole cell lysates with an anti-phospho-MAPK Ab, which exclusively recognized both tyrosine- and serine-phosphorylated MAPK. Treatment of parental Ba/F3 cells with IL-3 led to activation of STAT5 and MAPK but not of STAT1, STAT3, and PI3-K p85α (Fig. 2B). Meanwhile, STAT1, STAT3, PI3-K p85α, and MAPK were constitutively activated in v-src-transfected cells. In addition, tyrosine-phosphorylated PI3-K p110α was commmunoprecipitated with p85α in v-src-transformed cells. In v-srcΔSH2-transfected cells, on the other hand, significant phosphorylation of STATs, PI3-K, and MAPK was not detected. These data indicate that v-Src activates STAT1, STAT3, PI3-K, and MAPK in Ba/F3 cells either directly or indirectly through its SH2 domain.

Inducible Expression of Dominant-negative STAT3, Ras, and PI3-K in Ba/F3 Cells—To assess the function of these signaling molecules activated by v-Src, we inducibly expressed dominant-negative forms of STAT3 (STAT3D), Ras (N17), and PI3-K (Δp85) in parental Ba/F3 cells by using a Lac-inducible system, in which expression of the target protein was induced by IPTG treatment. The transfected clones were designated as Ba/F3/STAT3D, Ba/F3/N17, and Ba/F3/Δp85, respectively. As shown in Fig. 3, Western blot analysis on the whole cell lysates revealed that the addition of IPTG led to induction of STAT3D, N17, and Δp85 as early as 4 h, and their expression reached a maximum at about 24 h and was retained until 48 h. Furthermore, the amount of induced dominant-negative mutant proteins were found to be far more abundant than their respective endogenous proteins. Then, these clones were further transfected with v-src, and the obtained clones were designated as v-Src/STAT3D, v-Src/N17, and v-Src/Δp85, respectively.

Induced Expression of STAT3D, N17, and Δp85 Inhibits Their Corresponding Cellular Signaling Pathways—We first evaluated the inhibitory effects of dominant-negative mutants on their respective signaling pathways. The effectiveness of
STAT3D and N17 were measured by luciferase assays with reporter genes, 4x APRE-Luc and 3x AP-1-Luc, respectively. As shown in Fig. 4A (left panel), IPTG-induced STAT3D was able to disrupt v-Src-induced APRE-Luc activities (6.2-fold) almost completely in v-Src/STAT3D cells. Similarly, IPTG-induced N17 was found to repress IL-3- and v-Src-induced AP-1-Luc activities (7.8-fold for IL-3 and 10.2-fold for v-Src) to the basal level in Ba/F3/N17 and v-Src/N17 clones, respectively. We further evaluated the effects of N17 by examining phosphorylation of MAPK by Western blot analysis (Fig. 4B). Phosphorylated MAPK gradually decreased as N17 protein was inducibly expressed by IPTG treatment. Next, we assessed the efficacy of Δp85 with PI3-K assays (Fig. 4C). Without IPTG treatment, IL-3 stimulation was found to induce PI3-K activity (lane 1 versus lane 2), although we could not detect an apparent tyrosine phosphorylation of p85a and p110a on Western blot analysis (Fig. 2B). Because activation of PI3-K is sometimes difficult to detect by Western blot analysis in terms of its tyrosine phosphorylation, we assumed that PI3-K would be indeed activated in response to IL-3 in parental Ba/F3 cells. PI3-K activation was also observed in IPTG-untreated v-Src/Δp85 cells (lane 6). However, induced expression of Δp85 inhibited both IL-3- and v-Src-induced PI3-K activities (lanes 5 and 7) as efficiently as 100 nM of PI3-K inhibitor wortmannin in Δp85 and v-Src/Δp85 clones (lanes 3 and 8). These results suggest that inducibly expressed STAT3D, N17, and Δp85 are competent to inhibit their respective signaling pathways almost completely.

**STAT3, Ras, and PI3-K Pathways Are Independent of Each Other**—Because of the possibility of the cross-talk among v-Src-activated signaling pathways, we investigated the effects of N17, STAT3D, and Δp85 on the other signaling pathways. We first measured 4x APRE-Luc activities in v-Src/N17, v-Src/Δp85, and v-Src/STAT3D clones. As shown in Fig. 5A, APRE-Luc activity was disrupted in v-Src/STAT3D cells when STAT3D was inducibly expressed by IPTG. However, the induced N17 and Δp85 did not affect APRE-Luc activities. Consistently, a Western blot study revealed that the expression of N17 and Δp85 did not disrupt v-Src-induced phosphorylation of MAPK gradually decreased as N17 protein was inducibly expressed by IPTG. However, the induced N17 and Δp85 did not affect APRE-Luc activities. Consistently, a Western blot study revealed that the expression of N17 and Δp85 did not disrupt v-Src-induced phosphorylation of MAPK by Western blot analysis with an anti-PY Ab (4G10). Constitutive activation of STAT1, STAT3, PI3-K, and MAPK in v-Src-transformed Ba/F3 cells. Total cellular lysates were prepared as described above. STAT1, STAT3, STAT5, and PI3-K p85α were immunoprecipitated with respective Abs and subjected to Western blot analysis with an anti-PY Ab. MAPK activation was evaluated by Western blot analysis on the whole cell lysates with an anti-phospho-MAPK Ab. The filters were reprobed with corresponding Abs to confirm that the equal amounts of the proteins were loaded. IB, immunoblotting; IP, immunoprecipitation; α, anti.

**Effects of STAT3D, N17, and Δp85 on IL-3- and v-Src-dependent Proliferation of Ba/F3 Cells**—Next, we examined the effects of STAT3D, N17, and Δp85 on IL-3- and v-Src-dependent proliferation of Ba/F3 cells. The cells of indicated clones were cultured in the condition as indicated, and the total number of viable cells was counted. The induced expression of N17 inhibited IL-3-dependent proliferation by about 40% (at day 4), whereas that of STAT3D and Δp85 showed little effect (Fig. 6, left column). During the culture without IL-3, STAT3D and Δp85 inhibited v-Src-dependent proliferation by about 60% and 40% (at day 4), respectively, and N17 abolished v-Src-dependent cell proliferation by nearly 100% (Fig. 6, middle column). However, these growth inhibitory effects of N17, STAT3D, and Δp85 were canceled almost entirely by the addition of IL-3 (Fig. 6, right column).

**N17 Induces Apoptosis in v-Src-transformed Ba/F3 Cells**—To define the effects of STAT3D, Δp85, and N17 on v-Src-dependent proliferation, we performed cell cycle analysis on v-Src/STAT3D, v-Src/Δp85, and v-Src/N17 cells during the culture with IPTG in the presence or absence of IL-3. As shown in Fig. 7A, the induced expression of STAT3D, Δp85, and N17 led to a decrease in the proportions of proliferating cells detected in the S or G2/M phase (percentage of the cells in the S
or G2/M phase at 0 h versus 72 h: v-Src/STAT3D, 47% versus 23%; v-Src/N17, 45% versus 31%; v-Src/N17, 44% versus 7%). In addition, a noticeable proportion of v-Src/N17 cells underwent apoptosis, which was detected as subdiploid fractions, in response to the induced expression of N17 during the culture without IL-3 (percentage of the cells in subdiploid peaks: 28% at 36 h and 79% at 72 h). On the other hand, STAT3D and Δp85 did not induce apoptosis in v-Src/STAT3D and v-Src/Δp85 cells, respectively. Supplement of IL-3 to v-Src/N17 cells, however, abrogated both N17-induced growth suppression and apoptosis nearly completely. To further characterize N17-induced apoptosis in v-Src/N17 cells, we performed TUNEL assay and annexin-V staining, both of which are known to be useful for detecting cells undergoing apoptosis (Fig. 7B). A considerable proportion of v-Src/N17 cells was positive for TUNEL and Annexin-V staining after 48 h of IPTG treatment in the absence of IL-3; however, the cells were found negative for these stainings when they were cultured with IL-3 (percentage of TUNEL-positive cells: IL-3 (-) 61% versus IL-3 (+) 0.5%; and % annexin-V-positive cells: IL-3 (-) 75% versus IL-3 (+) 0.4%). Because caspase-3, a member of the ICE/Ced-3-family of cysteine proteases, is known as an important mediator of various types of apoptosis (as reviewed in Ref. 44), we examined whether caspase-3 was implicated in N17-induced apoptosis. v-Src/N17 cells were treated with IPTG for indicated times in the presence or absence of IL-3, and caspase-3 activity was evaluated by measuring fluorescence intensity derived from the caspase-3-cleaved substrate. As shown in Fig. 7C (left panels), caspase-3 activity was hardly observed in v-Src/N17 cells before the addition of IPTG. However, it was distinctly up-regulated after 24–72 h of treatment with IPTG (represented by fluorescence peaks on the right) in the absence of IL-3. In contrast, significant caspase-3 activation was not observed when the cells were cultured with IL-3 (Fig. 7C, right panels). These results imply that N17 might prompt apoptosis in v-Src/N17 cells via caspase-3 and that Ras might be a key mediator of v-Src-activated anti-apoptotic signaling in these cells.

**Bcl-2 Expression Is Down-regulated during N17-induced Apoptosis**—To clarify the mechanisms of N17-induced apoptosis, changes in mRNA expression of various apoptosis regulating genes were examined by Northern blot analysis (Fig. 8A). It has previously shown that Bcl-2 family members play crucial roles in the regulation of apoptosis. Bcl-2 and Bcl-XL are major anti-apoptotic mediators, whereas other members act as pro-apoptotic effectors (45, 46). After the addition of IPTG, bcl-2 mRNA was down-regulated within 24–36 h in v-Src/N17 cells when cultured without IL-3. The expression of bcl-XL mRNA was also down-regulated at 36 h to a lesser extent. Despite the putative pro-apoptotic effect, expression of *bim* mRNA was slightly down-regulated after 36 h with IPTG. The expression of other Bcl-2 family members was either undetectable (*bcl-1* and CIDE-A) or constant (*bax* and CIDE-B) during the culture. Although *c-myc* is known to promote apoptosis when overexpressed (47), its mRNA expression was clearly down-regulated at 24 h. In consistent with the result from Northern blot study, Western blot analysis demonstrated that expression of Bcl-2 protein was gradually reduced during IPTG treatment (Fig. 8B).

Based on the facts that the down-regulation of Bcl-2 expression was correlated with N17-induced apoptosis and that IL-3 could inhibit N17-induced apoptosis, we examined whether the expression of Bcl-2 would be restored by IL-3 when v-Src/N17 cells were cultured with IPTG. As demonstrated in Northern blot (Fig. 8C), the expression of *bcl-2* mRNA was severely down-regulated in v-Src/N17 cells after 36 h of culture with IPTG in the absence of IL-3 (lane 1 versus lane 2). However, its expression was maintained steadily even after the addition of IPTG when the cells were cultured in the presence of IL-3 (lane 3 versus lane 4). Moreover, stimulation by IL-3 alone was sufficient for inducing *bcl-2* mRNA expression in parental Ba/F3 cells (lane 5 versus lane 6). Because STAT5 was activated by IL-3 but not by v-Src (Fig. 2B), we speculated that STAT5 might be involved in IL-3-mediated *bcl-2* expression independently of the Ras pathway. To solve this question, we prepared a stable clone in which constitutively active STAT5 (*1*6-STAT5A) was overexpressed. In this clone, *bcl-2* expression was found even in the IL-3-deprived condition (lane 7). Considering these data, IL-3 might rescue v-Src/N17 cells from N17-induced apoptosis, at least partially through STAT5-mediated *bcl-2* induction.

To further investigate as to which downstream signaling of Ras was involved in transactivation of *bcl-2*, we established Ba/F3 transfectants expressing fully activated Ha-Ras (V12) or Ha-Ras effector loop mutants (V12S35, V12G37, and V12C40). The V12S35 mutant activates exclusively the Ras/MAPK pathway, the V12G37 mutant activates only the Raf guanine nucleotide dissociation stimulator, and the V12C40 mutant activates only P13-K (36, 37, 38, 39). These clones were deprived of IL-3 for 36 h and subjected to Northern blot analysis to examine *bcl-2* mRNA expression. As shown in Fig. 8C, *bcl-2* expression was detected in V12- and V12S35-transfected cells and slightly in V12C40-transfected cells. These results suggest that...
Ras might regulate bcl-2 expression primarily via the Raf/MAPK pathway and partially via the PI3-K pathway in Ba/F3 cells.

Ras Mediates v-Src-induced Growth and Anti-apoptotic Signals—Because the induced expression of N17 in v-Src/N17 cells resulted in severe apoptosis, we could not precisely evaluate the effects of N17 on v-Src-dependent cell growth. Therefore, Bcl-2 or caspase-3 inhibitors, cIAP1 or cIAP2 (31), was overexpressed in these cells to evade apoptosis, because N17-induced apoptosis was accompanied by down-regulation of Bcl-2 (Fig. 8) and activation of caspase-3 (Fig. 7C). v-Src/N17 cells were transfected with an expression vector containing Bcl-2, cIAP1, cIAP2, or an empty control vector, and the obtained clones were designated as v-Src/N17/Bcl-2, v-Src/N17/cIAP1, v-Src/N17/cIAP2, and v-Src/N17/Mock, respectively. Although the expression of Bcl-2 protein was reduced after 48 h of IPTG treatment in v-Src/N17/Mock cells, the Bcl-2 expression was enhanced and hardly affected by IPTG treatment in v-Src/N17/Bcl-2 cells (Fig. 9A). The overexpression of cIAP1 and cIAP2 proteins in v-Src/N17 cells was also verified by Western blot analysis (data not shown). These clones were then cultured in the absence of IL-3 with or without ITPG treatment and subjected to the measurement of total viable cell number and DNA content analysis at indicated times. As shown in Fig. 9B, viable cells were scarcely observed in mock-transfected v-Src/N17 cells but were detectable in bcl-2-, cIAP1-, or IAP2-transfected clones when cultured with ITPG (the total viable cells at day 4: v-Src/N17/Mock, None; v-Src/N17/Bcl-2, 0.60 × 10^3/μl; v-Src/N17/cIAP1, 0.45 × 10^3/μl; and v-Src/N17/IAP2, 0.55 × 10^3/μl). Consistent with these data, DNA content analysis revealed that the induced expression of N17 led to severe reduction of cells in the S or G2/M phase (Fig. 9C; percentage of the cells in the S or G2/M phase at 0 h versus 72 h: v-Src/N17/Bcl-2, 47% versus 11%; v-Src/N17/cIAP1, 52% versus 12%; and v-Src/N17/IAP2, 45% versus 11%). Furthermore, although the induced expression of N17 led to severe apoptosis in v-Src/N17/Mock cells (Fig. 9C; percentage subdiploid fraction at 72 h: 75%), N17-induced apoptosis was inhibited almost completely in the clones overexpressing Bcl-2, cIAP1, or cIAP2 (percentage of subdiploid fraction at 72 h: v-Src/N17/Bcl-2, 3.1%; v-Src/N17/cIAP1, 2.7%; and v-Src/N17/IAP2, 1.8%). These data indicate that Ras would be involved not only in v-Src-induced anti-apoptotic signals but also profoundly in growth signals.

Ras and STAT3 Regulate Partially Overlapping but Distinct Cell Cycle Regulatory Molecules in v-Src-dependent Cell Growth—Although v-Src-activated Ras and STAT3 promoted proliferation of Ba/F3 cells, it still remained to be determined how they mediated cell growth. Because cell proliferation is tightly controlled by cell cycle regulatory molecules such as cyclins, cyclin-dependent kinases (as reviewed in Ref. 48), and other types of regulatory molecules such as c-Myc, c-Fos, and c-Jun, we tried to determine which molecules were regulated by Ras and STAT3. First, to equalize v-Src/STAT3D clone and v-Src/N17/Bcl-2 clone in terms of Bcl-2 expression, we transfected an expression vector of Bcl-2 into v-Src/STAT3D and obtained v-Src/STAT3D/Bcl-2 clone, in which Bcl-2 was expressed at the similar level to that in v-Src/N17/Bcl-2 (data not shown). Then v-Src/N17/Bcl-2 and v-Src/STAT3D/Bcl-2 cells were cultured with ITPG for indicated times and subjected to Northern blot analysis. As shown in Fig. 10, in v-Src/N17/Bcl-2 cells, mRNA expression of c-myc and c-fos was reduced distinctly after 24 h of treatment with ITPG, and that of cyclin D2 and cyclin D3 began to decrease at 48 h. However, other cyclins and cyclin-dependent kinases were expressed constantly during 96 h of ITPG treatment. In v-Src/STAT3D/Bcl-2 cells, a decrease in c-myc mRNA expression was similarly found after...
24 h of treatment with IPTG. In addition, expression of cyclin E and cyclin D2 was gradually down-regulated after 72 and 96 h of IPTG treatment, respectively, whereas that of c-fos was markedly up-regulated from 48 to 96 h. The mRNA expression levels of other molecules remained constant throughout the test period in v-Src/STAT3D/Bcl-2 cells. Expression of cyclin D1 and c-jun was not detected in both clones. In addition, the introduced Bcl-2 was supposed to hardly affect the expression pattern of cell cycle regulatory molecules in v-Src/STAT3D/Bcl-2 cells, because the similar results were obtained from v-Src/STAT3D cells (data not shown). Taken together, these results suggest that Ras and STAT3 can either directly or indirectly regulate partially overlapping but distinct growth regulatory molecules in v-Src-dependent cell growth of Ba/F3 cells.

**DISCUSSION**

Numerous studies have shown that unrestrained activation of intracellular signaling pathways is involved in the pathogenesis of various types of malignancies. However, the precise mechanisms of tumorigenesis by the activation of multiple signaling pathways are still largely under investigation. To define the roles of individual signaling pathways in oncogene-induced growth and survival, we abrogated the function of key signaling mediators by introducing their dominant-negative mutants in v-Src-transformed Ba/F3 cells. Our experiments demonstrated that N17 induced severe apoptosis in these cells, possibly through down-regulation of Bcl-2 and activation of caspase-3. In addition, N17 repressed v-Src-dependent growth by 85% when N17-induced apoptosis was inhibited by overexpressing Bcl-2 or caspase-3 inhibitor. Similarly, STAT3D and Δp85 suppressed v-Src-dependent growth by about 60 and 40%, respectively, whereas both of them hardly affected v-Src-dependent survival. These results suggest that simultaneous activation of multiple signaling pathways including Ras, STAT3, and PI3-K is required for v-Src to exert full growth-promoting activities and that disruption of only one signaling cascade is considerably effective in suppressing v-Src-dependent proliferation of Ba/F3 cells. Moreover, it was speculated that the Ras
FIG. 7. Effects of STAT3D, Δp85, and N17 on cell cycle and characterization of N17-induced apoptosis. A, cell cycle analysis in v-Src/STAT3D, v-Src/Δp85, and v-Src/N17 cells during IPTG treatment. v-Src/STAT3D, v-Src/Δp85, and v-Src/N17 cells were cultured with IPTG for indicated times. The DNA content of the cultured cells was examined by propidium iodide staining and analyzed on FACS. B, characterization of N17-induced apoptosis by TUNEL and annexin-V stainings. v-Src/N17 cells were cultured in the presence or absence of IPTG for 48 h, subjected to the stainings for TUNEL and annexin-V, and analyzed on FACS. C, flow cytometric analysis on caspase-3 activities in v-Src/N17 cells. v-Src/N17 cells were cultured with IPTG for indicated times and subjected to FACS analysis to measure caspase-3 activities. Activation of caspase-3 was determined by a fluorescence intensity that derived from the caspase-3-cleaved substrate. Height of the right fluorescent peak indicates the degree of caspase-3 activation. All results shown are representative of triplicate experiments.

FIG. 8. Effects of N17 on expression of cell cycle regulatory molecules. A, changes in the mRNA expression of apoptosis regulatory genes in v-Src/N17 cells during IPTG treatment. Total RNA was isolated from v-Src/N17 cells that were cultured with IPTG for indicated times in the absence of IL-3 and analyzed by Northern blot analysis with various 32P-labeled probes. B, effect of N17 on Bcl-2 protein expression. v-Src/N17 cells were cultured with IPTG for indicated times in the absence of IL-3 and subjected to Western blot analysis with an anti-Bcl-2 Ab. C, regulation of bcl-2 mRNA expression in Ba/F3 cells. v-Src/N17, parental Ba/F3, and 1*6-STAT5A-transfected Ba/F3 cells were cultured with or without IPTG for 36 h in the presence or absence of IL-3. V12-, V12S35-, V12G37-, and V12C40-transfected cells were cultured in the absence of IL-3 for 24 h. Total RNA isolated from these cells was analyzed by Northern blot with 32P-labeled bcl-2 probe. The lower panel shows CHO-B mRNA as a loading control.

pathway would be essential for v-Src-induced anti-apoptotic signal.

Previously, Fukada et al. (25, 49) have investigated the molecular mechanisms by which gp130 induces cell growth by using Ba/F3 cells expressing a chimeric receptor composed of the extracellular domain of granulocyte colony-stimulating factor receptor and the transmembrane/cytoplasmic domains of gp130. They have reported that the chimeric receptor, which activates both STAT3 and SHP-2/Ras/MAPK pathways in response to granulocyte colony-stimulating factor, can neither induce cell proliferation nor survival when dominant-negative STAT3 is overexpressed and that this dominant-negative STAT3-induced apoptosis is accompanied by down-regulation of Bcl-2. These findings suggest that STAT3 activation is critical for gp130-mediated mitogenic and anti-apoptotic signals. In a paradox, we demonstrate here that Ras is essential for v-Src-mediated mitogenic and anti-apoptotic signals. Because N17 did not abrogate v-Src-induced STAT3 activities in our study, N17 would be considered to mediate down-regulation of Bcl-2 and to induce severe apoptosis in v-Src/N17 cells and marked growth suppression of v-Src/N17/Bcl-2 cells despite constitutive activation of STAT3 in these cells. This contradiction may be explained by the difference in the relative activation level of each signaling pathway. The preferential activation of STAT3 by gp130 and that of Ras by v-Src may destine the differential role of each signaling pathway.

Recently, Kiuchi et al. (24) have reported that gp130-mediated activation of STAT3 is enough to induce c-myc expression in Ba/F3 cells. Another group has previously demonstrated that a chimeric protein composed of the kinase domain of c-Raf-1 and the hormone binding domain of the estrogen receptor is able to induce c-myc expression in response to 4-hydroxytamoxifen even in serum-deprived NIH3T3 cells (51), indicating that activation of Ras/Raf/MAPK cascade alone is also sufficient to induce c-myc expression. Supporting these results, our present study demonstrates that the expression of c-myc is regulated by both STAT3 and Ras. However, because disruption of either STAT3 or Ras cascade led to severe reduction of c-myc expression in v-Src-transformed Ba/F3 cells, synergistic effects of STAT3 and Ras may be required for transactivating c-myc gene in these cells.

It has been established that activated forms of Ras, STAT3, and PI3-K are individually able to transform several fibroblast cell lines, NIH3T3, 3T3, and chicken embryo fibroblast, respectively (23, 51, 52). However, despite the activation of multiple signaling pathways by v-Src, suppression of Ras function by overexpressing dominant-negative Ras or GTPase-activating protein has been shown to inhibit v-Src-induced transformation in NIH3T3 cells (7, 35, 54). Moreover, several studies have demonstrated that suppression of STAT3 activities by overexpressing dominant-negative STAT3 also inhibits v-Src-induced transformation in NIH3T3 cells (55, 56). In addition, a recent study has shown that simultaneous inhibition of Ras/MAPK and PI3-K pathways can repress transforming activities of v-Src in chicken embryo fibroblast cells, in which inhibition of either pathway alone shows little or no effect on v-Src-induced transformation (6, 53). These data together with our experimental results suggest that v-Src may not be able to activate each of the signaling cascades to the full extent, and therefore,
simultaneous activation of multiple signaling pathways is re-
quired for effective transformation by v-Src.

Our data also indicated that v-Src-activated Ras pathway might be involved in transcriptional regulation of cyclin D2, cyclin D3, c-myc, and c-fos. The time course analysis demonstrated that expression of c-myc and c-fos was down-regulated by N17 as early as 24 h, whereas that of cyclin D2 and cyclin D3 decreased in the later phase, suggesting that Ras-mediated transcriptional control of these cell cycle regulatory molecules might be different. In other words, the Ras pathway is likely to control the expression of c-myc and c-fos directly but that of cyclin D2 and cyclin D3 indirectly. Similarly, STAT3 was considered to regulate the expression of c-myc directly and that of cyclin E and cyclin D2 indirectly based on observations of the early down-regulation of c-myc and the late down-regulation of cyclin E and cyclin D2 by STAT3D. In addition, it was speculated that STAT3 would be indirectly involved in negative regulation of c-fos, because its mRNA expression was up-regulated 48 h after the induction of STAT3D. These findings imply that Ras and STAT3 regulate partially overlapping but distinct cell cycle regulatory molecules. Furthermore, it would be of interest to note that the expression of cyclin A and cyclin B was retained steadily in v-Src/N17/Bcl-2 and v-Src/STAT3D/Bcl-2 cells despite severe growth inhibition by N17 and STAT3D. These results suggest that signaling pathway(s) other than the Ras and STAT3 pathways may constitutively activate transcription of cyclin A and cyclin B, thereby contributing to v-Src-induced proliferation.

In summary, we herein address that v-Src exerts its full growth-promoting activities through the simultaneous activation of multiple downstream signaling pathways. Ras, particularly, is a critical signal mediator as a potent suppressor of apoptosis. Our results suggest that although various signaling pathways are activated in cancer cells, inhibition of only one pathway that transmits anti-apoptotic signals might be considerably effective in suppressing cancer progression. Because Ras signaling is amplified in many human cancers with high frequency, effective anti-Ras therapeutics might throw light on anti-cancer therapy. It is also hoped that the dominant-negative mutant-expressing cell lines developed in this study will provide more useful information to understand the precise mechanisms of oncogenesis.

FIG. 9. Effect of N17 on v-Src-dependent cell growth. A, overexpression of Bcl-2 in v-Src/N17 cells. Mock- or bcl-2-transfected v-Src/N17 cells were cultured with or without IPTG for 48 h in the absence of IL-3. Total cellular lysates were isolated from these cells and analyzed by Western blot analysis with an anti-Bcl-2 Ab. B, effect of N17 on growth and survival of bcl-2-, cIAP1-, or cIAP2-transfected v-Src/N17 cells. The cells of indicated clones were seeded at a cell density of 100/μl, cultured with or without IPTG in the absence of IL-3, and the total number of viable cells was counted at the indicated times. The results are shown as the means ± S.D. of triplicated cultures. C, effect of N17 on the DNA content in bcl-2-, cIAP1-, or cIAP2-transfected v-Src/N17 cells. Cells cultured as described above were stained with propidium iodide and analyzed on FACSort for the DNA content. The results are representative of triplicate experiments. IB, immunoblotting.

FIG. 10. Effects of N17 and STAT3D on the expression of growth regulatory molecules. v-Src/N17/Bcl-2 and v-Src/STAT3D/Bcl-2 cells were cultured with IPTG for indicated times, and total cellular RNA was isolated. The mRNA expression of various growth regulatory molecules was analyzed by Northern blot analysis with 32P-labeled probes. CHO-B mRNA is shown as a loading control.
