Bi-directional Regulation between Tyrosine Kinase Etk/BMX and Tumor Suppressor p53 in Response to DNA Damage*

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Etk/Bmx, a member of the Tec family of nonreceptor tyrosine kinases, has been implicated in the regulation of various cellular processes including proliferation, differentiation, motility, and apoptosis. Here, we report the identification of Tec family kinases as the potential interacting proteins of the tumor suppressor p53 by an Src homology 3 domain array screening. Etk is physically associated with p53 through its Src homology 3 domain and the proline-rich domain of p53. Induction of p53 expression by DNA damage inhibits Etk activity in several cell types. Down-regulation of Etk expression by a specific small interfering RNA sensitizes prostate cancer cells to doxorubicin-induced apoptosis, suggesting that inhibition of Etk activity is required for apoptosis in response to DNA damage. We also show that Etk primarily interacts with p53 in the cytoplasm and that such interaction leads to bidirectional inhibition of the activities of both proteins. Overexpression of Etk in prostate cancer cells results in inhibition of p53 transcriptional activity and its interaction with the mitochondrial protein BAK and confers the resistance to doxorubicin. Therefore, we propose that the stoichiometry between p53 and the Tec family kinases in a given cell type may determine its sensitivity to chemo- therapeutic drugs.

DNA-damaging agents have been widely used for inducing apoptosis in tumor cells in anticancer therapy for decades. The tumor suppressor p53 is one of the key regulators of DNA damage-induced cell death. Upon DNA damage, the activity of p53 is rapidly increased through several post-translational mechanisms, including stabilization of the p53 protein by inactivation of MDM2 and direct modifications of p53 by phosphorylation, sumoylation, and acetylation (1–4). As a transcription factor, p53 induces the expression of the cell cycle regulator protein p21 and pro-apoptotic proteins such as Bax, NOXA, and PUMA (5–8) and inhibits the expression of anti-apoptotic proteins such as Bcl-2 and survivin (9–11). Moreover, p53-mediated cell death can also be achieved independently of its transcriptional activity by the binding of p53 to mitochondrial proteins such as Bcl-XL and BAK (12–14). The p53–BAK complex formation coincides with the loss of the interaction between BAK and an anti-apoptotic protein MCL-1 and leads to the oligomerization of BAK and the subsequent release of cytochrome c from mitochondria (14). The proline-rich domain of p53 is not required for its transcriptional activity but is essential for its apoptosis-inducing function in many cell types (12, 15, 16). There are two PXXPXP motifs, the perfect ligands for the Src homology 3 (SH3) domain (17–19), in the proline-rich domain of human p53. However, to date no SH3 domain-containing protein has been reported to bind to this region, although tyrosine kinase c-Abl has been shown to interact with the PXXP motif in p73, and such interaction is important for p73-mediated apoptosis in response to DNA damage (20–22).

Etk/BMX, a member of Tec Family tyrosine kinases, is shown to be involved in various cellular processes including proliferation, differentiation, motility, and apoptosis. Tec family tyrosine kinases share a high degree of homology and typically contain an N-terminal pleckstrin homology domain, an SH3 domain, and an SH2 domain in addition to the C-terminal kinase catalytic domain (23). Most of the Tec family members are restrictedly expressed in a certain lineage of hematopoietic cells. For example, Btk is primarily expressed in the B-cell lineage. Mutation of Btk is shown to associate with an inherited immunodeficiency disease known as X-linked agammaglobulinemia in human and the X-linked immunodeficiency (xid) in mouse (24–26). The xid mice carrying the mutant Btk gene lack peripheral matured B-cells (xid), and the pre-B-cells are prone to apoptosis, suggesting a role of Btk in maintaining the survival of B-cells (27). On the other hand, the expression profile of Etk is much broader than its counterparts. Etk is expressed in various tissues and cell types even though, in general, the expression level of Etk is relatively low in comparison to other non-receptor tyrosine kinases such as FAK and the Src family kinases (23). The expression and activity of Etk is induced by growth factors, cytokines, the extracellular matrix, and possibly by hormones (23, 28, 29), suggesting that the up-regulation of Etk activity may be part of its physiological needs in response to these stimuli. The elevated expression of Etk is also reported in several aggressive metastatic carcinoma cell lines (30). Etk has been shown to activate STAT3 and...
FIG. 1. Direct association of p53 with Tec family kinases. a, schematic structure of human p53. The amino acid sequence of the PRD of p53 is listed underneath the bar. The PXPFXP motifs are highlighted with boldface. AD, activation domain; DBD, DNA binding domain; NLS, nuclear localization signal; Oligo, oligomerization domain; BD, basic domain. b, SH3 domain array screening by the PRD of p53. Bacterial extracts containing the His-tagged, proline-rich domain of p53 were incubated with the SH3 domain array membrane. Protein interactions were visualized using a horseradish peroxidase-conjugated anti-His antibody and chemiluminescent detection. The amount of the recombinant GST-SH3 domain protein spotted on the membrane is 400 ng (H) and 80 ng (L). M, marker. The identity of each SH3 domain is as follows: lane 1, phosphatidylinositol 3-kinase regulatory α subunit; lane 2, channel-associated protein of synapse-110; lane 3, Stac protein; lane 4, tyrosine-protein kinase Tec; lane 5, Cdc42-interacting protein 4; lane 6, 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase γ2; lane 7, Rho GEF6; lane 8, CRK-associated substrate; lane 9, Myc box-dependent interacting protein 1; lane 10, embryonal Fyn-associated substrate. c, association of p53 with Tec family kinases. COS-1 cells were transiently transfected with p53 and the epitope-tagged Tec family kinases Etk, Tec, or Btk. Immunoprecipitation (IP) was performed using the polyclonal p53 antibody or an irrelevant polyclonal antibody as a control. The immunoprecipitates were subjected to immunoblotting (IB) with anti-T7 for Etk or anti-FLAG for Tec and Btk. The expression of the Tec family kinases was monitored by immunoblotting of the total cell lysates (TCL). d, the SH3 domain of Etk is required for binding to p53. T7-tagged Etk or Etk mutants were co-expressed with p53 in H1299 and PC3 cells. Immunoprecipitation was performed with the polyclonal p53 antibody and followed by immunoblotting with the anti-p53 antibody. e, direct interaction of Etk and p53 through SH3 domain and the proline-rich domain. Lysates from H1299 cells overexpressing p53 or p53 ΔPRD were incubated with the GST-EtkSH3 fusion protein bound to glutathione-Sepharose 4B beads at 4 °C for 1 h. The input of the cell lysates and the proteins associated with the beads were resolved on SDS-PAGE followed by immunoblotting with the anti-p53 antibody. The amount of the GST fusion proteins was determined by Coomassie Blue staining.
induce VEGF production in several cell types (31, 32) that are implicated in the resistance to various apoptosis inducers in many cancer cells. The overexpression of Etk in prostate cancer cells confers the resistance to apoptosis induced by photodynamic therapy and thapsigargin (33), suggesting a role of Etk in anti-apoptosis signaling.

Here, we report the identification of Tec family kinases as the potential interacting partners of the proline-rich domain of p53 by an SH3 domain array screening. We further show that Etk directly interacts with p53 in response to DNA damage and that such interaction leads to bidirectional inhibition of the activities of both proteins. Down-regulation of Etk activity by p53 was monitored by immunoblotting of total protein extracts with an anti-p53 antibody. TCL, total cell lysates. a, the interaction between Etk and p53 is necessary for p53 to inhibit tyrosine phosphorylation of Etk. H1299 cells were transiently transfected with the expression plasmids as indicated. At 24 h post-transfection, immunoprecipitation and immunoblotting were performed as in described for panel a.

**EXPERIMENTAL PROCEDURES**

**SH3 Domain Array Screening**—TranSignal SH3 domain array II was screened for the proteins binding to the proline-rich domain (PRD) of p53 following the manufacturer’s instruction (Panomics). Briefly, the cDNA encoding the PRD of human p53 (amino acids 47–92) was subcloned into the pExp vector. The His-tagged fusion protein was expressed in *Escherichia coli*. The ShanSignal SH3 domain array II membrane was incubated with the bacteria extract followed by incubation with a horseradish peroxidase-conjugated antibody. The protein-protein interactions were visualized by using chemiluminescent detection. Finally, the membrane was stained with Coomassie Blue to monitor the amount of spotted SH3 domain proteins.

**Cell Culture and Transfection**—COS-1, H1299, PC-3, and LNCaP cells were purchased from the American Type Culture Collection. LNCaP-Etk, the LNCaP cells stably expressing T7-tagged wild-type Etk, was established as described previously (33). COS-1 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. PC-3, LNCaP, and LNCaP-Etk cells were maintained in RPMI 1640 with 10% fetal bovine serum. H1299 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 9 g/liter glucose, 10 mM HEPES, 1 mM sodium pyruvate, and 0.15% sodium bicarbonate. Human umbilical vein endothelial cells were purchased from the Endothelial Cell Culture Facility of the University of Minnesota and maintained as described previously (30). The cells were transfected with FuGENE.
FIG. 3. Interaction between Etk and p53 inhibits the kinase activity and the biological functions of Etk. a, down-regulation of Etk kinase activity by p53. COS-1 cells were transiently transfected with Etk and p53. At 24 h post-transfection, the cells were lysed for immunoprecipitation (IP) with an anti-Etk antibody followed by an in vitro kinase (IVK) assay using purified GST-Gab1CT as a substrate. b, p53 inhibits Etk-induced Stat3 activity. H1299 and COS-1 cells were transfected with Etk alone or together with p53 wild type. At 24 h post-transfection, the cells were lysed and immunoprecipitation was performed with an anti-Stat3 antibody. The immunoprecipitates were subjected to immunoblotting (IB) with an anti-pStat3 Y705 antibody. c, p53 inhibits Etk-induced VEGF promoter activity. H1299 cells were transfected with 1.2 VEGF-luc, T7-tagged Etk, or its mutants and p53 or p53 ΔPRD. Luciferase activity was determined as described under “Experimental Procedures.” The results were expressed as mean ± S.D. of three independent experiments. The expression of Etk or p53 was monitored by immunoblotting with anti-T7 or anti-p53 antibodies.
annealed and ligated downstream of the U6 promoter, and the lentiviruses were prepared as described previously (36). The polyclonal Etk antibody was developed as described previously (34). The monoclonal anti-BMX antibody was from BD Transduction Laboratories. The anti-T7 antibody was purchased from Novagen. The anti-phosphotyrosine antibody (4G10) and anti-Bak were from Upstate Biotechnology Inc. Phospho-specific antibodies against Stat3 pY705 was from Cell Signaling Technology. Anti-p53 (FL-393, Pab240), anti-p21, anti-Stat3, and anti-actin (C2) were from Santa Cruz Biotechnology.

Immunoprecipitation and Western Blot—The cells were washed twice with ice-cold phosphate-buffered saline and then lysed with lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X-100, 2.5 mM sodium pyrophosphate, 1 mM Na3VO4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) at 4 °C for 30 min. The cell lysates were centrifuged to remove cell debris before incubation with the antibody at 4 °C for 1 h. The immunocomplexes were collected with protein A- or protein G-Sepharose beads. The immunoprecipitates were then suspended in SDS sample buffer and resolved by SDS-polyacrylamide gel electrophoresis. Immunoblotting was performed as described previously (38).

Extraction of Proteins from Mouse Tissue—The p53-heterozygous mice were purchased from the Jackson Laboratory and maintained in a pathogen-free animal facility. The mice were bred, and the genotypes of the offspring were determined by PCR following the provider’s instructions. The p53-null mice and their wild-type littermates were sacrificed, and the offspring were determined by PCR following the provider’s instructions. After centrifugation (13,000 g) for 10 min, the fusion proteins were collected by incubating the supernatants with glutathione-Sepharose 4B beads (Amersham Biosciences) at 4 °C for 1 h with continuous shaking. The beads were washed three times to remove the unbound proteins. The GST fusion proteins attached to the glutathione-Sepharose 4B beads were mixed with the lysates of H1299 cells transfected with p53 wild type or p53ΔPRD for 1 h at 4 °C by rotating. The beads were washed three times, and the bound proteins were immunoblotted with anti-p53 antibody.

DNA Damage Induction and Apoptotic Cell Death Assay—Parental LNCaP, LNCaP-Etk, or LNCaP cells infected with different viruses were seeded on coverslips and cultured 24 h before treatment with 0.5 μg/ml doxorubicin or 20 μg/ml cisplatin for 20 h. Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) using the In Situ Cell Death Detection kit (Roche Applied Science). Apoptotic cells were quantified by counting TUNEL-positive cells in 500 cells from three independent fields. The data were analyzed by the Student’s t test.

In Vitro Kinase Assay—COS-1 cells were transfected as described above. Immunoprecipitation was performed using an anti-Etk antibody. The immunoprecipitates for an in vitro kinase assay were washed twice with kinase buffer (50 mM Tris, pH 8, and 10 mM MgCl2) and then incubated at room temperature with kinase buffer containing 1 μg of GST-Gab1CT and 200 μM ATP (39). The reaction was terminated by adding an equal volume of 2× SDS sample buffer and boiling for 10 min. The reaction mixtures were separated by 10% SDS-polyacrylamide gel electrophoresis. The tyrosine phosphorylation of Gab1CT was detected with an antibody specific for phosphotyrosine (4G10).

Transient Transfection Reporter Assay—H1299 cells grown in 24-well plates were transfected with −1.2 VEGF-Luc reporter (32) or the pRL-TK control reporter, was cotransfected as an internal control. The total amount of DNA was kept constant by using an empty vector. Cells were lysed 24 h later, and the Dual-Luciferase assays were performed according to the protocol from the manufacturer (Promega, Madison, WI). The results were presented as fold induction compared with the control. The assays were carried out in triplicate.

Immunofluorescence Staining—The cells were treated as indicated and then fixed in 3.7% paraformaldehyde for 20 min and washed four times with phosphate-buffered saline. The cover slides were blocked in phosphate-buffered saline containing 0.3% Triton-X100, 1% bovine serum albumin, and 1% normal donkey serum for 1 h at room temperature. Mouse anti-T7 monoclonal antibody (10 μg/ml), rabbit polyclonal p53 antibody were added and incubated for 1 h at room temperature. After washing with phosphate-buffered saline, the cover slides were incubated with rhodamine-conjugated anti-mouse and fluorescein isothiocyanate-conjugated anti-rabbit for 45 min at room temperature. Finally, the cells were counterstained with 4′,6-diamidino-2-phenylindole to visualize nuclei before mounting. The cover slides were examined in an inverted microscope under a 60× oil immersion objective and a confocal laser microscope system.
RESULTS

To search potential interacting partners for the PRD of p53, we performed an SH3 domain array screening by using the bacteria-expressed, His-tagged PRD of human p53 (Fig 1a). Among the positive binders shown in Fig 1b, the SH3 domain of tyrosine kinase Tec (lane 4) appears to have the highest affinity to the p53PRD at both protein levels. Based on the high degree of homology among Tec family kinases, we reasoned that such interaction might also occur between p53 and other members of the Tec family of kinases. We proceeded to verify this interaction by co-expression of p53 with three members of the Tec kinases, namely Tec, Btk, and Etk in COS-1 cells. As expected, all of these kinases were efficiently co-immunoprecipitated with p53 (Fig 1c). Given that the Tec kinases are highly homologous, we subsequently focused our study on Etk, the most ubiquitously expressed member in this family. To confirm that the interaction between Etk and p53 is mediated by the SH3 domain, we co-expressed SH3 mutant, and p53 wild type. 24 h post-transfection, the cells were fixed and stained with anti-T7 and anti-p53 antibodies (visualized by rhodamine and fluorescein isothiocyanate-conjugated secondary antibody, respectively). Yellow indicates the colocalization of Etk and p53.

In addition, we examined the effect of wild-type p53 or p53 ΔPRD on the tyrosine phosphorylation of Etk and its mutant that lacks the SH3 domain. As shown in Fig. 1d, in contrast to the wild-type p53, p53 ΔPRD lost its inhibitory effect on Etk. Meanwhile, wild-type p53 was unable to inhibit tyrosine phosphorylation of the Etk mutant lacking an SH3 domain. These data suggest that the interaction between Etk and p53 is necessary for p53 to inhibit tyrosine phosphorylation of Etk.

To test whether the reduced tyrosine phosphorylation of Etk upon binding to p53 could be due to inhibition of the kinase activity of Etk, as the tyrosine phosphorylation of Etk is very often correlated with its kinase activity, we co-expressed Etk with p53 in COS-1 cells, and in vitro kinase assays were performed. Consistent with the above observations, co-expression of Etk with the wild-type p53 resulted in the loss of its in vitro kinase activity toward the exogenous substrate Gab1 (Fig 3a). Etk has been shown to activate STAT3 activity and induce the expression of VEGF in several cell types. We first examined the effect of p53 on Etk-induced STAT3 activity. Fig 3b shows that overexpression of Etk in COS-1 and H1299 cells resulted in increased phosphorylation of STAT3 and that the Etk-induced phosphorylation of STAT3 was completely abolished when p53 was co-expressed. As another measurement of the effect of p53 on Etk function, we also examined the effect of p53 on Etk-induced VEGF promoter activity. In Fig 3c, co-expression of Etk with the wild-type p53 led to a dramatic inhibition of the VEGF promoter activity, whereas the p53 mutant lacking the PRD virtually had no effect. Taken together, these results demonstrated that p53 inhibits Etk activity by directly binding to Etk in response to drug treatment in a manner dependent on the interaction between the SH3 domain of Etk and the proline-rich domain of p53.
To test whether inhibition of Etk activity is required for p53-dependent apoptosis, we examined the effects of siRNAs specific for Etk or p53 on doxorubicin-induced apoptosis. As shown in Fig. 4, the number of apoptotic cells was increased from 38% to 56% upon the treatment of Etk siRNA, suggesting that down-regulation of Etk expression sensitizes LNCaP cells to doxorubicin-induced cell death. Treatment with p53 siRNA virtually blocks the apoptosis in these cells. Interestingly, when the cells were treated by siRNAs for both p53 and Etk simultaneously, doxorubicin-induced apoptosis was partially resumed, suggesting that the inhibition of Etk activity by p53 may be required and might, at least in part, account for DNA damage-induced cell death. However, down-regulation of Etk alone is not sufficient for a full spectrum apoptotic response, suggesting that additional factors are needed for p53-dependent apoptosis.

It is known that p53 is primarily localized in the nucleus, whereas Etk is a cytoplasmic kinase. We would like to know where these two proteins interact. Immunofluorescence analysis of H1299 cells that were co-transfected with p53 and T7-Etk revealed that p53 was mainly in the nucleus when no Etk or only a low level of Etk is detected in the cell (Fig. 5). However, in the cells with high levels of Etk, p53 was primarily colocalized with Etk in the cytosol (Fig. 5). Deletion of either the SH3 domain of Etk or the PRD of p53 resulted in nuclear localization of p53. These results suggest that the cytoplasmic colocalization of Etk and p53 may depend on the integrity of both domains.

Given that p53 needs to enter the nucleus to exert its transcriptional activity, it is possible that the interaction between Etk and p53 in the cytoplasm may have inhibitory effects on the activity of p53 as well. We then set out to investigate the effects of Etk on the transcriptional activity of p53. It is known that p53 is involved in the induction of p21 and the inhibition of survivin in response to doxorubicin. As shown in Fig 6a, the induction of p21 expression induced by p53 is significantly diminished in LNCaP-Etk cells, whereas the repression of survivin expression by p53 was released in these cells. To further confirm the effect of Etk on p53-mediated transcription activity, we also cotransfected Etk and p53 with a luciferase reporter under the control of the survivin promoter into H1299 cells. Fig 6b shows that p53 suppresses the survivin promoter activity and that the p53-mediated inhibition was released by overexpression of the wild-type Etk but not of the mutant Etk lacking the SH3 domain, suggesting that Etk negatively regulates p53 transcriptional activity in an SH3-domain-dependent manner. Because p53 can also induce apoptosis by targeting at mitochondria, we also examined the effects of the overexpression of Etk on the association of p53 with the mitochondrial protein BAK. As shown in Fig 6c, doxorubicin treatment enhanced the association of BAK to p53 in LNCaP cells. However, this association was dramatically diminished in LNCaP-Etk cells, suggesting that overexpression of Etk prevents p53 from targeting to mitochondria.

Finally, we examined the effects of increased Etk expression on p53-mediated apoptosis. As shown in Fig. 7, Dox-induced apoptosis was significantly reduced in LNCaP-Etk cells in comparison to the parental LNCaP cells, suggesting that overexpression of Etk in prostate cancer cells confers resistance to DNA damage-induced cell death.

**DISCUSSION**

The tumor suppressor p53 can induce cell death through its transcriptional activity as well as its interaction with the proteins regulating the apoptotic machinery. In the current study, Tec family tyrosine kinases were identified as potential direct targets for p53 in response to DNA damage. We showed that
Etk is negatively regulated by p53 through an interaction between the SH3 domain of Etk and the PRD of p53. Inhibition of Etk activity by p53 may be an essential step, at least in prostate cancer cells, for DNA damage-induced apoptosis based on our observations as follows. 1) Etk activity is inhibited upon binding to p53 in response to DNA damage. 2) Down-regulation of Etk expression by the specific siRNA sensitizes prostate cancer cells to apoptosis induced by DNA damage; and 3) overexpression of Etk in prostate cancer cells confer the resistance to doxorubicin. Etk is known to be involved in the regulation of VEGF production and STAT3 activity in some cell types, which may contribute to its anti-apoptosis effects in these cells. However, it is still intriguing as to why p53 needs to inhibit Etk activity to induce apoptosis in response to DNA damage. One possible scenario might be that Etk may be involved in the integrin signaling responsible for maintaining the survival of adherent cells and that the inhibition of this pathway is essential for apoptosis. Another possibility could be Etk may regulate some cell surface proteins that are directly involved in drug response. Our previous study showed that the SH3 domain of Etk interacts with the endosome-associated protein RUFY1 and that such an interaction may play a role in the regulation of the plasma membrane presentation of the epidermal growth factor receptor and possibly some other cell surface proteins as well (40). Further experiments are necessary to investigate the potential competition between RUFY1 and p53 for the binding to the SH3 domain of Etk in response to DNA damage.

The interaction between p53 and Etk appears to occur in the cytosol and results in bidirectional inhibition of the activities of both proteins. The mechanisms underlying how the PRD of p53 inhibits Etk kinase activity and how Etk can retain p53 in the cytosol are still unclear and remain to be investigated. It is possible that the interaction between Etk and p53 may compete with its binding to the positive regulator(s) essential for Etk activation and prevent its plasma membrane translocation. Meanwhile, the cytoplasmic retention of p53 may result from the masking of the nuclear localization signal of p53 in the complex with Etk or the association of the complex with the cytoskeletal components present in the cytosol. Interestingly, we show that the interaction between Etk and p53 in the cytoplasm diminishes the transcriptional activity of p53 and its interaction with mitochondrial protein BAK, suggesting that the overexpression of Etk in cancer cells may have inhibitory effects on both the transcriptional and the mitochondrial functions of p53, the two major means for the induction of apoptosis by p53. Therefore, in addition to mutations of p53, the overexpression of some cytoplasmic “anchor” proteins like Etk in tumor cells may also be attributed to the cytoplasmic retention of p53, which has been reported in several types of cancers (41–43). Actually, elevated expression levels of Etk are observed in aggressive metastatic carcinoma cell lines, which are usually resistant to chemotherapeutic drugs (30). The deletion and the mutation of p53 are also associated with advance prostate cancers and many other cancers. Our data suggest that the stoichiometry or balance between p53 and Tec family kinases in a given cell type may determine its sensitivity to chemotherapeutic drugs. The intervention approach for down-regulation of Etk activity or expression would be expected to sensitize tumor cells to chemotherapeutic drugs.

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