We have identified and characterized a novel cysteine protease named CMH-1 that is a new member of the interleukin 1β converting enzyme (ICE) family of proteases with substrate specificity for Asp-X. CMH-1 has the highest similarity to CPP32 (52% amino acid identity) and MCH2 (31% identical). CMH-1 shares conserved amino acid residues that form the core structure of ICE as well as those residues involved in catalysis and in the P1 aspartate binding. Overexpression of CMH-1 in COS cells resulted in the processing of CMH-1 and the induction of apoptosis of transfected cells. Coexpression of CMH-1 with poly(ADP-ribose) polymerase (PARP) also resulted in a specific cleavage of PARP. Purified recombinant CMH-1 cleaved PARP but not interleukin 1β precursor in vitro.

Programmed cell death (apoptosis) plays an important role in embryonic development, homeostasis, and in diseases such as neurodegenerative disorders, autoimmune diseases, and cancer (for review, see Ellis et al. (1991)). Apoptosis has been characterized by a set of cellular events including cell shrinkage, chromatin condensation, and DNA fragmentation. It is becoming apparent that activation of proteases is a crucial event in the cellular execution of apoptosis (for review, see Martin and Green (1995)). Genetic studies in the nematode Caenorhabditis elegans have provided evidence that the Ced-3 gene is indispensable for cell death during worm development (Yuan et al., 1993). The Ced-3 protein sequence identity with mammalian interleukin-1β (IL-1β) converting enzyme (ICE) that cleaves the inactive IL-1β precursor to the proinflammatory cytokine (Thornberry et al., 1992). ICE and Ced-3 are members of a new cysteine protease family that also includes mammalian enzymes of T, ICE, MCH2, NEDD-2/C1H-1, CPP32, and MCH2 (Faucheu et al., 1995; Munday et al., 1995; Wang et al., 1994; Kumar et al., 1994; Fernandes-Alnemri et al., 1994, 1995). These cysteine proteases are composed of two subunits of approximately 20 kDa and 10 kDa derived from processing of precursor polypeptides. They share conserved amino acid residues for catalysis and binding of the P1 aspartate residue (Wilson et al., 1994; Walker et al., 1994). Overexpression of cysteine proteases in the ice/Ced-3 gene family leads to apoptosis of transfected cells (Miura et al., 1993; Faucheu et al., 1995; Fernandes-Alnemri et al., 1994, 1995; Gu et al., 1995a; Tewari et al., 1995), and ICE inhibitors block cell death of neurons deprived of neurotrophic factors (Gagliardini et al., 1994; Milligan et al., 1995). ICE or related proteases have also been implicated in Fas- and tumor necrosis factor α-mediated apoptosis (Kuida et al., 1995; Enari et al., 1995; Los et al., 1995; Tewari and Dixit, 1995; Miura et al., 1993), suggesting a physiological role for these cysteine proteases in apoptosis.

Recently, Nicholson et al. (1995) have identified CPP32 as a cysteine protease that is responsible for the cleavage of poly(ADP-ribose) polymerase (PARP), a DNA repair enzyme that is cleaved from a 116-kDa polypeptide into 31-kDa and 85-kDa polypeptides at the onset of apoptosis and in nuclei treated with apoptotic cytosolic extracts (Kaufmann et al., 1993; Lazebnik et al., 1994). Darmon et al. (1995) found that cytotoxic T lymphocyte-specific granzyme B can process and activate CPP32, resulting in PARP cleavage in target cells, suggesting that granzyme B-induced apoptosis may be mediated by a protease activation cascade involving CPP32. Using polymerase chain reaction, Fernandes-Alnemri et al. (1995) identified a second human Ced-3 homolog, MCH2, that is 38% identical with CPP32. Overexpression of MCH2 in insect cells induces apoptosis, and purified MCH2 cleaves PARP in vitro, suggesting a possible role of MCH2 in vertebrate apoptosis. We recently cloned a cDNA encoding a 34-kDa polypeptide that is highly homologous to CPP32 and Mch2. In this report we describe the cloning, expression, and characterization of this new cysteine protease named CMH-1 (CPP32/Mch2 homolog).

**MATERIALS AND METHODS**

Cloning of Cmh-1 cDNA—Sequence data base searching was performed using the BLAST algorithm (Altschul et al., 1990). Initial multiple sequence alignments of known ICE family protein peptide sequences were generated by the MACAW program (Schuler et al., 1991) and later refined by the combination of the PILEUP algorithm (Genetics Computer Group, 1995) and pairwise comparison. A TBLASTN search of the GenBankTM EST data base maintained by the National Center for Biotechnology Information (NCBI) using the ICE peptide sequence revealed limited similarity with the sequence of a clone deposited by the WashU-Merck EST project with an accession number of T50828. The partial human cDNA done 72778 corresponding to this EST sequence was obtained through the IMAGE Consortium (Lawrence Livermore National Laboratory), 5′ rapid amplification of cDNA ends-PCR (polym-erase chain reaction) generated a 1.0-kb fragment of the 5′ region of complete cDNA encoding the novel ICE homolog. In short, a human spleen cDNA pool was ligated with a DNA adapter (Clontech), and PCR was performed using a primer to the 5′ adapter (5′-CCATCCTAATAC- GACTCATATAGGCC) and a primer to the EST T50828 sequence (5′-GCAAAACTCTGTTACATTCCACC). A 0.8-kb Sad/HgaI fragment containing the 5′ coding region and a 1.55-kb HgaI-KpnI fragment from the 72778 clone containing the 3′ region were ligated together and subcloned into pBluescript SK (Stratagene) at Sad/KpnI to generate the full-length cDNA. The sequence of the novel cDNA named Cmh-1 was confirmed on both strands by ABI Prism Dye Deoxy Terminator sequencing using the ABI 373 DNA Sequencer.

**Northern blot Analysis of Cmh-1 mRNA—A human multiple tissue RNA blot (MNT blot, Clontech) containing 2 μg lane poly(A)+ RNA was**
residue peptide (MGSSHHHHHHSSGLVPRGSHMLE), where LVPRGS is the synthesis of a polypeptide of 303 amino acids consisting of a 23-rich Escherichia coli DNA fragment encoding the N-terminally T7-tagged CMH-1 lacking the first 23 amino acid residues was generated by PCR with primers 5'-GCTGAGCTATTGACTGAAGTAGAGTTC and then ligating the primers 5'-GGGCTCGAGCTATTGACTGAAGTAGAGTTC. Amplified DNA was digested with restriction enzymes and subcloned into the XbaI-cut pET-15b inducible expression vector (Novagen). The resulting plasmid directs expression of CMH-1 starting at Ala24, as confirmed by DNA sequencing and by N-terminal sequencing of the expressed proteins. E. coli BL21(DE3) carrying this plasmid was induced with 0.8 mM isopropyl-β-D-thiogalactopyranoside, harvested, and lysed in a microfluidizor (Microfluidic, Watertown, MA) in a buffer containing 20 mM sodium phosphate (pH 8.0), 300 mM NaCl, 2 mM dithiothreitol, 10% glycerol, 0.1 mM phenylmethylsulfonfyl fluoride, and 2.5 μg/ml leupeptin (Buffer A). Lysates were cleared by centrifugation at 100,000 × g for 30 min and the supernatant containing soluble CMH-1 was loaded onto a 0.5-mL nickel-NTA column (Qiagen) and washed with Buffer A extensively. The purified protein fraction contained three major polypeptides of approximately 34 kDa, 22 kDa, and 12 kDa. The N terminus of each of these peptides was analyzed using the ABI 477A Protein Sequencer. Cleavage of 35S-labeled IL-1β precursor and a truncated form of 35S-PARP by purified ICE or CMH-1 in vitro were carried out as described (Gu et al., 1995b).

RESULTS AND DISCUSSION

Cloning of Cmh-1, a Close Homolog of CPP32/Mch2—A TBLASTN search of the GenBank™ data base using the ICE peptide sequence revealed that an EST (expressed sequence tag) clone with the accession number T50828 showed limited sequence similarity. The clone corresponding to this sequence, human clone 72778, was obtained through the IMAGE Consortium, and its sequence was verified. The cDNA insert of clone 72778 contains a partial open reading frame for a novel ICE-like protein and 3'-untranslated region. Using rapid amplification of cDNA ends-PCR, we cloned the 5'-end of the full-length cDNA fragment from the clone 72778 to generate the full-length cDNA (Takebe et al., 1995). Expression, Purification, and Characterization of Recombinant (His)6-tagged CMH-1 Protein—An expression plasmid for the N-terminally His6-tagged CMH-1 (Ala24-Gln303) was constructed by introducing Xhol sites at the 5' and 3' ends of CMH-1 cDNA by PCR using primers 5'-GCTGAGCTATTGACTGAAGTAGAGTTC and 5'-GCTGAGCTATTGACTGAAGTAGAGTTC. Amplified DNA was digested with XbaI and subcloned into the XbaI-cut pCDLar vector (Takebe et al., 1988). The C186S/W232A/R233E mutant was generated by site-directed mutagenesis using oligonucleotides 5'-GGGCTCGAGCTATTGACTGAAGTAGAGTTC and 5'-GC-
The results suggest that the T7-tagged truncated CMH-1 protein is capable of autoprocessing itself into subunits equivalent to the P20 and P10 polypeptides of ICE in COS cells.

To determine if CMH-1 protein expressed in COS cells may have other detectable protease activity, we co-expressed CMH-1 in COS cells with a truncated form of PARP, a known substrate for CPP32 and other ICE-like proteases both in vivo and in vitro (Nicholson et al., 1995; Gu et al., 1995b). We found most of the 45-kDa PARP polypeptide was cleaved into a 31-kDa peptide when it is co-expressed with CMH-1, similar to the cleavage of PARP by ICE, Tx, and Nedd2. This cleavage was abolished when the mutant CMH-1 was used, indicating that the cleavage is specific and that CMH-1 encodes a protease that can either cleave PARP directly or activate an endogenous protease(s) that in turn cleaves PARP. A small amount of PARP cleavage was also observed when COS cells were transfected with PARP alone; this is most likely due to the presence of (an) endogenous ICE-like protease(s) in the cells.

Overexpression of the CMH-1 protein in COS cells also caused cells to round up, a sign of transfected cells undergoing apoptosis. To confirm this, we analyzed chromosomal DNA from cells transfected with the wild type CMH-1 and found internucleosomal DNA fragmentation (Fig. 2B). DNA fragmentation was absent from mock-transfected cells or cells transfected with the mutant CMH-1 (Fig. 2B). Thus, like other ICE-like proteases, CMH-1 is capable of inducing apoptosis when overexpressed in cells.

Expression and Purification of Recombinant CMH-1—To further characterize the activity of CMH-1 protein, we expressed an N-terminally (His)6-tagged CMH-1(Ala24-Qln303) in E. coli and purified the protein by Ni-affinity chromatography. The purified protein fraction contained three major polypeptides of approximately 34 kDa, 22 kDa, and 12 kDa (Fig. 3). An 11-kDa polypeptide was also present in some preparations. Immunoblotting with anti-(His)6-tag antibody and N-terminal sequencing analysis of these polypeptides indicated that the 34-kDa and 22-kDa polypeptides contain the (His)6-tag fused to the CMH-1 at Ala24 (Fig. 3). The N-termini of the 12-kDa and 11-kDa polypeptides start at Ser199 and Ala207 of CMH-1, respectively. Both residues are preceded by an aspartate, suggesting that 12-kDa and 11-kDa polypeptides are generated by processing of the CMH-1 protein at Asp206Ser199 and to a lesser extent at Asp206-Ala207. We propose that the 22-kDa and 12-kDa polypeptides represent the two subunits of the CMH-1 protease.

CMH-1 Is a CPP32-like Cysteine Protease That Cleaves PARP but Not IL-1 β Precursor—We further analyzed the protease activity of the purified CMH-1 protein using 35S-labeled PARP or IL-1β precursor prepared by in vitro transcription and translation as substrates. We found that CMH-1 at less than 3 nM concentration almost completely cleaved the PARP substrate within 30 min (Fig. 4A). No cleavage of IL-1β precursor was observed under similar conditions, indicating that CMH-1, like CPP32, has a preferred substrate specificity for PARP. The efficiency of CMH-1 cleaving PARP appears to be significantly higher than that observed for ICE in cleaving the same substrate under identical conditions (Gu et al., 1995b) and is close to the activity of CPP32 against PARP2 or ICE

\[ \text{Y. Gu, J. A. Lippke, C. Sarnecki, and M. S.-S. Su, unpublished results.} \]
against IL-1β precursor (Gu et al., 1995b). This deactivation activity of CMH-1 was inhibited by the tetrapeptide inhibitor DEVD-CHO (Nicholson et al., 1995; Wang et al., 1995) as well as the cysteine-alkylating reagents N-ethylmaleimide and iodoacetamide, but not by the ICE inhibitor YVAD-CHO (Thornberry et al., 1992; Gu et al., 1995b) or inhibitors of serine proteases or metalloproteases (Fig. 4B). Interestingly, CMH-1 also appears to be relatively insensitive to inhibition by crmA (cytokine response modifier A), a very potent ICE inhibitor synthesized by cowpox virus (Ray et al., 1992). At a crmA concentration of 16 μg/ml which is about 50-fold higher than that required to completely inhibit an equivalent amount of ICE activity. no inhibition of CMH-1 was observed. These results indicate that CMH-1 is a cysteine protease with properties closer to CPP32 than to ICE, consistent with its relative sequence identity to these two proteases.

CPP32 has been implicated to play a major role in apoptosis and cytotoxic T lymphocyte-mediated cell killing as well as cellular regulation of sterol metabolism (Nicholson et al., 1995; Darmon et al., 1995; Wang et al., 1995). CMH-1 and CPP32 share high amino acid similarity and conserved S1, S2, and S4 residues in the active site pocket. This sequence conservation might allow these two proteases to share an overlapping substrate profile and to have similar roles in apoptosis and other physiological processes. It is equally likely that CMH-1 protease has different preferred substrates that remain to be identified. The expression and purification of active CMH-1 protease should facilitate the characterization of the enzyme and its substrates.
Identification and Characterization of CPP32/Mch2 Homolog 1, a Novel Cysteine Protease Similar to CPP32
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