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Etk/Bmx Tyrosine Kinase Activates Pak1 and Regulates Tumorigenicity of Breast Cancer Cells*

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Etk/Bmx, a member of the Tec family of nonreceptor protein-tyrosine kinases, is characterized by an N-terminal pleckstrin homology domain and has been shown to be a downstream effector of phosphatidylinositol 3-kinase. P21-activated kinase 1 (Pak1), another well characterized effector of phosphatidylinositol 3-kinase, has been implicated in the progression of breast cancer cells. In this study, we characterized the role of Etk in mammary development and tumorigenesis and explored the functional interactions between Etk and Pak1. We report that Etk expression is developmentally regulated in the mammary gland. Using transient transfection, coimmunoprecipitation and glutathione *S*-transferase-pull down assays, we showed that Etk directly associates with Pak1 via its N-terminal pleckstrin homology domain and also phosphorylates Pak1 on tyrosine residues. The expression of wild-type Etk in a non-invasive human breast cancer MCF-7 cells significantly increased proliferation and anchorage-independent growth of epithelial cancer cells. Conversely, expression of kinase-inactive mutant Etk-KQ suppressed the proliferation, anchorage-independent growth, and tumorigenicity of human breast cancer MDA-MB435 cells. These results indicate that Pak1 is a target of Etk and that Etk controls the proliferation as well as the anchorage-independent and tumorigenic growth of mammary epithelial cancer cells.

Epithelial and endothelial tyrosine kinase (Etk, also called Bmx)[1] belongs to the Tec family of nonreceptor protein-tyrosine kinases that are characterized by an N-terminal Tec homology domain located downstream of a pleckstrin homology (PH) domain (1–3). In addition, Etk contains Src homology-3 (SH3) and -2 (SH2) domains, and a catalytic kinase domain (4). The PH domain protein module is commonly found in signal transduction proteins and is believed to help mediate lipid-protein or protein-protein interactions (5). The PH domains of Etk and Btk (a related Tec family member) have been shown to interact with heterotrimeric G protein and protein kinase C (6, 7), and these interactions are believed to regulate kinase activity. Recent studies suggest that activation of PI3-kinase stimulate Etk, probably because of the direct interaction between the lipid product resulting from PI3-kinase reaction and the PH domain of Etk (8). The PH domain is believed to be important because germline mutation in the PH domain of Btk leads to human X-linked agammaglobulinemia (1, 2, 9). In contrast, overexpression of kinase-active Btk induces cellular transformation and protects cells from apoptotic signals (10). Although most of the Tec family kinases such as Btk, Itk, and Tec are of hematopoietic origin, Etk is found to be expressed in a variety of tissues and cell types, including lung and prostate tissues and salivary epithelial and endothelial cells (3, 4, 11).

Because Etk is a cytoplasmic kinase with several motifs characteristic of signaling molecules, it has been implicated in signal transduction networks. For example, Etk/Bmx was shown to mediate activation of Rho and serum response factor in response to the heterotrimeric G proteins Gα-12 and -13 that can be activated by hormones and neurotransmitters (6). In addition, Etk was shown to be a substrate of Src kinases and to be responsible for Src activation of signal transducer and activator of transcription factor 3 (STAT3) and for cellular transformation (12). Experiments using a kinase inactive mutant Etk-KQ showed that Etk kinase activity was required for interleukin 6-induced neuroendocrine differentiation of prostate cancer cells (4). Furthermore, dominant-negative mutants of PI3-kinase blocked interleukin 6-induced stimulation of Etk in this system, suggesting that Etk is an effector of PI3-kinase (4).

In addition to Etk, the p21-activated kinases (Paks) represent another well characterized family of effectors of PI3-kinase. Pak1 is a direct target of the small GTPases Cdc42 and Rac1, and binding of GTPases to Pak1 stimulates its kinase activity via autophosphorylation (13). Expression of kinase-active Pak1 mutant triggers the formation of lamellipodia, dissolution of stress fibers, and dissolution of focal adhesion complexes in fibroblast cells (14, 15). Expression of another kinase-active Pak1 mutant with a mutation in GTPase binding sites triggers the formation of actin ruffles (15–17). Pak1 kinase activity is essential for the formation of polarized lamellipodia at the leading edge (18) and for actin-myosin-mediated cytoskeletal changes (19). Expression of the kinase-inactive Pak1 mutant blocks the ability of Ras to induce transformation of Rat1 fibroblast (20), suggesting that Pak1 plays a role in this cell transformation. Furthermore, expression of kinase-active Pak1 in breast cancer cells stimulates anchorage-independent growth (21, 22).

Despite the recent reports of the involvement of Etk in signaling cascades in human cancer cells and the fact both Etk

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[1] The abbreviations used are: Etk, epithelial and endothelial tyrosine kinase; PH, pleckstrin homology; Pak1, p21-activated kinase; Wt, wild type; aa, amino acid; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; mAb, monoclonal antibody; MBP, myelin basic protein; N-Ter, N-terminal; C-Ter, C-terminal; RT, reverse transcription; HRG, heregulin.

and Pak1 are downstream of PI3-kinase, the relationship between Etk and Pak1 kinase and the role of the Etk pathway in the biology of human mammary epithelial cancer cells remain unknown. We sought to determine the role of Etk pathway in breast cancer cells. We present new evidence that Etk is an upstream effector of Pak1 tyrosine phosphorylation and that it is directly associates with Pak1. Furthermore, we found that Etk activity is discovered to be required for the proliferation, anchorage-independent growth, and tumorigenicity of mammary epithelial cancer cells. These results indicate that Pak1 is a target of Etk and that Etk regulates the anchorage-independent and tumorigenic growth of mammary epithelial cancer cells.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—T7-tagged wild-type (Wt) pcDNA3-T7-Etk (T7-Etk), kinase-inactive pcDNA-T7-Etk (Etk-KQ), and N-terminal and C-terminal deletion mutants of Etk were previously described (4, 12). N-Ter ETK contains amino acids 1–240 and C-Ter has amino acid 243–674 (4, 12). Myc-tagged Pak1 Wt and Pak1 K299R mutants were generously provided by Jonathan Chernoff and have been earlier described (16, 18). To construct T7-tagged central inhibitory fragment of Pak1 (Pak aa 83–149, Ref. 18) Pak1 aa 83–149 domain was amplified by PCR and subcloned into pcDNA3.1 His (Invitrogen). Antibodies directed against T7, phosphotyrosine, Pak1, and c-Myc were purchased from Novagen, Upstate Biotechnology, Santa Cruz Biotechnology, and Neomarkers, respectively. Dr Hsing-Jien Kung kindly provided monoclonal antibody to Etk.

Cell Culture and Transfection—MCF-7, MDA-MB435 human breast cancer cell lines (18), were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) supplemented with 10% fetal bovine serum. Cells were transfected with the desired vector using Fugene-6 reagent (Roche, Nutley). Clonal stable cell lines overexpressing Wt-Etk or Etk-KQ or control pcDNA were selected in the presence of G418 resistance (500 μg/ml).

Immunoprecipitation and Kinase Assay—Cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% glycerol, 1% Nonidet P-40, 10 mM NaF, 1 mM NaVO4, and a mixture of protease inhibitors. The T7-tagged Etk or Myc-tagged Pak1 were immunoprecipitated from the cell lysates with mAbs directly against T7 or Myc, respectively, as described (23). When indicated, the immuncomplex was washed with kinase buffer (20 mM HEPES, pH 7.4, 1 mM dithiothreitol, 10 mM $MnCl_2$, and 10 mM $MgCl_2$). The kinase reaction was carried out in buffer supplemented with 7.5 μg of enolase (Sigma) for the Etk kinase assay and with myelin basic protein (MBP) for the Pak kinase assay.

GST Pull-down Assay—Wt-Etk, N-Ter, and C-terminal Etk cDNA (4, 12) were translated *in vitro* using the TNT reaction kit (Promega) in the presence of [^{35}S]methionine. Subsequently, 10 μl of reaction volume was diluted in 400 μl of protein-binding buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 10% glycerol, 10 mM NaF, 1% Nonidet P-40, 1 mM NaV04, and protease inhibitors) and incubated with 2 μg of Pak1-GST or GST protein beads at 4 °C for 4 h. The beads were washed six times with 1 ml of each binding buffer and eluted with 2× SDS-polyacrylamide gel electrophoresis sample buffer. Elutes were resolved onto a 10% SDS-polyacrylamide gel electrophoresis and visualized using a phosphorimager.

Cell Proliferation and Soft Agar Colony Formation Assays—MCF-7 and MDA-MB435 cells (2×10^4 cells per well in a 24-well plate) and counted daily for six days. Anchorage-independent colony assays were performed as described previously (21, 23). Briefly 1 ml of solution of 0.6% DIFCO Agar in DMEM supplemented with 10% fetal bovine serum was layered onto 60 × 15 mm tissue culture plates. MCF-7 or MDA-MB 435 cells (10,000 cells) were mixed with 1 ml of 0.36% Bactoagar solution in DMEM prepared in a similar manner and layered on top of the 0.6% Bactoagar layer. Plates were incubated at 37 °C in 5% CO_2 for two weeks. In dominant negative Pak1 experiment, MCF-7 cells or Wt-ETK cells were transfected with 10 μg of GFP or GFP-K299R Pak1 or GFP-Pak1 inhibitor and tested for anchorage-independent growth.

Tumorigenecity Studies—Exponentially growing cells (3×10^6) were injected into mammary fat pad (two sites/animal) of female athymic mice (Nu/Nu, 4 weeks old). Every fourth day, tumor volumes were measured with calipers along two major axes. Tumor volume was calculated as follows $V = (4/3)\pi R1^2 R2$, where $R1$ is radius 1 and $R2$ is radius 2 and $R1 < R2$ ($n = 10$ per group) (24).

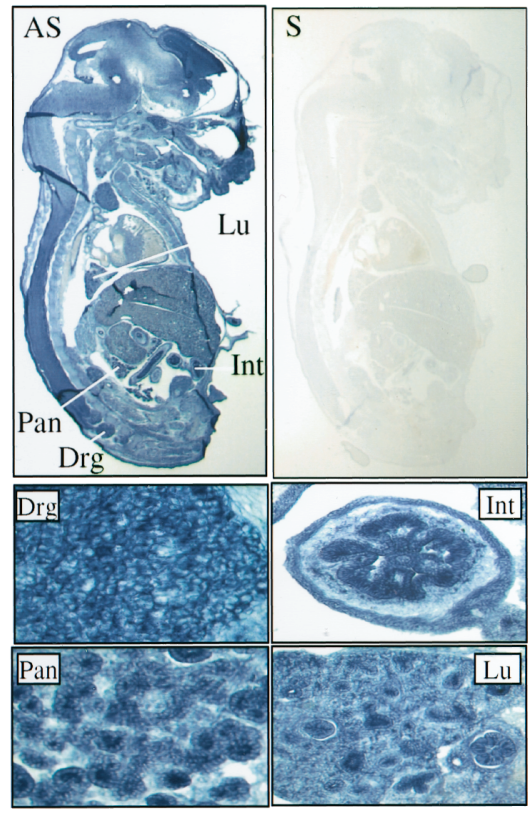

FIG. 1. **Etk expression during embryogenesis.** *In situ* hybridization using antisense (*AS*) or sense (*S*) probe. High Etk expression in high levels in the nervous and epithelial tissue, *i.e.* the dorsal root ganglia (*Drg*), the mucosa of the intestine (*Int*), the pancreas (*Pan*), and the lung (*Lu*).

Apoptosis—Tunnel method was used to detect DNA fragmentation as previously described by Gabriel *et al.* (25). Briefly, paraffin-embedded sections pretreated with protease were nicked and labeled with biotinylated poly(dU), introduced by terminal deoxy-transferase, and then stained using avidin-conjugated peroxidase.

Reverse Transcription (RT)-PCR and Southern Hybridization—Total cytoplasmic RNA was isolated from different stages of mice tissue using the Trizol Reagent (Life Technologies, Inc.) and 500 ng of RNA analyzed by RT-PCR. The forward primer for mEtk was 5′-CACACCACCTCAAAGATTTCATGG-3′ and the reverse primer was 5′-CATACTGCCCCTTCCACTTGC-3′. RT-PCR products were run onto 1% agarose gel, transferred to a blot, and probed with a 520-bp cDNA of mEtk.

In Situ Hybridization—For *in situ* hybridization, mouse mammary glands or 12-day-old embryos were dissected out and fixed with 4% paraformaldehyde. The tissues were processed into 10 μm of frozen sections, and *in situ* hybridization was performed as described (24). To make the probe, the Etk cDNA fragment was cloned to TOPO II vector, and RNA probe was labeled with digoxigenin and was synthesized by *in vitro* transcription. Sense-probe hybridization was used as background control.

RESULTS

Etk Expression during Embryogenesis and Mammary Gland Development—To explore the role of Etk during cell proliferation and differentiation, we first explored the expression profile of Etk during mouse embryonic development using *in situ* hybridization. As shown in Fig. 1, Etk mRNA was expressed in most tissues, with highest levels in the nervous and epithelial tissues, *i.e.* encephalon, dorsal root ganglia, pancreas, lung, and the intestinal mucosa. Etk mRNA signals in the vertebral column and hearts were significantly lower. *In situ* hybridization analysis of mouse mammary gland demonstrated that Etk mRNA signal was much stronger in the lactating alveoli than in the pregnant mammary gland (Fig. 2*A*).

Etk/Bmx is usually the only Tec family kinase expressed in

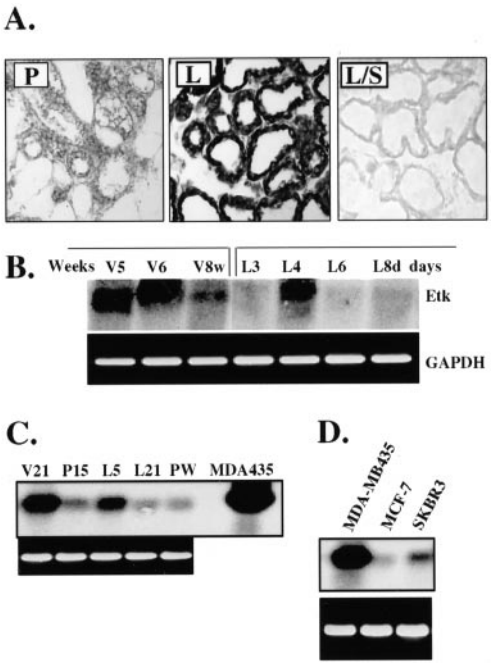

FIG. 2. **Etk expression during the mammary gland development.** *A, in situ* hybridization, a strong Etk signal was observed in the epithelial cells during lactation (*L*, day 4) but not pregnancy (*P*, day 15) stage. *L/S*, sense probe hybridization in the lactation mammary gland. *B,* RNA (1 μg) at various stages of mammary gland development (*V*, virgin weeks; *L*, lactation days) was analyzed for the expression of Etk and GAPDH control by RT-PCR followed by Southern hybridization. *C,* RNA at virgin (*V*), pregnancy (*P*), lactation (*L*) and post weaning (*PW*) stages (days) of mammary gland development was analyzed. *D,* expression of Etk in breast cancer cells. RNA (1 μg) from the indicated cell lines was analyzed for Etk expression by RT-PCR followed by Southern hybridization. The results are representative of two similar experiments.

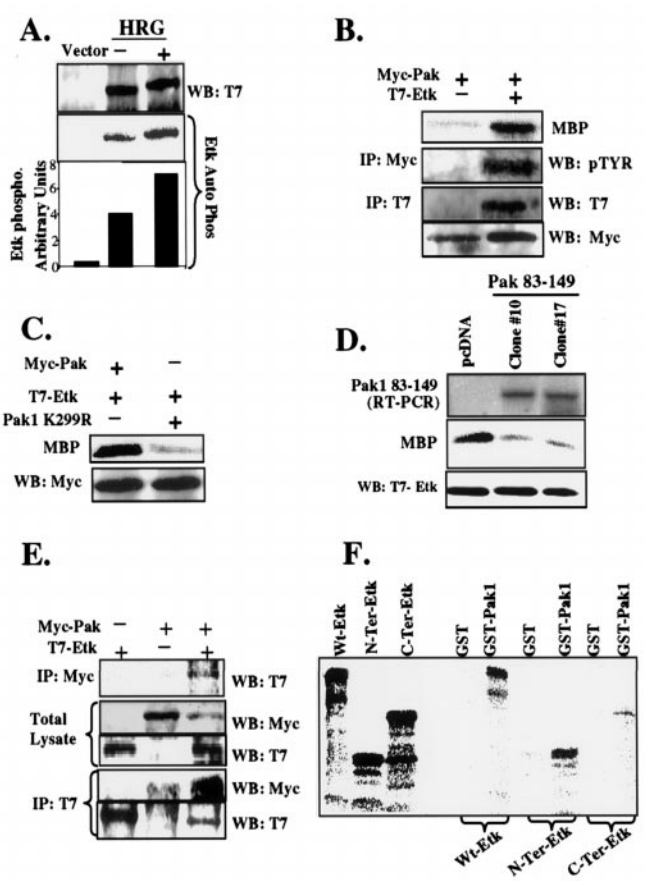

FIG. 3. **Etk regulation of Pak1 in MCF-7 breast cancer cells.** *A,* HRG stimulation of Etk kinase activity. T7-tagged Etk was transfected into cells (*lanes 2* and *3*), and cells were treated with HRG (30 ng/ml) for 10 min (*lane 3*). Immunoprecipitated (*IP*) Etk was used for an *in vitro* kinase, and the phosphorylated Etk is shown (*upper panel*). Immunoprecipitated Etk was immunoblotted with anti-T7 antibody (*lower panel*). *B,* Etk stimulates tyrosine phosphorylation and kinase activity of Pak1. Cells were transfected with Myc-Pak1 with or without T7-Etk. Cell lysates were immunoprecipitated with Myc antibody and subjected to *in vitro* kinase assay using MBP. The reaction products were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose and autoradiograph showing MBP phosphorylation (*first panel*). The blot was reprobed with mAb 4G10 to show Pak1 tyrosine phosphorylation (*second panel*) and then with Myc antibody (*fourth panel*). Cell lysates were immunoprecipitated with T7-antibody to show the expression of T7-Etk (*third panel*). *C,* cells were co-transfected with Myc-Pak1 and T7-Etk in the presence or absence of DN-Pak1. Cell lysates were immunoprecipitated with Myc antibody and subjected to *in vitro* kinase assay using MBP, and the blot was blotted with Myc antibody. *D,* Etk interacts with Pak1 *in vivo*. Cells were transfected with Myc-Pak1 or T7-Etk or both. Cell lysates were immunoprecipitated with antibodies directed against T7 or Myc and Western blotted with antibodies against T7 or Myc as indicated. *E,* stable clones expressing Pak1 inhibitors and pcDNA control cells were transfected with Wt-Etk, immunoprecipitated with Pak1, and subjected to *in vitro* kinase assay using MBP (*upper panel*). Cell lysates were immunoprecipitated with T7 antibody to show the expression of T7-Etk (*middle panel*). The expression of Pak1 inhibitors were verified by PCR (*lower panel*). *F,* GST pull-down assays to show the association of Pak1 with the *in vitro* translated Wt-Etk, N-Ter-Etk, or C-Ter-Etk. The *first three lanes* show the inputs. The results are representative of three similar experiments.

epithelial cells, but the expression level is generally low. Despite the low level, accumulating evidence suggests that it play an important role in the growth, differentiation, and apoptosis of epithelial cell (26). To understand the potential function of Etk in mammary gland, we investigated Etk expression during various stages of the mammary gland development by RT-PCR followed by Southern hybridization with a fragment of human Etk cDNA. Results indicated that Etk expression appears to undergo a cyclic change as the expression levels of Etk were down-regulated during pregnancy, early lactation, and again during the late stages of lactation (Fig. 2, *B* and *C*). Etk expression was lowest during pregnancy, a stage of high proliferation for mammary glands (Fig. 2, *B* and *C*), and highest in non-proliferative weaning and virgin mammary glands, suggesting that Etk may have an important role in the biology of mammary epithelial cells.

Expression and Activation of Etk in Breast Cancer Cells—Because the potential role of Etk in mammary epithelial cancer cells is not known, we next examined the expression of Etk in a panel of breast cancer cell lines by Southern hybridization. Among the breast cancer cell lines used, highly tumorigenic and metastatic MDA-MB435 cells exhibited the highest Etk content, whereas the Etk content of MCF-7 and SKBR-3 was lower (Fig. 2*D*). Because MCF-7 and SKBR-3 cells do not form tumors and metastasis *in vivo*, it appears that Etk expression may correlate with the degree of transformation of breast cancer cell lines used here.

To assess the physiological significance of Etk expression in breast cancer cells, we sought to determine whether Etk kinase activity can be stimulated by physiologically relevant molecules in mammary gland, as heregulin-β 1 (HRG), a polypeptide growth factor with a role in the development and tumorigenesis of mammary epithelial cells (18). Indeed, we found that HRG activated the autophosphorylation of Etk in MCF-7 breast cancer cells (Fig. 3*A*). Because of the low level of expression and relatively low avidity of the antibodies currently available, it has been difficult to visualize endogenous Etk in cell biology study. Therefore, we resorted to express T7-tagged Etk for this type of analysis.

Pak1 Phosphorylation and Activation by Etk in Breast Can-

cer Cells—Because both Etk and Pak1 are downstream of PI3-kinase, we sought to determine whether Etk could activate Pak1. Co-transfection of MCF-7 cells with T7-tagged Etk and Myc-tagged Pak1 constructs significantly increased Pak1 kinase activity as determined by an immune-complex kinase assay using MBP as a substrate (Fig. 3*B*, *upper panel*). Because Etk is a tyrosine kinase, we hypothesized that Etk phosphorylates Pak1 on tyrosine residues. Indeed, we discovered that co-transfection of Etk and Pak1 was accompanied by a substantial stimulation of tyrosine phosphorylation of Pak1 (Fig. 3*B*, *lower panel*). To verify that Pak1 is downstream of Etk, we next demonstrated that coexpression of a kinase-defective K299R Pak1 mutant protein (designated dominant-negative (DN)-Pak1) suppressed the ability of Etk to activate Pak1 kinase activity (Fig. 3*C*). In the targeted Pak1 K299R sequence, the lysine 299 ATP binding site was replaced by arginine, rendering Pak1 catalytically defective as shown by us and others in cell lines (16, 18). To rule out that the observed inhibitory effect of Pak1 K299R mutant was not due to ineffective transduction of Cdc42/Rac signals, we next generated stable MCF-7 clones expressing the central inhibitory fragment of Pak1 aa 83–149 (Fig. 3*D*, *upper panel*), which does not interfere with cdc42/Rac1 binding. These stable cell lines expressing control vector or the central Pak inhibitor were transfected with T7-tagged Etk, and the effect Etk on Pak1 kinase activity was measured by subjecting the immunoprecipitated T7-Etk to *in vitro* kinase assay using MBP as a substrate. The Etk was able to activate Pak1 activity in vector-transfected cells but not in the cells that express Pak inhibitor aa 83–149 (Fig. 3*D*). Together, these results confirm that Etk regulates Pak kinase activity. While at present, we do not know whether tyrosine phosphorylation of Pak1 induced by Etk contributes to its elevated activity. To our knowledge, this is the first report that shows that Pak1 is tyrosine-phosphorylated and serves as a downstream substrate of Tec family of kinase. While interesting and reproducible, at present we do not know the significance of the tyrosine phosphorylation, and experiments are planned to explore the significance of this finding.

Interaction of Etk with Pak1 in Vivo—To determine whether the observed activation of Pak1 by Etk is due to interactions between the two proteins, we examined the association between T7-tagged Etk and Myc-tagged Pak1 *in vivo* by reciprocal co-immunoprecipitation and Western blot assays. Results of a representative experiment are shown in Fig. *3E*. Transient expression of T7-tagged Etk, but not of control T7 vector, in MCF-7 cells was accompanied by the association of T7-tagged Etk with Myc-tagged Pak1 in both immunoprecipitation experiments. These results suggest that Etk associates with Pak1 and stimulates tyrosine phosphorylation and kinase activity of Pak1 (Fig. 3*E*).

To determine whether the observed association between Etk and Pak1 was direct or mediated via other proteins, we examined the ability of *in vitro* translated Wt-Etk or Etk-KQ protein to bind GST-Pak1 in GST pull-down assays. As shown in Fig. 3*F*, Etk and its N-terminal domain (aa 1–240) strongly interact with GST-Pak1; very little interaction was seen between Etk C-terminal domain (aa 243–674) and GST-Pak1, and GST alone provided a proper negative control.

Etk in Proliferation and Anchorage-independent Growth of Breast Cancer Cells—To further delineate the contribution of Etk in the biology of breast cancer cells, we established stable MCF-7 clones expressing T7-tagged Wt-Elk or kinase-inactive Etk-KQ or control vector. The results shown in Fig. 4*A* demonstrate the expression of tagged Etk in several representative clones. The functionality of Etk was assessed by performing *in vitro* kinase assays using enolase as an exogenous substrate (Fig. 4*B*). To determine how Etk affects the Pak1 pathway, MCF-7/Etk cells were transfected with Myc-Pak1. Overexpression of Etk in MCF-7 cells was associated with significant increases in the phosphorylation of Pak1 on tyrosine (Fig. 4*C*). MCF-7 cells expressing Wt-Etk were transfected with or without Myc-tagged Pak1, and tagged Pak1 was immunoprecipitated by an anti-Myc mAb and subjected to *in vitro* kinase assay. The *upper* and *lower* bands in Fig. 4*D* represent the autophosphorylated T7-Etk (73 kDa) and Myc-Pak1 (64 kDa), respectively. These protein bands were identified due to their differential electrophoretic mobilities in the gel.

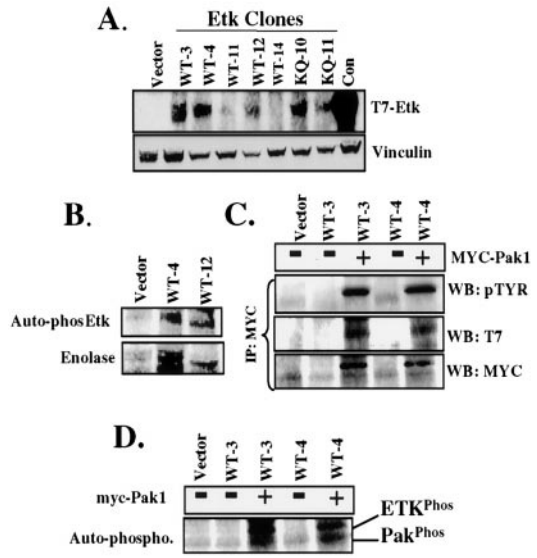

FIG. 4. **Overexpression of Etk stimulated Pak1 tyrosine phosphorylation.** *A,* characterization of MCF-7 clones expressing T7-tagged Wt or kinase-inactive (*KQ*) Etk by Western blotting by anti-T7 mAb or control vinculin Ab. *B,* baseline Etk kinase activity in Wt-Etk clones. Lysates of cells were immunoprecipitated with anti-Etk antibody, followed by *in vitro* kinase assays. The autophosphorylated Etk and enolase as an exogenous substrate of Etk are shown. *C,* the indicated Wt-Etk clones were transfected with or without Myc-tagged Pak1. The Myc-Pak1 was immunoprecipitated by an anti-Myc mAb, immunoblotted with antiphosphotyrosine Ab, and then immunoblotted with anti-T7 and Myc mAb. *D,* Wt clones were transfected with or without MYC-tagged Pak1, and tagged Pak1 was immunoprecipitated by an anti-Myc mAb subjected to *in vitro* kinase assay. Autophosphorylated Etk and Pak1 are also shown. Results shown are representative of three experiments.

To examine the influence of Etk expression on the growth characteristics of breast epithelial cancer cells, we measured the proliferation rate and the ability of cells to grow in an anchorage-independent manner. Compared with vector-transfected control cells, cells in which Wt-Etk was overexpressed demonstrated greater ability to form larger colonies in soft agar, and expression of Etk-KQ mutant led to a reduction in the anchorage-independent growth (Fig. 5,*A* and *B*). In addition, as shown in Fig. 5*C*, the growth rate of cells expressing Wt-Etk and Etk-KQ was affected 35–40% more than that of the control vector-transfected clone. Because Pak1 has been shown to promote the anchorage-independent growth (21, 22), we next determined whether dominant-negative Pak1 could modulate the ability of Wt-Etk cells to form colonies in soft agar. Wt-Etk cells were transfected with GFP-dominant-negative Pak1 and GFP-Pak1 inhibitor and used for soft agar assay. As shown in Fig. 6, *A* and *B*, Wt-Etk cells transfected with dominant-negative Pak1 or Pak1 inhibitor form small colonies compared with Wt-Etk control cells. Together, these findings suggested that Pak1 is a downstream effector of Etk pathway and may contribute to the observed phenotypic changes by Etk.

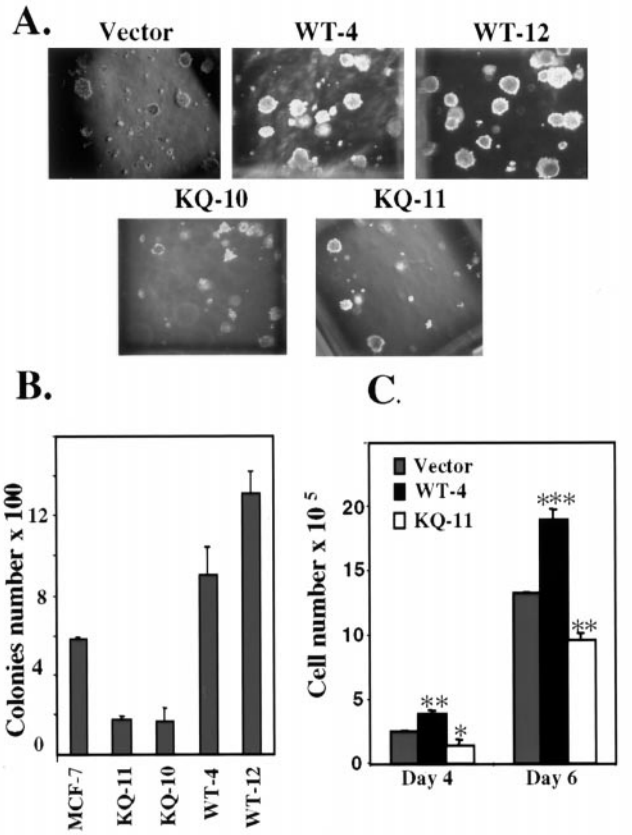

FIG. 5. **Etk regulation of cell growth and anchorage-independent growth of breast cancer cells.** The effects of Wt-Etk and Etk-KQ expression on anchorage-independent growth of MCF-7 cells. MCF-7 cells, Wt-Etk and Etk-KQ cells (10^4 cells/plate) were seeded in soft agar dishes (35-mm diameter) and colonies with diameters larger than 1 mm were counted after 2 weeks of incubation. The results are representative of three similar experiments. *B,* the numbers given are mean values ± S.E. of three independent experiments performed in triplicate. *C,* the effects of Wt-Etk or Etk-KQ expression on the growth rate of MCF-7 cells. Cells were seeded at a density of 2×10^4 in 24-well tissue culture plates in 10% fetal bovine serum/DMEM. After 4 and 6 days, cells were counted using a coulter counter. Each *point* represents the mean ± S.E. of two replicate wells. *Asterisks* indicate $p < 0.05$ by Student's *t* tests.

Effect of Overexpression of Kinase-inactive Etk of Breast Cancer Cells—We next sought to determine whether Etk activity is required for the maintenance of the transformed phenotypes in breast cancer cell lines. Highly tumorigenic and invasive MDA-MB435 cells were stably transfected with T7-tagged kinase-inactive Etk mutant (Etk-KQ) or with control pcDNA vector (Fig. 7*A*, *upper panel*). As expected from the results shown in Fig. 4, overexpression of Etk-KQ led to a significant reduction in Etk and Pak1 kinase activities (Fig. 7*B*). The expression of Etk-KQ was accompanied by a significant inhibition of the growth rate of MDA-MB435 cell (Fig. 7*C*). In addition, overexpression of kinase-inactive Etk-KQ reduced the ability of cells to grow in soft agar as compared with vector-transfected control cells (Fig. 7, *D* and *E*).

To investigate the significance of Etk expression *in vivo*, we next examined the ability of MDA-MB435 clones expressing kinase-inactive Etk-KQ or vector control in a xenograft model. In these experiments, cells were implanted into the mammary fat pad of athymic mice. Inactivation of Etk in MDA-MB435 cells severely affected the ability of cells to form tumors (*i.e.* by 55–70% compared with vector-transfected cells) (Fig. 8*A*). Histological examinations of tumors with hematoxylin and eosin staining revealed the presence of necrotic areas in tumors from Etk-KQ clones (data not shown). Reevaluation of these tumors with TUNEL staining confirmed that apoptosis was widespread in Etk-KQ tumors (Fig. 8*B*). Together, these findings suggest that Etk expression may be required for the maintenance of transformed phenotypes in breast cancer cells.

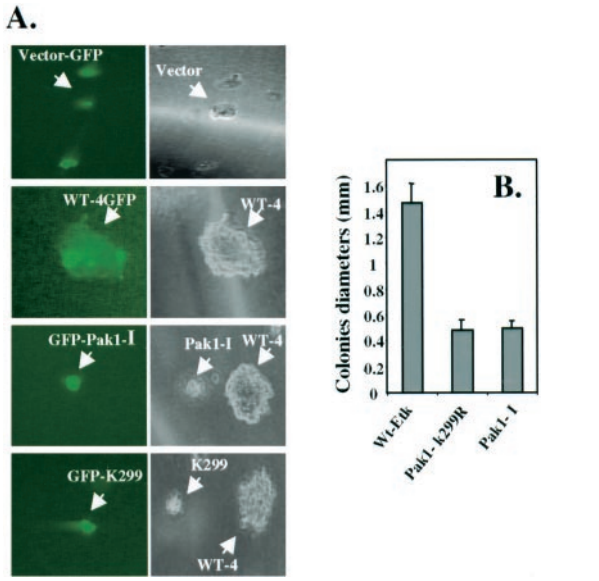

FIG. 6. **Dominant-negative inhibition of Pak1 reduces soft agar cloning efficiency of Wt-Etk cells.** *A,* MCF-7 cells and Wt-Etk cells were transfected with GFP-K299R Pak1 or GFP-Pak1 inhibitor and tested for anchorage-independent growth. Using a fluorescent lamp with excitation for GFP visualization captured the images presented on the *left panel,* and the *right panel* represents the phase contrast image of the same microscopic field to show total cells. *B,* the colony diameters of Wt-Etk cells transfected with Pak1 K299R and Pak1 inhibitor aa 83–149. Representation results from two independent experiments are shown here.

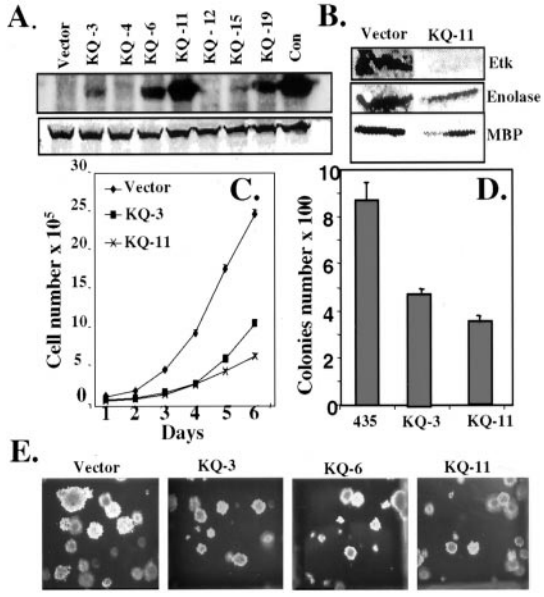

FIG. 7. **Kinase-defective Etk inhibits tumorigenicity of breast cancer cells.** *A,* characterization of MDA-MB435 clones expressing T7-Etk by Western blotting with anti-T7 mAb and with vinculin Ab. *B*, Etk and Pak1 kinase activities in Etk-KQ cells. Etk-KQ cells were immunoprecipitated with anti-Etk antibody or PAK1 antibody followed by *in vitro* kinase assays. The autophosphorylates Etk (*upper panel*) and enolase (substrate for Etk) or MBP (substrate for Pak1) (*lower panel*) are shown. *C,* effect of Etk-KQ on the growth rate of exponentially growing clones as determined by counting the numbers of cells. *D,* effect of Etk-KQ expression on anchorage-independent growth of MDA-MB435 cells over 14 days. *E,* representative results from two independent experiments are shown here.

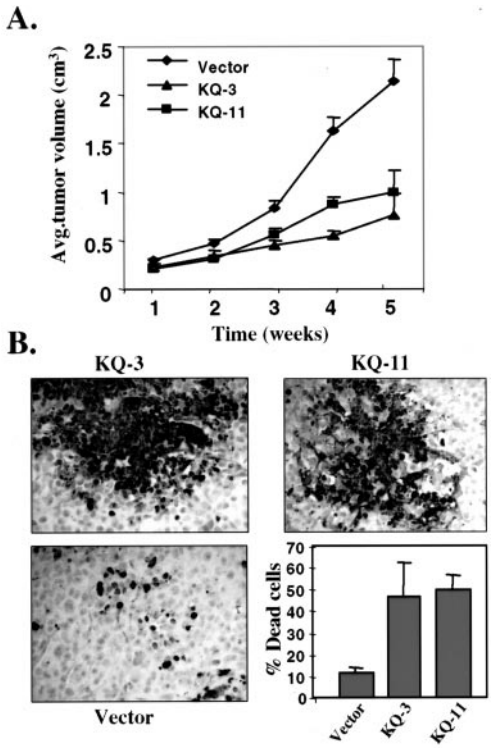

FIG. 8. **Effect of kinase-defective Etk on breast cancer tumorigenicity *in vivo*.** *A,* MDA-MB435 clones expressing control vector or Etk-KQ clones were injected subcutaneously in the mammary gland fat pad, and tumor volume was recorded (n = 10/group). *B,* histological examination of tumors by TUNEL staining. Quantitation of apoptotic cells using NIH image analysis program from 10 fields is shown in the *lower right panel*.

DISCUSSION

Etk is a member of the Tec family of the nonreceptor tyrosine kinases that are characterized by N-terminal PH domains. The PH domain is important in protein-protein interactions and is involved often in cytoplasmic signaling cascades. Etk is one of the few Tec family members, which are expressed in epithelial cells (10). Here we sought to determine the role of Etk in regulating breast cancer growth regulation. We report that: 1) Etk expression is developmentally regulated during mammary gland development; 2) Etk is expressed in the highly tumorigenic MDA-MB435 cell lines; 3) Etk is tyrosine-phosphorylated and is activated by physiologically relevant growth factor in breast cancer cells; 4) Etk phosphorylates Pak1 on tyrosine residues, and kinase-inactive Pak1 mutant blocked Etk activation of Pak1; 5) Etk directly interacts with Pak1 via the N-terminal PH domain-containing region; 6) overexpression of Wt-Etk in noninvasive breast cancer line enhanced the ability of the cells to grow in an anchorage-independent manner; and 7) expression of kinase-inactive Etk inhibits tumorigenic phenotypes in a highly tumorigenic breast cancer cell line. Taken together, these observations suggest that Etk play an important role in the regulation of mammary epithelial cancer cells.

The finding that Pak1 kinase activity is stimulated following Etk kinase activation is important, as it implies that Etk kinase constitutes an initial signal for Pak1 activation. This hypothesis is supported by several additional observations: 1) inhibition of Etk kinase by a kinase-defective mutant was accompanied by concurrent inhibition of Pak1 activity; 2) expression of dominant-negative Pak1 mutant blocked the ability of Etk to activate the Pak1 kinase and did not affect the Etk kinase; 3) Etk directly interacted with Pak1 via its N-terminal PH domain; and 4) Etk-mediated stimulation of anchorage-independent growth was blocked by dominant-negative Pak1. These results suggest that Pak1 may be downstream of Etk kinase in breast cancer cells. These findings are inconsistent with those in a recent report (6) that showed activation of Rho rather than of Cdc42 or Rac1 (upstream regulators of Pak1) by Tec family members in mouse 3T3 fibroblast cells. To reconcile these findings, we suggest that the Tec family may utilize distinct members of the small GTPase family members in epithelial cancer and fibroblast cells in humans and/or mice.

Another notable finding in this study was that Etk induced tyrosine phosphorylation of Pak1 and that Etk activity is required for the proliferation, anchorage-independent growth and tumorigenicity of breast cancer cells. This finding strongly suggests that Etk utilize Pak1 tyrosine phosphorylation to influence transformed phenotypes that are generally believed to be driven by tyrosine phosphorylation. This hypothesis is further supported by recent findings by us and by others that overexpression of kinase-active Pak1 mutant (T423E, predominantly serine phosphorylation) in breast cancer cells selectively enhanced the anchorage-independent growth of breast cancer cells (21, 22). Currently, we do not know the precise mechanism by which Etk exerts its profound stimulatory effects in the transformation functions of cancer cells. It is possible that in addition to Pak1, there are other unidentified downstream effectors for Etk pathway that contribute the observed phenotypic changes. Studies are in progress to investigate these and other possibilities.

Our findings clearly established, for the first time, that Etk kinase directly associates with Pak1 and stimulates Pak1 tyrosine phosphorylation and that Etk controls the anchorage-independent growth rate and tumorigenic behavior of human mammary epithelial cancer cells. These observations open a new avenue of investigation closely linking the Tec and Pak families with breast cancer cell activity.

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Etk/Bmx Tyrosine Kinase Activates Pak1 and Regulates Tumorigenicity of Breast Cancer Cells

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