Proteolytic Activation of Etk/Bmx Tyrosine Kinase by Caspses

Yi-Mi Wu‡§, Chia-Lin Huang‡, Hsing-Jien Kung‡§, and Chi-Ying F. Huang‡

From the §Division of Molecular and Genomic Medicine, National Health Research Institutes, Taipei 115, Taiwan, Republic of China and the $Department of Biological Chemistry and Cancer Center, University of California at Davis, Sacramento, CA 95817

Etk/Bmx is a member of the Btk/Tec family of kinases, which are characterized by having a pleckstrin homology domain at the N terminus, in addition to the Src homology 3 (SH3), SH2, and the catalytic domains, shared with the Src family kinases. Etk, or Btk kinases in general, has been implicated in the regulation of apoptosis. To test whether Etk is the substrate for caspases during apoptosis, in vitro translated [35S]methionine-labeled Etk was incubated with different apoptotic extracts and recombinant caspases, respectively. Results showed that Etk was proteolyzed in all conditions tested with identical cleavage patterns. Caspase-mediated cleavage of Etk generated a C-terminal fragment, containing the complete SH2 and tyrosine kinase domains, but without intact pleckstrin homology and SH3 domains. This fragment has 4-fold higher kinase activity than that of the full-length Etk. Ectopic expression of the C-terminal fragment of Etk sensitized the PC3 prostate cancer cells to apoptosis in response to apoptosis-inducing stimuli. The finding, together with an earlier report that Etk is potentially antiapoptotic, suggests that Etk may serve as an apoptotic switch, depending on the forms of Etk existing inside the cells. To our knowledge, this is the first case where the activity of a tyrosine kinase is induced by caspase cleavage.

Apoptosis (or programmed cell death) is a fundamental and complex biological process that enables an organism to eliminate unwanted or defective cells through an orderly process of cellular disintegration (1–3). Although apoptotic stimuli that elicit responses vary from cell to cell, there seems to be a basic biochemical machinery underlying the process of regulated programmed cell death. Growing evidence suggests that apoptosis is mediated by the activation of a family of cysteine proteases, known as caspases, which cleave target proteins immediately after specific aspartic acid residues (4–6). To date, a long list of caspase substrates has been identified (7–10). However, the functional significance of the majority of these cleavage events and their exact roles in the execution of apoptosis remain largely to be unraveled.

Caspase-mediated cleavages can initiate their deadly assault on the cell by inactivating proteins necessary for cell survival or structural integrity. Meanwhile, caspase-dependent cell killing also requires the activation of proapoptotic proteins. Protein kinases emerge as the direct substrates and effectors of caspases (11, 12). For example, two antiapoptotic protein kinases, Raf-1 and Akt, are inactivated by caspase-mediated proteolytic degradation (13). Proteolytic cleavage of ATM generates a kinase-inactive protein and prevents DNA repair and DNA damage signaling (14). Cleavage of focal adhesion kinase by caspases interrupts the assembly of the focal adhesion complex, resulting in cell death (15, 16). By contrast, the activities of several kinases are stimulated by caspase cleavage, such as MEKK-1 (17), PK2/human PAK65 (18, 19), MST1 (20), and protein kinase C isoforms δ (21, 22) and θ (23). In each case, caspase cleavage generates a constitutively active kinase by removing inhibitory domains from the proteins. Importantly, the active fragments then act as signals propagating the apoptosis processes.

Etk/Bmx (epithelial and endothelial tyrosine kinase or bone marrow tyrosine kinase gene in chromosome X) is a member of the Btk (Bruton’s tyrosine kinase) tyrosine kinase family (24, 25). Etk and three other members of this family, Btk (26, 27), Itk (28, 29), and Tec (29), share a common domain structure including a pleckstrin homology (PH) domain, an Src homology 3 (SH3) domain, an SH2 domain, and a catalytic tyrosine kinase domain (30, 31). Each kinase has a unique expression pattern, and Etk is commonly expressed in epithelial cells, endothelial cells, and monocytes/macrophages including prostate tissues (24, 25, 32–34). Recent studies suggest that various signals transmitted by the Btk family members play central but diverse modulatory roles in cell growth and differentiation (35–38).

Several lines of evidence implicate Btk family members in the apoptosis pathway. Interestingly, both Btk and Etk are reported to be able to induce pro-apoptotic signals. For instance, Btk and Itk are known to play vital roles in B cell and T cell development by protecting these cells from apoptosis (30, 39, 40). Btk is also shown to be an inhibitor of Fas-mediated apoptotic signal in B cells by its direct association with the death receptor Fas, blocking the Fas-FADD interaction (41). Consistent with its antiapoptotic role is the finding that Btk activates a survival signal pathway involving Akt and NFκB (42–44). In contrast, Btk is also involved in transmitting apoptosis signals, since an engineered variant of Btk line DT-40, which lacks both alleles of Btk, became resistant to UV-induced apoptosis (45). Etk also seems to play dual roles in apoptosis. Overexpression of Etk renders prostate cancer cell line LNCaP more resistant to photodynamic therapy or thapsigargin-induced apoptosis (46), whereas it sensi-

1 The abbreviations used are: PH (pleckstrin homology domain); SH2 and SH3, Src homology 2 and 3, respectively; Ab, antibody; HUVEC, human umbilical vein endothelial cells; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; CHO, Chinese hamster ovary; PAG, polyacrylamide gel electrophoresis; TNF, tumor necrosis factor.

* This work was supported by a National Health Research Institutes and Leukemia Society of America Career Development Award (to C. F. H.) and National Institutes of Health, Department of Defense, and CaPCURE grants (to H. J. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 886-226534401; Fax: 886-22789484; E-mail: chiying@nhri.org.tw.
tizes mast cell line 32D toward apoptosis upon treatment with G-CSF (47). How the Btk family kinases assume such a paradoxical role in apoptosis is unclear. One possibility we considered is that posttranslational modification converts these kinases into functionally opposite isoforms.

In this report, we explore the possibility that Etk can be converted to a proapoptotic form after caspase cleavage. We provide evidence that Etk is an in vitro and in vivo substrate for caspase 3 during apoptosis. Using point mutants, we mapped the cleavage site to be Asp424 in the SH3 domain. The cleavage separates the N-terminal PH and SH3 domains from the C-terminal SH2 and catalytic domains. The C-terminal fragment of Etk exhibits increased kinase activity and sensitizes prostate cancer PC3 cells toward apoptosis in response to apoptosis-inducing stimuli. To our knowledge, this is the first case where Btk family member is shown to be a caspase target. Our findings provide a mechanistic explanation as to how Etk can both be anti- and proapoptotic.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs—**Plasmids encoding the full-length Etk (Etk/wt, residues 1–674) (24), C terminus-truncated Etk (Etk/N-1 residues 1–242), and N terminus-truncated Etk (Etk/C, residues 243–675) were constructed in pcDNA3.1/Myc-His vector (Invitrogen). The kinase-dead mutant, Etk/K445Q, and two mutants for testing caspase cleavage, Etk/D242A and Etk/D295A, were generated by site-directed mutagenesis (Stratagene) employing Ekt/wt as the template. All of the Etk constructs have the T7 epitope at N termini and the Myc epitope at C termini except Etk/C, which only has the Myc epitope at the C terminus.

**Cell Culture and Transfections—**All cell lines were purchased from the ATCC and maintained at 37 °C with 5% CO2. Jurkat and A431 cells were grown in RPMI medium supplemented with 10% heat-inactivated fetal calf serum and 10 μg/ml penicillin/streptomycin. Human umbilical vein endothelial cells (HUVEC) were grown in an endothelial cell culture medium supplemented with epidermal growth factor and endothelial cell growth supplement (E-STIM; Becton Dickinson). Human prostate cancer PC3 cells were grown in RPMI medium supplemented with 10% fetal bovine serum and 100 μg/ml each of leupeptin, aprotinin, chymostatin, and pepstatin. After incubation at 4 °C for 30 min, cellular debris was removed by centrifugation. Protein concentrations were determined by Bradford assay, and equal amounts of total lysates were used for further analyses.

**Immunoprecipitation and Western Blot Analysis—**Immunoprecipitations were performed with anti-T7 tag monoclonal antibody (Novagen) or anti-Etk polyclonal antibody (against Etk N terminus) (24) at 4 °C for 2 h. Protein A-agarose beads (Upstate Biotechnology, Inc., Lake Placid, NY) were added and incubated for another 2 h at 4 °C. Beads were washed three times with buffer containing 10 mM HEPES, pH 7.0, 2 mM MgCl2, 50 mM NaCl, 5 mM EGTA, 0.1% Triton X-100, and 60 mM 2-glycerophosphate. Immune complexes or total cell lysates were resuspended in SDS sample buffer and resolved by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Millipore Corp.) and detected with anti-T7 antibody, anti-Etk antibody, or anti-α-tubulin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The complexed IgGs were detected by incubation with secondary antibodies conjugated to horseradish peroxidase and developed using the ECL system (Amersham Pharmacia Biotech).

**Proteolytic Cleavage of Etk in PC3 Cells—**PC3 cells were transiently transfected with plasmids encoding Etk/wt or Etk/D242A proteins. Twenty-four hours after transfection, PC3 cells were treated with 1 μg/ml anti-Fas antibody for various time periods. Both the adherent and detached cells were harvested, washed with phosphate-buffered saline, and lysed in lysis buffer as described above. Equal amounts of total lysates were immunoprecipitated with anti-Etk antibody. The immune complexes were resolved by SDS-PAGE followed by immunoblotting with anti-T7 antibody.

In *Vitro* Kinase Assay—Etk/wt, Etk/K445Q, Etk/D242A, and Etk/C were in vitro translated in the presence of [35S]methionine. Etk/N-1 was in vitro translated in the absence of [35S]methionine. Various Etk proteins were immunoprecipitated with anti-Myc antibody. These immune complexes were washed three times with wash buffer as described above. Etk kinase activity assays were carried out in kinase reaction buffer containing 30 mM PIPES, pH 7.0, 10 mM MnCl2, 30 μM ATP, 1 μCi of [γ-32P]ATP, 1 mM Na3VO4, and immunoprecipitated Etk/N-1 as the substrate. Following incubation for 10 min at 30 °C, kinase reactions were terminated by adding SDS sample buffer and analyzed by SDS-PAGE and autoradiography.

**Flow Cytometry—**Parental PC3 and various PC3 Etk stable clones were either untreated or treated with 250 ng/ml anti-Fas antibody, UV irradiation (180 J/m2), or 10 ng/ml TNF-α (Upstate Biotechnology) plus 10 μg/ml cycloheximide (Sigma) for 24 h. Both floating and adherent cells were collected and washed once with phosphate-buffered saline. Apoptotic cells were quantified by flow cytometry analysis (Becton Dickinson FACStar Plus flow cytometer) after cells were stained with fluorescein isothiocyanate-conjugated annexin V and counterstained with propidium iodide as described by the manufacturer (ApoAlert Annexin V Apoptosis Kit, CLONTECH).

**RESULTS**

**Etk Is Proteolytically Cleaved in Apoptotic Extracts—**To examine whether Etk might serve as a substrate for caspases during apoptosis, we first prepared the cell-free apoptotic extracts from either Jurkat T cells or A431 cells as the source of caspases. Previously, Jurkat T cells were shown to be hypersensitive to a wide variety of apoptotic inducers, such as anti-Fas antibody (48) and staurosporine (49). Similarly, apoptosis and caspase activation were observed when human epidermoid carcinoma A431 cells were UV-irradiated (50). In our study, treatment of Jurkat T cells with either anti-Fas antibody or staurosporine and A431 cells with UV irradiation resulted in ~40–60% cell death, with concurrent elevation of the activities (3–5-fold) of caspases 3, 6, 8, and 9 (data not shown). The in *vivo* translated [35S]methionine-labeled Etk was incubated, respectively, with these apoptotic extracts. As shown in Fig. 1, Etk was proteolytically degraded in all three apoptotic extracts but remained intact in untreated extracts. Strikingly, the cleavage patterns of Etk were identical in all three extracts, as immune complexes resolved by a doublet around 50 kDa and a single fragment around 35 kDa. We presumed that the elevated caspase activities in these apoptotic extracts were responsible for the cleavage of Etk.

To test whether the cleavage of endogenous Etk also occurs in cells undergoing apoptosis, the HUVEC were examined. The reason is that although Etk is expressed in multiple tissues, its...
protein level is generally low. Among all of the cell lines we surveyed, HUVEC contain the highest level of endogenous Etk, which increased the sensitivity of our assay. HUVEC were treated with either staurosporine or actinomycin D to induce apoptosis (51, 52). Cell lysates were prepared and immunoprecipitated with anti-Etk antibody raised against the PH domain of Etk (24). Immunoblot analysis of immunoprecipitated complexes with the same Etk antibody revealed a dramatic decrease of full-length Etk in staurosporine and actinomycin D-treated cells (Fig. 2). However, we were unable to detect the cleavage products, presumably due to the instability of the fragments in this cell type under the experimental conditions.

Etk Is a Direct Substrate for Caspase 3—Given the existence of at least 14 caspases of diverse specificity (6), the specific cleavage pattern of Etk (Fig. 1) points to the action of a selected set of caspases. This prompted us to investigate which caspase(s) is responsible for Etk degradation during apoptosis. Two approaches were taken. First, caspase inhibitors at different concentrations were used to discern the possible caspase(s) involved. As shown in Fig. 3A, cleavage of [35S]methionine-labeled Etk in anti-Fas antibody or staurosporine-treated apoptotic extracts (not shown) could be totally blocked by Ac-DEVD-CHO (an inhibitor for caspase 3) at a concentration as low as 0.1 μM, whereas it took a higher concentration of caspase 1 inhibitor (Ac-YVAD-CHO) to achieve the same goal. These data suggest that Etk cleavage is preferentially carried out by DEVD-sensitive caspase(s) such as caspase 3 or caspase 3-like proteases.

We also examined whether Etk could be cleaved by purified recombinant caspases in vitro and, if so, whether the degradation patterns mirror that observed in apoptotic extracts. Fig. 3B showed that [35S]methionine-labeled Etk could be cleaved by caspase 3 at a concentration as low as 2 ng. By contrast, caspases 6 and 7 have no effect on Etk at the concentrations tested, and caspase 8 is able to cleave Etk only at a relatively high concentration. These results strongly suggest that caspase 3 (or caspase 3-like proteases) is more likely the enzyme that cleaves Etk and that the proteolytic cleavage of Etk is a general feature in apoptotic cells.

In addition, it has been shown previously that phosphorylation of presenilin-2 regulates its cleavage by caspases (53). To verify if the autophosphorylation of Etk is required for caspase-mediated cleavage, the invariant lysine in the ATP binding pocket of Etk was mutated to glutamine. The resultant mutant, Etk/K445Q, has neither kinase activity (see Fig. 8) nor autophosphorylation ability (data not shown) as determined by an in vitro kinase activity assay. This mutant was in vitro translated and treated with either apoptotic extracts or recombinant caspases. The kinetics of Etk/K445Q degradation was identical to that when Etk/wt was used as the target (data not shown), indicating that neither the kinase activity nor autophosphoryl-
Methionine-labeled Etk and D4GDI were incubated alone or with increasing amounts of recombinant caspase 3 for 1 h at 37 °C, respectively. Reaction mixtures were separated by SDS-PAGE followed by autoradiography.

To address the relative sensitivity of Etk cleavage by caspase 3, we compared the cleavage kinetics with one of the well-known caspase 3 targets, D4GDI (49). [35S]methionine-labeled Etk and D4GDI (with similar molar ratio) were incubated respectively with increasing amounts of recombinant caspase 3. The results showed that both Etk and D4GDI were partially cleaved under low concentrations of caspase 3. Etk was almost completely cleaved by 5 ng of caspase 3, whereas the cleavage of D4GDI was only about 30% under the same conditions. Even with an excess amount of caspase 3 (10 ng), only about 50% of D4GDI was cleaved (Fig. 4). The data indicate that Etk is a better substrate for caspase 3 when compared with D4GDI.

Mapping the Caspase Cleavage Site in Etk—When [35S]methionine-labeled Etk was incubated with either apoptotic extracts or recombinant caspase 3, a closely spaced 50-kDa doublet was generated (Figs. 1 and 3). The doublet may represent two distinct cleavage products with similar sizes or the same product with a different extent of posttranslational modification such as phosphorylation. To distinguish these possibilities, λ-phosphatase treatment was performed on either uncleaved or recombinant caspase 3-cleaved [35S]methionine-labeled Etk. Both the doublets at 85 kDa (full-length Etk) and 50 kDa (cleavage products) became single bands, indicating that the upper bands are the phosphorylated forms of Etk (Fig. 5A). These data suggest that the the 50-kDa doublet and the 35-kDa fragment were derived from a single caspase cleavage event.

We noticed that Etk contains three potential caspase 3 recognition motifs, DXDX, which were found at the cleavage site of many proteins during apoptosis (8). These sequences are DFPD<sup>242</sup> W, DDYD<sup>285</sup> W, and DLYD<sup>317</sup> N (the arrows indicate the potential cleavage sites in the amino acid sequence). A diagrammatic representation of the protein domain structure and potential cleavage sites of Etk is shown in Fig. 6A. Based on the size of cleavage products, D<sup>242</sup> W and D<sup>285</sup> W are likely candidate cleavage sites. These two sites were therefore individually mutated to alanines, and their susceptibility to cleavage by caspases was tested. Etk/wt and these two mutants, Etk/D242A and Etk/D285A, were in vitro translated and incubated with recombinant caspases. Etk/wt and Etk/D295A, but not Etk/D242A, were cleaved by either caspase 3 or 8 (Fig. 5B and data not shown). Similar experiments were carried out using cell-free apoptotic extracts. Again, Etk/wt and Etk/D295A, but not Etk/D242A, were proteolysed in these apoptotic extracts (Fig. 5C and data not shown).

To further examine whether Asp<sup>242</sup> is the bona fide cleavage site in vivo during apoptosis, Etk/wt and Etk/D242A, which were N-terminally tagged with T7 epitope, were ectopically expressed in cultured cells. Due to the low transfection efficiency of HUVEC, we used PC3, a prostate cancer cell line known to express a low level of Etk, as the test system. Transfected PC3 cells were treated with anti-Fas antibody for various time points to induce apoptosis. Cell lysates prepared from different time points were immunoprecipitated with anti-Etk antibody and immunoblotted with anti-T7 antibody. The time course data revealed that Etk/wt but not Etk/D242A was degraded within 2 h upon anti-Fas antibody treatment as illustrated by the appearance of cleavage products, Etk/N-I and Etk/N-II (Fig. 7). The sizes of Etk/N-I and Etk/N-II were identical to those bands cleaved by a high concentration of recombinant caspase 3 (Fig. 3B). Taken together, the data confirm the identity of the cleavage site mapped in vitro. The absence of Etk/N-II in D242A-expressing samples suggests that caspase cleavage of Etk after Asp<sup>242</sup> is necessary for the exposure of the second caspase cleavage site. The second cleavage site, however, remains unclear at this time.

Activation of Etk after Caspase 3-mediated Proteolysis—Caspase 3-mediated cleavage of Etk generated two fragments. The N-terminal fragment contains the complete PH and partial SH3 domains (amino acids 1–242, referred to as Etk/N-I). The C-terminal fragment contains partial SH3, complete SH2, and tyrosine kinase domains (amino acids 243–675, termed Etk/C) (Fig. 6A). Based on the current model of regulation of Btk family kinases (54) in the uninduced state, the C-terminal catalytic domain interacts with the N-terminal PH domain, thus assuming a “closed” form. The removal of the N-terminal 1–242 residues from the C-terminal catalytic domain by caspase cleavage might result in kinase activation of Etk. To test this possibility, in vitro kinase activity assays were performed by using a peptide (Etk/N-I), which carries the auto-phosphorylation site of Etk, as a substrate. This experiment was modeled after a similar one used successfully to measure the kinase activity of Btk (55, 56). A series of C-terminally Myc-tagged Etk constructs, including Etk/wt, Etk/K445Q, Etk/D242A, and Etk/C (Fig. 6A), were in vitro translated in the presence of [35S]methionine. This strategy overcame the difficulty encountered in the immunoprecipitation experiment (57) that was due to comigration of IgG heavy chain with Etk/C in SDS-PAGE. This system permits quantification of the amount of each sample as illustrated by Fig. 8. All of these samples were immunoprecipitated with anti-Myc antibody, and the immunocomplexes were subjected to kinase assay. The kinase activity of Etk/C was about 4-fold higher than that of Etk/wt or Etk/D242A (after calibration of the methionine contents of Etk/wt and Etk/C) (Fig. 8). The most plausible explanation of these results is that caspase cleavage removes an inhibitory N-terminal regulatory domain (containing the PH domain) from Etk, thereby generating an active kinase. This is consistent with our earlier data showing that deletion of the PH domain leads to a constitutively active Etk (24, 58).

Proteolytic Activation of Etk Potentiates Apoptosis—We next investigated whether constitutively active Etk/C might contribute to the demise of the cells. Initial experiments showed that ectopic expression of Etk/C alone either in PC3, HeLa, or 293T cells did not induce apoptosis, suggesting that the truncated Etk itself is not a trigger for apoptosis (data not shown). As a result, we were able to isolate stable clones of PC3 transfected with either Etk/wt or Etk/C. Three clones with comparable protein expression levels (as judged by Western blots) were selected for each construct. PC3 is known to be rather resistant to apoptosis (59). We therefore asked whether Etk/C could...
sensitize PC3 cells toward apoptosis induced by Fas antibody, UV, or TNF-α plus cycloheximide. Cells were either untreated or treated with these agents, and the extent of apoptosis was determined by annexin V and propidium iodide binding assay. Results showed that the Etk/C-overexpressing clones are significantly more sensitive to apoptotic stimuli than Etk/wt with a consistent 63–73% increase in the number of dying cells (Fig. 9). The fractional increase of apoptotic cells in this experiment is comparable with those reported previously with other caspase-activated kinases (19, 20). The finding suggests that the truncated Etk is proapoptotic, at least under the conditions we analyzed.

**DISCUSSION**

There is very strong evidence that caspases participate in the apoptotic process. The identification of caspase substrates provides a means to understand the often complex and diverse apoptotic pathways. In this study, we describe a tyrosine kinase Etk as a direct substrate for caspases. Both recombinant caspases 3 and 8 cleave Etk, although with different efficiency, and generate an identical cleavage pattern, suggesting some degree of functional redundancy within the caspase family (8). The data also raise the possibility that Etk can alternatively be proteolyzed by caspase 8 in some caspase 3-deficient cells. A single amino acid change from Asp242 to Ala abolishes such a
cleavage and unambiguously identifies the cleavage site to be DFPD\(^242\) \(\downarrow\) W. While this sequence does not match any known caspase cleavage sequence, DXXD is a consensus recognition motif of caspase (8, 60). It is interesting that among the Btk family kinases, the DFPD \(\downarrow\) W sequence is uniquely present in Etk despite the overall structural homology of Btk family kinases. There are, however, two putative caspase 3 cleavage motifs in Btk. One of them, DPKD\(^401\) \(\downarrow\) W, is located in the proximity of the kinase domain (Fig. 6B), the cleavage of which should in theory also generate an active kinase without a PH domain. The kinase activity of Etk/C is 4 times higher than that of the wild type. This activation is similar in magnitude to that caused by either deletion of the PH domain (24) or competitive binding of PTPD1 to the PH domain (61). All of these data are consistent with the model that the PH domain negatively regulates the kinase activity of Etk in a manner similar to that proposed for Itk (54).

The proteolysis of ectopically expressed Etk/wt occurs within 2 h in response to anti-Fas antibody treatment (Fig. 7). However, at this time point, no biochemical or morphological manifestations of apoptotic cell death could be observed, suggesting that Etk cleavage and its activation may be involved in the apoptotic process. This is reminiscent of several other protein kinases, such as MEKK-1 (17) and PAK2/human PAK65 (18, 19), that are also activated by caspase-mediated cleavage. A common feature of these kinases is that overexpression of caspase-truncated kinase induces apoptosis of cells. In our case, however, introduction of caspase-truncated Etk/C does not commit the cell to apoptotic demise but enhances apoptosis induced by extracellular stimuli (Fig. 9). These data suggest that Etk might not play a role in the initiation of apoptosis but contributes to the propagation or amplification of the apoptotic signal. How Etk does this is presently unclear. It is conceivable that the cleavage and activation of Etk might deregulate the activity of downstream targets. This is supported by the findings that apoptosis induced by Fas ligation (62, 63) and TNF (64, 65) results in rapid tyrosine kinase activation and tyrosine phosphorylation of multiple cellular proteins and that tyrosine kinase inhibitors can significantly reduce apoptosis (62, 63).

Etk and Btk were shown to have both antiapoptotic (41–44, 46) and proapoptotic (45) potential (37). This suggests that Btk family kinases may act as apoptotic switches and that their activities can influence both pro- and antiapoptotic signal molecules. Depending on the cell contexts and the forms of the kinases, as the present study suggests, the balance could be tilted in either direction. In this regard, it is interesting to note that Btk was shown to inhibit B cell apoptosis induced by Fas ligation (41). This inhibition is due to a direct interaction between Fas and Btk, via the PH domain, which interferes with Fas-FADD association. The activated and associated Btk kinase is apparently important in this inhibition process. If, however, Btk is cleaved by a caspase, which separates the PH domain from the kinase domain, the apoptosis process will be enhanced. This could be one reason why, in Fas-mediated apoptosis, Btk family kinases could be both anti- and proapoptotic.

In summary, we show in this report that a tyrosine kinase, Etk/Bmx, can be a substrate of a caspase(s). The resulting truncated molecule contains an intact SH2 domain and kinase domain. This molecule has an enhanced kinase activity and, while not apoptotic on its own, possesses an ability to enhance apoptosis induced by other agents. Our findings offer an alternative mechanism to explain the dual roles of Etk (or Btk family kinases in general) in apoptosis. The identification of new substrate(s) and interacting protein(s) as the consequence of Etk cleavage should provide insights into the apoptosis mediated by these family kinases.

**Acknowledgment—**We thank Dr. Jau-Song Yu for providing technical advice.

**REFERENCES**

1. Evan, G., and Littlewood, T. (1998) *Science* 281, 1317–1322
2. Jacobson, M. D., Weil, M., and Raff, M. C. (1997) *Cell* 88, 347–354
3. Steller, H. (1985) *Science* 228, 1445–1449
4. Budihardjo, I., Oliver, H., Luttiger, M., Luo, X., and Wang, X. (1999) *Annu. Rev. Cell Biol.* 15, 269–290
5. Earnshaw, W. C., Martins, L. M., and Kaufmann, S. H. (1999) *Annu. Rev. Biochem.* 68, 383–424
6. Thornberry, N. A., and Lazebnik, Y. (1998) *Science* 281, 1312–1316
7. Cynys, V., and Yuan, J. (1998) *Genes Dev.* 12, 1551–1570
8. Nicholson, D. W., and Thornberry, N. A. (1997) *Trends Biochem. Sci.* 22, 299–306
9. Strehl, C., and Schulze-Osthoff, K. (1998) *Cell Death Differ.* 5, 997–1000
10. Tan, X., and Wang, J. Y. (1998) *Trends Cell Biol.* 8, 116–120
11. Bokoch, G. M. (1998) *Cell Death Differ.* 5, 837–845
12. Cross, T. G., Scheel-Toellner, D., Henriquez, N. V., Deacon, E., Salmon, M., and Lord, J. M. (2000) *Exp. Cell Res.* 256, 34–41
13. Widmann, C., Gibson, S., and Johnson, G. L. (1998) *J. Biol. Chem.* 273, 7414–7417
14. Smith, G. C., di Faagna, F., Larkin, N. D., and Jackson, S. P. (1999) *Mol. Cell. Biol.* 19, 6076–6084
15. Levkau, B., Herren, B., Koyama, H., Ross, R., and Baines, E. W. (1998) *J. Exp. Med.* 187, 579–586
16. Wen, L. P., Fahrai, J. A., Troie, S., Guan, J. L., Orth, K., and Rosen, G. D. (1997) *J. Biol. Chem.* 272, 26056–26061
17. Cardone, M. H., Salvesen, G. S., Widmann, C., Johnson, G., and Frisch, S. M. (1997) *Cell* 90, 315–323
18. Lee, N., MacDonald, H., Reinhard, C., Halenbeck, R., Roulston, A., Shi, T., and Williams, L. T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 13642–13647
19. Rudel, T., and Bokoch, G. M. (1997) *Science* 274, 1571–1574
20. Graves, J. D., Gotob, Y., Draves, K. E., Ambrose, D., Han, D. K., Wright, M., Chernoff, J., Clark, E. A., and Krebs, E. G. (1998) *EMBO J.* 17, 2222–2234
21. Emoto, Y., Manome, Y., Meinhardi, G., Kisaki, H., Kharbanda, S., Robertson, M., Ghayur, T., Wong, W. W., Kamen, R., and Weichselbaum, R. (1995) *EMBO J.* 14, 6148–6155
22. Ghayur, T., Hugunin, M., Talanian, R. V., Ratnoff, S., Quinlan, C., Emoto, Y., Pandey, P., Datta, R., Huang, Y., Kharbanda, S., Allen, H., Kamen, R., Wong, W., and Kufe, D. (1996) *J. Exp. Med.* 184, 2399–2404
23. Datta, R., Rejima, H., Yoshida, K., and Kufe, D. (1997) *J. Biol. Chem.* 272, 20317–20320
24. Qiu, Y., Robinson, D., Pretlow, T. G., and Kung, H. J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 6544–6549
25. Tamagnone, L., Lahtinen, I., Mustonen, T., Virtaneva, K., Francis, F., Muscatelli, F., Alitalo, R., Smith, C. I., Larsson, C., and Alitalo, K. (1994) * Oncogene* 9, 3683–3688
26. Chukada, S., Saffran, D. C., Rawlings, D. J., Parolini, O., Allen, R. C., Klimas, I., Sparkes, R. S., Kubagawa, H., Mohandas, T., and Quan, S. (1993) *Cell* 72, 279–290
27. Vetere, D., Vorechovych, I., Siders, P., Holland, J., Davies, A., Flinter, F., Hammarsrom, L., Kinnon, C., Levinsky, R., Bobrow, M., Smith, C. I., and Bentley, D. R. (1993) *Nature* 361, 226–233
28. Silicione, J. D., Morrow, T. A., and Desiderio, S. V. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 11914–11918
29. Sato, K., Mano, H., Aiyama, T., Iinawah, J., Yazaki, Y., and Hirai, H. (1994) *Leukemia* 8, 1663–1672
30. Rawlings, D. J., and Witte, O. N. (1995) *Semin. Immunol.* 7, 237–246
31. Robinson, D., Wu, Y. W., and Lin, S. F. (2000) *Oncogene* **19**, 5548–5557
32. Ekman, N., Lymboossaki, A., Vastrik, I., Sarvas, K., Kaipainen, A., and Alitalo, K. (1997) *Circulation* **96**, 1729–1732
33. Kaukonen, J., Lahtinen, I., Laine, S., Alitalo, K., and Palotie, A. (1996) *Br. J. Haematol.* **94**, 455–460
34. Weil, D., Power, M. A., Smith, S. I., and Li, C. L. (1997) *Blood* **90**, 4332–4340
35. Hsueh, R. C., and Scheuermann, R. H. (2000) *Adv. Immunol.* **75**, 283–316
36. Mano, H. (1999) *Cytokine Growth Factor Rev.* **10**, 267–280
37. Qiu, Y., and Kung, H. J. (2000) *Oncogene* **19**, 5651–5661
38. Satterthwaite, A. B., and Witte, O. N. (2000) *Immunol. Rev.* **175**, 120–127
39. Schaeffer, E. M., Debnath, J., Yap, G., McVicar, D., Liao, X. C., Littman, D. R., Sher, A., Varmus, H. E., Lenardo, M. J., and Schwartzberg, P. L. (1999) *Science* **284**, 638–641
40. Uckun, F. M. (1998) *Biochem. Pharmacol* **56**, 683–691
41. Vassilev, A., Ozer, Z., Navara, C., Mahajan, S., and Uckun, F. M. (1999) *J. Biol. Chem.* **274**, 1646–1650
42. Petro, J. B., Rahman, S. M., Ballard, D. W., and Khan, W. N. (2000) *J. Exp. Med.* **191**, 1745–1754
43. Xue, L. Y., Qiu, Y., He, J., Kung, H. J., and Oleinick, N. L. (1999) *Oncogene* **18**, 3391–3398
44. Enari, M., Talanian, R. V., Wong, W. W., and Nagata, S. (1996) *Nature* **380**, 723–726
45. Na, S., Chuang, T. H., Cunningham, A., Turi, T. G., Hanke, J. H., Bokoch, G. M., and Danley, D. E. (1996) *J. Biol. Chem.* **271**, 11209–11213
46. Tang, T. K., Chang, W. C., Chan, W. H., Yang, S. D., Ni, M. H., and Yu, J. S. (1998) *J. Cell. Biochem.* **70**, 442–454
47. Bajpai, U. D., Zhang, K., Teutsch, M., Sen, R., and Wortis, H. H. (2000) *J. Exp. Med.* **191**, 1735–1744
48. Rokhlin, O. W., Hostager, B. S., Bishop, G. A., Sidenenko, S. P., Glover, R. A., Gudkov, A. V., and Cohen, M. B. (1997) *Cancer Res.* **57**, 3941–3943
49. Andreotti, A. H., Bunnell, S. C., Feng, S., Berg, L. J., and Schreiber, S. L. (1997) *Nature* **385**, 93–97
50. Park, H., Wahl, M. I., Afar, D. E., Turek, C. W., Rawlings, D. J., Tam, C., Scharenberg, A. M., Kinet, J. P., and Witte, O. N. (1996) *Immunity* **4**, 515–525
51. Jui, H. Y., Tseng, R. J., Wen, X., Fang, H. I., Huang, L. M., Chen, K. Y., Kung, H. J., Ann, D. K. (1998) *J. Biol. Chem.* **273**, 11204–11207
52. Simon, H. U., Yousefi, S., Dibbert, B., Hebestreit, H., Weber, M., Branch, D. R., Blaser, K., Levi-Schaffer, F., and Anderson, G. P. (1998) *Blood* **92**, 547–557
53. Stein, H., and Kiewnick, S. (1998) *J. Cell. Biochem.* **71**, 122–129
54. Weber, C., Negrescu, E., Ertl, W., Pletsch, A., Frankenberger, M., Ziegler-Heitbrock, H. W., Siess, W., and Weber, P. C. (1995) *J. Immunol.* **155**, 445–451