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MCL-1V, a novel mouse antiapoptotic MCL-1 variant, generated by RNA splicing at a non-canonical splicing pair

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ABSTRACT

Myeloid cell leukemia-1 (MCL-1) that belongs to BCL-2 family is essential for survival of hematopoietic stem cells. It is upregulated in various types of cancer and promotes cancer cell metastasis. It is known that human *MCL-1* gene undergoes differential splicing and yields three mRNAs encoding antiapoptotic MCL-1L and proapoptotic MCL-1S and MCL-1ES. However, no MCL-1 variants have been reported in mouse cells. We report here a new splicing variant of mouse *Mcl-1*, *Mcl-1V*, that is expressed in a variety of mouse normal and tumor cell lines and tissues. Comparative sequence analysis of the full-length *Mcl-1* and *Mcl-1V* cDNAs suggested that *Mcl-1V* mRNA results from splicing within the first coding exon of *Mcl-1* gene at a non-canonical donor–acceptor pair. MCL-1V lacks 46 amino acid residues within the N-terminal region of MCL-1. It localizes in mitochondria and inhibits anoxia- and anticancer drug-induced apoptosis as potent as MCL-1, and decayed less rapidly than MCL-1 in the cells undergoing apoptosis. Collectively, our results show that mouse cells ubiquitously express antiapoptotic MCL-1V that may play a role in mitochondrial cell death.

Introduction

Myeloid cell leukemia-1 (MCL-1) is initially identified in differentiating myeloblastic leukemia cells [1]. It belongs to BCL-2 antiapoptotic family members and has four BCL-2 homology (BH) domains (BH1-4) and transmembrane (TM) domain. It is essential for hematopoietic stem cell survival, maintenance and development of B and T cells and embryogenesis, but on the other hand it is expressed abundantly in various types of cancer including leukemia/lymphoma and multiple myeloma and several non-hematopoietic cancers [2–11]. MCL-1 overexpression is also associated with cancer metastasis [7,8]. Its expression is enhanced by hypoxia that has been shown to correlate with poor outcome of cancer patients [7,12]. Therefore, MCL-1 is suggested to be involved in the etiology and malignant progression of cancer cells.

The human *MCL-1* gene consists of three exons and generates three mRNAs, *MCL-1L*, *MCL-1S* and *MCL-1ES*, at canonical and non-canonical splice sites. *MCL-1L* mRNA encodes 350-amino acid-long antiapoptotic MCL-1L containing BH1-4 and TM domains. *MCL-1S* mRNA results from exon 2 skipping and encodes 271-amino acid-long proapoptotic MCL-1S lacking BH1, BH2 and TM domains. MCL-1S promotes apoptosis by suppression of MCL-1L activity through interaction with the BH3 domain of MCL-1L [13,14]. *MCL-1ES* mRNA results from splicing within the first exon of *MCL-1* gene, encodes 197-amino acid-long proapoptotic MCL-1ES lacking PEST motifs (sequences rich in proline, glutamic acid, serine, and threonine) which have been proposed to constitute protein instability determinants. It also interacts with MCL-1L, and induces mitochondrial cell death [15].

The mouse *Mcl-1* gene also consists of three exons and generates mRNA encoding 331-amino acid-long MCL-1, but no MCL-1 splicing variants have so far been reported. However, we have been demonstrated that, besides full-length MCL-1 (~40-kDa), a closely spaced band (~37-kDa) is detected in immunoblots using MCL-1 antibody, the expression of which is repressed by *Mcl-1* siRNA transfection [7]. However, it remained to be examined whether it is a post-translationally modified MCL-1 or a splice variant. We report here that the 37-kDa protein is encoded by a new splice variant of mouse *Mcl-1*, which we named *Mcl-1V* (*Mcl-1* variant; GenBank Accession No. GU182318) and shows antiapoptotic activity.

Materials and methods

Cell lines and cell culture. The characteristics of low-metastatic P29 and high-metastatic A11 cells derived from Lewis lung carci-

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noma, low-metastatic NM11 and high-metastatic LuM1 cells derived from Colon26, B16F1 cells derived from B16 melanoma are described elsewhere [16–19]. They were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich) supplemented with 10% heat inactivated (56 °C, 30 min) fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Balb/c 3T3 A31 fibroblasts, NIH3T3 fibroblasts, COS7 fibroblasts, human lung adenocarcinoma A549 cells and cervical carcinoma HeLa cells were also cultured under the same conditions.

Tissue samples. Various tissues (brain, lung, liver, heart, spleen, stomach, and kidney) were excised from a C57BL/6 mouse (Nippon SLC). Experiments were performed according to the guidelines for animal use and experimentation of Chiba Cancer Center.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from cells and tissues with QIAquick RNeasy Mini Kit (QIAGEN) and guanidium–CsCl ultracentrifugation method, respectively. RNAs were first reverse-transcribed using oligo(dT) primers, and the resulting cDNA was used for amplification of target cDNAs using *Ex* Taq DNA polymerase (TaKaRa BIO) or *Go*Taq DNA polymerase (Promega). The 5′ and the 3′ primers used are shown in Table S1.

Cloning of cDNAs and plasmid construction. cDNAs were generated by RT-PCR described as above. Full-length *Mcl-1* and *Mcl-1V* cDNAs were amplified by PCR, and inserted into the EcoRI–BamHI site of pEGFP-N1 (Clontech), generating pEGFP-Mcl-1 and pEGFP-Mcl-1V constructs. They were then digested with EcoRI and BamHI, and the inserts were ligated into the corresponding sites of pcDNA3.1(−)/Myc-His(A) (Invitrogen).

Plasmid transfection. Transfections of the plasmids into A11 cells were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Forty-eight hours after the transfection, the cells were subjected to immunoblot and immunofluorescent analyses.

Western blotting. Cells were lysed in 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF and protease inhibitor cocktail (Sigma–Aldrich), or directly dissolved in SDS sample buffer. Proteins were separated by SDS–PAGE under reducing conditions and transferred to a nitrocellulose membrane. The membrane was incubated with first antibodies, washed, and then with species-appropriate HRP-conjugated secondary antibodies. As the first antibodies, anti-MCL-1 antibody (Santa Cruz Biotechnologies), anti-mouse MCL-1 antibody (Acris Antibodies), anti-MYC antibody (Invitrogen), anti-cleaved caspase-3 (Asp175) antibody (Cell Signaling Technology) and anti-β-actin antibody (Sigma–Aldrich) were used. Immunodetection was performed using the enhanced chemiluminescence system (ECL; Amersham Bioscience).

Assay of apoptosis. To induce apoptosis, P29 cells were cultured in AnaeroPack (Mitsubishi Gas) for 18 h or cultured in the presence of adriamycin (0.5 μg/ml) or cisplatin (200 μg/ml). Apoptosis was assessed by using Annexin V-Cy3 Apoptosis Detection Kit (BioVision). In some experiments, P29 cells transfected with pcDNA3.1(−)Myc-His(A)/Mcl-1, pcDNA3.1(−)Myc-His(A)/Mcl-1V or vector alone were fixed with 4% formaldehyde/PBS for 10 min, permeabilized with 3% BSA/0.1% glycine/PBS for 30 min, and incubated with anti-MYC antibody (Invitrogen) followed by FITC conjugated anti-mouse IgG antibody (Sigma–Aldrich). The cells were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) (1 μg/ml). The number of cells with fragmented and condensed nuclei in FITC-positive cells was counted under an inverted fluorescence microscopy (Olympus). Cleaved caspse-3 was detected as described above.

Assay of stability of MCL-1 and MCL-1V. A11 cells were treated with cycloheximide (200 μg/ml) (Calbiochem), or they were cultured in the presence of adriamycin (0.5 μg/ml) or cisplatin (200 μg/ml) to induce apoptosis. Cell lysates were prepared as above at each time point and then subjected to immunoblot analysis using anti-MCL-1 antibody.

Results

Expression of MCL-1 in mouse cell lines and tissues

We previously reported that two closely spaced doublet of approximate molecular weight of ∼40-kDa and ∼37-kDa were detected in Lewis lung carcinoma cells using anti-MCL-1 antibody in immunoblots [7]. To examine whether the two bands can be detected in other immortalized and transformed mouse cells, we first carried out Western blot analysis using a panel of cell lines (P29 and A11 cells derived from Lewis lung carcinoma, NM11 and LuM1 cells derived from Colon26 carcinoma, B16F1 cells, and Balb/c 3T3 A31 cells). The results showed that the doublet was detected in all cell lines examined (Fig. 1A). Of interest, the expression levels of the proteins, in particular the 37-kDa protein, were higher in high-metastatic A11 and LuM1 cells than in their low-metastatic counterparts. The difference in the molecular weight between the two bands was apparently smaller than that between human MCL-1L and MCL-1S (∼8-kDa) (Fig. 1B). Since the 37-kDa protein could be a post-translationally modified product, we then examined whether the cells express one or two *Mcl-1* mRNAs. By performing RT-PCR using primers flanking the open reading frame of the *Mcl-1* transcript, two products were detected in all of the cell lines and tissues examined (Fig. 1C and D). The difference in size between the longer and the shorter transcripts was approximately 130-bp (Fig. 1E). In the case of human *MCL-1L* and *MCL-1S* transcripts, the difference in size was approximately 250-bp (Fig. 1E). As the length of exon 2 of mouse *Mcl-1* gene is equal to that of human *MCL-1* gene (247-bp), the shorter one seemed not to be resulted from exon 2 skipping.

Cloning of MCL-1V cDNA

To determine whether the 37-kDa protein is a previously uncharacterized variant, we tried to isolate cDNA clones corresponding to the two transcripts using total RNA isolated from A11 cells, and could isolate the longer and the shorter ones. Sequencing of the open reading frame of the cDNA clones revealed that the longer one was 996 bp in length and identical to the mouse *Mcl-1* cDNA (Accession No. NM_008562), and the shorter one was 858 bp in length and lacked 138 bp within the 5′-terminal region of the *Mcl-1* cDNA, which we named *Mcl-1V* (Fig. S1). Identical *Mcl-1V* transcripts were also present in mouse lung tissues (data not shown). Of interest, the region absent in *Mcl-1V* transcript was found to reside within the exon 1 of *Mcl-1* gene (Fig. S1). To examine whether *Mcl-1V* transcript is produced from the *bona fide Mcl-1* gene, we performed PCR using genomic DNA isolated from P29 and A11 cells and the two primer sets flanking the region absent in *Mcl-1V*. Irrespective of the primer set, we detected only one PCR product (Fig. S2). Thus, *Mcl-1V* mRNA is suggested to be generated from the *Mcl-1* gene by splicing within the first coding exon at a non-canonical donor–acceptor pair (GG–TG).

Comparison of the amino acid sequence between MCL-1 and MCL-1V indicated that MCL-1V lacks 46 amino acids (amino acids 18–63) within the N-terminal region of MCL-1 (Fig. 2). Mouse MCL-1 has BH and TM domains. Besides, it has two PEST-like motifs (amino acids 58R to 76R and 132A to 158Q) (Fig. 2) [20]. MCL-1V lacks a short stretch (RPPPV) of the N-terminal PEST-like motif (Fig. 3).

MCL-1V localizes in mitochondria as well as MCL-1

It has been shown that MCL-1L localizes in mitochondria and that N-terminus 79 amino acids are required for localization [21]. To assess subcellular localization of MCL-1V, we overexpressed MCL-1V-MYC in COS7 cells and stained the cells with anti-MYC

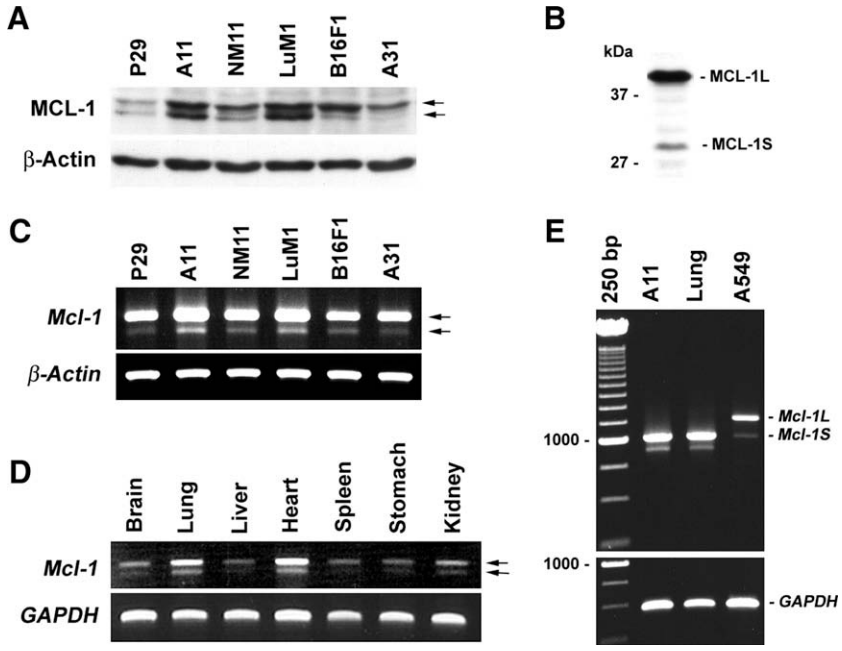

Fig. 1. Expressions of Mcl-1 in several mouse cell lines and tissues. (A) Expression of MCL-1 protein in Lewis lung carcinoma cells (P29 and A11), Colon26 cancer cells (LuM1 and NM11), melanoma cells (B16F1), and normal fibroblasts (Balb/c 3T3 A31). MCL-1 expression was examined by Western blot analysis using anti-MCL-1 antibody. β-Actin served as a loading control. Arrows indicate closely spaced bands. (B) Expression of MCL-1L and MCL-1S in HeLa cells. MCL-1 expression was examined by Western blot analysis using anti-MCL-1 antibody. (C) Expression of *Mcl-1* mRNA in the indicated cell lines. mRNA expression levels were analyzed by RT-PCR using the primers flanking the entire coding region of the *Mcl-1* gene. β-Actin served as a control. Arrows indicate two *Mcl-1* mRNAs. (D) Expression of *Mcl-1* mRNA in mouse tissues. Total RNA was prepared from the indicated tissues and used for RT-PCR analysis of *Mcl-1* mRNA expression. *GAPDH* served as a control. (E) The difference in size of *Mcl-1* cDNAs in A11 cells, the lung, and human A549 cells. The 250 bp size marker was used to check the approximate size of cDNAs. *GAPDH* served as a control.

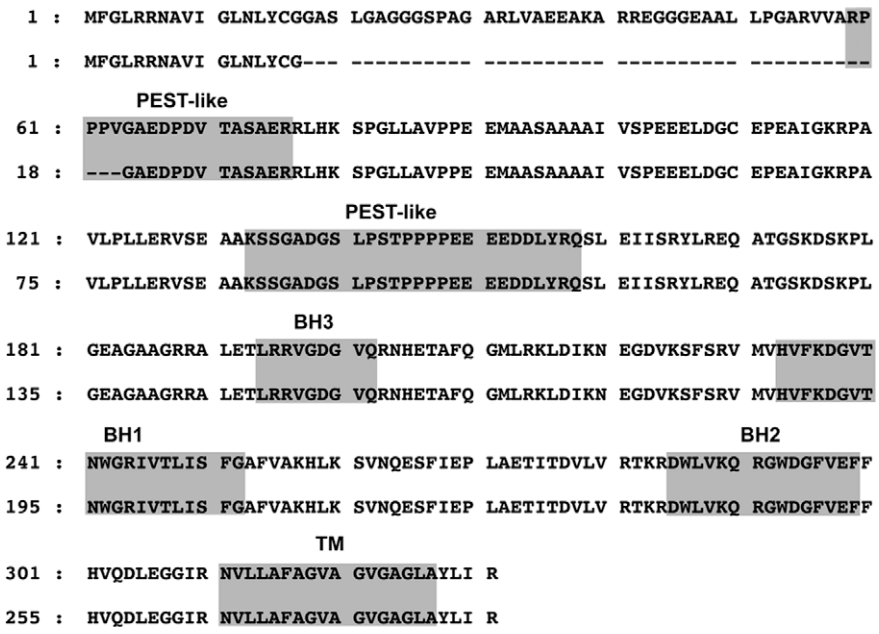

Fig. 2. Comparison of the amino acid sequences of MCL-1 and MCL-1V. PEST-like motifs, Bcl-2 homology (BH) domains, and TM domain are shown by gray boxes. PEST-like motifs were referred to the previous report [23].

antibody and MitoTracker Red. MCL-1V-MYC was found to be localized in mitochondria (Fig. S3).

MCL-1V has an antiapoptotic activity as potent as MCL-1

It has been reported that MCL-1L has an antiapoptotic activity whereas MCL-1S and MCL-1ES have a proapoptotic activity. To determine whether MCL-1V is antiapoptotic or proapoptotic, we transfected pEGFP-Mcl-1, and pEGFP-Mcl-1V into P29 cells which express low levels of MCL-1 and MCL-1V (Fig. 1B). We transfected pEGFP-N1 as a control. Since P29 cells are susceptible to anoxia-induced apoptosis [7], we cultured the transfected cells under anoxic conditions for 18 h, and assessed the number of Annexin V-positive cells within EGFP-positive cells. MCL-1-EGFP and MCL-1V-EGFP-

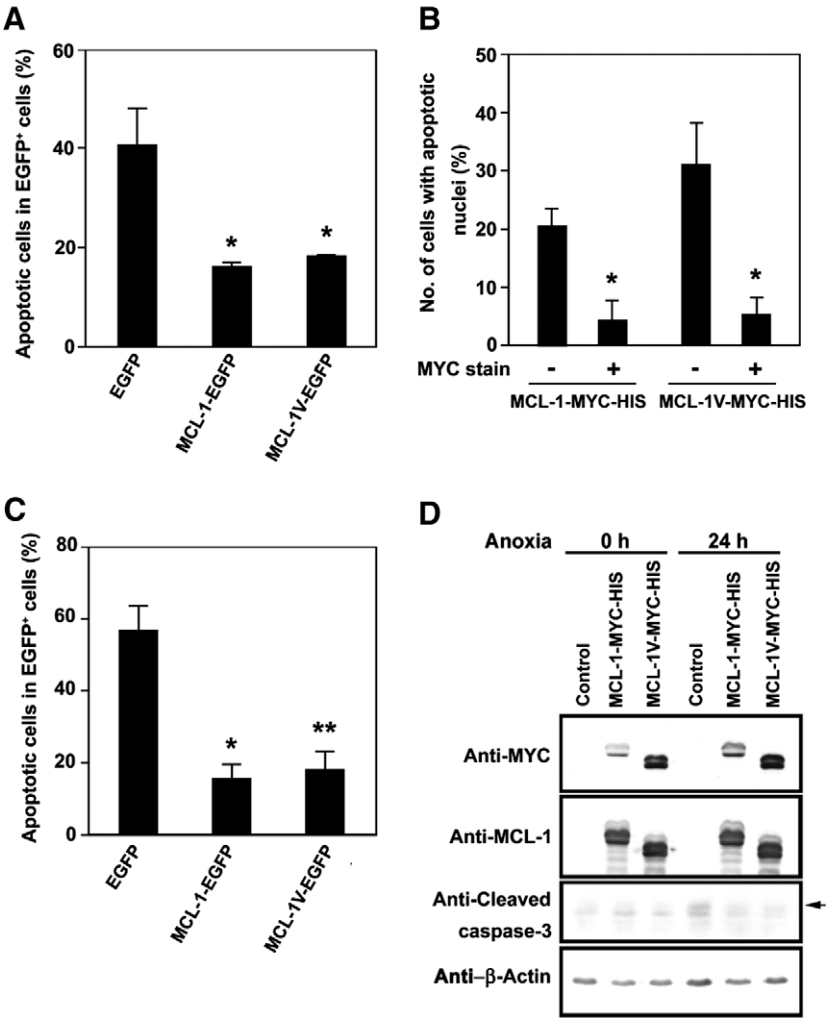

Fig. 3. MCL-1V shows antiapoptotic activity as potent as MCL-1. (A) Suppression of anoxia-induced apoptosis of P29 cells by MCL-1 and MCL-1V assessed by Annexin V staining. P29 cells expressing EGFP, MCL-1-EGFP, or MCL-1V-EGFP were cultured in anoxia (0.1% oxygen) for 18 h and then stained with Annexin V-Cy3 Apoptosis Detection Kit. The number of Annexin V-positive cells in P29 cells expressing EGFP, MCL-1-EGFP, or MCL-1V-EGFP were counted and then the percentage of Annexin V-positive cells within EGFP-positive cells was calculated. (B) Suppression of anoxia-induced apoptosis of P29 cells by MCL-1 and MCL-1V assessed by nuclear fragmentation and condensation. P29 cells expressing MCL-1-MYC-HIS or MCL-1V-MYC-HIS were cultured under anoxic conditions for 18 h. The cells were stained with anti-MYC antibody followed by FITC-conjugated secondary antibody, and then counterstained with DAPI. Nuclear morphologies of MYC-negative or MYC-positive P29 cells were observed, and the percentage of cells with aberrant nuclear morphology was calculated. (C) Suppression of cisplatin-induced apoptosis of P29 cells by MCL-1 and MCL-1V assessed by Annexin V staining. P29 cells expressing EGFP, MCL-1-EGFP, or MCL-1V-EGFP were cultured in the presence of 200 μg/ml cisplatin for 18 h and then stained for Annexin V. The number of Annexin V-positive cells in P29 cells expressing EGFP, MCL-1-EGFP, or MCL-1V-EGFP were counted as above (A). (D) Suppression of anoxia-induced apoptosis of NIH3T3 cells by MCL-1 and MCL-1V assessed by caspase-3 cleavage. NIH3T3 cells expressing MCL-1-MYC-HIS or MCL-1V-MYC-HIS were cultured under anoxic conditions for 24 h. The level of cleaved caspase-3 was examined by Western blot analysis. β-Actin served as a loading control. The arrow indicates the position of cleaved caspase-3. $^{*}P < 0.001$, $^{**}P < 0.01$.

expressing cells showed lower frequency of apoptosis than EGFP-expressing cells. MCL-1V was as effective as MCL-1 in suppressing anoxia-induced apoptosis (Figs. 4A and S4A). Next, we exposed P29 cells that had been transfected with pcDNA3.1(−)Myc-His(A)-Mcl-1, pcDNA3.1(−)Myc-His(A)-Mcl-1V, or pcDNA3.1(−)Myc-His(A) as a control, to anoxia, and counted the number of cells with nuclear fragmentation and condensation in both MYC-positive and MYC-negative cells. The rates of apoptotic cells within MYC-negative cells were about 20–30% whereas those in MCL-1-MYC-HIS- and MCL-1V-MYC-HIS-expressing cells were significantly lower (~5%) (Figs. 4B and S4B). Again, MCL-1V was as effective as MCL-1 in suppressing anoxia-induced apoptosis. MCL-1- and MCL-1V-over-expressing P29 cells were also more resistant to cisplatin (Figs. 4C and S4C). The antiapoptotic activity of MCL-1V was further corroborated by the detection of cleaved caspase-3. In this case, we used NIH3T3 cells instead of P29 cells, because the transfection efficiency of P29 cells is low. After culturing the cells for 24 h under anoxic conditions, cleaved caspase-3 was detected in control cells but not in MCL-1 and MCL-1V expressing cells (Fig. 4D). Together, these results suggest that MCL-1V is as potent as MCL-1 in suppressing apoptosis.

MCL-1V is more stable than MCL-1 in the cells undergoing apoptosis

The fact that MCL-1V lacks a part of PEST-like motif suggests that the stability of MCL-1V might be different from that of MCL-1. The observation that the ratio of Mcl-1V/Mcl-1 in A11 cells was considerably lower at the protein level than the mRNA level seemed to support this notion (Fig. 1). Then, to compare the stability between MCL-1 and MCL-1V, we first treated A11 cells with cycloheximide (CHX) for up to 6 h, and the amounts of MCL-1 and MCL-1V were compared. The expression level of MCL-1 was gradually reduced in a time-dependent manner whereas that of MCL-1V remained fairly constant until 6 h after the treatment (Fig. 4A). There was no difference in the mRNA levels when the cells were treated with CHX (Fig. 4B). These results suggested that MCL-1V is more stable than MCL-1.

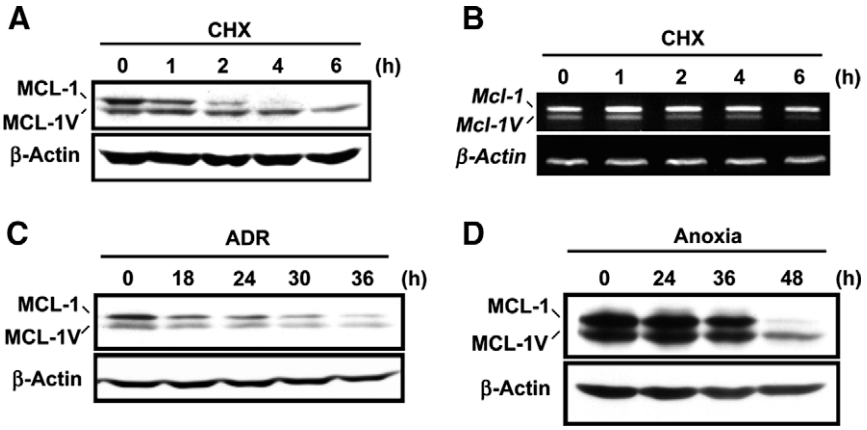

Fig. 4. Comparison of the stability between MCL-1 and MCL-1V. (A) Stability of MCL-1 and MCL-1V in A11 cells treated with cycloheximide (CHX) (200 μg/ml). A11 cells were treated with CHX for up to 6 h. MCL-1 and MCL-1V were detected by Western blot analysis. β-Actin served as a loading control. (B) Expression of *Mcl-1* and *Mcl-1V* mRNAs in A11 cells treated with CHX. A11 cells were treated with CHX for up to 6 h. Total RNAs were extracted from the cells at the indicated time, and then RT-PCR was performed. (C) Expression of MCL-1 and MCL-1V in A11 cells treated with ADR. A11 cells were treated with ADR (0.5 μg/ml) for up to 36 h. The expression levels of MCL-1 and MCL-1V in A11 cells treated with ADR were detected by Western blot analysis. β-Actin served as a loading control. (D) Expression of MCL-1 and MCL-1V in A11 cells cultured in anoxia for up to 48 h. MCL-1 and MCL-1V were detected by Western blot analysis. β-Actin served as a loading control.

Then, we next examined whether there is any difference in the stability between MCL-1 and MCL-1V in the cells undergoing apoptosis. For this, we treated A11 cells with adriamycin for 18–36 h. A11 cells underwent apoptosis by 36 h after the treatment (Fig. S5). Under these conditions, MCL-1V was less rapidly decayed than MCL-1 (Fig. 4C). Upon exposure to anoxia, MCL-1V was also retained longer than MCL-1 in A11 cells (Fig. 4D). Thus, MCL-1V is more stable than MCL-1 in the cells undergoing apoptosis.

Discussion

In the present study, we identified and characterized a novel MCL-1 variant (MCL-1V) expressed ubiquitously in mouse cell lines and tissues. It showed antiapoptotic activity comparable to MCL-1, and is more stable than MCL-1 in the cells undergoing apoptosis. We also revealed that *Mcl-1V* mRNA is generated by splicing within the first coding exon of *Mcl-1* gene at a non-canonical donor–acceptor pair (GG–TG). According to the Information for the Coordinates of Exons (ICE): a human splice sites database [22], the GG–TG pair is rare but is utilized for a splice site.

It has been suggested that the N-terminus of MCL-1L is important for the intracellular localization and the antiapoptotic function [21]. Since MCL-1V lacks 46 amino acids within the N-terminal region of MCL-1, we evaluated the subcellular localization and the function of MCL-1V, and found that it localizes in mitochondria and exhibits antiapoptotic activity as well as MCL-1. The most prominent difference between MCL-1V and MCL-1 was their stability in the cell. We found that MCL-1V has a longer half-life than MCL-1 in cycloheximide-treated A11 cells. Of particular note is that MCL-1V is more stable than MCL-1 in A11 cells undergoing apoptosis under anoxic conditions or after treatment with cisplatin. Actually, A11 cells can survive longer than P29 cells after the treatment with various drugs and under anoxic conditions [7]. The reason why MCL-1V is more stable in the cells is presently obscure. The amino acid sequence (amino acids 18–63) absent in MCL-1V corresponds to a part of weak PEST-like motif. The PEST motifs of MCL-1L are reported to be frequently phosphorylated by ERK, GSK-3, CDK, or JNK [20,23–25]. Some reports indicated that this phosphorylation is critical for MCL-1 degradation through ubiquitin-proteasome pathway [23,25]. Therefore, the sequence absent in MCL-1V might contain important amino acid residues to be phosphorylated for degradation. It is reported that the N-terminus of human MCL-1 contains a short stretch (VARPPPIGAEVPDVTATPARLLFFA) for binding to tankyrase 1 that downregulates the MCL-1 expression level [26]. The consensus tankyrase-binding motif is RXXPDG [27]. Because MCL-1V lacks a part of this stretch (underlined), it is possible that tankyrase 1 cannot bind to MCL-1V and, as such, MCL-1V is more stable than MCL-1. Further studies are required to solve this issue.

It has previously shown that the N-terminal truncated form of MCL-1L is generated by post-translational modification of MCL-1L in normal lymphoid tissues and lymphomas. Of interest, this truncated form of MCL-1L is turned over less rapidly than MCL-1L and retained antiapoptotic activity [28]. Therefore, the properties of MCL-1V are similar to those of the N-terminal truncated form of MCL-1L.

The property of MCL-1V with regard to its stability has pathophysiological implications. It may be advantageous for tumor cells to survive longer under severe environment such as hypoxic conditions and nutrient-starving conditions. In this context, it is of interest to note that MCL-1V is overexpressed in high-metastatic A11 cells compared to low-metastatic P29 cells, and the former is more resistant to anoxia and glucose deprivation than the latter [7]. The differential expression of MCL-1V and MCL-1 was also observed in mouse colon carcinoma cell lines that have different metastatic potential. Therefore, MCL-1V may play an important role in controlling metastatic potential of some mouse tumors. Since the N-terminal truncated form of MCL-1L acts like MCL-1V as described above, it might be related to metastatic potential of human tumor cells. This possibility may be an interesting issue to be pursued.

Conflict of Interest disclosure

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.086.

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