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MCL-1S, a Splicing Variant of the Antiapoptotic BCL-2 Family Member MCL-1, Encodes a Proapoptotic Protein Possessing Only the BH3 Domain*

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MCL-1 (myeloid cell leukemia-1) is an antiapoptotic BCL-2 family protein discovered as an early induction gene during myeloblastic leukemia cell differentiation. This survival protein has the BCL-2 homology (BH) domains 1, 2, and 3 and a C-terminal transmembrane region. We identified a short splicing variant of the *MCL-1* mRNA in the human placenta encoding a protein, termed MCL-1 short (MCL-1S), with an altered C terminus as compared with the full-length MCL-1 long (MCL-1L), leading to the loss of BH1, BH2, and the transmembrane domains. Analysis of the human *MCL-1* gene indicated that MCL-1S results from the splicing out of exon 2 during mRNA processing. MCL-1S, unlike MCL-1L, does not interact with diverse proapoptotic BCL-2-related proteins in the yeast two-hybrid system. In contrast, MCL-1S dimerizes with MCL-1L in the yeast assay and coprecipitates with MCL-1L in transfected mammalian cells. Overexpression of MCL-1S induces apoptosis in transfected Chinese hamster ovary cells, and the MCL-1S action was antagonized by the antiapoptotic MCL-1L. Thus, the naturally occurring MCL-1S variant represents a new proapoptotic BH3 domain-only protein capable of dimerizing with the antiapoptotic MCL-1L. The fate of MCL-1-expressing cells could be regulated through alternative splicing mechanisms and interactions of the resulting anti- and proapoptotic gene products.

Apoptotic cell death is critical for the maintenance of tissue homeostasis in a healthy organism as well as for pathogenesis during disease states including cancer, neurodegenerative disorders, autoimmune diseases, and viral infection (1). It is becoming clear that BCL-2 proteins play a pivotal role in the intracellular mechanisms of apoptosis regulation. The *bcl-2* gene was first isolated as a proto-oncogene at the breakpoint of a t(14,18) chromosomal translocation associated with follicular B-cell lymphoma (2, 3). This protein is localized to the mitochondria, perinuclear membrane, and smooth endoplasmic reticulum (4, 5). When overexpressed, the BCL-2 protein suppresses apoptosis induced by a variety of agents both *in vivo* and *in vitro* (6, 7). By using homologous gene isolation, protein-protein interaction screening, and subtraction cloning, an expanding family of BCL-2-related proteins has been identified in organisms from viruses to mammals (8, 9).

The members of the BCL-2 family can be subdivided into antiapoptotic proteins such as BCL-2, BCL-xL, and BCL-w and proapoptotic proteins such as BAX, BOD, BOK, and BAD. It has been proposed that anti- and proapoptotic BCL-2 proteins regulate cell death by binding to each other and forming heterodimers (10, 11). A delicate balance between anti- and proapoptotic BCL-2 family members exists in each cell, and the relative concentration of these two groups of proteins determines whether the cell survives or undergoes apoptosis. *MCL-1* was first discovered as an early induction gene during the differentiation of a human myeloid leukemia cell line (12). Although the MCL-1[1] protein possesses the BCL-2 homology (BH)1, BH2, BH3, and transmembrane (TM) domains found in other BCL-2-related proteins, it is distinguished by a unique PEST sequence in its N-terminal region. Subsequent studies established MCL-1 as an antiapoptotic BCL-2 family protein with an expression pattern differing from that of BCL-2 and capable of suppressing cell death induced by various stimuli including growth factor deprivation and exposure to chemotherapeutic agents or UV irradiation (13, 14).

In addition to having evolved into a family of homologous proteins with distinct functions, some of the *bcl-2*-related genes encode different isoforms that vary in subcellular localization, function, and levels of expression (15). For example, both the *bcl-2* and *bax* genes encode a dominant form containing a TM domain and a short splicing variant lacking this region (10, 16). The BCL-2 variant, without the TM domain, appears to have either decreased or no antiapoptotic activity and a different subcellular localization (5, 17). In addition, multiple splicing variants for the *bcl-x* gene have been identified, and the BCL-xS variant lacks the BH1 and BH2 domains but retains the TM region. Although it does not possess proapoptotic activity in the absence of an antiapoptotic signal, BCL-xS antagonizes the survival action of BCL-xL when BCL-xS is stably transfected (18, 19). When expressed at high levels using a BCL-xS adenovirus system, apoptosis was selectively induced in cancerous but not normal bone marrow cells (20). Here, we

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF203373, AF162676, and AF162677.

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[1] The abbreviations used are: MCL-1, myeloid cell leukemia-1; BH domain, BCL-2 homology domain; TM, transmembrane; MCL-1L, MCL-1 long; MCL-1S, MCL-1 short; BAC, bacterial artificial chromosome; PCR, polymerase chain reaction; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; bp, base pair; EST, expressed sequence tag; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine.

identified a novel apoptosis-regulatory mechanism in which the alternative splicing of *MCL-1* leads to the generation of both anti- and proapoptotic proteins capable of heterodimerization. The short form of MCL-1 (MCL-1S), unlike the originally identified antiapoptotic MCL-1 long (MCL-1L), is a BH3 domain-only proapoptotic protein, and its action is antagonized by MCL-1L.

EXPERIMENTAL PROCEDURES

Identification of a Novel Splicing Variant of Human MCL-l cDNA—The expressed sequence tag (EST) division of the GenBank™ data base was searched using the human MCL-1 cDNA sequence as a query. This search identified a total of three independent partial nucleotide sequences from an ovary tumor library (GenBank™ accession number AA464453) and a germinal center B-cell library (GenBank™ accession numbers AA521010 and AA749362) that were homologous to the known human MCL-1 cDNA except for a deletion of 248 nucleotides from the coding sequence (nucleotide numbers 689–936). The original cDNA clones from which these ESTs had been derived were obtained from the IMAGE consortium (Genome Systems, St. Louis), and their sequence was verified.

Cloning of cDNAs Encoding MCL-1S—Reverse transcription of human placenta total RNA (CLONTECH, Palo Alto, CA), total RNA isolated from cultured human placenta cell line Hs 732.Pl (ATCC, Manassas, VA), or human myeloid leukemia cell line K-562 (ATCC) was performed using oligo$(dT)_{18}$ primer and recombinant Moloney-murine leukemia virus reverse transcriptase as described in the manufacturer's protocol (CLONTECH). For polymerase chain reaction (PCR) amplification of MCL-1 cDNAs, the DNA amount equivalent to 0.1 μg of total RNA was used in a 50-μl reaction. As a control, the same PCR was performed in the absence of cDNAs. The primers used were 5′-AGGAATTCGATGTTTGGCCTCAAAAGAAACGCGGTA-3′ and 5′-GAATTCGGAAGTTACAGCTTGGAGTCCAACTGC-3′. The latter primer corresponds to sequences of 78 or 89 nucleotides downstream from the predicted stop codon of MCL-1S and MCL-1L, respectively. MCL-1 cDNAs were amplified by 35 thermal cycles (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min) from the human placenta cDNAs using *Pfu* DNA polymerase (Stratagene, La Jolla, CA). The predicted MCL-1S band was eluted and purified using a gel extraction kit (Qiagen, Chatsworth, CA), and the cDNA was sequenced. The sequences of the cDNA from human placenta matched with the postulated MCL-1 splicing variant from the EST data base, and the cDNA was named as MCL-1S. The known MCL-1 product was renamed MCL-1L.

Genomic Structure of Human MCL-1—The Genomic Survey Sequence division of the GenBank™ data base was searched for genomic sequences representing the human *MCL-1* gene. A Genomic Survey Sequence (accession number AQ192046) was found, and the original BAC clone was sequenced to verify its identity (Research Genetics, Huntsville, AL). In order to determine the structure of the human *MCL-1* gene, the BAC clone was additionally sequenced using custom primers derived from parts of the coding sequence adjacent to the exon-intron junctions predicted from the MCL-1S sequence.

Analysis of MCL-1S Interactions with Diverse Anti- and Proapoptotic Proteins in the Yeast Two-hybrid System—To study the interactions between two human MCL-1 variants (MCL-1L and MCL-1S) and diverse other BCL-2 family proteins, cDNAs encoding MCL-1L and MCL-1S were subcloned into the *Eco*RI site of the activation domain of GAL4 in a yeast shuttle vector, pGADGH, and cDNAs encoding various pro- and antiapoptotic BCL-2 family proteins were fused to the GAL-4 binding domain of the pGBT9 vector (CLONTECH) (21). Specific binding of different protein pairs was evaluated based on the activation of the *GAL1-HIS3* reporter gene in medium lacking leucine, tryptophan, and histidine but in the presence of 30 mM 3-aminotriazole (22). At least 10 different colonies expressing each pair of fusion protein were tested.

Assessment of Apoptosis in Transfected CHO Cells—Apoptosis was monitored following transfection of different cDNAs as described previously (21) with modifications. Chinese hamster ovary (CHO) cells (2×10^5 cells/35-mm well) were cultured in Dulbecco's modified Eagle's medium/F-12 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. After 24 h of incubation, CHO cells were transfected using the LipofectAMINE procedure (Life Technologies, Inc.) with the pcDNA3 expression vector (Invitrogen, Carlsbad, CA) with or without different cDNA inserts, together with 0.1 amounts of the indicator plasmid pCMVβ (CLONTECH) to allow the identification of transfected cells. Inclusion of a 10-fold excess of expression vectors as compared with the pCMVβ reporter plasmid ensured that most of the β-galactosidase-expressing cells also expressed the protein(s) under investigation. After a 4-h incubation of cells with the transfection mixtures in a serum-free medium, the medium was replaced with the fresh medium containing 10% fetal bovine serum and changed again 24 h later. Forty eight h after transfection, cells were fixed in 0.3% glutaraldehyde and stained with 0.4 mg/ml 5-bromo-4-chloro-3-indolyl-D-galactoside (Life Technologies, Inc.) to detect β-galactosidase expression. The number of blue cells was counted by microscopic examination. Data were expressed as the percentage (mean ± S.E.) of viable cells as compared with the control group.

Analysis of in Vivo Binding of MCL-1L and -S—After 24 h of culture, CHO cells (1.5×10^6) were transfected with 3 μg each of pcDNA3-MCL-1L or -MCL-1S, alone or together with FLAG epitope-tagged MCL-1S or MCL-1L, using the LipofectAMINE procedure. CHO cells were harvested at 24 h posttransfection and lysed in prechilled 1% Nonidet P-40 lysis buffer containing 10% of a eukaryotic cell protease inhibitor mixture (Sigma). In a control group, cells were transfected with MCL-1S together with FLAG-tagged BCL-xL. Following 30 min of incubation in the lysis buffer, the lysates were centrifuged at $10,000 \times g$ for 10 min at 4 °C, and the supernatants were collected. Aliquots of the lysates were precleared by incubation with normal mouse IgG and protein A-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at 4 °C. The precleared lysates were then incubated with 1 μg/ml anti-mouse FLAG M2 monoclonal antibody (Sigma) for 2 h and subsequently incubated with protein A-agarose for an additional 2 h at 4 °C. The immune complexes were centrifuged for 5 min at $2,000 \times g$ and washed five times with 1% Nonidet P-40 lysis buffer.

The immunoprecipitates and aliquots of total lysates were boiled in SDS sample buffer for 5 min, subjected to 10% Tricine/SDS-polyacrylamide gel electrophoresis (PAGE) (23), and electroblotted onto 0.22-μm nitrocellulose membranes. The membrane was blocked in 5% nonfat dry milk in Tris-buffered saline solution with 1% Tween 20 for 1 h followed by incubation with 0.2 μg/ml of the anti-rabbit MCL-1 polyclonal antibody (Santa Cruz Biotechnology) for 1 h at room temperature. The blot was then incubated for 10 min with 0.1 μg/ml of anti-rabbit IgG-horseradish peroxidase conjugate (Promega, Madison, WI) as a secondary antibody, before visualization by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). The same membrane was stripped and incubated with 0.2 μg/ml of anti-mouse FLAG M2 monoclonal antibody (Sigma) for 1 h at room temperature. The blot was exposed to the same secondary antibody and visualized by ECL. Due to the instability of MCL-1L and MCL-1S when expressed alone, twice the amount of total protein was loaded into SDS-PAGE. Also, five times of total protein was loaded when MCL-1S was coexpressed with FLAG-tagged BCL-xL.

RESULTS

Identification of MCL-1 Splicing Variants and Isolation of MCL-1S—The existence of a short variant of MCL-1 was postulated based on sequences from the EST data base of GenBank™ identical to the known human MCL-1 cDNA except for the deletion of 248 nucleotides near the 3′-end of the coding sequence. To confirm the existence of this novel splicing variant, the MCL-1S cDNA was amplified from human placenta, a human placental cell line Hs 732.Pl, and a human myeloid leukemia cell line K-562 following reverse transcription-PCR using specific MCL-1 primers. As shown in Fig. 1*A*, a minor transcript of 907 bp was amplified together with the known MCL-1 transcript of 1155 bp (*lane 2,* human placenta; *lane 4,* placenta cell line; *lane 5,* myeloid leukemia cell line). We named the truncated transcript as MCL-1S (short) to distinguish it from the known MCL-1L (long) transcript. The deduced amino acid sequence of MCL-1S encodes a protein of 271 amino acids long as compared with MCL-1L which has 350 amino acids (Fig. 1*B*). Comparison of the two human splicing variants of MCL-1 indicated that the missing nucleotide stretch leads to a shift in the reading frame of the deduced protein after the BH3 domain. As a result, the short transcript lacks the BH1, BH2, and TM domains. Of interest, the unique C terminus of MCL-1S, a stretch of 41 amino acid residues, contains six cysteine and multiple basic residues, likely to be important for secondary structures.

Structure of the Human MCL-1 Gene and the Predicted Derivation of Splicing Variants—To understand the transcrip-

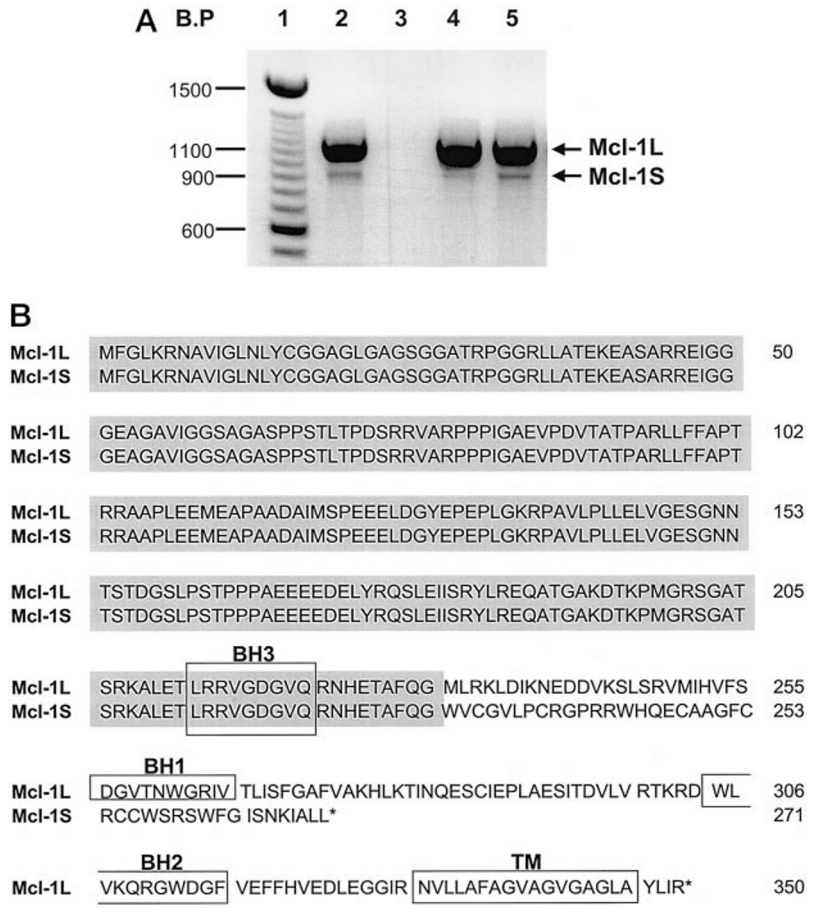

FIG. 1. **Characterization of the MCL-1S transcript from human placenta and myeloid cells: comparison of the deduced sequence and domain arrangement between MCL-1S and MCL-1L.** *A,* reverse transcription-PCR amplification of MCL-1S and MCL-1L transcripts expressed in human placenta and myeloid leukemia cells. Molecular weight markers are shown in *lane 1. Lanes 2, 4,* and *5* show MCL-1S (907 bp) and MCL-1L (1155 bp) cDNAs amplified from human placenta, a placental cell line Hs 732.Pl, and myeloid leukemia cell line K-562, respectively. *Lane 3* shows the lack of a PCR product under the same reaction conditions in the absence of cDNA template. *B,* comparison of deduced amino acid sequences for MCL-1L and MCL-1S. Identical residues of MCL-1L and MCL-1S are *shaded.* The open reading frame for MCL-1S predicts a protein of 271 amino acids in length, whereas MCL-1L consists of 350 amino acids. The BH1, BH2, and TM domains are missing in MCL-1S, resulting in a novel BH3 domain-only BCL-2 family protein. The GenBank™ accession number for MCL-1S is AF203373.

tional mechanisms underlying the derivation of the MCL-1S transcript, a BAC clone containing the human *MCL-1* gene was identified from the Genome Survey Sequence division of the GenBank™. This clone was obtained and sequenced to reveal the intron-exon junctional sequences (Fig. 2*A*) that conform to the so-called GT-AG rule (24). This analysis revealed that the coding sequence of MCL-1L is derived from three exons, separated by introns of 351 bp and more than 1-kilobase pair in size, respectively. Based on the intron-exon structure, it is apparent that MCL-1S results from the splicing out of the second exon of the gene, leading to a frameshift downstream from the BH3 domain and the loss of the BH1, BH2, and TM domains in the translated product (Fig. 2*B*).

MCL-1L Interacts with Diverse Proapoptotic BCL-2 Family Proteins, Whereas MCL-1S Only Dimerizes with MCL-1L in the Yeast Two-hybrid System—The dimerization properties of human MCL-1S and MCL-1L with different pro- and antiapoptotic BCL-2 members were assessed in the yeast two-hybrid system (Fig. 3). In agreement with our earlier report using rat MCL-1(L) (25), human MCL-1L interacted strongly with the proapoptotic BCL-2 family members BAX, BAK, and BOD/BIM isoforms but showed negligible interactions with BCL-xL, BCL-2, and BCL-w in the yeast system. In addition, MCL-1L did not homodimerize in yeast cells. In contrast, MCL-1S showed negligible interaction with any of the pro- or antiapoptotic BCL-2 family members tested with the exception of MCL-1L (of either human or rat origin). MCL-1S also did not homodimerize in the yeast system (data not shown). These data suggest that the two splicing variants of MCL-1 could form heterodimers.

Coprecipitation of MCL-1S and MCL-1L in Mammalian Cells—To confirm the dimerization of MCL-1S and MCL-1L in mammalian cells *in vivo*, we further investigated the binding between the MCL-1 splicing variants in CHO cells transiently transfected with the MCL-1S and MCL-1L cDNAs. As shown in Fig. 4, immunoprecipitation experiments indicated that MCL-1L could be coprecipitated with MCL-1S tagged with a FLAG epitope when the cell lysate was treated with the M2 antibody against the FLAG epitope. Likewise, MCL-1S was coprecipitated with MCL-1L when the latter protein was tagged with the FLAG epitope. In both tests, comparable amounts of the two splicing variants were detected using the MCL-1 antibody in immunoblots, suggesting a stoichiometric interaction between these proteins. In contrast, MCL-1S did not coprecipitate with FLAG-tagged BCL-xL, demonstrating the specificity of MCL-1S binding to MCL-1L. Of interest, when MCL-1S and MCL-1L were coexpressed, higher amounts of both proteins were detected, suggesting an enhancement of protein stabilization.

MCL-1S Is a Proapoptotic Protein Capable of Being Antagonized by the Survival Action of MCL-1L—It has been shown previously that the region encompassing the BH1 and BH2 domains of BCL-2 proteins is important for pore formation and for heterodimerization with other BCL-2 members (26, 27), whereas the TM region is important for the anchoring of BCL-2 proteins to mitochondria and other cellular organelles (10, 18, 28). Several BH3 domain-only BCL-2 family proteins were found to be proapoptotic "ligands" capable of suppressing the function of membrane-bound antiapoptotic BCL-2 proteins following heterodimerization (11, 29, 30). Because the MCL-1S protein lacks the pore-forming region and the TM domains but retains the BH3 dimerization domain, we tested the role of MCL-1S as a proapoptotic BH3-only protein. As shown in Fig. 5*A*, transfection of increasing amounts of the MCL-1S expression plasmid into CHO cells led to dose-dependent cell killing. The proapoptotic effect of MCL-1S was blocked following cotransfection with P35, a pan-specific caspase inhibitor of

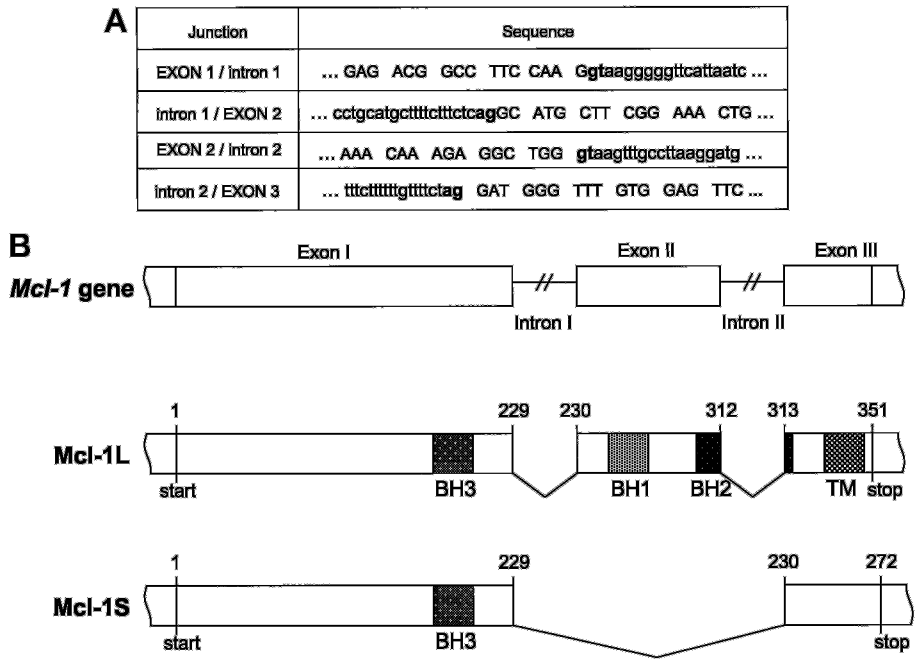

FIG. 2. **Organization of the human *MCL-1* gene and the postulated derivation of MCL-1S following alternative splicing.** *A,* partial genomic sequence of the *MCL-1* gene in the junctions of exon 1/intron 1, intron 1/exon 2, and exon 2/intron 2. Exon nucleotides of the human *MCL-1* gene are shown in *uppercase letters*, and 5′- and 3′-splice site sequences are shown in *lowercase letters*, with the GT and AC dinucleotides in *boldface type. B,* diagrammatic representation of the human *MCL-1* gene and the proposed derivation of two MCL-1 splicing variants. The regions of conserved BH domains (*BH1–3*) and a TM anchor region are depicted. Corresponding amino acid residue numbers are also shown. *1* denotes the start codon.

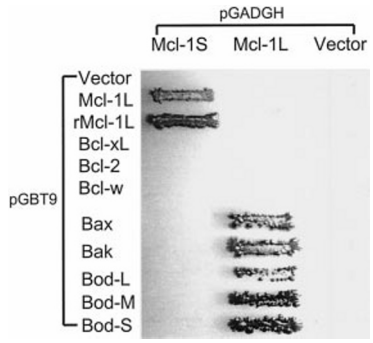

FIG. 3. **Differential interaction of MCL-1S and MCL-1L with diverse antiapoptotic and proapoptotic BCL-2 proteins in the yeast two-hybrid system and dimerization between MCL-1S and MCL-1L.** Yeast cells were grown in selective media containing 30 mM 3-aminotriazole and lacking Trp, Leu, and His. The prominent growth of colonies expressing MCL-1S and human or rat (*r*) MCL-1L fused to the GAL4 activation domain and binding domain, respectively, indicates a strong interaction between the fusion proteins in the yeast assay. The BCL-2 proteins tested also include the antiapoptotic BCL-xL, BCL-2, and BCL-w as well as the proapoptotic BAX, BAK, and three BOD variants. No growth of yeast colonies was found in cells expressing only MCL-1L, rat MCL-1L, BCL-xL, BCL-2, BCL-w, BAX, BAK, or BOD variants, ruling out self-activation of these constructs. The picture is a representative of 10 different colonies tested.

baculoviral origin (31), suggesting MCL-1S-induced cell killing is mediated by downstream effector caspase. Furthermore, cotransfection with different amounts of the plasmid encoding MCL-1L blocked the proapoptotic effect of MCL-1S, suggesting antagonistic actions between the two splicing variants of the *MCL-1* gene (Fig. 5*B*). MCL-1S-induced cell killing was completely inhibited by MCL-1L at a plasmid ratio of 3:1. In contrast, two antiapoptotic BCL-2 proteins (BCL-xL and BCL-w) not capable of interacting with MCL-1S were less effective in antagonizing the action of MCL-1S (Fig. 5*B*). At a high plasmid concentration (0.7 μg/culture), both BCL-xL and BCL-w reduced the number of viable cells in the present assay. In contrast to its complete antagonism of MCL-1S action, MCL-1L was minimally effective in antagonizing the action of another BH3 domain-only protein BOD-L even when 700-fold excess of MCL-1L plasmid was used (Fig. 5*B*).

DISCUSSION

We have isolated a naturally occurring alternatively spliced variant of MCL-1 that has different structural features and a diametrically opposing action as compared with the known antiapoptotic MCL-1 protein, now named MCL-1 long (L). The short splicing variant, MCL-1S, originally identified in germinal center B-cell and ovarian tumor cDNA libraries as partial sequences, and isolated as a full-length coding sequence from both human placenta and human myeloid leukemia cells, results from the deletion of 248 nucleotides from the full-length MCL-1L cDNA. A shift in the open reading frame leads to the complete loss of the BH1, BH2, and TM domains and the derivation of an altered C-terminal sequence. The two splicing variants of the *MCL-1* gene are capable of forming dimers, and the proapoptotic action of MCL-1S can be antagonized by MCL-1L.

Based on their structural and functional attributes, BCL-2 proteins can be divided into three subgroups as follows: 1) the antiapoptotic channel-forming BCL-2 proteins with three or four BH domains (BH1 to 3/4) and a TM anchor sequence; 2) the proapoptotic channel-forming proteins with BH1 to BH3 domains; and 3) the proapoptotic ligands containing only the BH3 domain (9, 32, 33). The first two subgroups of proteins are believed to be anchored to the outer mitochondrial membrane, whereas the third subgroup of proteins acts as ligands that dimerize with the membrane-anchored, channel-forming BCL-2 "receptors" (34, 35). The BH3 domains in the third subgroup are essential for the binding activity of these ligands. MCL-1S is missing the BH1, BH2, and TM domains but retains the BH3 domain, whereas MCL-1L contains all of these domains. Thus, MCL-1S resembles other proapoptotic BH3 domain-only family proteins, and the *MCL-1* gene encodes proteins belonging to two of the three subgroups of BCL-2 family proteins. Overexpression of MCL-1S decreases the viability of CHO cells in a dose-dependent manner, and its cell-killing effect is mediated by caspase activation because P35 effectively inhibits the proapoptotic activity of MCL-1S. Furthermore, the apoptotic action of MCL-1S was completely antagonized by MCL-1L but partially blocked by noninteracting proteins BCL-xL and BCL-w. In addition to the derivation of proteins of opposing functions, the alternative splicing of MCL-1S could also utilize overlapping transcriptional machinery for the synthesis of MCL-1L, leading

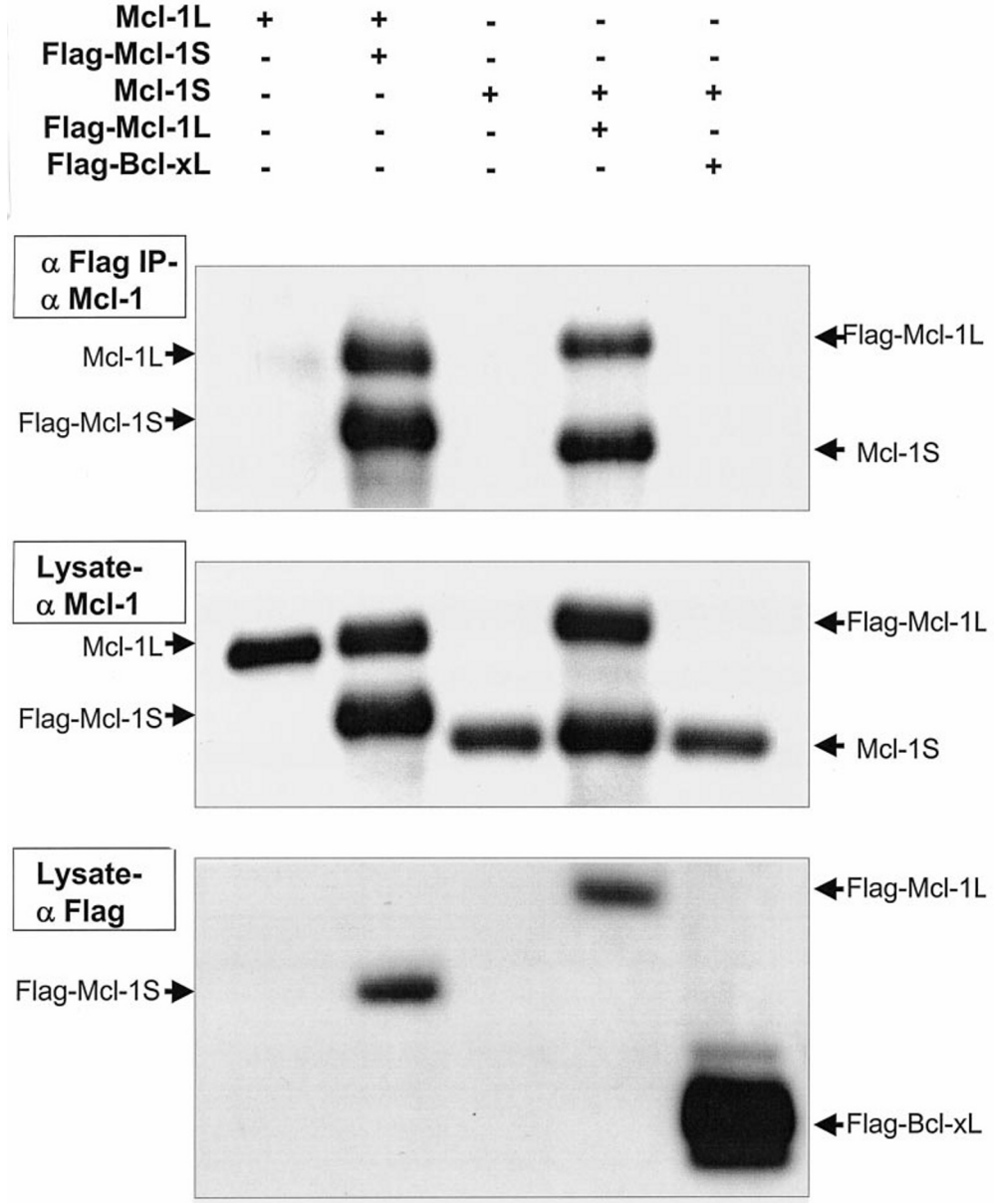

FIG. 4. ***In vivo* dimerization of the two MCL-1 variants in mammalian cells.** Binding between MCL-1 variants expressed in CHO cells was tested by immunoprecipitation. Cells were transfected with MCL-1L with or without FLAG-tagged MCL-1S as indicated. Conversely, cells were transfected with MCL-1S with or without FLAG-tagged MCL-1L. As a negative control, MCL-1S was also cotransfected with FLAG-tagged BCL-xL. Equal amounts (3 μg) of different MCL-1 constructs or FLAG-tagged BCL-xL were used. At 24 h after transfection, the cell lysate was used either for immunoblots using a specific antibody to determine MCL-1 protein expression (*middle panel*) or for immunoprecipitation (IP) tests using M2 antibodies against the FLAG epitope. Following immunoprecipitation of tagged proteins, immunoblotting with MCL-1 antibodies was performed to demonstrate the interaction between MCL-1L and MCL-1S (*upper panel*). The membrane used for immunoblotting with anti-MCL-1 antibody was also used for the detection of FLAG-tagged proteins using M2 antibodies (*lower panel*). Due to the instability of MCL-1L and MCL-1S when expressed alone, twice the amount of total protein was loaded into SDS-PAGE. Also, five times of the total protein was loaded when MCL-1S was coexpressed with FLAG-tagged BCL-xL.

to decreased levels of MCL-1L transcripts.

In eukaryotic cells, MCL-1L was capable of interacting with other proapoptotic BCL-2 family proteins (BAX, BAK, and BOD variants) but not with antiapoptotic proteins (BCL-2, BCL-w, and BCL-xL), as demonstrated by the yeast two-hybrid assay. In contrast, MCL-1S did not interact with any other member of BCL-2 family that was tested but exhibited a strong interaction with MCL-1L in the yeast assay. Furthermore, the binding between MCL-1S and MCL-1L was confirmed in mammalian cells based on immunoprecipitation tests. The observed heterodimerization appears to be stoichiometric because comparable amounts of MCL-1L and MCL-1S proteins were detected following coprecipitation. These findings suggest that the balance between antiapoptotic MCL-1L and proapoptotic MCL-1S could determine the fate of cells that express both proteins.

Dimer formation between pro- and antiapoptotic BCL-2 family proteins, mediated by their highly conserved BH domains, is thought to serve as a "rheostat" for the determination of cell fate through mutual antagonism. The observations that MCL-1L interacts widely with different proapoptotic BCL-2-related proteins, whereas MCL-1S interacts only with MCL-1L, suggest that the alternative splicing mechanism in an MCL-1L-expressing cell could be regulated under certain physiological or pathological conditions thus leading to the synthesis of the proapoptotic MCL-1S protein to induce cell death. We tested possible changes in MCL-1 variant expression using both the human placental cell line Hs732.P1 and the human

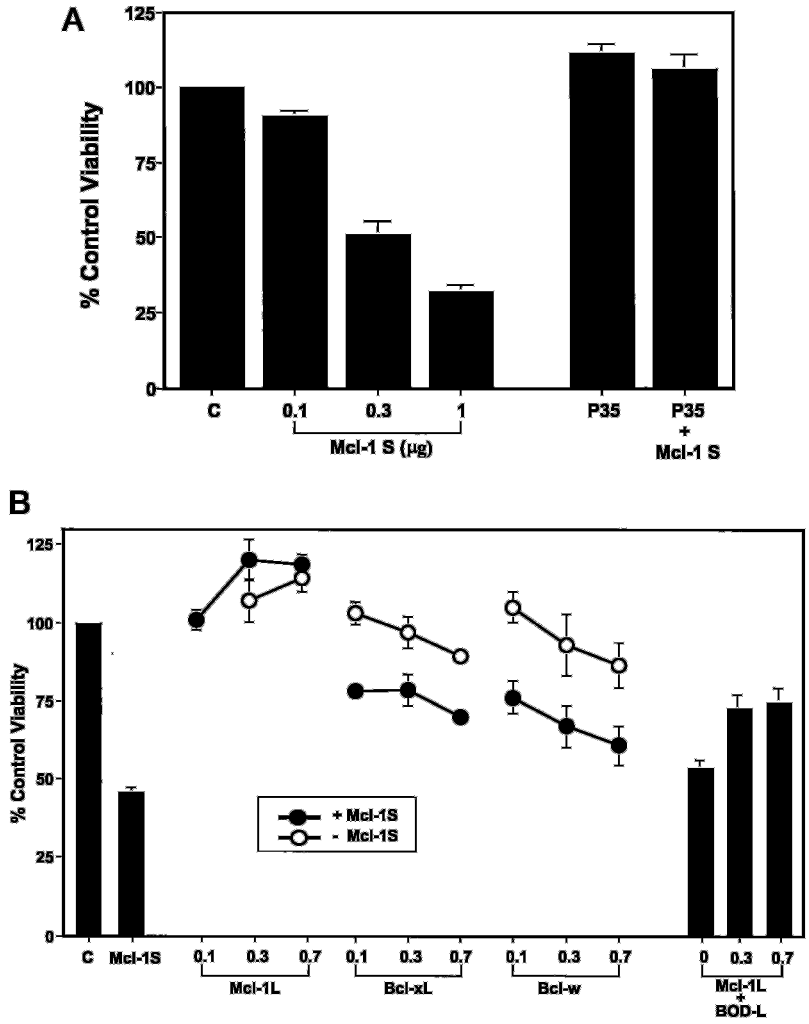

FIG. 5. **MCL-1S is a proapoptotic protein in mammalian cells: blockage of MCL-1S-induced cell killing by MCL-1L and the caspase inhibitor P35.** Quantitative analysis was performed to test cell killing by MCL-1S and the antagonistic effects of MCL-1L and P35. All CHO cells were transfected with a total of 1.1 μg of plasmid DNA including 1.0 μg of pcDNA3 expression constructs and 0.1 μg of the pCMV-β-gal reporter. The number of β-gal-expressing cells was determined at 48 h after transfection. Data (mean ± S.E., $n = 9$) are from three different experiments performed as triplicates. For the control (*C*) group, 1.0 μg of the empty pcDNA3 vector was used. *A,* dose dependence of MCL-1S-induced cell killing. When increasing amounts (0.1, 0.3, or 1 μg) of MCL-1S were used for transfection, the empty vector was added to ensure the use of the same amount (1.1 μg) of total plasmid. To determine that MCL-1S-induced cell death is mediated by caspases, cells were transfected with a mixture containing 0.3 μg of MCL-1S and 0.7 μg of P35. *B,* antagonism of MCL-1S-induced cell killing by MCL-1L. In all experiments, 0.3 μg of plasmid coding MCL-1S was used. Apoptosis induced by MCL-1S was dose-dependently blocked by MCL-1L (0.1, 0.3, or 0.7 μg). In contrast, 0.1, 0.3, and 0.7 μg BCL-xL and BCL-w were less effective in blocking the cell killing induced by MCL-1S. At 0.7 μg, both BCL-xL and BCL-w led to nonspecific cell killing. Furthermore, BOD-L (0.001 μg) was capable of inducing cell killing, but the BOD-L action was minimally prevented by MCL-1L (0.3 or 0.7 μg).

myeloid leukemia cell line K-562. The ratio of MCL-1L/MCL-1S mRNAs was determined by reverse transcription-PCR after treatment of cultured cells with staurosporine or etoposide or after serum deprivation. However, these treatments did not affect the ratio of MCL-1L/MCL-1S mRNA levels. In addition, endogenous MCL-1S protein expression in the immature rat ovary, spleen, and uterus could not be detected in immunoblots (data not shown). Future studies are needed to identify the mechanisms and conditions regulating the alternative splicing of the *MCL-1* gene.

Analysis of *MCL-1* gene genomic structure and its comparison with MCL-1L and MCL-1S cDNAs indicate the skipping of exon 2, which is also observed in other BCL-2 family members such as the variants of BCL-2, BOK, and BAX (10, 16, 36). Alternatively spliced products have been found for several BCL-2-related proteins, caspases, and death receptors in the apoptosis regulatory pathway (15). BCL-2 exists in two forms as the result of alternative splicing, BCL-2α and BCL-2β (16). The mouse *bcl-x* gene encodes five variants, BCL-xL, BCL-xS, BCL-xβ, BCL-xγ, and BCL-xΔTM, each possessing different domains (18, 37, 38). *BCL-w* was also shown to have variants BCL-w and BCL-w-rox (39). In addition to the antiapoptotic subfamily of BCL-2, splicing variants of proapoptotic BCL-2 family proteins (*e.g.* BAX, BOK, and BOD/BIM) have also been isolated (10, 36, 40–43). However, compared with other known variants of BCL-2 family proteins, the splicing of MCL-1S from the *MCL-1* gene is unique in its consequences for domain structure and biological function. Indeed, MCL-1S is the only known splicing variant of a *bcl-2* family gene that possesses intrinsic proapoptotic activity in normal cells and dimerizes with another variant of the same gene.

The discovery of MCL-1S with proapoptotic activity expands the BH3 domain-only BCL-2 subfamily. Multiple members of this subfamily have been reported, including mammalian BAD, BID, BIK/NBK, BLK, BOD/BIM, HRK, NIP3, and NIX (11, 29, 30, 41, 44–46). In addition, EGL-1, the nematode counterpart of BH3-only cell death activator, was also identified (47). EGL-1 is required for programmed cell death in *Caenorhabditis elegans* because gain-of-function mutations of *egl-1* cause apoptosis in hermaphrodite-specific neurons, and a loss-of-function *egl-1* mutation prevents most of the somatic programmed cell death (47). The binding of EGL-1 to the nematode BCL-2 homolog, CED-9, disrupts the interaction of CED-9 with CED-4, a homolog to human APAF-1, and promotes the activation of a caspase CED-9 (48). Based on the present finding of MCL-1 variants with characteristics similar to both *ced-9* and *egl-1*, one can postulate that these two types of genes are derived from a common ancestry through gene duplication and exon loss.

BH-3 domain-only BCL-2 subfamily proteins can be further divided into two groups based on the presence or absence of the TM anchor region. Like MCL-1S, BAD and BID have the BH3 domain but without the TM region. Under a survival signal, BAD is phosphorylated on two serine residues and sequestered by the cytoplasmic 14-3-3 proteins in an inactive form (35, 49). Following a death signal, BAD is dephosphorylated and translocated to the mitochondria membrane where it interacts with BCL-xL or BCL-2 (50, 51). In contrast, another BH3 domain-

only protein, BID, translocates from cytosol to mitochondria following caspase-8-mediated cleavage at its N terminus, and the truncated BID releases cytochrome *c* to induce apoptosis (52, 53). Because most BH3-only proteins interact with at least one of the antiapoptotic BCL-2 family proteins, it is reasonable to suggest that the heterodimerization and antagonism between MCL-1S and MCL-1L observed here are of physiological significance.

Several anti- and proapoptotic BCL-2 family genes (*bax, bcl-2, bcl-xL, bfl-1/A1, egl-1, hrk,* and *MCL-1*) are transcriptionally inducible. Survival factors that induce MCL-1 in hematopoietic cells include granulocyte-macrophage colony-stimulating factor, interleukin-1β, and interleukin-3 (54–56). We recently demonstrated that gonadotropins stimulate *MCL-1* mRNA expression in ovarian granulosa and theca cells to suppress follicle cell apoptosis (25). The MCL-1 protein is induced rapidly and has a high turnover rate that may be related to its unique PEST motifs not found in other BCL-2 members (12, 13). Even though MCL-1 shares many common properties with other BCL-2 family proteins, its rapid turnover rate and transcription into variants exhibiting either anti- or proapoptotic actions are unique for regulating apoptosis in tissues that require rapid adaptation and turnover of cells. The present demonstration of the expression of splicing variants of MCL-1 encoding proteins with diametrically opposing actions raises the possibility that the regulation of apoptosis in a given cell can be controlled by the expression of varying levels of the antiapoptotic MCL-1L and the proapoptotic MCL-1S transcripts. The present model underscores the importance of alternative splicing events in the regulation of cell function.

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