Mch3, a Novel Human Apoptotic Cysteine Protease Highly Related to CPP32


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Abstract

Recent evidence suggests that mammalian cysteine proteases related to Caenorhabditis elegans CED-3 are key components of mammalian programmed cell death or apoptosis. We have shown recently that the CPP32 and Mch2a cysteine proteases cleave the apoptotic markers poly(ADP-ribose) polymerase (PARP) and lamins, respectively. Here we report the cloning of a new Ced-3/interleukin 1β-converting enzyme-related gene, designated Mch3, that encodes a protein with the highest degree of homology to CPP32 compared to other family members. An alternatively spliced isoform, named Mch3β, was also identified. Bacterially expressed recombinant Mch3 has intrinsic autokatalytic/autoactivation activity. The specific activity of Mch3α toward the peptide substrate DEVD-7-amino-4-methylcoumarin and PARP resembles that of CPP32. Like interleukin 1β-converting enzyme and CPP32, the active Mch3α is made of two subunits derived from a precursor (proMch3α). It was of interest that recombinant CPP32-p17 subunit can form an active heteromeric enzyme complex with recombinant Mch3-p12 subunit and vice versa, as determined by the ability of the heteromeric complexes to induce apoptosis in SF2 cells. These data suggest that proMch3α and proCPP32 can interact to form an active Mch3α/CPP32 heteromeric complex. We also provide evidence that CPP32 can efficiently cleave proMch3α, but not the opposite, suggesting that Mch3α activation in vivo may depend in part on CPP32 activity. The high degree of conservation in structure and specific activity and the coexistence of Mch3α and CPP32 in the same cell suggests that the PARP cleavage activity observed during apoptosis cannot solely be attributed to CPP32 but could also be an activity of Mch3α.

Introduction

The growing ICE/Ced-3 family of cysteine proteases include mammalian ICE (1, 2), NEDD2 (ICH-1; Refs. 3 and 4), CPP32 (5), Mch2 (6), TX (ICH-2, ICEα; Refs. 7–9), and ICEα-III (9) proteins as well as the C. elegans Ced-3 cell death protein (10). These proteases fall into two subfamilies composed of ICE, NEDD2, TX, and ICEα-III on the one hand and Ced-3, CPP32, and Mch2 on the other. Common features of this family include conservation of the active site QACRG pentapeptide, ability to induce apoptosis when overexpressed in a heterologous system, and requirement for proteolytic cleavage of the proenzymes at conserved aspartate cleavage sites.

Cloning of Human Mch3. A 10-μl aliquot of human Jurkat A Uni-ZAP XR cDNA library (5) containing ~105 plaque-forming units was denatured at 99°C for 5 min and used as a template for PCR amplification with a degenerate primer encoding the pentapeptide GSFW/SGSYY1 and T3 vector-specific primer (Stratagene) as described previously (6). A 10-μl aliquot of the primary amplification product was then used as a template for a secondary PCR amplification with primer T50-p1 (CCCTGGAAATAGGCAGAA) derived from GenBank sequence T50828 and a second vector-specific primer SK-Zap (CAGGAATTCGACGAC) located downstream of the T3 primer. The secondary amplification products were cloned into a Smal cut pBluescript II KS+ vector. All clones were screened by PCR using the QACRG degenerate primer and SK-Zap primer. Clones that were positive for the presence of the QACRG coding sequence were then subjected to DNA sequencing using the T3 and T7 sequencing primers (Stratagene). This resulted in identification of a Ced3α-like partial cDNA with high homology to CPP32. The partial cDNA was then excised from the vector, radiolabeled, and used to screen the original Jurkat A Uni-ZAP XR cDNA library. Positive clones were purified, rescued into the pBluescript II SK+ plasmid vector, and sequenced.

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3 The abbreviations used are: ICE, interleukin 1β-converting enzyme; Mch3, mammalian Ced-3 homologue; PARP, poly(ADP-ribose) polymerase; ITPG, isopropyl-1-thio-β-D-galactopyranose; GST, glutathione S-transferase; AMC, 7-amino-4-methylcoumarin; ACP, Autographa californica nuclear polyhedrosis virus; SF9, Spodoptera frugiperda cells; kDa, kilodalton.

4 The sequences reported in this paper have been deposited in the GenBank data base (accession numbers u37448 and u37449).

Materials and Methods

Cloning of Human Mch3. A 10-μl aliquot of human Jurkat A Uni-ZAP XR cDNA library (5) containing ~105 plaque-forming units was denatured at 99°C for 5 min and used as a template for PCR amplification with a degenerate primer encoding the pentapeptide GSFW/SGSYY1 and T3 vector-specific primer (Stratagene) as described previously (6). A 10-μl aliquot of the primary amplification product was then used as a template for a secondary PCR amplification with primer T50-p1 (CCCTGGAAATAGGCAGAA) derived from GenBank sequence T50828 and a second vector-specific primer SK-Zap (CAGGAATTCGACGAC) located downstream of the T3 primer. The secondary amplification products were cloned into a Smal cut pBluescript II KS+ vector. All clones were screened by PCR using the QACRG degenerate primer and SK-Zap primer. Clones that were positive for the presence of the QACRG coding sequence were then subjected to DNA sequencing using the T3 and T7 sequencing primers (Stratagene). This resulted in identification of a Ced3α-ICE-like partial cDNA with high homology to CPP32. The partial cDNA was then excised from the vector, radiolabeled, and used to screen the original Jurkat A Uni-ZAP XR cDNA library. Positive clones were purified, rescued into the pBluescript II SK+ plasmid vector, and sequenced.

Construction of Transfer Vectors and Recombinant Baculoviruses. The Mch3 cDNA was amplified by PCR using primers T50-p3 (GCTCAT-zAACATCTCTCCTCATCT) and T50-p4 (ATGCCAGATGATCATGCGGC) and subcloned into the pBluescript II SK+ vector. The Mch3 sequence was then excised with BamHI and subcloned into a BamHI cut pVL1393 to generate pVL-Mch3a transfer vector. The cDNAs encoding the p20 and p12 subunits of Mch3α were amplified by PCR using the following primers: p20 subunit, T50-p4 and Mch3-p20-tag (CTACTCCGGCTTCCAGGAC); p12 subunit,
Cloning of Mch3. An approach combining information from the GenBank database of human expressed sequence tags and PCR was used to identify and clone a novel member of the family of Ced-3/ICE-like apoptotic cysteine proteases from the human Jurkat T-cell line. Human Ced-3/ICE-like sequences present in a unidirectional Jurkat cDNA library were first enriched using degenerate PCR primers encoding the conserved GSWFIJGSWYI pentapeptides. The enriched library was amplified with a primer derived from an expressed sequence tag (T50828), and the resulting partial cDNA was sequenced to confirm its identity and then used as a probe to obtain the full-length cDNA as described in “Materials and Methods.” Several cDNA clones were isolated. One cDNA, named Mch3a, contains an open reading frame of 909 bp that encodes a 303-amino acid protein with a predicted molecular mass of ~34 kDa (Fig. 1). The initiator methionine at nucleotide 44 conforms to the consensus Kozak translation initiation sequence. A second cDNA clone, named Mch3b, is different from that of Mch3a and is shown below the nucleotide sequence of Mch3a. The predicted amino acid sequence of Mch3b is shown above the nucleotide sequence. The predicted amino acid sequence of Mch3b that is different from that of Mch3a is shown below the nucleotide sequence. Vertical arrow, intron location. Amino acid and nucleotide residues are numbered to the right of each sequence.

Northern Blot Analysis. Tissue distribution analysis of Mch3 mRNA was performed on Northern blots prepared by Clontech containing 2 μg/lane of poly(A)+ RNA. A radioactive Mch3 riboprobe was prepared using a Smal linearized pBluescript II SK-Mch3a as a template for T7 RNA polymerase in the presence of [α-32P]ATP. The blots were hybridized, washed, and then visualized by autoradiography.

Expression of Mch3 in Bacteria and Assay of Enzyme Activity. The GST-Mch3a1 and GST-Mch3a2 cDNAs were subcloned in-frame into the BamHI site of the bacterial expression vector pGEX-2T (Pharmacia Biotech, Inc.). The GST-Mch3a3 cDNA was subcloned in-frame into the BamHI/EcoRI site of pGEX-2T. Bacterial extracts were prepared and assayed as described recently (6). YVAD-CHO and -AMC were purchased from Bachem Bioscience (King of Prussia, PA). Recombinant His-tagged Crm A was purified as described (11). Synthesis of DEVD-CHO and -AMC will be described elsewhere.5

In Vitro Transcription and Translation. Mch3a, Mch3b, GST-Mch3a, and GST-CPP32 cDNAs were subcloned into the pBluescript II KS+ plasmid under the T7 promoter. The vectors were linearized with the appropriate restriction enzyme and used as templates for T7 RNA polymerase. The in vitro synthesized mRNAs were then used for in vitro translation with reticulocyte lysates as described previously (15).

Results and Discussion

Cloning of Mch3. An approach combining information from the GenBank database of human expressed sequence tags and PCR was used to identify and clone a novel member of the family of Ced-3/ICE-like apoptotic cysteine proteases from the human Jurkat T-cell line. Human Ced-3/ICE-like sequences present in a unidirectional Jurkat cDNA library were first enriched using degenerate PCR primers encoding the conserved GSWFIJGSWYI pentapeptides (6). The enriched library was amplified with a primer derived from an expressed sequence tag (T50828), and the resulting partial cDNA was sequenced to confirm its identity and then used as a probe to obtain the full-length cDNA as described in “Materials and Methods.” Several cDNA clones were isolated. One cDNA, named Mch3a, contains an open reading frame of 909 bp that encodes a 303-amino acid protein with a predicted molecular mass of ~34 kDa (Fig. 1). The initiator methionine at nucleotide 44 conforms to the consensus Kozak translation initiation sequence (16). A second cDNA clone, named Mch3b, is different from that of Mch3a and is shown below the nucleotide sequence of Mch3a. The predicted amino acid sequence of Mch3b is shown above the nucleotide sequence. The predicted amino acid sequence of Mch3b that is different from that of Mch3a is shown below the nucleotide sequence. Vertical arrow, intron location. Amino acid and nucleotide residues are numbered to the right of each sequence.
Mch3β was also identified and found to contain a deletion and insertion corresponding to nucleotides 488—592 of the Mch3α sequence (amino acids 149—183; Fig. 1). Mch3β also has a longer 5'-nontranslated sequence. Exon/intron analysis of the Mch3 genomic region that corresponds to the deletion/insertion in Mch3β revealed that the Mch3β mRNA resulted from two simultaneous alternative splicing events involving an exon and an intron shown as exon A and intron A in Fig. 2. The first event caused the deletion of nucleotides 488—592 of the Mch3α sequence due to the use of an alternative splice donor located within the coding region of exon A and an alternative splice acceptor located within intron A (Fig. 2). The second splicing event caused an insertion of a 74-bp intronic sequence due to the use of an alternative splice donor located within intron A and the normal splice acceptor of intron A. All the alternative splice donor/acceptor sites used in these events conform to the GT/AG rule (Fig. 2). As a result of the deletion and insertion, Mch3β cDNA did not maintain the same reading frame as Mch3α after amino acid 148. The new reading frame in Mch3β does not encode a QACRG pentapeptide sequence, and it terminates with a TGA stop codon corresponding to bp 837—839 of Mch3α (Fig. 1). Mch3β encodes a protein of 253 amino acids with a predicted molecular mass of ~28 kDa. In vitro translated Mch3α and Mch3β migrate as 36- and 33-kDa protein products, respectively (Fig. 2). The smaller translation products seen in the Mch3α and Mch3β translation reactions are probably internally translated products. We do not yet know the function of Mch3β. However, similar to the alternatively spliced Ich-1 isoform (Ich-1s; Ref. 4), Mch3β could be a negative regulator of apoptosis and could inhibit the activity of the parental enzyme by acting as a dominant inhibitor.

Mch3 Is a Cysteine Protease Highly Related to CPP32. The predicted full-length Mch3α protein sequence shows the highest homology to human CPP32 (5). Mch3α shares an overall ~53% identity (67% similarity) with CPP32 (Fig. 3), compared to ~37% identity (55% similarity) with CED-3 (10). Mch3α shows less than 30% identity with other family members such as ICE, NEDD/ICH-1, TX (ICH-2, ICErel- II; Refs. 7—9), or ICErel- III (9). In addition to the conservation of the active site QACRG pentapeptide, the predicted structure of Mch3α appears to be similar to CPP32 (5, 12). CPP32 is cleaved at Asp28 and Asp175 to generate two polypeptides with molecular masses of 17 (p17) and 12 (p12) kDa that form the active CPP32 enzyme complex (12). On the basis of the high homology between Mch3α and CPP32, the most probable cleavage sites in Mch3α are Asp53 and Asp198 (Figs. 1 and 3). Cleavage at these sites would generate two polypeptides equivalent to the p17 and p12 subunits of CPP32. However, there are three potential aspartic acid cleavage sites at positions 15, 20, and 23 that could be used to remove a short propeptide during processing of Mch3α to the active enzyme. In fact, the tetrapeptide DSVD (amino acids 20—23 of Mch3α) is very similar to the DEVD tetrapeptide substrate of CPP32 (6, 12). Hence, the aspartyl protease activity of CPP32 and Mch3α has been confirmed by in vitro methods. In addition, three Asp cleavage sites (Asp193, Asp204, and Asp206) located between the two subunits may serve as potential processing sites to separate the two subunits.

Tissue Distribution of Mch3. The tissue distribution of Mch3 was analyzed by Northern blot analysis of poly(A)⁺ RNA isolated from different human tissues. As shown in Fig. 4, a major 2.4-kb Mch3 message is detectable in all tissues examined. The lowest expression of Mch3 mRNA is seen in whole brain. Examination of Mch3 mRNA in different regions of the brain also shows low but detectable expression. A similar tissue distribution was also seen with CPP32 mRNA, although the CPP32 mRNA is more abundant than Mch3 mRNA in brain tissues (data not shown). The size of the Mch3 mRNA is consistent with the length of the cloned Mch3α and β cDNAs (Fig. 1). Two less abundant messages of (0.8 and 3.3 kb) are also detectable in some tissues such as the small intestine. The larger message could be an incompletely processed Mch3 mRNA or an alternatively spliced version of Mch3 mRNA containing a frameshift of 767 bp.
Expression and Autoprocessing of Mch3 in Escherichia coli.

We have shown recently that expression of ICE, CPP32, and Mch2α in bacteria as fusion proteins with GST results in processing and formation of active enzymes (6). This process is associated with cleavage of the NH2-terminal GST prodomain from the active enzyme. The ability to easily purify and estimate the size of the GST prodomain cleavage product allows prediction of the autoprocessing site(s). To express Mch3α in E. coli, three GST-Mch3α expression vectors were constructed and transformed into DH5α bacteria. The first construct (GST-Mch3α1) encodes GST fused at its COOH terminus to amino acids 1–303 of Mch3α (Fig. 5A). The second construct (GST-Mch3α2) is similar to the first construct; however, it has an additional 16 amino acids fused in-frame to the COOH terminus of GST. These 16 amino acids are encoded by bp -48 to -1 (relative to the ATG start site) of Mch3α cDNA (see Fig. 1). The third construct encodes GST fused at its COOH terminus to amino acids 24–303 of Mch3a (Fig. 5A). After induction with IPTG, bacterial extracts were prepared from E. coli expressing the recombinant fusion proteins. The extracts were adsorbed to glutathione-Sepharose resin, washed several times, and then analyzed by SDS-PAGE. As shown in Fig. 5B, compared to the GST nonfusion protein control that migrates as a ~28-kDa protein (Fig. 5B, Lane GST), the Mch3α1 preparation (Fig.
5B, Lane 1) contains a major GST prodomain cleavage product that migrates as a ~30-kDa band. However, because of the additional 16 amino acids in GST-Mch3a2, its major GST prodomain cleavage product (Fig. 5B, Lane 2) migrates as a ~32-kDa band. The same ~32-kDa GST prodomain product was also obtained when an in vitro translated GST-Mch3a2 was incubated with CPP32 or Mch3a (see below). Cleavage at Asp23 would generate products that have calculated molecular masses of ~28.8 and ~31.1 kDa (Fig. 5A). These sizes are consistent with the ~30- and ~32-kDa GST-prodomain products seen in the GST-Mch3a1 and GST-Mch3a2 preparations, respectively (Fig. 5B, Lanes 1 and 2). As expected, removal of amino acids 1–23 as in GST-Mch3a1 resulted in expression of a major 59-kDa unprocessed GST-Mch3a fusion protein (Fig. 5B, Lane 3). Two additional GST-fusion protein products, one migrating as a ~47-kDa band and the other as a ~29-kDa band, were also produced. The ~47-kDa band may represent a partially processed GST-Mch3a without the COOH-terminal p12 subunit, probably as a result of cleavage at Asp198. The ~29-kDa band may represent a cleavage product generated by cleavage at a site COOH terminal to Asp23. This suggests that Mch3a can still be cleaved, albeit less efficiently at a site COOH terminal to Asp23. Therefore, because our data suggest that Asp23 and Asp198 are the most likely processing sites, we will refer to the large subunit of Mch3a as p20 and the small subunit as p12.

**Kinetic Properties and Enzymatic Activity of Mch3a and CPP32.** The kinetic properties of bacterially expressed recombinant Mch3a and CPP32 were determined using the peptide substrate DEVD-AMC in a continuous fluorescent assay. The DEVD-AMC substrate is the PARP cleavage site P1–P4 tetrapeptide. Both Mch3a and CPP32 exhibited Michaelis-Menten kinetics in cleaving this substrate with $K_m$ of 51 and 13 μM, respectively (Table 1). The $K_m$ of recombinant CPP32 (13 μM) is comparable to the $K_m$ of purified human CPP32 (9.7 ± 1.0 μM) reported recently (12). The peptide aldehyde DEVD-CHO is a potent inhibitor of both Mch3a and CPP32 at low μM concentrations ($K_{i}$ of 1.8 nM; $K_{i}$ of 0.59 nM). In contrast, the ICE inhibitor peptide aldehyde YVAD-CHO ($K_{i}$ of 0.76 nM; Ref. 2) is a very weak inhibitor of both Mch3a and CPP32 ($K_{i}$ > 10 μM). The ICE inhibitor cowpox serpin, CrmA (17), is also a very weak inhibitor of Mch3a and CPP32 ($K_{i}$ > 1 μM; $K_{i}$ of 0.56 μM). These data confirm that the two enzymes, Mch3a and CPP32, are functionally similar to each other and suggest that they have similar substrate specificity. The high concentration of CrmA required to inhibit either CPP32 or Mch3a (Table 1) suggests that the target of CrmA inhibition in apoptosis is most probably not CPP32 or Mch3a. In addition, it has been shown recently that CrmA has 10,000-fold preference for ICE over CPP32 (12). Therefore, we believe that the target(s) of CrmA inhibition of apoptosis is ICE or an ICE-related protease(s) and not CPP32, as claimed recently (11).

**CPP32 also has been claimed recently to be the sole PARP-cleaving enzyme in apoptosis (12). However, our data show that Mch3a has the same substrate specificity toward PARP as does CPP32. Incubation of purified bovine PARP or human Hela nuclei with Mch3a resulted in a complete cleavage of PARP in less than 15 min (Fig. 6A). Similar activity was also observed with recombinant CPP32 and with S/M extracts derived from chicken DU249 cells in the committed phase of apoptosis (Ref. 13; Fig. 6B). Inhibition studies with the serine protease inhibitors TLCK and TPCK revealed interesting results. At 1 mM DTT, TPCK was able to inhibit both Mch3a and CPP32 PARP cleaving activity (Fig. 6C, Lanes 4 and 3, respectively). Under these conditions, TLCK did not inhibit Mch3a activity but it completely inhibited CPP32 activity (Fig. 6C, Lanes 3 and 2). In contrast, at 5 mM DTT, the inhibition of Mch3a and CPP32 by TLCK and TPCK was much reduced, although a slight inhibition of CPP32 was ob-

**Table 1. Kinetic Parameters of Mch3a and CPP32.**

The activity of recombinant Mch3a and CPP32 was measured using bacterial lysates prepared with ICE buffer (25 mM HEPES-1 mM EDTA-5 mM DTT-0.1% CHAPS-10% sucrose (pH 7.5)) at room temperature. The $K_m$ for Mch3a and CPP32 were determined from the hydrolysis rate of 50 and 10 μM DEVD-AMC, respectively, after a 30-min preincubation of the enzyme with inhibitor. Before incubation with enzymes, purified CrmA was activated by incubation with 5 mM DTT for 10 min at 37°C.
Fig. 6. Western blot analysis of PARP cleavage by Mch3α and CPP32. A, cleavage of bovine and human PARP by Mch3α. Purified bovine PARP (200 ng; Lanes 2–8) or isolated HeLa nuclei (1 × 10⁶; Lanes 9–15) were incubated at 37°C with buffer (Lanes 2 and 9) or with lysates (50 μg) from untransformed DH5α E. coli (Lanes 3 and 10) or transformed with a cDNA expressing Mch3α (Lanes 4–8 and 11–15). After the time indicated above Lanes 2–15, the cleavage products were analyzed by Western blotting. Mch3α-containing lysate (50 μg) incubated for 60 min in the absence of PARP was also blotted for comparison (Lane 1). B, cleavage of PARP by CPP32 and S/M extract. Purified bovine PARP (200 ng; Lanes 2–15) was incubated with buffer (Lane 2), with CPP32-containing lysate (30 μg; Lanes 4–9), and with S/M extracts (140 μg; Lanes 10–15) for the time indicated. C, effect of DTT concentration on the sensitivity of Mch3α and CPP32 to TLCK and TPCK. E. coli lysates (30 μg) containing Mch3α (Lanes 2–7) or CPP32 (Lanes 8–13) were preincubated with 0.5% methanol (to control for the effect of solvent; Lanes 2, 5, 8, and 11), with 100 μM TLCK (Lanes 3, 6, 9, and 12), and with 100 μM TPCK (lanes 4, 7, 10, and 13) for 15 min at 37°C in the presence of 1 (Lanes 2–4 and 11–13) or 5 (Lanes 5–10) mM DTT. Purified bovine PARP (200 ng) was added to preincubated lysates (Lanes 2–13) or to buffer preincubated in the presence of 5 mM DTT for 15 min (Lane 1) and was resolved by immunoblotting after 10 min of reaction.

served. This suggests that the concentration of thiol agents may influence significantly the ability of TLCK and TPCK to inhibit cysteine proteases. Furthermore, these drug studies reveal slight but significant differences between the closely related Mch3α and CPP32 enzymes.

Functional Interchangeability of Mch3α and CPP32 Subunits in Induction of Apoptosis. To test the ability of Mch3α to induce apoptosis, we took advantage of the ability of baculovirus-expressed ICE-like cysteine proteases to induce early apoptosis of infected Sf9 cells (5, 6, 8, 9, 15). Sf9 cells were infected with recombinant baculoviruses encoding full-length Mch3α or truncated Mch3α or CPP32 variants, separately or in various combinations. Cells were then examined by light microscopy for morphological signs of apoptosis, such as blebbing of the cytoplasmic membrane, condensation of nuclear chromatin, and the release of small apoptotic bodies. In addition, the genomic DNA was examined for internucleosomal DNA cleavage. Expression of full-length Mch3α in Sf9 cells caused approximately 50% of the cells to undergo apoptosis 42 h postinfection. This was accompanied by induction of internucleosomal DNA cleavage (Fig. 7A, Lane 3). This result was similar to those obtained previously with ICE, CPP32, and Mch2α (5, 6, 15). In control experiments, truncated Mch3α that encodes either the p20 subunit (amino acids 1–198) or the p12 subunit (amino acids 199–303) were unable to induce apoptosis in Sf9 cells when expressed separately (Fig. 7B, Columns 1 and 2). However, when these two subunits were coexpressed, ~49% of the cells died by apoptosis (Fig. 7B, Column 3). Similarly, the two subunits of CPP32 were not apoptotic when expressed separately (Fig. 7B, Columns 4 and 5) but were apoptotic when coexpressed together (Fig. 7B, Column 6). Particularly interesting results were obtained when the Mch3-p20 subunit was coexpressed with the CPP32-p12 subunit or vice versa (i.e., CPP32-p17 with Mch3-p12). These combinations were able to cause apoptosis in more than 50% of the cells (Fig. 7B, Columns 9 and 10). No significant induction of apoptosis was observed in control cells coexpressing Mch3-p20 and CPP32-p17 together or cells coexpressing Mch3-p12 and CPP32-p12 together (Fig. 7B, Columns 7 and 8). In addition, we have shown recently that coexpression of CPP32 and ICE subunits in any combination does not induce apoptosis in Sf9 cells (5).

Our data suggest the possibility that Mch3α and CPP32 may heterodimerize in vivo in mammalian cells to form active proapoptotic
Fig. 7. Induction of apoptosis in Sf9 cells by Mch3α and CPP32. A, expression of Mch3α in Sf9 cells induces internucleosomal DNA cleavage. Total cellular DNA was isolated at 42 h postinfection from Sf9 cells infected with the wild-type baculovirus (Lane 1) or the recombinant baculoviruses AcNPV-Mch3α (Lane 2) or AcNPV-ICE (Lane 3). The DNA samples were analyzed by electrophoresis in a 1.8% agarose gel containing ethidium bromide. Lane M, molecular mass markers. B, Sf9 cells were infected with the following recombinant baculoviruses: Column 1, AcNPV-Mch3α-p20; Column 2, AcNPV-Mch3α-p12; Column 3, AcNPV-Mch3α-p17; Column 4, AcNPV-CPP32-p17; Column 5, AcNPV-CPP32-p12; Column 6, AcNPV-CPP32-p17 and AcNPV-CPP32-p12; Column 7, AcNPV-Mch3α-p20 and AcNPV-CPP32-p12; Column 8, AcNPV-Mch3α-p12 and AcNPV-CPP32-p12; Column 9, AcNPV-Mch3α-p20 and AcNPV-CPP32-p12; Column 10, AcNPV-CPP32-p17 and AcNPV-Mch3α-p12. Forty-two h postinfection, cells were examined microscopically, and several fields were counted (average 1500 cells/condition), and the number of apoptotic cells was expressed as a percentage of total cells counted. These data were confirmed using the DNA cleavage assay as shown in A.

Fig. 8. Cleavage of ProMch3α by CPP32. A, pBluescript vectors containing a GST-Mch3α2 or a GST-CPP32 insert under the T7 promoter were linearized with the appropriate restriction enzymes as indicated by arrows and then used as templates for in vitro transcription and translation in the presence of [35S]-methionine. B, Lanes 1 and 2, the GST-Mch3α2 DNA template was linearized with EcoRI before transcription/translation to allow translation of GST only, and the products of translation were incubated with buffer (Lane 1) or CPP32 (Lane 2) for 30 min at 30°C. The major 28-kDa band in both lanes is GST, and the minor 64-kDa band in Lane 1 is the full-length GST-Mch3α2 fusion protein produced as a result of incomplete digestion of the DNA template with EcoRI. This 64-kDa band is cleaved by CPP32 in Lane 2 to produce the 28-kDa GST band seen above the 28-kDa GST band. Lanes 3–6, the GST-Mch3α2 DNA template was linearized with XhoI before transcription/translation, and the product of translation (full-length GST-Mch3α2 fusion protein) was incubated for 30 min on ice with buffer (Lane 3) or at 30°C with buffer (Lane 4), CPP32 (Lane 5), or Mch3α (Lane 6). C, the GST-CPP32 DNA template was linearized with EcoRI before transcription/translation, and the product of translation (full-length GST-CPP32 fusion protein) was incubated for 30 min at 30°C with buffer (Lane 1), Mch3α (Lane 2), or CPP32 (Lane 3). D, the GST-Mch3α2 fusion protein was immobilized on a GST-Sepharose resin, and the resin-GST-Mch3α2 was incubated for 1 h on ice with buffer (Lane 1) or at 30°C with CPP32 (Lane 2). The protein products in B and C were analyzed on a 14% SDS-gels, and in D on a 10–20% gradient SDS-gel. Arrows in B and C, cleavage products.
CLONING OF Mch3

The possibility that Mch3α is downstream of CPP32 suggests that CPP32 could be the PARP-cleaving enzyme during the early stages of apoptosis but that Mch3α may be involved in the final stages of PARP cleavage and apoptosis. We suggest that activation of the death program in mammalian cells is regulated by multiple pathways, and that execution of apoptosis may involve different cascades of cysteine proteases.

References


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