Cleavage of PITSLRE Kinases by ICE/CASP-1 and CPP32/CASP-3 during Apoptosis Induced by Tumor Necrosis Factor*

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Emerging evidence suggests that multiple aspartate-specific cysteine proteases (caspases (CASPs)) play a crucial role in programmed cell death. Many cellular proteins have been identified as their substrates and serve as markers to assay the activation of CASPs during the death process. However, no substrate has yet been unambiguously identified as an effector molecule in apoptosis. PITSLRE kinases are a superfamily of Cdc2-like kinases that have been implicated in apoptotic signaling and tumorigenesis. In this paper we report that tumor necrosis factor (TNF)-mediated apoptosis is associated with a CrmA- and Bel-2-inhibitable cleavage of PITSLRE kinases, indicating a role for CASPs. Testing of seven murine CASPs for their ability to cleave p110 PITSLRE kinase a2-1 in vitro revealed that only CASP-1 (ICE (interleukin-1β-converting enzyme)) and CASP-3 (CPP32) were able to produce the same 45-kDa cleavage product as observed in cells undergoing TNF-induced apoptosis. Mutational analysis revealed that cleavage of p110 PITSLRE kinase a2-1 occurred at Asp393 within the sequence YVPDS, which is similar to that involved in the CASP-1-mediated cleavage of prointerleukin-1β. TNF-induced proteolysis of PITSLRE kinases was still observed in fibroblasts from CASP-10/0 mice. These data implicate CASP-3 as a potential important CASP family protease responsible for the cleavage of PITSLRE kinases during TNF-induced apoptosis.

Apoptosis is a fundamental process for normal development of multicellular organisms and is involved in the regulation of the immune system, normal morphogenesis, and maintenance of homeostasis (1). Aspartate-specific cysteine proteases belonging to the interleukin-1β-converting enzyme (ICE) family, recently renamed the caspase (CASP) family (2), have been implicated as principal effectors of apoptosis, presumably by their proteolytic action on specific targets, including members of the ICE-related protease family themselves, poly(ADP-ribose) polymerase, DNA-dependent protein kinase, the 70-kDa small U1 ribonucleoprotein, lamins, protein kinase Cα, D4-GDP dissociation inhibitor, and various components of the cytoskeleton (3, 4). Many of these proteins are likely to be involved in the morphological and biochemical changes that accompany apoptosis, or in aspects of DNA damage sensing and repair, and are used as markers to assay the activation of CASPs during the death process. However, no substrate has yet been unambiguously identified as a downstream effector molecule in apoptosis.

PITSLRE kinases are a superfamily of protein kinases related to the master mitotic protein kinase Cdc2 (4–6). Ectopic expression of the smallest member of this superfamily has previously been shown to induce apoptosis (5). In addition, deletion of the PITSLRE gene complex and complete loss of expression of specific isoforms occur in many neuroblastoma cell lines and is frequently observed in human cancers (8–10). Induction of apoptosis via the Fas receptor in human T cells has recently been shown to be correlated with proteolysis and increased activity of PITSLRE kinases (7). Triggering of Fas or the related TNF receptor is known to induce the activation of several CASPs (3, 4). Hence we examined the possibility that PITSLRE kinases are substrates for CASP-1 (ICE) and six other members of the murine CASP family in vitro as well as in vivo and demonstrate that CASP-3 (CPP32) is likely to be a crucial CASP responsible for PITSLRE kinase cleavage during TNF-induced apoptosis.

MATERIALS AND METHODS

Cell Lines and Transfections—All cell lines were cultured in appropriate media using standard tissue culture conditions. PC60p55p75 is a rodent T cell hybridoma that has been transfected with the p55 and p75 human TNF receptors (11). HeLaH21 is a human cervix carcinoma. Transfection, isolation, and characterization of Bel-2-expressing PC60p55p75 cells were described previously (11). HeLaH21 cells expressing CrmA were obtained by transfection with 30 μg of pCAGGS-CrmA and 1 μg of pSV2neo using the calcium phosphate-DNA coprecipitation method and selecting G418-resistant transformants by culturing cells in medium containing 500 μg/ml G418 for 3 weeks. pCAGGS-CrmA was constructed by inserting the cowpox virus DNA fragment encoding CrmA, provided by Dr. J. Pickup (12), into the mammalian expression vector pCAGGS (13). CrmA expression was confirmed by Western blotting. Embryonic fibroblasts were prepared from wild-type and CASP-10/0 mice as described previously (14).

Cell Lysis and Immunoblotting—These were performed essentially as described previously (6, 7). PITSLRE kinases were detected using antibody P2N100 directed against the first 50 amino acids of the p110 isoforms or antibody GN1 directed against the first 72 amino acids of p58 PITSLRE kinase p1 (6). ECL (Amersham Life Science, Amersham, U.K.)

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The abbreviations used are: ICE, interleukin-1β-converting enzyme; AMC, aminomethylcoumarin; CASP, caspase; IL-1β, interleukin-1β; TNF, tumor necrosis factor; CHAPS, 3-(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.
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United Kingdom) was used for visualization.

Protein Assays—p110 PITSLRE kinase α2-1 and proIL-1β cDNAs were subcloned into the pGEM1Zf(+) plasmid (Promega, Madison, WI) behind the T7 and SP6 promoter, respectively, using routine recombiant DNA techniques. 35S-Labeled proIL-1β and p110 PITSLRE kinase α2-1 were prepared by the in vitro coupled transcription-translation reticulocyte lysate system (Promega) with SP6 and T7 polymerase, respectively, and incubated for 70 min at 30 °C with either purified recombinant human CASP-1 (a gift from Drs. N. Thornberry (Merck Research Laboratories) and R. Talanian (BASF Bioresearch Corp., Worcester, MA)), murine CASP-1 or CASP-3, or crude cell extracts from Escherichia coli transformed with different murine CASP expression plasmids, in a total volume of 50 μl of CASP reaction buffer containing 10 mM HEPES, pH 7.5, 2 mM EDTA, 0.1% CHAPS, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 10 μg/ml aprotinin. In some experiments, p110 PITSLRE kinase was first purified from the reticulocyte lysate by immunoprecipitation with antibody GNI as described (6). Reactions were stopped by the addition of SDS-PAGE sample buffer. Cleavage products were analyzed by SDS-PAGE and fluorography.

Site-directed Mutagenesis—The p110 PITSLRE kinase α2-1 D393A mutant was generated by PCR methodology using the mutagenic oligonucleotide primer TTCGACCGAGCTTCCGGGGA and a kit from CLONTECH. The presence of the introduced mutation (underlined) and fidelity of PCR replication were confirmed by sequence analysis.

Bacterial Expression and Purification of Murine CASPs—The full-length cDNA clones of murine, CASP-1, CASP-2, CASP-3, CASP-6, CASP-7, CASP-11, and CASP-12 (16), were used as a template for classical PCR amplification with Vent polymerase and primers designed to generate CASPs lacking the N-terminal prodomain and appending a 10-amino acid Streptag to the C terminus. The resulting products were cloned into the bacterial expression vector pLT10TH downstream of the His tag and transformed in E. coli strain MC1061pT7POL23 (17). Empty pLT10TH was used as a negative control.

CASP Reaction Buffer and either used as active enzyme preparations for in vitro cleavage experiments or further purified by TALON immobilized metal affinity chromatography (CLONTECH) and elution with 20 mM Tris-HCl, pH 7.5, 100 mM imidazole, 50 mM NaCl, 10% glycerol, and 1 mM glutathione. All CASPs were expressed at approximately equal levels, except for CASP-7 which was about 5-fold higher.

RESULTS AND DISCUSSION

We first studied the cleavage of PITSLRE kinases and the role of CASPs in the killing of a rodent PC60p55p75 T cell hybridoma by human TNF. Expression and proteolysis of PITSLRE kinases were examined by Western blotting using PITSLRE kinase-specific antibodies P2N100 and GN1 (6, 7). In the cells studied, the former recognized the p110 PITSLRE kinase α2 and β2 isoforms (Fig. 1A), while the latter reacted with a p90 PITSLRE kinase isoform, which has been tentatively identified as an alternatively spliced PITSLRE kinase isoform lacking much of exon 2, but containing all other exons (6), and a 170-kDa protein, which is presumably another unknown PITSLRE kinase isoform (Fig. 1B). The PITSLRE kinase α1 (p65) and β1 (p58) isoforms could not be detected and are presumably not present in the cells used. Following treatment of PC60p55p75 cells with TNF, the p110 PITSLRE kinase isoform was cleaved to fragments of 60 and 43 kDa and a minor 52-kDa product (Fig. 1A). These processing products seemed to be very unstable, because they were only detectable when p110 cleavage was still incomplete and after long exposure times of the blots. In parallel, the p170 isoform was cleaved to a 130-kDa protein, which accumulated with increasing TNF exposure times, while the p90 isoform remained unaffected (Fig. 1B). Proteolysis of PITSLRE kinases was already detectable after 2-h TNF treatment, slightly preceding the onset of TNF-induced apoptosis. Similar results were obtained in HeLa cells that had been treated with anti-Fas antibody or TNF (data not shown). Nonspecific proteolysis could be excluded by the fact that several other proteins were not degraded (data not shown). As mentioned above, the p90 PITSLRE kinase isoform lacks exon 2, corresponding to amino acids 375-415 of the PITSLRE kinase α2-1 110-kDa isoform. This region of the protein contains a putative cleavage site for proteases of the CASP family (YVPD393S). The observation that the p90 PITSLRE kinase isoform, in contrast to the p110 and p170 PITSLRE kinase isoforms, was not cleaved after TNF treatment, suggests a possible role for CASPs in TNF-induced cleavage of the p110 and p170 PITSLRE kinase isoforms. The latter hypothesis was further substantiated by investigating the effect of TNF in cells stably transfected with either the human bcl-2 or cowpox virus crmA gene, which encode proteins known to act as upstream (18) or direct (12) inhibitors, respectively, of at least some CASPs. Overexpression of these genes has been shown previously to protect cells against TNF-induced apoptosis (11, 19, 20). Both CrmA and Bcl-2 expression completely inhibited the TNF-induced cleavage of PITSLRE kinases as well as cell death (Fig. 2). Similarly, pretreatment of cells with the CASP-specific inhibitor benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Ref. 4; Enzyme Systems Products, Dublin, CA), in contrast with inhibitors of granzyme B, cathsipin B, serine, and cysteine proteases, completely protected cells from TNF-induced apoptosis and PITSLRE kinase cleavage (data not shown). These results further indicate a role for CASPs in the cleavage of PITSLRE kinases during the TNF-induced cell death pathway.

In the human system, the CASP family comprises seven different members that share a conserved QACRG pentapeptide containing the active site cysteine (2-4). More recently,

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three additional CASPs have been described, viz. CASP-8 (MACH = FLICE = Mch5; Refs. 21–23), CASP-9 (ICE-LAP6; Ref. 24), and CASP-10 (Mch4; Ref. 21). These differ from the other CASPs by a nonconservative substitution in the active site QACRG pentapeptide, and the presence of a FADD-like effector domain in the case of CASP-8 and CASP-10. In mice, only the cloning of CASP-1 (ICE), CASP-2 (NEDD-2 = human Icch1) and CASP-11 (Ich3) has been reported so far (2–4, 25). These differ from the other CASPs by a nonconservative substitution in the active site QACRG pentapeptide, and the presence of a FADD-like effector domain in the case of CASP-8 and CASP-10. In mice, only the cloning of CASP-1 (ICE), CASP-2 (NEDD-2 = human Icch1) and CASP-11 (Ich3) has been reported so far (2–4, 25). We have recently isolated and characterized seven murine CASPs containing the conserved QACRG box (16): CASP-1 (ICE), CASP-2 (Ich1), CASP-3 (CPP32, Yama, or apopain), CASP-6 (Mch2), CASP-7 (Mch3, ICE-LAP3, or CMH-1), CASP-11 (presumably the murine homolog of human CASP-4, TX, Ich2, or ICP30), and CASP-12 (related but presumably not homologous to human CASP-5 or ICP30). To analyze whether one of these CASPs can directly cleave PITSLRE kinases, we treated in vitro translated p110 PITSLRE kinase α2-1 with crude cell extracts of bacteria expressing the specific murine CASPs. Interestingly, CASP-1 and CASP-3 were the only CASPs that could cleave p110 PITSLRE kinase to a 43-kDa processed product (Fig. 3). In some experiments, weak 60- and 80-kDa cleavage products could also be observed (see also Fig. 4). The size of the 43- and 60-kDa PITSLRE kinase cleavage products corresponds to the size of the protein bands that were observed after TNF-induced cleavage of the p110 PITSLRE kinase in vivo (Fig. 1A), further suggesting that the latter is mediated by CASP-1 or CASP-3. None of the other CASPs displayed any effect on the PITSLRE kinase, although the latter is mediated by CASP-1 or CASP-3. None of the other CASPs by a nonconservative substitution in the active site.

Examination of the amino acid sequence of the p110 PITSLRE kinase revealed that the 43-kDa protein generated upon incubation with CASP-1 or CASP-3 could be generated by cleavage at the sequence YYVHDH50 | S, which is very similar to the YYVHD | A cleavage site for CASP-1 in proIL-1β (26) and which conforms to the specific hydrolysis of YYVAD-AMC by CASP-1 and CASP-3 as described above. Indeed, in vitro translated p110 PITSLRE kinase α2-1 and p110 PITSLRE kinase α2 were cleaved with similar efficiency by purified CASP-1, requiring a minimal CASP-1 concentration of only 0.25 ng (Fig. 4, A and B). PITSLRE kinase cleavage by CASP-3 required about 25 times higher concentrations than CASP-1 (Fig. 4C). Moreover, a p110 PITSLRE kinase, in which Asp9| S had been mutated to Ala, was no longer cleaved by CASP-1 or CASP-3 (Fig. 4, B and 4C), although some residual nonspecific cleavage resulting in a 43-kDa degradation product could still...
Primary embryonic fibroblasts from wild-type (CASP-1−/−) or CASP-1−/− mice were either left untreated or treated with 10,000 IU ml−1 human TNF in the presence of 0.1 μg ml−1 actinomycin D for 8 h. Expression and cleavage of PITSLRE kinases was assessed by Western blotting with P2N100 (A) and GN1 (B) antibodies, respectively. Cell death is indicated at the bottom of each lane and represents the mean of triplicate samples with an S.D. <10%.

We finally investigated whether in vivo CASP-1 itself is required for TNF-induced cleavage of PITSLRE kinases; thus we analyzed the effect of TNF in embryonic fibroblasts obtained from CASP-1−/− mice (27, 28). As shown in Fig. 5, TNF-induced cell death and PITSLRE kinase cleavage was similar in wild-type and CASP-1-deficient fibroblasts. Taking into account that CASP-3 was the only other CASP that was found to cleave p110 PITSLRE kinase in vitro, the above results suggest a crucial role for CASP-3-mediated cleavage of PITSLRE kinases in the TNF-induced cell death pathway. We cannot, however, exclude an involvement of other newly identified (CASP-8, CASP-9, CASP-10; Refs. 21–24) or still unidentified CASPs (although the inhibition by CrmA limits possible candidates), or a more complex scenario involving two or more proteases which function redundantly or concomitantly, such that elimination of CASP-1 alone has no effect. The biological implication for CASP-3-mediated cleavage of PITSLRE kinases in TNF-induced apoptosis is still unclear. To date, no specific substrates for PITSLRE kinases have been identified. Cleavage by CASP-1 and CASP-3 occurs at a site that separates the C-terminal domain from the N-terminal part that harbors two nuclear localization signals as well as a region that has been shown to bind SH2 domains (29). Therefore, CASP-3-mediated cleavage of PITSLRE kinases might be a mechanism to modulate their localization or interaction with other proteins. It should also be mentioned that in addition to PITSLRE kinases, Cdc2 itself and other Cdc2-like enzymes have been shown to play a role in apoptosis induced by TNF and granzyme B (30, 31).

In conclusion, we have shown that TNF-induced apoptosis is associated with CASP-3-specific proteolysis of PITSLRE kinases. This processing does not occur in cells in which the apoptotic pathway is blocked by Bcl-2 or by CrmA expression. Since the PITSLRE kinases represent the first CASP substrates for which a direct apoptotic function and a role in tumorigenesis have been described (5–10), our findings might have important implications for understanding the mechanism of action of TNF, as well as the regulation of apoptosis in human cancer and other diseases.

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