Molecular Ordering of Apoptotic Mammalian CED-3/ICE-like Proteases*

(Received for publication, July 2, 1996)

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Apoptosis is executed by cysteine proteases belonging to the CED-3/ICE family, which, unlike other mammalian cysteine proteases, cleave their substrates following aspartate residues. Proteases belonging to this family exist in the cytosol aszymogens that require accurate processing at internal aspartate residues to generate the two-chain active enzymes. As such, CED-3/ICE family members are capable of activating each other in a manner analogous to the proteasezymogens of the coagulation or complement cascades. At present, it is unknown whether such mutual processing exists in vivo, and if so whether it is sequential, implying an order to the death pathway. Using a cell-free apoptosis system, recombinant ICE proteases and both biochemical and morphological criteria, we demonstrate an ordering of the mammalian ICEs that are most related to the Caenorhabditis elegans death protease CED-3.

Programmed cell death, or apoptosis, a process of fundamental importance to all multicellular organisms, occurs in a morphologically distinct fashion in the absence of a concomitant inflammatory response (1, 2). Identification of genes involved in cell death in Caenorhabditis elegans has been of pivotal importance in deciphering components of the death pathway (3). The discovery, in particular, that the C. elegans death gene ced-3 possesses homology to interleukin-1-β (IL-1β)α-converting enzyme (ICE), a mammalian protease with a very unusual specificity for cleaving substrates after aspartate residues, has implicated this class of protease in the death pathway (4, 5). While there is one ced-3 gene in C. elegans, mammals possess numerous related genes, not all of which appear to play a prominent role in apoptosis (6). Mammalian ICE itself, for example, appears to be primarily involved in the proteolytic maturation of the cytokine IL-1β (7). Phylogenetic analysis of all known mammalian ICEs does, however, suggest the existence of subfamilies (8). Importantly, mammalian ICEs most related to CED-3 (8–15), including Yama (CPP32, aopapain), LAP3 (Mch3, CMH1, SREBP-2), and Mch2 form a unique subfamily (CED-3 subfamily) that are activated at a similar point in the death pathway downstream of both mammalian death suppressors (Bcl-2 and Bcl-xL) and a viral death inhibitor (CrmA) (16, 17). Acting in concert, this subfamily of proteases likely represents the functional equivalent of CED-3. Upon receipt of an apoptotic signal, the endogenous zymogen form of these enzymes is rapidly converted to the active dimeric species by cleavage into large and small subunits whose intimate association is required for proteolytic activity (18). To determine the ordering of the ICE proteases most related to CED-3, we have utilized a cell-free system of apoptosis. In keeping with prior studies (16, 19), addition of purified and characterized active recombinant CED-3/ICE proteases to a cytosolic extract from untreated cells (naïve extract) engaged the death pathway as evidenced by the induction of morphological changes in added indicator nuclei. Concurrently, we assessed DNA laddering and, by immunoblotting, the state of the endogenous death proteases (single polypeptide zymogen or active processed species) and cleavage of apoptotic substrates.

MATERIALS AND METHODS

Cell Lines—Jurkat, a human T-cell line, was cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, nonessential amino acids, L-glutamine, and penicillin/streptomycin. 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—Lysates were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and analyzed essentially as described previously for Yama, LAP3, and Mch2 (8, 16, 17). Nuclei were solubilized in 4 M urea/SDS sample buffer, resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and analyzed using an affinity-purified rabbit anti-lamin A (generously provided by Brian Burke, University of Calgary) at a dilution of 1:500, followed by a 1:6,700 dilution of secondary goat anti-rabbit horseradish peroxidase conjugate (Amersham Corp.). Immuno blotting of nuclei was performed with antibodies to poly(ADP-ribose) polymerase (PARP) and U1–70 small ribonucleoprotein (U1–70 kDa) as described previously (Refs. 14 and 20, respectively). Bound antibody was visualized using the ECL kit (Amersham) according to the manufacturer’s instructions.

Protease Assays—Recombinant active forms of Yama, LAP3, and Mch2 were expressed using the pET 23b vector (Novagen) and purified with the QiAexpress system (Qiagen). N-terminal sequencing of each recombinant protease on an Applied Biosystems model 476A sequencer confirmed purity and demonstrated that the following cleavage sites resulted in removal of the N-terminal (pro) peptides and generation of the large and small catalytic subunits: Mch2 at aspartates 28 and 175 (mature cleavage at aspartate 179); LAP3 at aspartates 23 and 206 (minor cleavage at aspartate 198). Cytosolic extracts were prepared from nonapoptotic Jurkat cells as described (21) and from apoptotic Jurkat cells treated for 2 h at 37 °C with 100 ng/ml Fas/Apo1 as described (22). Nuclei were prepared from HeLa cells as described (23). The in vitro apoptotic reactions were performed by the addition of 105 HeLa nuclei to naïve or apoptotic extract (~8 mg/ml) in the presence or absence of recombinant protease. DNA fragmentation analysis was performed as described (21).

RESULTS AND DISCUSSION

DNA Fragmentation and a Distinct Morphology Are Observed in Indicator HeLa Nuclei upon the Addition of Recombinant CED-3-like Proteases—Recombinant CED-3-like proteases added directly to indicator HeLa nuclei did not induce DNA fragmentation (Fig. 1, lanes 4, 6, and 8). In the presence of naïve extract, however, the active proteases Mch2 (Fig. 1, lane 3), Yama (Fig. 1, lane 5), or LAP3 (Fig. 1, lane 7) induced

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DNA fragmentation. As nonspecific proteolysis occurs in the presence of excess protease, a careful titration of each preparation of purified recombinant protease was performed to determine the minimal amount capable of triggering the DNA fragmentation.

Surprisingly, the morphology of the indicator HeLa nuclei varied depending on the protease used to trigger DNA fragmentation. As expected, HeLa nuclei incubated with naive extract (Fig. 2, A and F) displayed normal morphology, whereas HeLa nuclei incubated with apoptotic extracts (Fig. 2, B and G) exhibited a classic apoptotic morphology where condensed chromatin formed apoptotic bodies. Similarly, a combination of Mch2 (Fig. 2, C and H) and naive extract induced the appearance of apoptotic nuclear morphology. In contrast, a distinct intermediate morphology of margination was observed when naive extract and HeLa nuclei were incubated with either Yama (Fig. 2, D and I) or LAP3 (Fig. 2, E and J). The chromatin was condensed at the nuclear rim, producing a halo effect, but there was little evidence of apoptotic body formation. These results indicated for the first time that the activity of each CED-3-like protease was potentially unique. In addition, the production of overt apoptotic morphology by Mch2 indicates that this protease either has the ability to cleave most of the substrates required to produce the morphology, or that it acts in concert with other downstream proteases to bring this about.

Distinct Cleavage Profiles of Apoptotic Substrates by CED-3-like Proteases in the Presence or Absence of Naive Extract—To further investigate distinct properties of the CED-3-like proteases, we analyzed the state of the indicator apoptotic substrates PARP (14, 24), U1–70 kDa (20, 25), and lamin A (26) in the presence or absence of naive extract. As expected, these substrates remained intact upon incubation with naive extract (Fig. 3, A–C, lane 1), but apoptotic extracts induced cleavage to the indicated signature apoptotic fragments (Fig. 3, A–C, lane 2). Cleavage of both PARP and U1–70 kDa, but not lamin A, was induced upon addition of Yama (Fig. 3, A–C, lanes 3 and 4) or LAP3 (Fig. 3, A–C, lanes 5 and 6), regardless of the presence or absence of naive extract. Importantly, Mch2 alone, in the absence of any extract, induced cleavage of lamin A (Fig. 3C, lane 7) and partial cleavage of PARP (Fig. 3A, lane 7), but did not cleave U1–70 kDa (Fig. 3B, lane 7). In contrast, Mch2 endowed upon the naive extract the ability to cleave all three substrates to their respective diagnostic apoptotic fragments (Fig. 3, A–C, lane 8). This result is consistent with the notion that Mch2-mediated cleavage of U1–70 kDa and PARP is indirectly being propagated by the activation of downstream proteases present in the naive extract (possibly Yama or LAP3).

Specific Activation of Endogenous CED-3-like Zymogens upon Addition of Recombinant Active Proteases to Naive Extract—In such an experimental paradigm, it appeared reasonable to assume that if the added exogenous protease did not process a particular endogenous ICE-zymogen then this zymogen was unlikely to be a physiological substrate for the added protease. Conversely, if the added protease processed the ICE-zymogen into an active dimeric species, then this zymogen could potentially be a physiologic substrate for the added protease. As expected, all three endogenous CED-3-like proteases were present as single chains in naive cytosolic extract, indicative of zymogen status, (Fig. 4, A–C, lane 1) but became...
Fig. 3. Specific cleavage of apoptotic substrates by CED-3-like proteases in the presence or absence of naive extract. Western blot analyses of the cleavage of PARP (A), U1–70 kDa (B), and lamin A (C). Substrates within HeLa nuclei incubated with naive extract were not cleaved (lane 1). Incubation with apoptotic extract resulted in cleavage to signature apoptotic fragments (lane 2). Recombinant Yama with (lane 3) or without naive extract (lane 4) induced the cleavage of PARP and U1–70 kDa, but not lamin A. Similarly, recombinant LAP3 with (lane 5) or without naive extract (lane 6) induced the cleavage of PARP and U1–70 kDa, but not lamin A. Recombinant Mch2 alone significantly cleaved lamin A and partially cleaved PARP, but did not cleave U1–70 kDa (lane 7). Incubation of recombinant Mch2 with naive extract (lane 8) induced the cleavage of all three substrates.

Fig. 4. Differential activation of endogenous CED-3-like zymogens following addition of recombinant active proteases to naive extract. Western blot analyses of the activation of Mch2 (A), Yama (B), and LAP3 (C). The zymogen form of each protease (lane 1) is detected in naive extract by an antibody directed against the respective large subunit. The presence of the large subunit indicates activation and, as expected, is observed for each of the CED-3-like proteases in apoptotic extract (lane 2). Recombinant Mch2 incubated with naive extract (lane 3) generated the mature processed large subunit as well as intermediates; pro + large subunit for Yama and pro (lacking the pro-domain) for LAP3. Similarly, recombinant Yama incubated with naive extract processed LAP3, which generated the Δpro intermediate (lane 4) and the large subunit (visible on a longer exposure) but Mch2 was not cleaved and remained as a zymogen. Incubation of recombinant LAP3 with naive extract (lane 5) did not induce the cleavage of either endogenous Yama or Mch2 zymogens. As endogenous forms of the proteases could not be distinguished from their added recombinant counterparts by immunoblotting, these lanes (A, lane 3; B, lane 4; and C, lane 5) were not included.

processed in Fas/Apo1-stimulated apoptotic extracts (Fig. 4, A–C, lane 2). Addition of Mch2 to naive extract resulted in cleavage of both endogenous Yama (Fig. 4B, lane 3) and LAP3 (Fig. 4C, lane 3) consistent with their activation. When Yama was added, a small fraction of endogenous LAP3 was processed (Fig. 4C, lane 4), but Mch2 remained in itszymogen form (Fig. 4A, lane 4). Significantly, however, when LAP3 was added, both endogenous Mch2 (Fig. 2A, lane 5) and Yama (Fig. 2B, lane 5) remained in thezymogen form, despite the induction of DNA fragmentation (Fig. 1, lane 7). Therefore, the zymogen substrate specificity of the CED-3-like proteases is consistent with Mch2 acting upstream of both Yama and LAP3. These results support the finding that Mch2 directly activates Yama in cell extracts (27). Since recombinant LAP3 cannot cleave pro-Yama but Yama can cleave pro-LAP3, we do not rule out the possibility that Yama can act as an intermediate between Mch2 and pro-LAP3. Future studies will delineate the upstream activators of both Mch2 and LAP3.

Our results are consistent with a sequential pathway that allows Mch2 to initiate the activation of pro-Yama and pro-LAP3, thereby amplifying the range of death substrates cleaved during apoptosis (Fig. 5). These studies provide an experimental and conceptual framework that should allow the positioning of other ICE members in the enzymatic death cascade.

Acknowledgments—We thank Hangjun Duan and Manish Garg for technical assistance and Claudia Vincenz and David Beidler for helpful discussions. We are grateful to Ian J ones for his expertise in preparing the figures. We also thank Brian Burke for the generous gift of the affinity purified rabbit anti-lamin A antibody, Guy Poirier for the generous gift of the anti-PARP antibody, and Antony Rosen for the generous gift of the anti-U1–70 kDa antibody.

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J. Biol. Chem. 1996, 271:20977-20980.
doi: 10.1074/jbc.271.35.20977

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