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Etk, a Btk Family Tyrosine Kinase, Mediates Cellular Transformation by Linking Src to STAT3 Activation

YUH-TYNG TSAI,[1] YI-HSIEN SU,[1] SHIH-SHUAN FANG,[1] TZYE-NAN HUANG,[1] YUN QIU,[2] YUH-SHAN JOU,[3] HSIU-MING SHIH,[3] HSING-JIEN KUNG,[3,4] AND RUEY-HWA CHEN[1]*

Institute of Molecular Medicine, College of Medicine, National Taiwan University,[1] and Division of Molecular and Genomic Medicine, National Health Research Institute,[3] Taipei, Taiwan; Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, Minnesota[2]; and Cancer Center, University of California at Davis, Sacramento, California[4]

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Etk (also called Bmx) is a member of the Btk tyrosine kinase family and is expressed in a variety of hematopoietic, epithelial, and endothelial cells. We have explored biological functions, regulators, and effectors of Etk. Coexpression of v-Src and Etk led to a transphosphorylation on tyrosine 566 of Etk and subsequent autophosphorylation. These events correlated with a substantial increase in the kinase activity of Etk. STAT3, which was previously shown to be activated by Etk, associated with Etk in vivo. To investigate whether Etk could mediate v-Src-induced activation of STAT3 and cell transformation, we overexpressed a dominant-negative mutant of Etk in an immortalized, untransformed rat liver epithelial cell line, WB, which contains endogenous Etk. Dominant-negative inactivation of Etk not only blocked v-Src-induced tyrosine phosphorylation and activation of STAT3 but also caused a great reduction in the transforming activity of v-Src. In NIH3T3 cells, although Etk did not itself induce transformation, it effectively enhanced the transforming ability of a partially active c-Src mutant (c-Src378G). Furthermore, Etk activated STAT3-mediated gene expression in synergy with this Src mutant. Our findings thus indicate that Etk is a critical mediator of Src-induced cell transformation and STAT3 activation. The role of STAT3 in Etk-mediated transformation was also examined. Expression of Etk in a human hepatoma cell line Hep3B resulted in a significant increase in its transforming ability, and this effect was abrogated by dominant-negative inhibition of STAT3. These data strongly suggest that Etk links Src to STAT3 activation. Furthermore, Src-Etk-STAT3 is an important pathway in cellular transformation.

Etk (also called Bmx) is a member of the Btk nonreceptor tyrosine kinase family (48, 51, 57). This family of kinases is characterized by a modular structure, including an N-terminal pleckstrin homology (PH) domain, a Tec homology domain, Src homology 2 (SH2) and SH3 domains, and a C-terminal kinase domain. Many members of the Btk family are predominantly expressed in cells of hematopoietic origin. Btk is expressed in B cells and myeloid cell lineages and is essential for B-cell development and signaling. Loss-of-function mutations in Btk result in human X-linked agammaglobulinemia and murine X-linked B-cell immunodeficiency (50, 53). Itk is expressed in T cells and activated by various T-cell receptors and by FcεRII stimulation in mast cells (2, 25, 30). Mice lacking Itk have defects in T-cell development and signaling (34). Tec, expressed in various hematopoietic cells and liver, is involved in the intracellular signaling of T-cell receptors and many cytokines, such as c-Kit, granulocyte colony-stimulating factor, interleukin-3 (IL-3), IL-6, and erythropoietin (37, 39, 42, 44, 58). In contrast to these family members, Etk is expressed in a variety of tissues and cell types including hematopoietic, epithelial, and endothelial cells as well as several prostate cancer cell lines and tissues (19, 48, 57, 65). Little is known about the biological function of Etk and the signaling pathways in which Etk is involved. So far, Etk is only known to be required for IL-6-induced differentiation of prostate cancer cells (48) and Gα12/13-induced activation of serum response factor in fibroblasts (40). No downstream effectors for Etk have been clearly defined. Although a recent report indicated the activation of STAT3 by overexpressed Etk in Cos cells (52), whether this involves a direct or an indirect mechanism is not clear. Furthermore, current knowledge on the role of Btk family kinases in signal transduction is largely limited to cells of hematopoietic origin. It would be important to elucidate the signaling mechanism of Etk in other cell types.

Btk family kinases are subject to several modes of regulation. They are activated by Src family kinases (16, 27, 49), phosphatidylinositol 3-kinase (PI 3-kinase) (3, 33, 48), and the α subunits of G proteins, such as Gα12/13 and Gαq (4, 28, 40). Src family kinases have been shown to directly bind and phosphorylate Btk or Itk on a tyrosine residue in the activation loop of the kinase domain. This phosphorylation leads to autophosphorylation with eventual full activation of Btk or Itk (27, 47, 49). The full activation of Btk and Itk is also dependent on the interaction between their PH domains and phosphatidylinositol 3′-polyphosphates, products of the PI 3-kinase (3, 33). A previous study showed that Etk, being analogous to other Btk kinases, is a downstream effector of PI 3-kinase (48). Etk is somewhat atypical in that it lacks a proline-rich region present in the Tec homology domain of all other Btk kinases (48). This region was suggested to mediate an interaction with the SH3 domain of certain Src family kinases (41). Thus, whether Etk is a substrate for Src has yet to be experimentally demonstrated.

v-Src is a potent oncogene which mediates cellular transformation by engaging several signaling pathways. In addition to the well-established Shc-Grb2-Ras-Raf-Erk connection, recent reports indicated that STAT3 is also activated by v-Src (10, 69),

* Corresponding author. Mailing address: 7 Chung Shan S. Rd., Institute of Molecular Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan. Phone: 886-2-23970800, ext. 5700. Fax: 886-2-23957801. E-mail: rhchen@ha.mc.ntu.edu.tw.

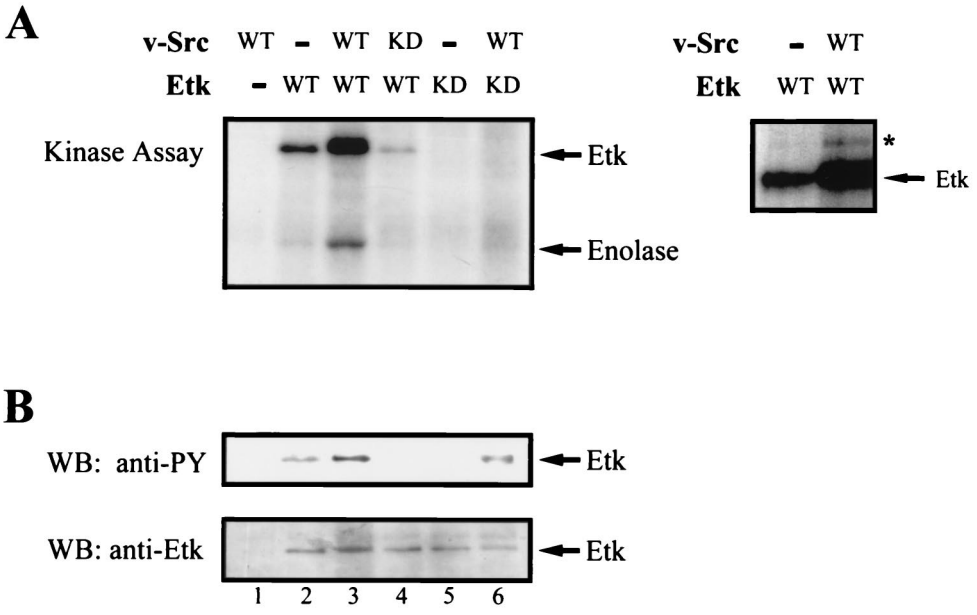


FIG. 1. Tyrosine phosphorylation and activation of Etk upon coexpression with v-Src in 293 cells. 293 cells were transiently transfected with wild-type (WT) or kinase-defective (KD), T7-tagged Etk and/or v-Src as indicated above each lane. Two days later, cells were lysed and the cell lysates were used for immunoprecipitations with anti-T7 antibody. (A) As shown on the left, the immunocomplexes were subjected to in vitro kinase assays in the presence of [γ-^{32}P]ATP and enolase as an exogenous substrate. Phosphorylated proteins were separated by SDS-PAGE and detected by autoradiography. Kinase assay products similar to those of lanes 2 and 3 of the blot shown on the left were separated on a longer gel and detected by autoradiography. The estimated migration of STAT3 protein is indicated by the asterisk. (B) Immunocomplexes (lanes are as described for panel A) were resolved by SDS-PAGE and subjected to Western blotting (WB) with antiphosphotyrosine antibody (anti-PY) or anti-Etk antibody.

which is independent of the Ras signaling pathway (35). Importantly, STAT3 activation is required for v-Src-mediated transformation of NIH3T3 cells (7, 60). Consistent with this view is the increasing body of evidence indicating the roles of STAT3 in cell growth, antiapoptosis, and malignant transformation. For instances, constitutive activation of STAT3 has been detected in cells transformed with various oncoproteins and tumor viruses (10, 36, 43, 69, 71) as well as in many human cancer cell lines and tissues (21, 24, 26, 63, 70). Introducing a constitutively dimerizeable STAT3 into immortalized fibroblasts causes cellular transformation and tumor formation (8). Furthermore, STAT3-deficient mouse embryos implant but fail to grow, suggesting a role in cell proliferation and/or survival (56). In addition, STAT3 is required for the cytokine receptor gp130-mediated G1-to-S phase progression of a pro-B-cell line by regulating several cyclins and cyclin-dependent kinase inhibitors (23) and it mediates cytokine-induced survival of the cell by upregulating Bcl-2 (22). In cell lines derived from multiple myelomas, constitutively activated STAT3 is essential to protect against apoptosis (10).

Not only v-Src is able to activate STAT3; c-Src also plays a role in IL-3- (12) and epidermal growth factor- (46) induced STAT3 activation. A key question, then, is how v-Src or activated c-Src mediates the activation of STAT3. One report showed that v-Src forms a complex with STAT3, suggesting a direct role (10). Other studies, however, imply a more indirect mechanism involving other tyrosine kinases, such as JAK (9). It is likely that different mechanisms are utilized by different cell types.

As part of our effort to understand the mechanisms underlying cellular transformation by Src-like kinases, we investigated the role of Etk in this process. Here, we present evidence which indicates that v-Src is able to transform rat liver epithelial cells and that it does so by activating STAT3 via Etk tyrosine kinase. We show that Etk is a downstream effector of Src; upon activation by Src, it associates with and phosphorylates STAT3. This pathway is crucial for transformation, as dominant-negative mutants of Etk or STAT3 interfere with the transformation process. Furthermore, coexpression of Etk and a weakly transformed Src mutant, c-Src378G, synergistically induces transformation and STAT3 activation. The present work not only illuminates the Etk pathway in several nonhematopoietic cell types but also provides one molecular link between Src and STAT3.

MATERIALS AND METHODS

Plasmids. pcDNA3T7Etk and pcDNA3T7EtkKQ are expression plasmids for T7-tagged wild type and kinase-defective Etk, respectively (48). pRK5MEtk and pRK5MEtkKQ, expression plasmids for myc-tagged Etk and EtkKQ, respectively, were constructed by PCR and cloned into pRK5M (20). Deletion mutants of Etk were generated by cloning PCR fragments corresponding to each deletion to pCDNA3T7. To construct pBabevSrc, a retrovirus-based expression plasmid for v-Src, the coding region of v-Src was PCR amplified from pMvSrc (a gift from H.-F. Yang-Yen) and cloned to pBabepuro3. The same PCR fragment was cloned to pRK5 or pBabepuro3 to generate pRKvSrc or pBabevSrc, respectively. pRK5vSrcKD, a kinase-defective v-Src (v-SrcKD) mutant carrying a K-to-R mutation at position 297, was generated by in vitro mutagenesis with the QuickChange site-directed mutagenesis kit (Strategene). To construct pRKSrc527F, a retroviral vector for a constitutively active c-Src, the coding region of c-Src was excised from pUSESrc (Upstate Biotechnology) and cloned to pBluescript. The resulting plasmid was used as a template for in vitro mutagenesis to create pBSSrc527F. pBabecSrc378G, a retroviral vector for a weakly active c-Src, was also constructed by in vitro mutagenesis. The mutagenized Src was then subcloned to pRK5. pCAGGSSTAT3D, an expression plasmid for a dominant-negative STAT3 (45), was kindly provided by T. Hirano. pMc/CMVSTAT3, an expression plasmid for wild-type STAT3, was from X.-Y. Fu.

Antibodies. Polyclonal antibody to Etk was described previously (48). The anti-phosphotyrosine antibody (RC20) was purchased from Santa Cruz. Antibody specific to STAT3 phosphorylated at position 705 was from New England Biolabs. Anti-T7 antibody was from Novagen. Antibodies to JAK2, v-Src, c-Src, STAT1, and STAT3 were from Upstate Biotechnology.

Cell culture and transfection. NIH3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. WB-F344 rat liver

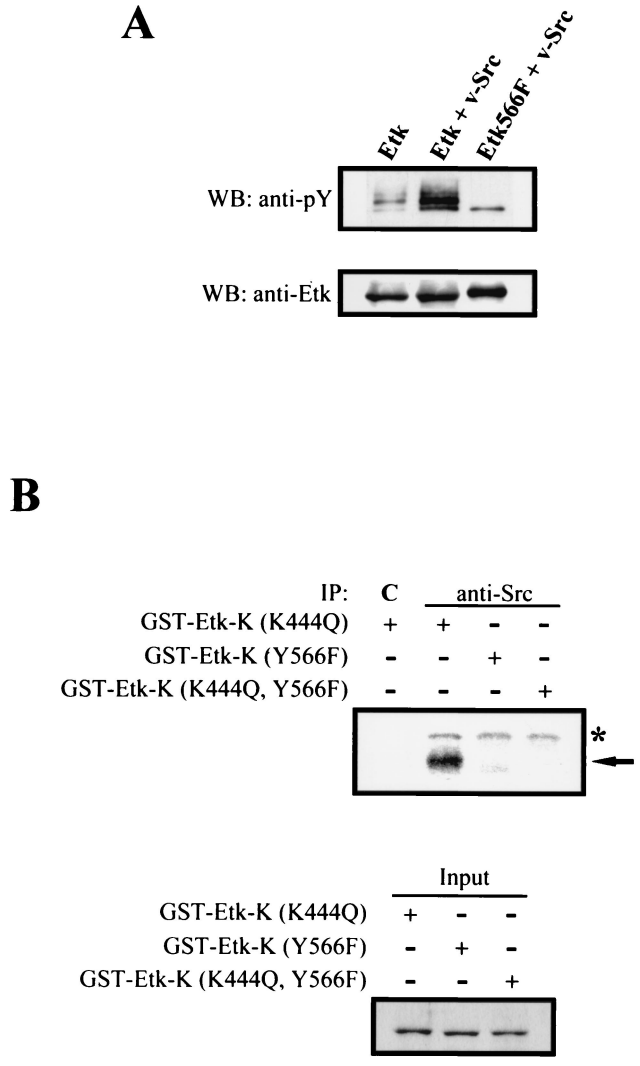


FIG. 2. Src phosphorylates Etk at Y566. (A) Mutation at Y566 decreases the Src-stimulated tyrosine phosphorylation of Etk. 293 cells were cotransfected with T7 tagged-wild type or mutant Etk and v-Src as indicated. Two days after transfection, cell lysates were subjected to immunoprecipitations with anti-T7 antibody followed by Western blotting (WB) with antiphosphotyrosine antibody (anti-PY) or anti-Etk antibody. (B) Src phosphorylates Etk on Y566 in vitro. A constitutively active Src (c-Src 527F) was transfected into 293 cells. Cell lysates were subjected to immunoprecipitations (IP) with anti-c-Src antibody (anti-Src) or a control antibody (C), and the precipitated proteins were used for in vitro kinase assays in the presence of GST-Etk-K(K445Q), GST-Etk-K(Y566F), or GST-Etk-K(K445Q, Y566F) as the substrate. Phosphorylated proteins were separated by SDS-PAGE and detected by autoradiography, as shown at the top of the panel. The asterisk and arrow indicate the phosphorylated Src and GST fusion proteins, respectively. The amount of each GST fusion protein used in the kinase assay is shown at the bottom of the panel to demonstrate an equal input.

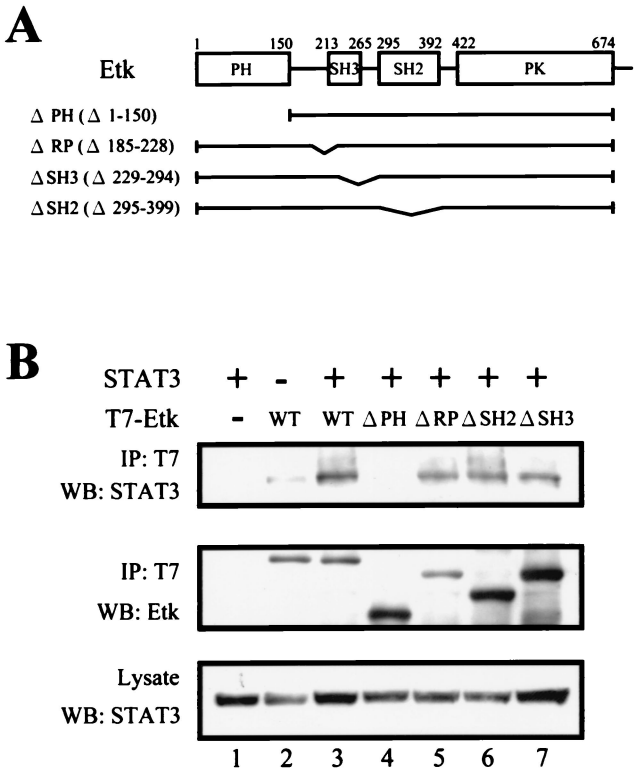


FIG. 3. Etk associates with STAT3 in vivo via the PH domain of Etk. (A) Schematic representation of the Etk protein and its mutants. Various structural domains are also labeled. RP, the two direct repeats in the Etk sequence (48); PK, protein kinase domain. (B) Association of STAT3 with Etk or its deletion mutants. 293 cells were transfected with STAT3 and/or T7-tagged wild-type or mutant Etk as indicated. Cell lysates were immunoprecipitated with anti-T7 antibody, followed by Western blotting with anti-STAT3 antibody to detect Etk-bound STAT3 (top) or with anti-Etk antibody to demonstrate the expression of Etk and its mutants (middle). Similar cell lysates were subjected to Western blotting with anti-STAT3 antibody to detect the expression of STAT3 (bottom).

epithelial cells (59) were cultured as described previously (29). Human hepatoma cell line Hep3B was cultured as described previously (14). Transfection of NIH3T3 and Hep3B cells was performed using Lipofectamine reagent (Life Technologies) according to the manufacturer's instructions. For transient transfection, cells were harvested 48 h after transfection. For selecting stable clones, G418 (700 μg/ml) was added to culture medium 48 h after transfection.

Construction of recombinant retrovirus and infection. Production of recombinant retrovirus and infection of host cells were carried out following procedures essentially as described by Kitamura et al. (32). Briefly, package cell line Phoenix-Eco was transfected by calcium phosphate method. The supernatant containing viral particles was harvested 48 h after transfection. For infection of WB cells, 2×10^5 cells were seeded onto a 60-mm plate the night prior to infection and incubated with 2 ml of viral stock in the presence of polybrene (6 μg/ml for WB and 4 μg/ml for NIH3T3). The medium was changed to fresh culture medium 18 to 24 h after the beginning of the infection. Two days after infection, cells were selected in culture medium containing 600 μg of G418 per ml or 2 μg of puromycin per ml.

Fusion protein construction and purification. Glutathione *S*-transferase (GST)-Etk-K(K445Q) was constructed by inserting a PCR fragment corresponding to the kinase domain of Etk (Etk-K) containing a mutation at position 445 into pGEX-4T vector. Mutation of Y566 was introduced by site-directed mutagenesis, and the corresponding PCR fragments were cloned to pGEX-4T to make GST-Etk-K(Y566F) and GST-Etk-K(K445Q, Y566F). Expression of the fusion proteins was induced by 0.1 mM isopropyl-β-D-thiogalactopyranoside for 3 h, and the fusion proteins present in the inclusion bodies were solubilized by denaturation with 6 M urea. The fusion proteins were allowed to refold by dialysis to remove urea and then purified by incubating with glutathione-Sepharose beads. The beads were washed five times with wash buffer containing 10 mM Tris-HCl (pH 8.0), 120 mM NaCl, 1 mM EDTA, and 0.1% NP-40, and the fusion proteins were eluted by elution buffer containing 50 mM Tris-HCl (pH 8.0) and 5 mM reduced glutathione.

Immunoprecipitations and in vitro kinase assays. Cells were lysed in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% NP-40, 10 mM NaF, 1 mM $NaVO_4$, 1 mM sodium pyrophosphate, 2 μM aprotinin, 2 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The T7-tagged Etk, JAK2, or Src was precipitated from cell lysates containing equivalent amounts of proteins with anti-T7, anti-JAK2, or anti-c-Src antibody, respectively, followed by protein A-Sepharose conjugated with rabbit anti-mouse antibody as described previously (15). The immunocomplex recovered was washed three times with lysis buffer, boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and subjected to SDS-PAGE. Alternatively, the immunocomplex was washed twice with lysis buffer and twice with kinase buffer containing 20 mM HEPES (pH 7.4), 1 mM dithiothreitol, 10 mM $MnCl_2$, 10 mM $MgCl_2$. The kinase reaction was carried out in kinase buffer supplemented with 7.5 μg of acid-denatured enolase or 2 μg of each GST-Etk-K

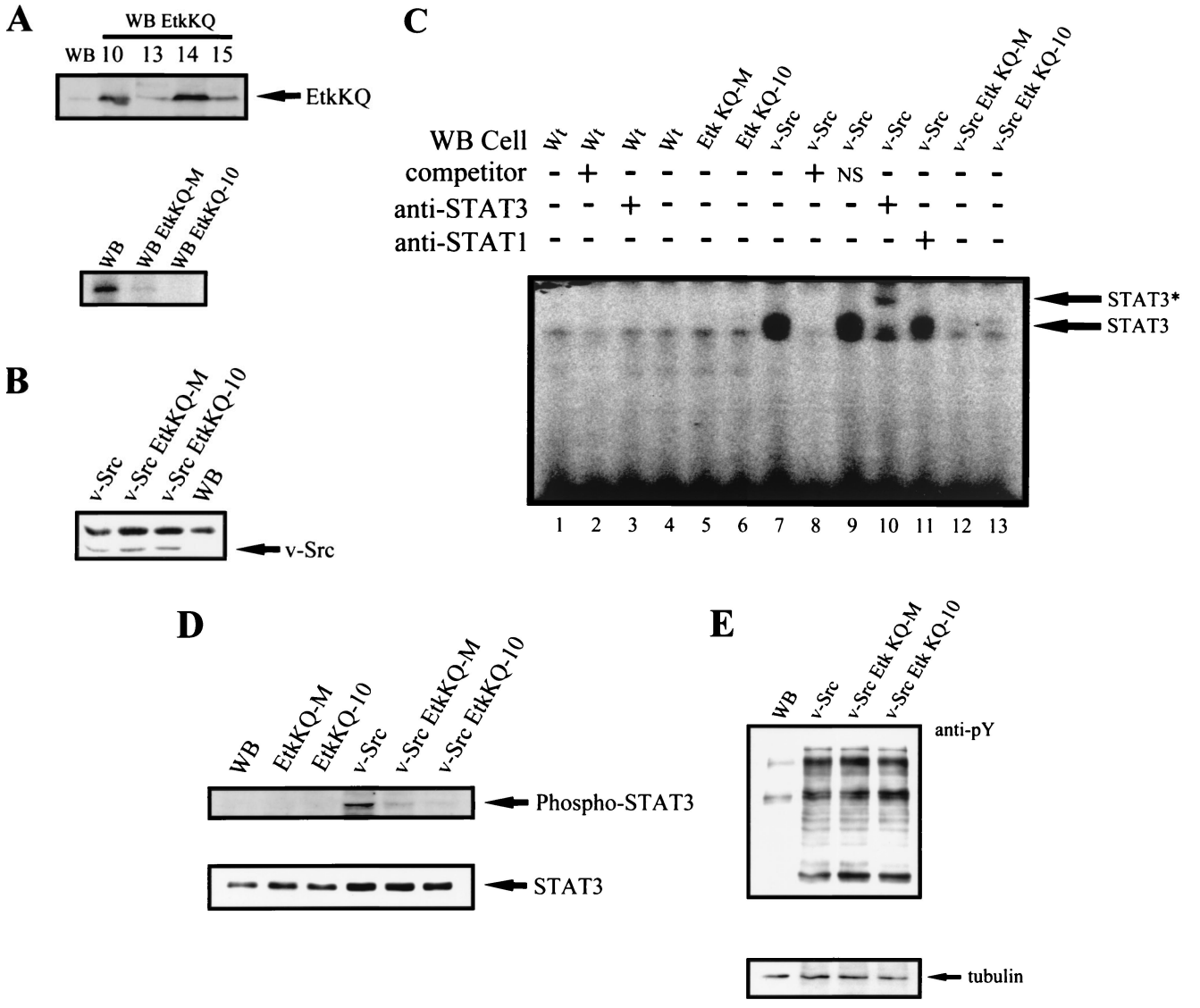


FIG. 4. Etk mediates the activation of STAT3 by v-Src. (A) The kinase-defective mutant of Etk (EtkKQ) functions as a dominant-negative mutant. The upper blot shows the expression levels of Etk and EtkKQ in parental WB cell and four stable cell lines as demonstrated by immunoblotting with anti-Etk antibody. The lower blot shows Etk kinase activities in WB and its stable lines. EtkKQ-M is a mixture of all four stable clones. Lysates of cells were immunoprecipitated with anti-Etk antibody, followed by in vitro kinase assays. The autophosphorylated Etk is shown. (B) Introducing v-Src into WB and its derivatives by recombinant retrovirus. WB and its derivatives were infected by retrovirus containing v-Src and the puromycin resistance gene. Lysates from equal numbers of puromycin resistant cells from each infection were used for Western blotting with antibody specific to v-Src. (C) EtkKQ blocks STAT3 DNA binding activity induced by v-Src. Nuclear extracts from WB and its derivatives as indicated were subjected to EMSA using a ^{32}P-labeled hSIE probe. A 133-fold molar excess of cold hSIE or a nonspecific oligonucleotide (NS) was used as the competitor, and supershifting (STAT3* indicates supershifted complex) was performed with anti-STAT3 antibody. The anti-STAT1 antibody was included as a control. (D) EtkKQ inhibits v-Src-induced tyrosine phosphorylation of STAT3. Lysates from cells as indicated were used for Western blotting with an antibody specific to STAT3 phosphorylated at position 705 (phospho-STAT) (upper blot) or the anti-STAT3 antibody (lower blot). (E) EtkKQ does not generally block tyrosine phosphorylation on v-Src targets. Lysates from cells as indicated were used for Western blotting with antiphosphotyrosine antibody (anti-pY) or antitubulin antibody.

fusion protein and 10 μCi of [γ-^{32}P]ATP at 30°C for 30 min. The kinase reaction was terminated by addition of an equal volume of 2× SDS-PAGE sample buffer. The samples were boiled for 5 min prior to electrophoretic separation by SDS-PAGE.

EMSA. Electrophoretic mobility shift assay (EMSA) was performed according to the procedures described previously (31). Briefly, nuclear extracts containing equal amount of proteins were incubated with ^{32}P-labeled hSIE probe (62) in the presence or absence of cold hSIE or a nonspecific oligonucleotide. The samples were electrophoresed on a 5% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer. For supershift, nuclear extracts were incubated with anti-STAT3 or anti-STAT1 for 20 min before the addition of labeled probe.

Luciferase assay. NIH3T3 and its derivatives were transfected with pGASLuc and pRKβ-gal. One day after transfection, cells were serum starved overnight and then harvested. Luciferase and β-galactosidase activities were quantitated by the Luciferase Assay System and the β-galactosidase Enzyme Assay System (Promega), respectively. The luciferase activity was normalized to β-galactosidase activity to account for transfection efficiency.

Soft agar colony formation assay. WB, NIH3T3, or Hep3B cells stably expressing various proteins were trypsinized, diluted in 0.3% of top agar and spread onto 60-mm plates containing 0.5% of bottom agar. For cell lines WB and NIH3T3 and their derivatives, 5×10^4 cells were seeded in each plate. For Hep3B and its derivatives, 10^4 cells were seeded. Colonies formed after 1 (for WB) or 4 (for Hep3B and NIH3T3) weeks were visualized by staining with 0.05% *p*-iodonitrotetrazolium violet dye.

RESULTS

Etk is phosphorylated and activated by v-Src. Src family kinases have been shown to activate Btk family kinases, includ-

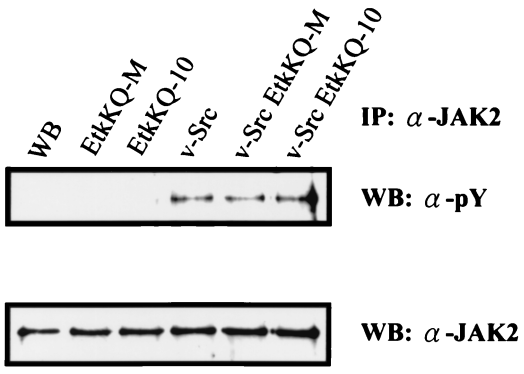


FIG. 5. Src induces tyrosine phosphorylation of JAK2. JAK2 was immunoprecipitated from lysates of WB and its derivatives. The immunoprecipitates (IP) were used for Western blotting (WB) with antiphosphotyrosine (α-pY) or anti-JAK2 antibody.

ing Btk, Itk, Tec, and Txk (16, 27, 38, 49). This, however, has not been tested for Etk. To explore the upstream regulators of Etk, we investigated whether Etk could be activated by oncogenic v-Src. Coexpression of v-Src and Etk in 293 cells led to a substantial increase in the in vivo tyrosine phosphorylation of wild-type Etk (Fig. 1B, lane 3). Furthermore, v-Src stimulated the kinase activity of Etk, as judged by autophosphorylation of Etk and phosphorylation of exogenous substrates such as enolase in an in vitro kinase assay (Fig. 1A, lane 3). This increase in tyrosine phosphorylation and kinase activity was not observed upon coexpression of a v-SrcKD with Etk (Fig. 1, lane 4), indicating that these events are dependent on the kinase activity of v-Src. Coexpression of v-Src with a kinase-defective Etk (EtkKQ) did not result in an in vitro phosphorylation on enolase or Etk (Fig. 1A, lane 6). Therefore, the in vitro phosphorylation seen with wild-type Etk (Fig. 1A, lane 3) was carried out predominantly, if not exclusively, by Etk, rather than by v-Src or other contaminating kinases in the immunoprecipitates. v-Src also increased tyrosine phosphorylation for the kinase-defective Etk, although the amount was less prominent than that observed for wild-type Etk (Fig. 1B, lanes 3 and 6). This suggests that the increase in tyrosine phosphorylation of wild-type Etk upon coexpression of v-Src is due to the combination of phosphorylation of Etk by v-Src and Etk autophosphorylation.

To further investigate the mechanism of Etk activation by v-Src, we determined the Src phosphorylation site on Etk. Previous studies showed that Src family kinase phosphorylates Btk at Y551 (47, 49). Mutation of an analogous tyrosine residue (Y566) on Etk almost completely abolished the Src-induced tyrosine phosphorylation of Etk in cotransfected 293 cells (Fig. 2A). Phosphorylation of Y566 by Src was also tested

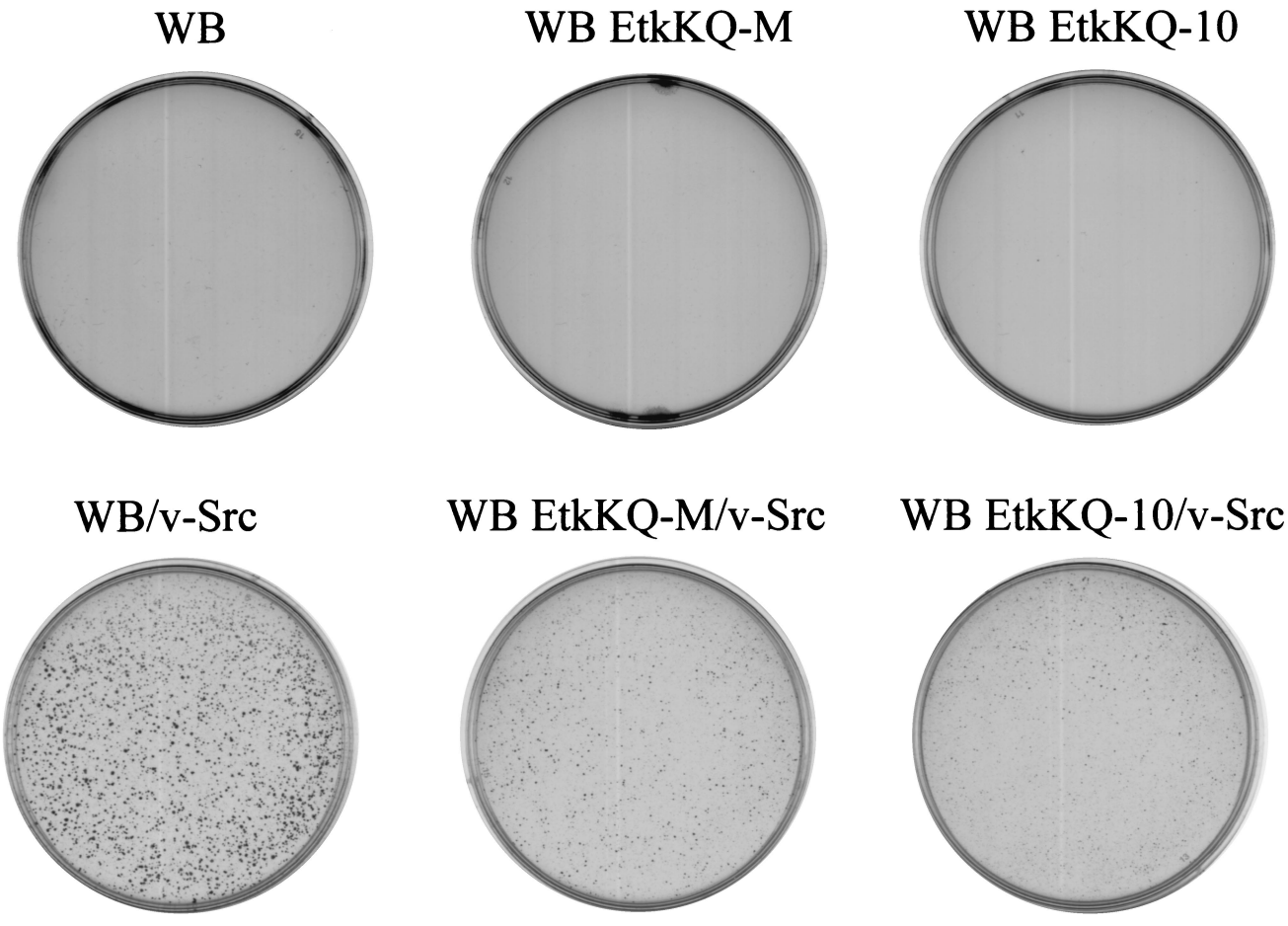
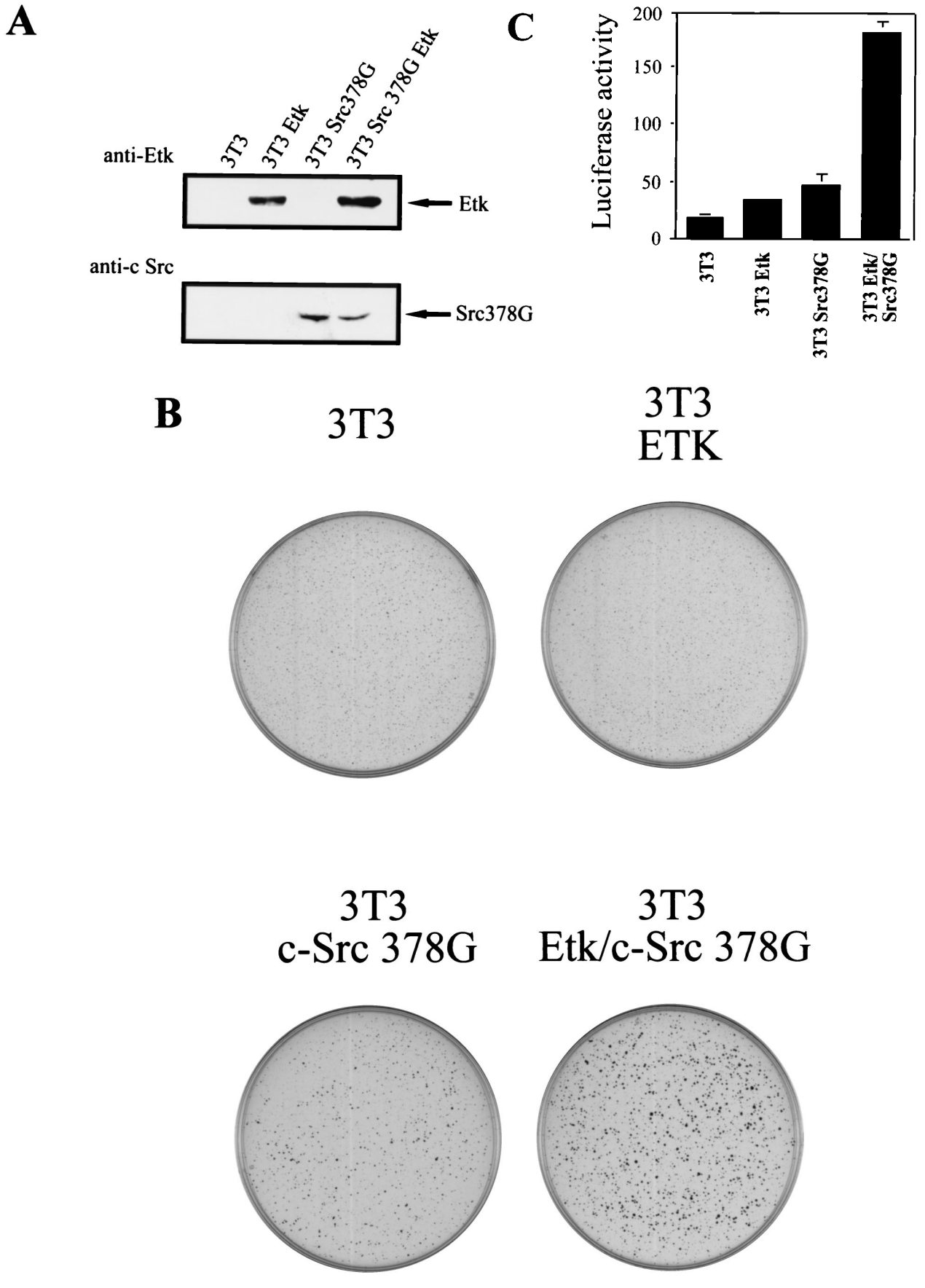


FIG. 6. Dominant-negative inhibition of Etk reduces soft agar cloning efficiency of v-Src-transformed WB cells. Cells (5×10^4) of each type as described for Fig. 3 were seeded in soft agar dishes (60-mm diameter). Colonies were stained and counted after 1 week.



in the in vitro kinase assay using chimeric proteins consisting of Etk-K fused with GST as the substrate. To distinguish the event of transphosphorylation of Etk by Src from its autophosphorylation, several GST-Etk-K mutants, in which K445 and/or Y566 were replaced, were compared as the substrates of Src. Because the lack of antibody can effectively immunoprecipitate v-Src, a constitutively active Src (c-Src 527F) was utilized in the assay. As shown in Fig. 2B, c-Src 527F purified by immunoprecipitation from transfected 293 cells effectively phosphorylated GST-Etk-K(K445Q). Phosphorylation of GST-Etk-K(Y566F) was much less efficient, whereas the GST-Etk-K(K445Q, Y566F) double mutant was not phosphorylated. Altogether, these results strongly suggest that Etk is activated by Src via a mechanism similar to that of other Btk kinases, i.e., by direct phosphorylation by Src on Y566, followed by Etk autophosphorylation.

Etk associates with STAT3 in vivo. Having identified Src as an upstream regulator of Etk, we wished to test whether STAT3 is its immediate downstream effector. To investigate whether Etk acts directly on STAT3, we examined their association in vivo. Lysates from 293 cells cotransfected with a T7-tagged Etk and STAT3 were immunoprecipitated with anti-T7 antibody, and coprecipitation of STAT3 was examined by Western blotting. This assay demonstrated the formation of a stable complex between Etk and STAT3 (Fig. 3B, lane 3). Furthermore, association of Etk with endogenous STAT3 was also observed (Fig. 3B, lane 2), indicating that this association occurs under physiological conditions. The associated STAT3 is likely to be phosphorylated by Etk directly, as is evident by the existence of a 90-kDa phosphorylated band corresponding to the size of STAT3 in the anti-Etk immunoprecipitates subjected to an in vitro kinase assay (Fig. 1A). To map the domain in Etk required for this association, we constructed a panel of Etk deletion mutants lacking each of the potential protein-protein interaction domains (Fig. 3A). Coimmunoprecipitation analysis showed that the PH domain deletion mutant could no longer associate with STAT3, suggesting that this domain is required for interacting with STAT3 (Fig. 3B).

Etk mediates v-Src-induced activation of STAT3. STAT3 was shown to be activated in v-Src transformed cells and required for v-Src-mediated transformation (7, 60). The identification of v-Src as an upstream activator and STAT3 as a downstream effector of Etk prompted us to investigate whether Etk is involved in v-Src-induced activation of STAT3 and whether Etk is required for v-Src-mediated transformation. To test these possibilities, a rat liver epithelial cell line, WB, was chosen for the following reasons. First, WB cells are nontumorigenic but can be transformed by many oncogenes including v-Src (17). Secondly, this cell line expresses a relatively high level of Etk as determined by reverse transcription-PCR (data not shown) and Western blotting (Fig. 4A upper panel, lane 1). We therefore tested whether the endogenous Etk could be inhibited by overexpression of the EtkKQ. WB cells were infected with recombinant retrovirus carrying EtkKQ, and four stable lines expressing EtkKQ were obtained (Fig. 4A). A high expressor, EtkKQ-10, and a mixture of all four clones (EtkKQ-M) were chosen for further analysis. In vitro kinase assays showed that expression of EtkKQ significantly decreased the kinase activity of endogenous Etk (Fig. 4A), indicating that EtkKQ functions as a dominant-negative mutant. The parental WB cells and stable clones expressing EtkKQ were infected by recombinant retrovirus containing v-Src and the puromycin resistance gene. After selection with puromycin, three pools of drug-resistant cells (WB v-Src, WB v-Src EtkKQ-10, and WB vSrc EtkKQ-M) expressing comparable amounts of v-Src were generated (Fig. 4B).

To assess the activity of STAT3 in WB and its derivatives, nuclear extracts were prepared and used for EMSA with the ^{32}P-labeled hSIE probe that binds STAT1 and STAT3 with high affinity. Similar to what was found in fibroblasts, v-Src expression in WB cells markedly increased the DNA binding activity of STAT3 (Fig. 4C, lanes 7 to 10). Importantly, this v-Src-induced activation of STAT3 was inhibited in cells stably expressing EtkKQ (Fig. 4C, lanes 12 and 13). To further characterize the role of Etk in activation of STAT3 by v-Src, lysates from these cells were used for Western blot analysis with an antibody specific to STAT3 phosphorylated at tyrosine 705, an event required for its dimerization and nuclear translocation (54, 66). As shown in Fig. 4D, dominant-negative Etk blocked v-Src-induced tyrosine phosphorylation of STAT3. The expression levels of STAT3 were comparable in all cell lines (Fig. 4D). Noted that the dominant-negative Etk did not generally affect v-Src-induced tyrosine phosphorylation on many cellular proteins (Fig. 4E), indicating that Etk is likely to transduce v-Src signals to only few targets, such as STAT3. We conclude from these observations that Etk links v-Src to the activation of STAT3.

Increased tyrosine phosphorylation on JAK2 in v-Src-transformed WB cells. Previous study showed that JAK1 and JAK2 are strongly and moderately activated in v-Src-transformed fibroblasts, respectively, suggesting that these kinases are involved in mediating STAT3 activation by v-Src (9). To test whether JAKs are activated in v-Src transformed WB cells and whether inhibition of Etk affects JAK activation, we examined the tyrosine phosphorylation of JAKs in WB and its derivatives. Western blot analysis showed WB contains a much higher level of JAK2 than JAK1 (data not shown). Consistent with a previous report (9), JAK2 phosphorylation was indeed higher in v-Src transformed cells (Fig. 5). This increase of the phosphorylation, however, is only moderate, since a large amount (~1 mg) of protein from cell extracts was required to generate the results shown in Fig. 5. The mechanism of JAK2 activation by v-Src remains obscure. It is, however, clear that this activation is not mediated by Etk, since expression of EtkKQ did not affect this phosphorylation of JAK2 (Fig. 5). These data suggest that JAK and Etk act in parallel in mediating v-Src-induced STAT3 activation. Nevertheless, the contribution of JAK2 to v-Src-induced STAT3 activation is minor, since dominant-negative Etk almost completely abolished the STAT3 phosphorylation and activity (Fig. 4C and D). Therefore, we conclude that, in this cell type, Etk is the primary mediator to transduce v-Src signal to STAT3.

Etk is required for transformation of WB epithelial cells by v-Src. To investigate the role of Etk in v-Src-induced cell

FIG. 7. Etk and c-Src 378G synergize in STAT3 activation and transformation of NIH3T3 cells. (A) Expression levels of Etk (upper blot) or c-Src mutant (lower blot) in stable cell lines. NIH3T3 cells stably expressing Etk and c-Src378G were generated by retrovirus-mediated gene transfer. Pools of drug-resistant cells were lysed, and the cell lysates were used for Western blotting with anti-Etk antibody or anti c-Src antibody. (B) Etk potentiates the transforming activity of c-Src 378G. Cells as described in for panel A were used for soft agar colony formation assay. Cells (5×10^5) were seeded in each dish, and colonies were stained and counted after 4 weeks. (C) Etk activates STAT3 in synergy with c-Src378G. Parental NIH3T3 cells and stable lines as described for Fig. 6A were transiently transfected with 1.5 μg of pGASLuc STAT3 reporter and 0.5 μg of pRKβ-gal. Each experiment was carried out in triplicate, and the error bars represent standard deviations. For each experiment, the luciferase activity was normalized to β-galactosidase activity to account for transfection efficiency.

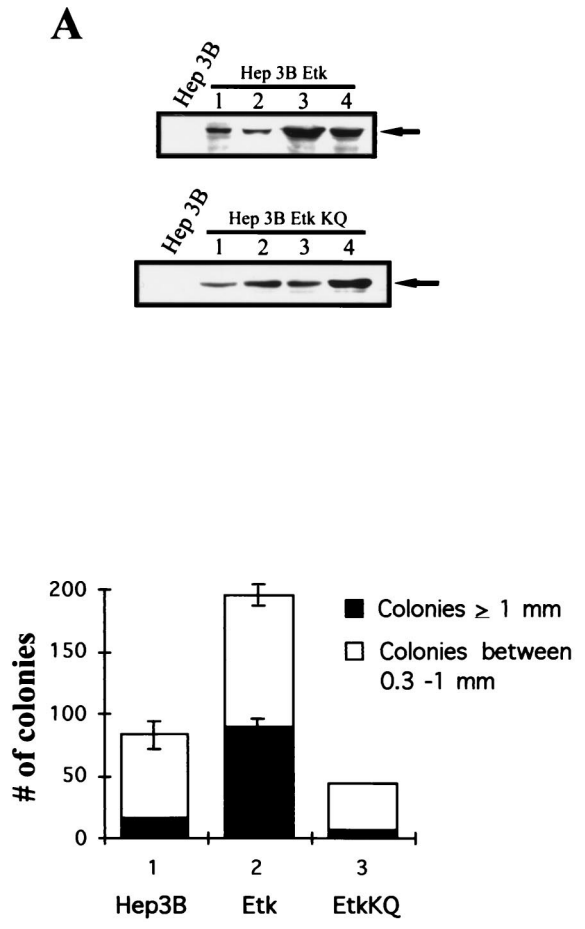


FIG. 8. Expression of Etk enhances the soft agar cloning efficiency of Hep3B cells. (A) Overexpression of Etk (upper blot) or EtkKQ (lower blot) in Hep3B stable transfectants. Cell lysates from Hep3B or its stable transfectants were subjected to Western blotting with anti-Etk antibody. The positions of Etk and EtkKQ are indicated. (B) Soft agar colony formation assay for Hep3B and stable transfectants. Hep3B Etk and Hep3B EtkKQ are mixtures of the four stable lines shown in panel A. For each experiment, 10^4 cells were seeded. Colonies with diameters larger than 1 mm and between 0.3 and 1 mm were separately counted after 4 weeks of incubation, and the numbers given are averages of at least two independent experiments.

transformation, we performed soft agar colony formation assays on WB and its derivatives. Consistent with previous studies (17), WB cells expressing v-Src efficiently formed colonies in soft agar. However, the soft agar colony forming ability of v-Src was markedly diminished by dominant-negative inhibition of Etk (Fig. 6). Furthermore, the high expressor EtkKQ-10 exhibited a higher degree of inhibition than EtkKQ-M, indicating a dose-dependent effect. These findings demonstrate a significant role of Etk in v-Src-induced transformation of epithelial cells.

Etk and Src synergistically activate STAT3 and transform NIH3T3 cells. Having demonstrated an inhibition by dominant-negative Etk in v-Src-induced transformation and STAT3 activation, we next investigated whether overexpression of Etk could enhance these effects of Src. The already high endogenous level of Etk impeded our effort to overexpress Etk in this cell line. We thus performed studies in NIH3T3 cells, which contain a much lower level of Etk (data not shown). Etk and/or a partially activated Src mutant (c-Src378G) were introduced into NIH3T3 cells by retrovirus-mediated gene transfer. Pools of infected cells were assayed for their transforming activities. As shown in Fig. 7B, cells overexpressing Etk did not grow in soft agar, and cells expressing Src378G were only weakly transformed. However, coexpression of Src378G with Etk resulted in a significant increase in both the number and size of the colonies formed in soft agar. This enhancement in transforming activity could not be attributed to the expression level of the c-Src378G, since the two pools of infected cells contain a similar level of this Src mutant (Fig. 7A). Our result is consistent with previous study in which Btk requires the partially active Src to affect its transforming activity (1).

To further demonstrate an interaction between Src and Etk signaling, we determined the activity of STAT3 in NIH3T3 cells stably expressing Etk and/or Src378G. These cells were transiently transfected with the pGASLuc, a STAT3-driven luciferase reporter plasmid (7). Src378G or Etk alone was capable of driving a moderate increase in the transcription of reporter gene, whereas coexpression of the two genes led to a synergistic activation of STAT3 (Fig. 7C). Taken together, these findings verifies the notion that Src and Etk act together in mediating STAT3 activation and cellular transformation.

STAT3 is a critical component of Etk signaling leading to transformation. The results derived from previous sections provide strong evidence that Etk mediates both STAT3 activation and cellular transformation induced by v-Src. The remaining task was then to demonstrate that the role of Etk in cellular transformation is due, at least in part, to its signaling to STAT3. Our strategy was to investigate the effect of STAT3 inactivation on the Etk-mediated transformation. For this purpose, we needed a cell line that could be readily transformed by Etk overexpression. Human hepatoma cell line Hep3B was a good candidate (see below). Hep3B cells were transfected with expression vectors for wild-type Etk or EtkKQ, and several stable clones were generated. Figure 8A shows the expression levels of Etk or EtkKQ in these clones. The stable clones expressing Etk or EtkKQ were respectively pooled and subjected to soft agar assays. While parental Hep3B cells did not efficiently form colonies in soft agar when plated at a low density (10^4 cells/plate), overexpression of Etk increased its transforming ability. This enhancement in transformation was not observed with the EtkKQ (Fig. 8B). To assess the role of STAT3 in this effect, we transfected Hep3BEtk cells with a dominant-negative mutant of STAT3, STAT3D, carrying substitutions of A for E at positions 434 and 435 (45). Several stable cell lines were generated and pooled for soft agar assay. A pool of Hep3B stable clones expressing STAT3D generated previously (13) was also included in the assay. As shown in Fig. 9B, overexpression of STAT3D abrogated the effect of Etk on the transforming ability of Hep3B cells. This difference is unlikely due to the difference in expression levels of transfected genes, since the two Etk- and the two STAT3D-expressing lines contain similar amounts of Etk and STAT3D, respectively (Fig. 9A). Thus, STAT3 is involved in Etk signaling leading to transformation. Taken together, the above data show that the Src-Etk-STAT3 pathway plays an important role in the transformation of rat, mouse, and human cells.

DISCUSSION

The role of Etk in Src-induced activation of STAT3. In this report, we demonstrate v-Src to be an upstream activator and STAT3 to be an immediate downstream effector of Etk. Furthermore, we provide evidence indicating the involvement of Etk in Src-induced activation of STAT3. Several lines of evidence are presented to support this notion. First, we found that expression of v-Src resulted in an activation of Etk and that

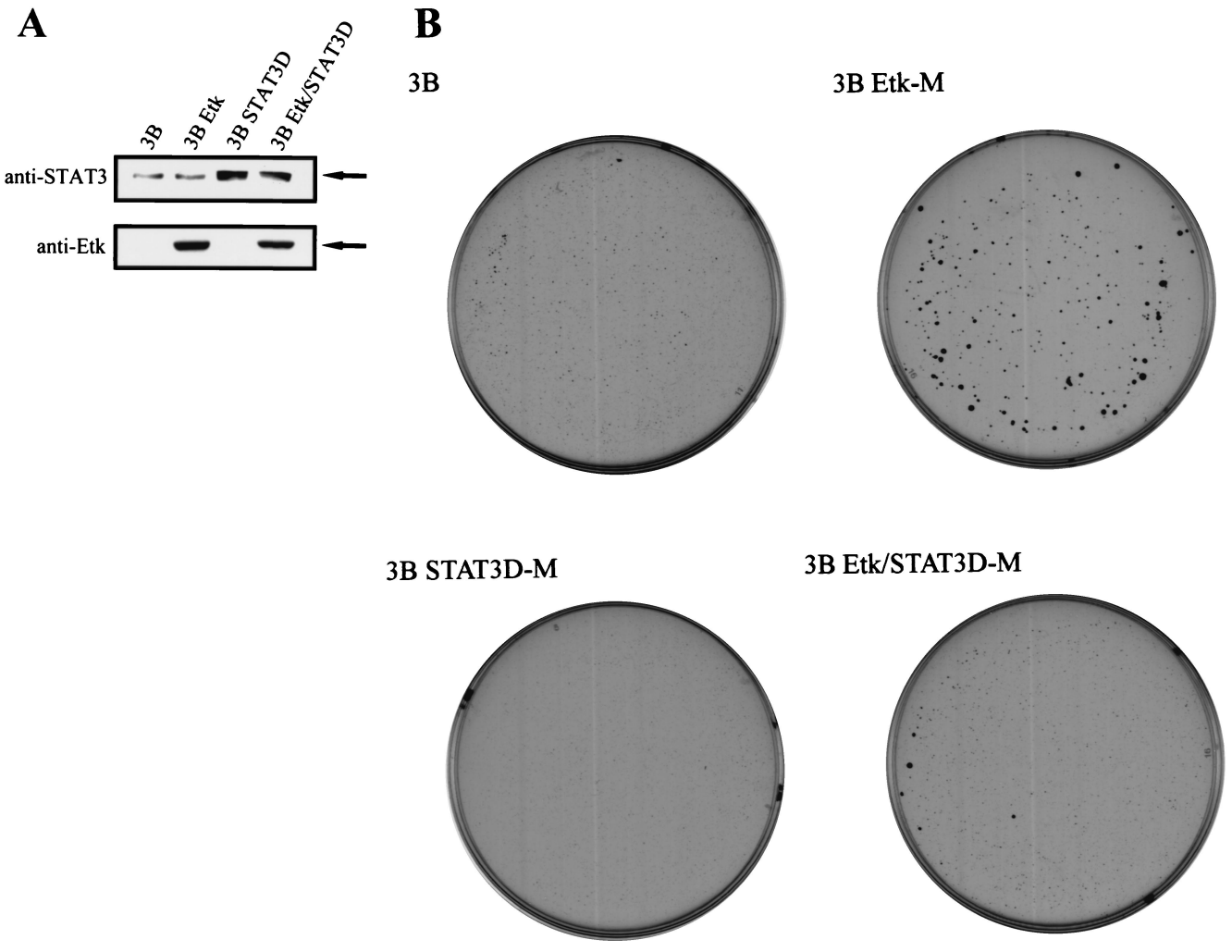


FIG. 9. Dominant-negative inhibition of STAT3 abrogates the effect of Etk on transformation of Hep3B cells. (A) Expression levels of Etk and STAT3D in Hep3B stable transfectants. Each stable transfectant was a mixture of several clones. Western blot analysis with anti-Etk (upper blot) or anti-STAT3 (lower blot) was performed with cell lysates containing equal amounts of proteins. The positions of Etk and STAT3 are indicated. (B) Soft agar colony formation assay. Cells described above were seeded as described for Fig. 7A. Pictures were taken after 4 weeks of incubation.

expression of Etk led to STAT3 activation. Secondly, Src and Etk acted synergistically in the activation of STAT3, suggesting an interaction between Src and Etk signaling. Thirdly, Etk directly complexed with and tyrosine phosphorylated STAT3. Finally and most importantly, dominant-negative inhibition of Etk activity by a kinase-defective mutant was able to inhibit STAT3 activation by v-Src. Thus, these results firmly establish a cascade link of v-Src-Etk-STAT3. Previous study revealed that v-Src associates in a complex with STAT3 (10), suggesting a direct phosphorylation and activation of STAT3 by v-Src. Another study found a constitutive activation of JAK family kinase in Src-transformed cells (9), implicating a role of JAK in v-Src-induced activation of STAT3. Yet, another report showed that it is the Src-like activity, not JAK activity, which mediates IL-3-induced activation of STAT3 (12). However, these mechanisms of STAT3 activation by v-Src need not be mutually exclusive and may vary according to cell types. In this report, we describe a new mechanism of STAT3 activation by v-Src, through the action of Etk. We show here that in epithelial cells, such as WB cells, containing a relatively high level of Etk, v-Src-induced STAT3 activation is primarily mediated through Etk. Although in this cell line JAK2 is modestly activated by Src, our finding that dominant-negative Etk nearly completely abolished v-Src-induced STAT3 activation indicates that other kinases, such as JAK2, play only minor role in linking Src signaling to STAT3.

Most interestingly, a recent study has demonstrated a requirement of STAT3 serine phosphorylation in Src transformation (61). The Ras- and Rac-1-mediated p38 and JNK pathways are activated by Src and responsible for this serine phosphorylation. Our results, which revealed the involvement of Etk in Src signaling leading to STAT3 tyrosine phosphorylation, complement well with this finding and suggest multiple signaling pathways induced by Src converged on the activation of STAT3.

We demonstrate that coexpression of v-Src and Etk led to an increased phosphorylation and activation of Etk. Furthermore, tyrosine phosphorylation in the EtkKQ expressed with v-Src was significantly reduced compared to that in the wild-type Etk expressed with v-Src. In addition, Src directly phosphorylated Etk at Y566, a position equivalent to the Src kinase phosphor-

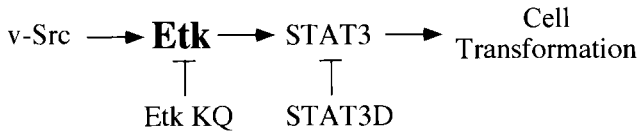


FIG. 10. Model depicting participation of Etk in cell transformation (see text).

ylation site on Btk. These findings strongly suggest that Etk is activated by Src in a fashion similar to that in which other Btk family kinases are activated (27, 47, 49). Src is likely to phosphorylate Etk on Y566, which leads to its autophosphorylation and eventually complete activation. In other Btk family kinases, the proline-rich region is implicated in the association with Src family kinases (41). Etk, however, does not contain a typical proline-rich region. The association of Src and Etk could not be reproducibly detected by coimmunoprecipitation analysis (data not shown), which might be due to a transient nature of this interaction. Alternatively, additional components might be required to stabilize this association.

Our observation that Etk associates with STAT3 in vivo suggests that STAT3 is directly tyrosine phosphorylated and activated by Etk. In support of this notion, STAT3 coimmunoprecipitated with Etk was phosphorylated by Etk in an in vitro kinase assay. The interaction of Etk and STAT3 is presumably mediated through the PH domain of Etk, since deletion of this domain abrogated interaction. Nevertheless, it cannot be excluded that such a deletion affects Etk's membrane localization or protein conformation, thus resulting in the disruption of its interaction with STAT3. Interestingly, the PH domain of Etk contains a YPFQ motif which is also found in several other Btk family kinases. Similar sequences (YXXQ) have been shown to function as STAT3 docking sites in many cytokine and growth factor receptors, such as the IL-10 receptor (64), the granulocyte colony-stimulating factor receptor (17), gp130 (55, 68), c-Met (6), and v-Eyk (5). Additional studies will be required to determine precise residues in Etk required for STAT3 binding.

Role of Etk in transformation. This report reveal that Etk is involved not only in v-Src-induced STAT3 activation but also in v-Src-induced cell transformation. The ability of Etk in mediating v-Src-induced transformation is due, at least in part, to its induction of STAT3 activity, since dominant-negative inhibition of STAT3 blocked the enhancement of transformation of Hep3B cells by Etk. Therefore, the v-Src-Etk-STAT3 pathway is important for cell transformation (Fig. 10). Emerging evidence suggests that STAT3 plays a critical role in cellular transformation by several oncogenic receptor and nonreceptor tyrosine kinases, including v-Src (7, 60), v-Eyk (5), Ros, and insulin-like growth factor receptor (72). Furthermore, recent studies demonstrated that a constitutively activated STAT3 is capable of inducing cell transformation and tumor formation, indicating the function of STAT3 as an oncogene (8). Although the mechanism by which STAT3 contributes to oncogenesis is not fully understood, its involvement in cell transformation is consistent with several lines of evidence for its roles in proliferation (22, 23, 56, 72) and antiapoptosis (10, 22). In addition, previous studies revealed that the function of STAT3 seems to be more important in anchorage-independent growth than in monolayer growth, implying that STAT3 signaling can fulfill certain adhesion-triggered signaling (72).

Although Etk is clearly required for transforming WB cells by v-Src, it is insufficient to trigger transformation by itself when introduced into untransformed cells such as NIH3T3 cells. The requirement of a partially activated Src as the cooperating oncogene for Etk to efficiently transform NIH3T3 cells resembles the case of Btk (1). Given that malignant transformation of mammalian cells is a complex process involving multiple changes, additional molecules and signaling pathways are likely to cooperate with Etk in transformation. In this regard, we found that Etk is capable of enhancing the transforming efficiency of Hep3B. This hepatoma cell line is marginally transformed and presumably have other cooperating pathways activated already. Experiments are underway to investigate the cooperating pathway to achieve a full understanding of Etk-mediated transformation process.

The present report, taken in the light of a recent finding that Etk is critically involved in an antiapoptotic pathway that protects prostate cancer cells from radiation and thapsigargin (67), suggests a possible role for Etk in the transformation and progression of human malignancies. Although the activity of Etk in various tumors has not been determined, Etk is widely expressed in many epithelial tumors, including hepatoma and prostate cancers (data not shown). Additional studies are needed to further investigate the contribution of Etk in human epithelial malignancies.

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Yuh-Tyng Tsai and Yi-Hsien Su contributed equally to this work.

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