Chapter II

Role of tyrosine kinase Etk/Bmx in EGF-induced apoptosis of MDA-MB-468 breast cancer cells
Abstract

Etk/Bmx, a member of the Tec family of tyrosine kinases, mediates various signaling pathways and confers several cellular functions. In the present study, we have explored the functional role of Etk in mediating EGF-induced apoptosis, using MDA-MB-468 cell line as a model. We first demonstrated that EGF-treatment induces Etk tyrosine phosphorylation in both HeLa and MDA-MB-468 cells. Overexpression of Etk by recombinant adenovirus in MDA-MB-468 cells potentiates the extent of EGF-induced cell apoptosis. The observed Etk-enhanced MDA-MB-468 cell apoptosis is associated with the Stat1 activation, as demonstrated by electrophoresis mobility shift assays and reporter gene assays. By contrast, a kinase domain deletion mutant $\Delta K$, functioning as a dominant negative mutant, ameliorates EGF-induced Stat1 activation and apoptosis in MDA-MB-468 cells. To explore whether the activated Etk alone is sufficient for inducing apoptosis, a conditionally activated Etk ($\Delta$Etk-ER), a chimeric fusion protein of PH domain-truncated Etk and ligand binding domain of estrogen receptor, was introduced into MDA-MB-468 cells. Upon $\beta$-estradiol ligand activation, the $\Delta$Etk-ER could stimulate Stat1 activity and confer cell apoptosis independent of EGF-treatment. Taken together, our findings indicate that Etk is a downstream signaling molecule of EGF receptor and suggest that Etk activation is essential for transducing the EGF-induced apoptotic signaling.
Introduction

It is well recognized that EGF and TGFα are among the most potent growth factors (76-78), which by engaging EGF receptor (EGFR), transmits signals for growth, survival and in some cases, motility (79-82). Overexpression of EGF receptor is common among human epithelial malignancies (83-87). Amplification and mutation of EGFR locus have also been found in a variety of tumors including glioblastoma (88-92). Yet, paradoxically, in some EGF-receptor overexpressing cancer cells exemplified by breast cancer cell line MDA-MB-468 and vulvar carcinoma derived cell line A431, EGF treatment induces growth arrest and apoptosis (93, 94). The signals that trigger apoptosis are likely to be different from those involved in other EGF responses. For example, the PI3 kinase pathway and Ras-Raf-MAPK kinase pathways activated by EGFR have been shown to mediate cell survival and mitogenic responses (79-81). PI-3 kinase and phospholipase C-dependent pathways were reported to mediate EGF-induced cell migration (82). Activation of c-Src and Stat3 by EGF was shown to be associated with cellular transformation in certain cell types (95, 96). Little is known about the pathway leading to apoptosis. Fu and coworkers provided some insights into this process. These authors showed that EGF-induced growth arrest and apoptosis correlate with the activation of Stat-1, with consequent activation of p21/WAF1 and caspase 1(97, 98). In addition, Darnell and colleagues demonstrated that a dominant-negative mutant of Stat1 inhibits EGF-induced growth arrest in A431 cells, suggesting the essential role of activated Stat1 in EGF-evoked arrest of growth (99). How Stat-1 is activated by overexpressed EGFR however is not clear. In this report, we provide evidence that tyrosine kinase Etk/BMX is involved in the EGF-induced activation of Stat1 and apoptosis.

Etk/Bmx is a member of the Tec family non-receptor tyrosine kinases, including Btk, Itk, Tec, and Txk (reviewed in (5, 9, 18)). Members of this family share an amino-terminal pleckstrin homology (PH) domain, a Tec homology (TH) domain, a SH3 domain, a SH2 domain, and a catalytic domain. The expression of Tec family kinases has been primarily identified in the
hematopoietic cells. Btk is preferentially expressed in B cells and is necessary for B cell development (100, 101). Mutations in the Btk gene result in human X-linked agammaglobulinemia and murine X-linked immunodeficiency (37, 102, 103). Likewise, Itk is predominately expressed in T cells and is crucial in T cell development and T cell receptor-initiated signaling cascade (26, 104-106). Tec is expressed in most hematopoietic cells and is involved in a variety of signal pathways including interleukin-3, stem-cell factor, and granulocyte colony stimulating factor (107-109). Etk/Bmx was initially identified in bone marrow and subsequently in epithelial cells, fibroblast and endothelial cells (31, 43, 110). Previous studies showed that Etk plays a pivotal role in IL-6-induced neuroendocrine differentiation and neuropeptide-induced androgen independent growth of prostate cancer cells (110, 111). In prostate cancer cells, Etk can be activated by PI-3 kinase and protects LNCaP from radiation induced apoptosis (110, 112). Interestingly, upon caspase cleavage, the constitutively activated Etk becomes proapoptotic (113). Etk has also been shown to mediate Src-induced transformation of epithelial cells and fibroblasts through activation of Stat3 and to transform the breast cancer cells via activation of Pak1 (31, 48). In addition, Etk was also reported to be activated by IL-3 and GM-CSF in mast cells and by VEGF and angiopoietin in endothelial cells (114-116). Most recently, Etk has been found to play a critical role in cell migration of endothelial cells in the signaling of integrin and TNF receptor type 2 (50, 51). Thus, Etk seems to be an integrator of several signals with diverse outcomes.

We report here that Etk is activated by EGF treatment of breast cancer cell MDA-MB468 and is involved in EGF-induced apoptosis. Overexpression of Etk sensitizes cells toward EGF-induced apoptosis, while expression of a dominant negative Etk blocks such a process. We also found that Etk expression activates Stat1 activity, which in turn activates the expression of p21, accounting for the growth arrest and apoptosis induced by EGF in this cell type. Our studies provide a mechanism whereby overexpressed EGF-receptor induces apoptosis.
Results

EGF stimulates tyrosine phosphorylation of Etk- To investigate whether Etk is a downstream signaling molecule of EGFR *in vivo*, both HeLa cells and MDA-MD-468 cells were treated with 100 ng/ml EGF for various time periods as indicated and assayed for Etk tyrosine phosphorylation, a hallmark of Etk activation. Cells were harvested and then equal amount of cell lysates was subjected to Western blot analyses with an antibody against tyrosine-phosphorylated Etk. Upon EGF treatment, the tyrosine phosphorylation of endogenous Etk in HeLa cells increased within 5 min and rapidly declined back to basal level at 30 min (Fig. 1A). Likewise, the tyrosine phosphorylation of Etk could be induced by EGF in MDA-MB-468 cells in a similar fashion except that the activation was sustained at least up to 120 min (Fig. 1B). To further substantiate this prolonged activation, MDA-MB-468 cells were infected with a recombinant adenovirus expressing T7-tagged Etk (Ad-Etk) or a control virus (Ad-vec). After 48 h, cells were stimulated with EGF and followed by the analyses of Etk activation. Similar to endogenous Etk, ectopically expressed T7-tagged Etk was persistently activated up to 120 min, as evidenced by increased tyrosine phosphorylation levels, in EGF-treated cells (Fig. 1C). Together, these findings demonstrate that Etk is activated in cells in response to EGF-treatment and this activation is not a cell-type specific event. However, the duration of the Etk activation was more pronounced in MDA-MB-468 cells, expressing high levels of EGF receptor, than that in the HeLa cells, expressing low levels of EGF receptor (117).

To further demonstrate that the activation of the Etk induced by EGF is dependent on EGFR activity, an EGFR-specific inhibitor, AG1498, was utilized in the EGF-treated cells. As expected, the EGF-induced phosphorylation of the Etk was markedly abrogated in MDA-MB-468 cells treated with AG1498 (Fig. 1D, *lane 3 versus lane 4*), suggesting the essential role of the EGFR in Etk activation. Etk was previously reported to be activated by PI3-kinase and/or Src kinase (31, 110). To test whether these kinases are involved in the Etk activation by EGF, MDA-MB-468 cells
were pretreated with a Src kinase inhibitor, PP2, or a PI3-kinase inhibitor, LY294002, followed by the EGF stimulation. As shown in Fig. 1D, both inhibitors could attenuate the degree of the Etk tyrosine phosphorylation, indicating that both Src and PI3-kinases partially mediate the Etk activation in response to EGF.

_Etk potentiates EGF-induced apoptosis of MDA-MB-468 cells-_ It has been shown that EGF stimulation induces apoptosis in MDA-MB-468 cells (94). Since Etk was activated by EGF-treatment in a sustained manner in MDA-MB-468 cells, we next examined whether Etk is involved in EGF-induced MDA-MB-468 apoptosis. To test this possibility, MDA-MB-468 cells were infected with a recombinant adenovirus expressing T7-tagged Etk (Ad-Etk) or a control virus (Ad-vec). After 48 h, cells were stimulated with EGF and followed by the analyses of cell apoptosis. EGF-treated MDA-MB-468 cells displayed markedly morphological changes, reflecting adherent cell apoptosis, such as cell condensation, rounding up, and detachment from the culture dishes (Fig. 2A). Particularly, the extent of cell detachment was more prominent in EGF-treated and Ad-Etk-infected MDA-MB-468 cells. This is not due to the adenovirus infection _per se_ since the same titer of the vector-infected cells displayed a lower level of cell detachment comparable to mock infected cells. The extent of cells undergoing apoptosis was further quantified by the propidium iodide (PI) staining followed by the FACS analysis of the sub-G1 fraction. As shown in Fig. 2B, the amount of EGF-induced apoptosis was significantly increased to ~30% in cells overexpressing Etk, compared to ~10% in mock and vector-infected cells. These findings suggest that the overexpression of Etk could potentiate the EGF-induced MDA-MB-468 apoptosis. Intriguingly, in the absence of EGF-treatment, Etk overexpression alone was sufficient to confer cell apoptosis to a level comparable to that observed in EGF-treated control cells. This is probably due to the activation of Etk kinase activity by its overexpression (Fig. 1C, lane 2), leading to the activation of the apoptotic signaling pathway(s) in MDA-MB-468 cells.

_Etk potentiates EGF-induced Stat1 activation-_ Previously, it has been demonstrated that the
induction of Stat1 activity and p21 expression is essential and indispensable for EGF-induced MDA-MB-468 cell apoptosis (97, 98). Hence, we next explored the possibility that the effect of Etk on the EGF-induced apoptosis is mediated through Stat1 activation. To test this possibility, we have performed electrophoresis mobility shift assays with a $^{32}$P-labeled SIE oligonucleotide to assay for Stat1 activation in the respective nuclear extract prepared from cells expressing Etk in the absence or presence of EGF stimulation. As a control, EGF-treatment apparently induced the DNA-protein complex formation, including Stat3-Stat3 homodimer, Stat1-Stat3 heterodimer, and Stat1-Stat1 homodimer, in both mock and vector-infected cells (Fig. 3A, lane 2-5). Among these complexes, formation of Stat1-Sat3 and Stat1-Stat1 dimers were robustly increased in Etk-overexpressing cells (lane 7), suggesting that Etk is capable of enhancing EGF-induced Stat1 activation. To further substantiate this notion, we have performed the Stat1-responsive reporter gene assays. An approximate 25-fold induction of Stat1-mediated reporter activation was detected in Etk-overexpressing cells by EGF-treatment, whereas a 9-fold induction was observed in the control cells (Fig. 3B, left panel, lane 2 and lane 4). In addition, overexpression of Etk alone was sufficient to activate the Stat1 reporter (lane 3) to the level comparable to EGF-treated control cells. These results confirm that Etk induces Stat1 activity and promotes the EGF effect on Stat1-mediated transcriptional activation. A similar scenario was also observed using p21 promoter linked to a luciferase reporter (Fig. 3B, right panel). Taken together, these findings indicate that the effect of EGF on cell apoptosis could be mediated by Etk via the Stat1 activation and p21 induction.

A dominant negative mutant of Etk inhibits EGF-induced Stat1 activation and cell apoptosis- To further demonstrate the necessity of Etk in EGF-induced Stat1 activation and apoptosis, we investigated the effect by a dominant negative mutant of Etk on EGF-dependent events. We first constructed a kinase deletion mutant of Etk, EtkΔK, (Fig. 4A) and tested its efficacy in functioning as a dominant negative mutant in Stat1-mediated reporter gene activation. As shown in Fig 4B, the wild-type Etk potentiated Stat1 activity, in a dose-dependent manner, in
response to EGF-treatment (*lanes 4, 6, 8, and 10 versus lane 2*). By contrast, increased amount of Ad-EtkΔK proportionally inhibited EGF-induced Stat1 transactivation (Fig. 4B, *lanes 12, 14, 16, and 18 versus lane 2*), indicating that EtkΔK functions as an Etk dominant negative mutant. We next tested whether this mutant could attenuate EGF-induced apoptosis using the recombinant Ad-EtkΔK. As expected, the EGF-induced MDA-MB-468 apoptosis was drastically reduced in EtkΔK-overexpressing cells (Fig 4C). Altogether, these observations suggest that Etk plays an essential role in mediating apoptotic pathway activation in EGF-treated MDA-MB-468 cells.

*Conditionally activated Etk induces both Stat1 activation and cell apoptosis:* Since the kinase domain of Etk is critical for EGF-evoked Stat1 activation and apoptosis, we wondered whether the activation of Etk kinase activity, in the absence of EGF stimulation, is sufficient to activate Stat1 and p21, leading to cell apoptosis. To test this possibility, we utilized an inducible Etk (ΔEtk-ER), a chimeric fusion of a PH domain-truncated Etk linked to the ligand binding domain of estrogen receptor, to activate Etk activity conditionally. Upon the β-estradiol (E2) treatment, the kinase activity of ΔEtk-ER was activated in a time-dependent fashion in MDA-MB-468 cells infected with adenovirus carrying ΔEtk-ER, as evidenced by Western blot analysis of the increased amount of tyrosine phosphorylation of ΔEtk-ER (Fig. 5A). The inducible activation of ΔEtk-ER was in a sustained manner in MDA-MB-468 cells, as previously reported by Wen et al., in Pa-4 and A549 cells (118). We next assessed whether the ΔEtk-ER activation alone could stimulate gene expression, independent of EGF-treatment, by electrophoresis mobility shift assays and transient transfection of reporter assays. Upon the E2 treatment, the extent of detected SIE-Stat1 complexes in nuclear extracts from cells infected with Ad-ΔEtk-ER was comparable to that observed in EGF-treated control cells (Fig. 5B, *lane 5 and lane 8*). The increased Stat1 activity was primarily due to the Etk activation, but not the E2 treatment, since that the observed Stat1 activation was not detectable in the control cells treated with E2 (*lane 3*). Furthermore, ΔEtk-ER was capable of activating both Stat1 and p21 reporter gene activities in
the presence of E2 (Fig. 5C, lane 6 in both panels). In the presence of EGF, the activation of Stat1 in ΔEtk-ER-infected cells was further enhanced (Fig. 5C, lane 8). This is probably resulted from the activation of endogenous Etk (Fig. 1) and/or additional potentiation of ΔEtk-ER upon EGF treatment (Fig. 5C, bottom panel, lane 4). It should be noted that in the absence of E2, we failed to detect the tyrosine phosphorylated-ΔEtk-ER in EGF-treated cells (Fig. 5C, lane 2, lower panel), suggesting that further activation of ΔEtk-ER by EGF-treatment is dependent on the activated state of ΔEtk-ER. It is possible that the initial activation by E2 renders the Etk in ΔEtk-ER accessible for further activation by EGF-evoked signaling. Taken together, these findings suggest that activation of Etk kinase by itself is sufficient for induction of Stat1 activity.

We next sought to determine whether conditionally activated ΔEtk-ER could induce MDA-MB-468 cell apoptosis. As expected, upon E2 stimulation, cells expressing ΔEtk-ER exhibited cell apoptosis to a level comparable to EGF-treated control cells (Fig. 5D), suggesting that activation of Etk is sufficient for triggering MDA-MB-468 cell apoptosis. However, EGF-treatment of activated ΔEtk-ER cells further promoted the apoptosis. Together, our findings clearly provide a functional role of Etk in mediating EGF-induced Stat1 activation and cell apoptosis in MDA-MB-468 breast cancer cells.
Discussion

In the present study, we provide evidence that Etk plays an important role in mediating the EGF-elicited apoptotic signals. First, EGF stimulation rendered Etk tyrosine phosphorylation, suggesting that Etk is a downstream signaling molecule of EGFR. Secondly, overexpression of Etk led to cell death and enhanced the ability of EGF to induce apoptosis via the activation of Stat1 and the induction of p21 promoter activity. Thirdly, a dominant negative Etk, EtkΔK, abrogated the afore-mentioned effects of EGF on gene regulation and apoptosis. Finally, conditionally activated Etk could mimic EGF effect on Stat1 activation and p21 promoter, leading to the cell apoptosis. To our knowledge, this is the first report in which activation of Etk is able to induce apoptosis in breast carcinoma cells.

The biological outcomes of the various signals, including growth, differentiation, and apoptosis, transmitted by the Tec family kinases are in a cell context-dependent fashion (119). There are number of reports suggesting a role of Tec family kinases in growth and anti-apoptosis. For example, Etk has been shown to be a critical mediator of cellular transformation through a cascade link of v-Src-Etk-Stat3 (31) in fibroblast and epithelial cells. Overexpression of Etk stimulates the proliferation of MCF-7 breast cancer cells and protects prostate cancer cells from ionization- and thapsigargin-induced apoptosis (48, 112). Targeted disruption of Btk has been shown to lead the B-cells toward apoptosis (120) and overexpression of Btk in chicken B-cells suppresses the Fas-mediated apoptotic signal via the disruption of the FAS-FADD interaction. By contrast, Btk has also been demonstrated to induce apoptosis in HeLa cells (121) and to mediate the radiation-induced apoptosis in DT-40 lymphoma B-cells (122). Our findings reported here that Etk sensitizes MDA-MB-468 cells toward EGF-induced apoptosis provide additional evidence that Tec family kinases are involved in pro-apoptotic functions. These data together suggest that Tec family kinases are bi-directional switches of cell growth and apoptosis. While the mechanism of this switch is presently unclear, we noted that Etk assumes two forms inside the cells: the wild type form which requires upstream signals such as PI-3 kinase, FAK, PTPD1 and
Src for its activation (31, 47, 51, 114), and the caspase-8 truncated form which is constitutively active. We previously showed that constitutively activated form induced apoptosis (113). It is possible that sustained active Etk either by very strong upstream signals as overexpressed EGF receptor or by caspase cleavage drives cells to apoptosis pathway. The \(\Delta\)Etk-ER used in this study mimics the caspase-activated Etk, and its activation alone is sufficient to induce apoptosis is consistent with this hypothesis. The fact that EGF treatment of \(\Delta\)Etk-ER cells gave rise to super-induction of apoptosis suggests that the \(\Delta\)PH-Etk can still respond to EGF receptor signals such as Src, consistent with the increased tyrosine phosphorylation of \(\Delta\)PH-Etk upon EGF treatment.

As to how hyperactive Etk activity leads to apoptosis is not completely understood. We found that Etk’s ability to induce apoptosis and to activate Stat activities (based on SIE-gel shift or SIE reporter assays) seemed to go hand-in-hand. Furthermore, dominant-negative kinase-dead Etk\(\Delta\)K which negated EGF-induced apoptosis also down-modulated SIE-promoter. This observation is echoed by the finding that the kinase activity-defective mutant of Btk is compromised by its capacity in mediating HeLa cell apoptosis (121). Both studies suggest that the kinase activity is crucial for transducing the apoptotic signals to the downstream effectors. The downstream signaling pathway of Btk-mediated apoptosis in HeLa cells is through p38 MAPK activation (121), whereas that of Etk-mediated apoptosis in MDA-MB-468 cells is through Stat1 activation. Recently, p38 MAPK has been demonstrated to enhance Stat1-dependent gene transcription and apoptosis through serine 727 phosphorylation (123-125). Thus, there is a likelihood that a common pathway is utilized. Further experiments are required to support or refute this notion.

In addition to MDA-MB-468 cells, EGFR overexpression leading to ligand-dependent apoptosis has also been observed in other cell types, including A431, 293, mesenchymal Rat-1 fibroblasts, U-87 MG, and C6 glial cells (126, 127). Furthermore, T. Hognason et al. have demonstrated that EGFR overexpression also induces a ligand-independent apoptosis in 293 EBNA cells, suggesting that apoptosis is a direct result of EGFR overexpression (126).
Although the correlation between EGFR overexpression and cell apoptosis has been well established in these cell lines, the underlying mechanism for EGFR-induced apoptosis is largely unclear. Studies in MDA-MB-468 and A431 cells revealed that activation of Stat1 is important in EGF-induced cell growth arrest and apoptosis (97). In these cell types, expression of p21/WAF1 and caspase 1 is upregulated by Stat1-dependent manner, leading to cell growth and apoptosis (98). Stat1-mediated apoptosis was abrogated in Stat1-deficient cell line, suggesting that activation of Stat1 pathway is essential for induction of apoptosis (97). Chin, et. al. previously suggested that the ability of overexpressed EGFR to activate Stat1 was critical in EGF-induced apoptosis (127). We wish to extend this hypothesis by suggesting that the sustained activation of Etk which leads to sustained Stat1 is the underlying cause of the EGF-induced apoptosis. Consistent with this notion is that finding that in HeLa cells the activation of Etk is not sustained and the cells do not undergo apoptosis.

Recent studies revealed that Etk could be activated by a wide range of cell-surface receptors through coupling with different signaling molecules. For example, PI3-kinase has been shown to be required for IL-6-mediated Etk activation in prostate cancer cells (110). Both Src and FAK activities are necessary for Etk activation by neurotropic factor bombesin in LNCaP prostate cancer cells (111). Furthermore, integrin-engaged Etk activation in epithelial cell and endothelial cells has also been reported to be FAK dependent (51). Etk activated by these signaling molecules was thought to be via lipid-protein and protein-protein interactions. Presumably, binding of the lipid products of PI3-kinase to the PH domain of Etk recruits Etk to plasma membrane, where the active Src kinase located in order to activate Etk through phosphorylation. Protein-protein interaction between FAK and the PH domain of Etk appears to disrupt the intramolecular interaction of Etk protein, leading to unfolding of the kinase domain and leaving Etk available for upstream regulators. Our results that both Src and PI3-kinase inhibitors partially blocked the Etk activation by EGF-treatment (Fig. 1D) suggest that the activation of the Etk by EGFR is at least through the signaling pathways mediated by PI3-kinase and Src kinase. These findings do not exclude the possibility that other signaling molecules such as FAK are also involved in the
EGFR-Etk activation. For instance, FAK could form complexes with EGFR in the absence of EGF in A431 cells (48). David et al. (128) have demonstrated that FAK forms a complex with the activated EGFR and is required for EGF-stimulated cell motility. Currently, we are in the process of elucidating the possible pathways in the EGFR-mediated Etk activation in MDA-MB-468 cells.

In summary, we have demonstrated Etk functions as an important signaling molecule in the process of regulating EGF-induced cell apoptosis of MDA-MB-468 breast cancer cells. More importantly, the conditionally activated Etk alone is sufficient to trigger the apoptosis of such breast cancer cells. Thus, our studies provide a potential strategy for developing an effective treatment on breast cancers overexpressing EGFR.
Materials and methods

Plasmid construction- T7-tagged Etk lacking the kinase domain and ΔEtk-ER were amplified by polymerase chain reaction with the template of pCMV-T7-Etk and pLNCX- ΔEtk-ER (118), respectively, and cloned into pcDNA3 (Invitrogen) mammalian expression plasmid to generate pCMV-T7-EtkΔK and ΔEtk-ER.

Recombinant adenovirus construction- The recombinant adenoviruses expressing T7-tagged Etk wild-type and mutants were constructed and produced according to manufacture's instruction of adenovirus expression vector kit (TaKaRa). Briefly, the cDNA coding the T7-Etk was inserted into the Swa I site of pAxCAwt cosmid vector. The resulting cosmid was then co-transfected with the complexes of adenovirus genomic DNA and terminal protein into 293 cells to generate the recombinant virus. After confirmation, each recombinant virus was subjected to virus amplification and titration. 10 MOI of each recombinant virus was used to infect MDA-MB-468 cells.

Cell culture, Western blot analysis, transient transfection and luciferase assays- HeLa cells and human adenocarcinoma cell line MDA-MB-468 were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. For endogenous Etk phosphorylation, cells were plated into 10-cm Petri dishes and then treated with EGF 100ng/ml for different time as indicated. 40 µg of each cell lysate was subjected to Western blot analysis with anti-phospho-Etk (specifically recognized phosphorylation of the Etk Y40) and anti-Etk antibodies (Cell Signaling). For ectopic Etk phosphorylation, 10 MOI of Ad-Etk recombinant adenovirus was added to MDA-MB-468 cells. After 48 h, cells were stimulated with EGF for different time course and then harvested for immunoprecipitation and Western blot analysis. For the inhibitor experiments, cells expressing T7-tagged Etk were pretreated with AG1498 (Sigma), PP2 (Calbiochem) or LY294002 (Sigma) for 30 min, followed by EGF stimulation for 30 min and then harvested for immunoprecipitation and Western blot analysis. All the transient transfection experiments were performed by using FuGENE 6 transfection reagent (Roche
Molecular Biochemicals) as manufacture instructed. Cells (2x10^5) were transfected with expression vector of wild-type T7-tagged Etk or its derived mutants along with p3xLy6E-Luc or p21-Luc reporter construct (a gift from Dr. S.-Y. Hsieh) as well as pRL-TK plasmid as an internal control for normalization of transfection efficiency. Following transfection, cells were cultured in medium containing charcoal-stripped 0.05% (V/V) FBS for starvation. Cells were further treated with 100 ng/ml EGF in combination with beta-estradiol (E2) (1µM) as indicated. Cell lysates were harvested and assayed for relative activity (Firefly-luciferase for the reporter and Renilla luciferase for the indicator) as manufacture instructed (Parkard BioScience).

Flow cytometric analysis of apoptosis- Cells (5x10^5) were grown in 6-well plates for recombinant adenovirus infection. After 24 h, cells were treated with or without EGF (100 ng/ml) for different times. For analysis of cell apoptosis, detached and attached cells were pelleted and resuspended in 70% EtOH for fixation. After fixation, the cells were stained by the fluorescent dye propidium iodide (PI) for 30 min and further analyzed by FACS (Becton Dickinson, Franklin Lakes, NJ USA)

Electrophoretic mobility shift assay (EMSA)- Human SIE (5’-AGCTTCATTCCGCATCCCTAAAAAGCT-3’) was synthesized and annealed to its corresponding oligonucleotide to form a double-strand. EMSA was performed according to the procedure described by Vignais et al. {Vignais, 1999 #29}. Briefly, DNA-protein binding reactions (20µl) were performed by incubation of the nuclear extracts (6µg) in a solution containing 13 mM HEPES (pH7.9), 65 mM NaCl, 0.5 mM EDTA, 2% Ficoll 400, 5% glycerol, 50 µg of poly (dl-dC)/ml, and 0.5 mM dithiothreitol for 10 min at room temperature, followed by an addition 30-min incubation with 50,000 cpm of ^32P-labeled human SIE probe at room temperature. For supershift assay, the nuclear extracts were pre-incubated with anti-Stat1 (Upstate Biotechnology) or anti-Stat3 (Upstate Biotechnology) antibody for 20 min before addition of SIE probe. The DNA-protein complexes were separated on 5% nondenaturing polyacrylamide gel in 0.5x TBE buffer. Gels were dried and subsequently detected by phosphorimagener analysis (Bio-imaging Analyser BSA 1500, Fuji).