The CED-3/ICE-like Protease Mch2 Is Activated during Apoptosis and Cleaves the Death Substrate Lamin A*

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Apoptosis, or programmed cell death, is required for embryonic development and tissue homeostasis (1). Dysregulated apoptosis can result in cancer, autoimmune diseases, or neurodegenerative disorders (2, 3). Distinct from necrosis, apoptosis is characterized morphologically by cell shrinkage, membrane blebbing, and chromatin condensation (4).

Genetic analyses in Caenorhabditis elegans provided the first inkling about the nature of the death pathway (5). One of the genes identified, ced-3, was required for cell death in the developing nematode (6) and found to have significant homology to the mammalian protein interleukin-1β (IL-1β)γ converting enzyme (ICE) which proteolytically processes pro-IL-1β to an active cytokine (7, 8). ICE and ICE-like enzymes are cysteine proteases that possess a very unusual substrate specificity in that, other than granzyme B, they are the only mammalian proteases known to cleave substrates following aspartate residues (9). Overexpression of both CED-3 and ICE proteins induces apoptosis in cultured cells (9), supporting the notion that this family of cysteine proteases plays a critical role in the death pathway. Subsequently, a number of CED-3/ICE homologues have been identified and phylogenetically ordered based on their homology to one another (10). The CED-3/ICE subfamilies include the CED-3 subfamily, consisting of Yama (CPP32, apopain) (11–13), LAP3 (Mch3, CMH1) (10, 14, 15), and Mch2 (16); the ICE subfamily, which consists of ICE (7, 8), ICE rel II (Tx, ICH-2) (17–19), and ICE rel III (17); and the NEDD-2 subfamily, mNedd-2 (20) and Ichi-ch-1 (21).

In an attempt to identify which members of the CED-3/ICE family are involved in apoptosis, we have used, as a criterion, activation of the endogenous zymogen upon receipt of an apoptotic signal from a single polypeptide to a proteolytically competent, active dimeric species. Since proteolytic activation of zymogenic forms of the CED-3/ICE family into active p20/p10 subunits involves cleavage following aspartate residues, it follows that the convertase must be either another CED-3/ICE family member or granzyme B, an aspartate-specific serine protease. Indeed, two of the three CED-3/Yama subfamily members (Yama and LAP3) are rapidly activated upon receipt of a death stimulus; the inactive endogenous zymogen being cleaved to generate an active (p20/p10) dimeric species (10, 22).

In addition, both Yama and LAP3 function downstream of the death inhibitors Bcl-2, Bcl-xL, and CrmA. Importantly, Mch2, but not Yama or LAP3, is capable of cleaving lamin A to its signature apoptotic fragment, indicating that Mch2 is an apoptotic laminase.

Materials and Methods

Cell Lines—J urkat, a human T cell line, and BJAB, a human B cell line, were cultured in RPMI 1640 media containing 10% heat-inactivated fetal bovine serum, nonessential amino acids, l-glutamine, and penicillin/streptomycin. pEBs-Bcl-2-, pEBs-Bcl-xL-, and pZEM-CrmA-expressing J urkat cell lines have been described previously (22). HeLa cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, nonessential amino acids, l-glutamine, and penicillin/streptomycin.

Immunoblotting—This was performed essentially as described previously for Yama and LAP3 (10, 22). Briefly, cells (2 × 10⁶) were either not treated or treated with 100 ng/ml anti-Apo1 (IgG3) (24) or 2 μM staurosporine (Sigma). Cells were pelleted, lysed in 0.5% Nonidet P-40, clarified by centrifugation and the supernatant resolved on a 14% polyacrylamide gel and analyzed by autoradiography.

Protease Assays—cDNAs encoding Mch2, Yama, PARP, and lamin A were cloned into pET 23b (Invitrogen) and expressed in BL21(DE3)λlysS, and the C-terminal (His)₆-tagged proteins were purified by affinity chromatography with Ni²⁺-NTA-agarose (Qiagen) according to the manufacturer's instructions.

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1The abbreviations used are: IL-1β, interleukin-1β; PARP, polype (ADP-ribosyl) polymerase; TLCK, Tos-Lys-CH₂Cl; ICE, interleukin-1β converting enzyme; PAGE, polyacrylamide gel electrophoresis.

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Mch2 Is Activated during \( \alpha \)-Fas/\( \alpha \)-Apoptin- and Staurosporine-Induced Cell Death—Fas is a cell surface receptor that, upon activation by cognate ligand or agonist antibody, engages the death pathway, resulting in the activation of Yama and LAP3 death proteases. To investigate the possible activation of Mch2 from its pro-form to a p20/p10 active species upon receipt of a death signal, a rabbit anti-peptide antibody was generated that was specific for the p20 subunit. As expected, in naive untreated Jurkat T cells, only the pro-form of Mch2 was observed (Fig. 1A). However, upon engagement of the Fas death receptor, the zymogen form converted to the p20 subunit form, indicative of activation (Fig. 1A). A similar transition was observed in BJ AB cells. Exposure of cells (Jurkat, HeLa, or BJ AB) to an alternative death stimulus, staurosporine, similarly led to the processing of Mch2, implying that activation of Mch2 was not specific for any particular inducer of apoptosis (Fig. 1B).

Mch2, Yama, and LAP3 Function at a Similar Point in the Death Pathway—As Mch2 appeared to be activated in a manner analogous to other members of the CED-3 subfamily, we asked if it, like Yama and LAP3, functioned downstream of the general mammalian death inhibitors (Bcl-2 and Bcl-x\(_L\)) and the cowpox virus encoded serpin CrmA, a potent inhibitor of certain ICE family members (22). It had been shown previously that while Bcl-2 and Bcl-x\(_L\) could inhibit staurosporine-induced apoptosis in leukemic cell lines, they did not attenuate Fas-induced death. Conversely, CrmA potently inhibited Fas-induced cell death, but not staurosporine-induced apoptosis, implying that Bcl-2, Bcl-x\(_L\), and CrmA have different target specificities. In cells overexpressing either Bcl-2 or Bcl-x\(_L\), Mch2 was not activated on exposure to staurosporine (Fig. 2A). However, when the apoptotic stimulus was mediated through the Fas/\( \alpha \)-Apoptin receptor (which is not inhibited by Bcl-2 or Bcl-x\(_L\)), Mch2 was clearly activated (Fig. 2A). In contrast, CrmA was unable to inhibit Mch2 activation in response to staurosporine, but effectively attenuated Mch2 processing in response to activation of the Fas/\( \alpha \)-Apoptin receptor (Fig. 2B). Taken together, these data suggest that upstream of Mch2 activation in the Fas death pathway is a CrmA-inhibitable protease that is not inhibited by either Bcl-2 or Bcl-x\(_L\). This CrmA-inhibitable protease is presumably lacking, bypassed, or not essential in the staurosporine-induced death pathway (and hence the inability of CrmA to inhibit staurosporine-induced cell death).

The staurosporine pathway, however, must contain a Bcl-2 and Bcl-x\(_L\) inhibitable step that is upstream of Mch2 activation as they completely block staurosporine-mediated Mch2 activation. Therefore, Mch2, like Yama and LAP3, is activated at a point in the death pathway that is downstream of both mammalian and viral inhibitors of cell death.

Granzyme B Functionally Activates Mch2—Cytotoxic T lymphocytes trigger cell death by either introducing granzyme B into target cells through perforin channels and/or by expressing Fas ligand that engages the Fas/\( \alpha \)-Apoptin death receptor on the surface of target cells. Despite being a serine protease, granzyme B, like CED-3/ICE family members, possesses the ability to cleave substrates following aspartate residues. Previous studies have demonstrated that granzyme B proteolytically processes and activates pro-Yama and pro-LAP3 (10, 23), ICE family members most related to the C. elegans death gene cd-3. However, this does not represent indiscriminate activation as, when ICE itself is proteolytically processed by granzyme B, it does not reconstitute a functional enzyme. As shown in Fig. 3A, granzyme B proteolytically processed both endogenous pro-Mch2 (in naive cell extracts) and recombinant pro-Mch2 to a dimeric species, indicating that it could potentially be activated by granzyme B. This was confirmed when granzyme B-processed Mch2 was found to be proteolytically competent, capable of cleaving the death substrate PARP to its signature apoptotic fragment (Fig. 3B). Therefore, all known
Mch2, an Apoptotic Laminase

inhibitors, Bcl-2 and Bcl-xL and of the viral serpin, CrmA. and LAP3, functions downstream of the mammalian cell death machinery. Mch2 possesses an unique substrate specificity, distinct from other subfamily members. of the CED-3 subfamily are proteolytically processed by granzyme B to generate active proteases.

Mch2 is an Apoptotic Laminase—CED-3/ICE proteases have been implicated in the cleavage of a number of apoptotic substrates including PARP (28), SREBP (29), U1–70 kDa (30), Gas2 (31), and actin (32). It is unlikely that cleavage of a single substrate represents a lethal hit. Rather, it is more appealing to consider that it is the coordinated, concerted, and hierarchical cleavage of a number of different substrates that leads to the eventual demise of a cell. Nevertheless, monitoring the cleavage of specific substrates provides a useful biochemical marker for the apoptotic process. The picture that is beginning to emerge is that certain CED-3/ICE members demonstrate marked substrate preference and that they are probably less promiscuous than previously thought. For example, Yama and LAP3 cleave PARP, but are unable to proteolytically process pro-Ill-1β, the substrate for ICE (13, 14). Likewise, ICE will effectively cleave pro-Ill-1β, but at physiological concentrations is unable to cleave PARP (13). The protease responsible for the cleavage of lamin A, however, has remained elusive (33). Mch2, like Yama and LAP3, is able to cleave either endogenous PARP or recombinant PARP to a signature apoptotic fragment (Fig. 4A). In stark contrast, Mch2, but not Yama or LAP3, cleaved endogenous lamin A and recombinant lamin A to the signature apoptotic form (Fig. 4B). An identical lamin A cleavage profile was observed when HeLa nuclei were incubated with apoptotic extract (Fig. 4B). Additionally, Mch2-mediated lamin A cleavage was inhibited by TLCK, a characteristic of the putative apoptotic form (Fig. 4B). Therefore, Mch2 possesses an unique substrate specificity, distinct from other subfamily members.

In conclusion, we have demonstrated that Mch2, like Yama and LAP3, functions downstream of the mammalian cell death inhibitors, Bcl-2 and Bcl-xL and of the viral serpin, CrmA. Further, granzyme B can functionally activate Mch2, supporting the contention that granzyme B kills cells by activating downstream components of the CED-3/ICE death machinery. Finally, Mch2, unlike Yama or LAP3, can cleave lamin A to its signature apoptotic fragment, demonstrating substrate specificity within the CED-3-like subfamily.

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Mch2, an Apoptotic Laminase

16446

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