The N Terminus of the Anti-apoptotic BCL-2 Homologue MCL-1 Regulates Its Localization and Function*

Marc Germain1 and Vincent Duronio2
From the Department of Medicine, University of British Columbia and Vancouver Coastal Health Research Institute, Jack Bell Research Centre, Vancouver, British Columbia V6H 3Z6, Canada

The BCL-2 homologue MCL-1 plays an important role in the regulation of cell fate by blocking apoptosis as well as regulating cell cycle. MCL-1 has an unusual N-terminal extension, which contains a PEST domain and several phosphorylation sites that have been suggested to regulate its turnover. Here we report that the first 79 amino acids of MCL-1 regulate its subcellular localization. Deletion of this domain impairs both its mitochondrial localization and its anti-apoptotic activity. Conversely, expression of the N terminus of MCL-1 promotes both the association of MCL-1 with mitochondria and cell survival in a fashion that is dependent on the presence of endogenous MCL-1. In addition, the N terminus of MCL-1 has an antagonistic effect on proliferation. Although MCL-1 decreases proliferation through binding to proliferating cell nuclear antigen and cyclin-dependent kinase 1 in the nucleus, the N terminus of MCL-1 accelerates cell division. On the other hand, deletion of this region further increases the anti-proliferative activity of MCL-1. These results suggest that the N terminus of MCL-1 plays a major regulatory role, regulating coordinately the mitochondrial (anti-apoptotic) and nuclear (anti-proliferative) functions of MCL-1.

Apoptosis is a genetically conserved form of programmed cell death required for normal development and homeostasis of multicellular organisms. As such, it plays a major role in the prevention of cancer by causing the destruction of damaged or otherwise altered cells (1, 2). Induction of apoptosis after such damage is usually achieved through the intrinsic, or mitochondria, pathway. In this pathway loss of mitochondrial integrity results in cytochrome c (cyt c)3 release and subsequent activation of a series of apoptotic proteases, known as caspases, through the formation of the apoptosome, a protein complex composed of APAF-1, caspase-9, and cyt c (1).

Events leading to the release of cyt c from mitochondria are controlled by the BCL-2 family of proteins that are characterized by the presence of at least one BCL-2 homology (BH) domain (1, 3). Several BCL-2 homologues also possess a C-terminal transmembrane domain that mediates their association with mitochondria (4, 5). Pro-apoptotic BAX and BAK, containing BH1–3, are required for permeabilization of mitochondria, although their exact mechanism of action is still debated. Both proteins are kept inactive in healthy cells and become activated through a change in conformation upon induction of apoptosis, leading to their oligomerization (1, 3). This activation is regulated by a balance between two antagonistic classes of BCL-2 homologues: BH3-only and anti-apoptotic proteins. Anti-apoptotic BCL-2 proteins such as BCL-2 itself, BCL-XL, and MCL-1 inhibit apoptosis by blocking either directly or indirectly the activation of BAX and BAK (1, 3). Interaction between BCL-2 family members is mediated by a groove formed by the BH1–3 domains in the BCL-2 homology region on the one hand and the α-helix formed by the BH3 of the pro-apoptotic proteins on the other hand (2). These interactions play a requisite role in the regulation of BCL-2 family proteins.

MCL-1 is an anti-apoptotic BCL-2 homologue that is necessary for early embryonic development as well as for the generation and maintenance of hematopoietic cell lineages (6, 7). MCL-1 has a short half-life, and its protein levels are tightly regulated both transcriptionally and through proteasomal degradation (8). Degradation of MCL-1 after UV irradiation is required for induction of apoptosis (9). Conversely, MCL-1 protein levels are increased in several human cancers, and its overexpression in transgenic mice leads to malignancies (8, 10). Paradoxically, MCL-1 has also been reported to delay cell cycle progression in S/G2 through interaction with proliferating cell nuclear antigen (PCNA) and cyclin-dependent kinase 1 (CDK1) (11, 12). This has been associated with a nuclear localization of MCL-1, as opposed to its mitochondrial anti-apoptotic function. MCL-1 is, thus, an important regulatory protein in the context of development and oncogenesis.

Structurally, MCL-1 differs from other anti-apoptotic BCL-2 proteins in two respects. First, differences in amino acid charge distribution in the BCL-2 homology region allow for a different

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specification toward BH3-only proteins as compared with BCL-2 and BCL-X(L) (13). Second, MCL-1 possesses a 170-amino acid extension at its N terminus that contains two PEST domains and several phosphorylation sites. This N-terminal extension is a potential regulatory domain for the degradation of MCL-1 through its PEST sequences (14), which contain phosphorylation sites that promote the recruitment of the E3 ligase β-TRCP (15, 16) as well as caspase cleavage sites (17).

Here we report that the first 79 amino acids of MCL-1 regulate its subcellular localization, promoting its association with mitochondria. Overexpression of the N terminus of MCL-1 (Nt-MCL) promoted cell survival by recruiting more MCL-1 at this organelle, whereas deletion of this sequence (MCL-1ΔN79) partially impaired both its mitochondrial localization and anti-apoptotic activity. Conversely, MCL-1ΔN79 inhibited cell growth more potently than MCL-1, whereas Nt-MCL increased proliferation. These results suggest that the N terminus of MCL-1 plays an important regulatory role in the function of MCL-1.

MATERIALS AND METHODS

MCL-1 Constructs—To generate Nt-MCL, full-length MCL-1 in pcDNA3.1/V5-His(A) (cloned between BamHI and EcoRI) was digested using BamHI and NotI and cloned into the same vector to give a fusion protein expressing the first 79 amino acids of MCL-1 with a His and a V5 tag at its C terminus. V5-MCL-1ΔN79 and V5-MCL-1ΔN54 were generated by replacing the first 79 or 54 amino acids of MCL-1 by a V5 tag using PCR and cloned into pcDNA3.1/His-V5(A) between BamHI and EcoRI restriction sites. MCL-1ΔTM was generated by removing amino acids 321–350 using PCR and cloned into pcDNA3.1/His-V5. Nt-MCL-GFP was generated by fusing the first 79 amino acids of MCL-1 to the N terminus of GFP using PCR. Both GFP and Nt-MCL-GFP were cloned into pcDNA3.1/His-V5 vector.

Cell Culture, Transfections, and Infections with Adenoviral Vectors—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 20 mM l-glutamine. To generate cell lines stably expressing full-length MCL-1 or the deletion mutants, HeLa cells were transfected with the various constructs using Lipofectamine 2000 (Invitrogen) and selected with G418. For UV irradiation, cells were grown to 95% confluency (or 50% confluence) using 200 pfu/cell Ad tBID in the presence of 50 μM zVAD-FMK.

Antibodies and Immunoblots—The following antibodies were used: mouse anti-β-actin, mouse anti-BrdUrd, and mouse anti-FLAG (Sigma-Aldrich), rabbit anti-MCL-1 and mouse anti-BCL-X (Santa Cruz Biotechnology), mouse anti-MCL-1 (Pharmingen), mouse anti-cyt c (78H.2C12, Pharmingen), rabbit anti-BAK, and rabbit anti-p85 PI3K (Upstate), mouse anti V5 antibody (Invitrogen), rabbit anti-GFP (Molecular Probes), rabbit anti-TOM20 (a gift from Dr. Gordon Shore (19)). For immunoprecipitation, cells were lysed in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 supplemented with a protease inhibitor mixture (Sigma-Aldrich). Lysates were incubated for 2 h with the antibody and precipitated with protein A-Sepharose. For immunoblot analysis, proteins were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and blotted with specific antibodies. Blots were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence (Amer sham Biosciences).

Half-life Determination—To determine the half-life of MCL-1, HeLa cells were transiently transfected with the various constructs for 24 h, after which they were either irradiated using 200 mJ/cm² UVB or treated with 10 μM cycloheximide (CHX). The experiment was also repeated in the presence of 10 μM MG132 as a control. Cells were harvested at the indicated times and analyzed by Western blot using a MCL-1-specific antibody as well as a p85 PI3K antibody as a loading control. The density of the bands was quantified using Image J software and normalized using the p85 PI3K blot.

Immunofluorescence—For immunofluorescence, cells were grown on coverslips and treated as indicated in the figure legends. Cells were then fixed with 4% paraformaldehyde and analyzed by immunofluorescence using AlexaFluor 488 and AlexaFluor 594 secondary antibodies (Molecular Probes). Coverslips were then visualized using an Axioplan 2 microscope (Carl Zeiss MicroImaging, Inc.) with a 100 × 1.30 oil objective (Carl Zeiss MicroImaging, Inc.). Images were captured and overlaid using Northern Eclipse software.

siRNA—Cells were transfected with siRNA specific for GFP, BCL-X, and MCL-1 (Santa Cruz Biotechnology) using SilentiFect (Bio-Rad) transfection reagent. 10 nm siRNA was used for siRNA GFP and siRNA BCL-X, whereas 6.67 nm was used for siRNA MCL-1 to reduce toxicity. Cells were collected after 24 h analyzed for protein expression by Western blot.

Isolation of Mitochondria and Alkaline Extraction—Mitochondria were isolated as previously described (19) with the following modifications. Cells were harvested and resuspended in HIM buffer (200 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.5, 1 mM EGTA). Cells were broken by passing them 24 times through a 25-gauge needle. Nucleus and cell debris were removed by centrifugation at 1000 × g. The resulting supernatant was centrifuged at 9000 × g to pellet the heavy
membrane fraction containing mitochondria. For alkaline extraction, 30 μg of heavy membrane were incubated for 30 min on ice with 0.1 M Na2CO3 followed by centrifugation at 50 000 × g to recover the membranes (19).

**RESULTS**

The First 79 Amino Acids of MCL-1 Regulate Its Anti-apoptotic Activity after UV Irradiation—MCL-1 has a long N-terminal region containing a PEST domain, which is not required for binding to other BCL-2 homologues (13). Caspase-mediated cleavage of this domain (at Asp-127 and Asp-157) has been reported to impair the anti-apoptotic function of MCL-1 (17, 20). To test the anti-apoptotic activity of different N-terminal deletions of MCL-1 that do not disrupt its PEST domain and, therefore, should not affect its half-life, HeLa cells were transiently transfected with several MCL-1 deletion mutants, and cyt c release was analyzed by immunofluorescence after irradiation of the cells with 200 mJ/cm2 UV. As shown in Fig. 1A, MCL-1 almost completely blocked cyt c release in this context, whereas the activity of mutants lacking the first 54 (MCL-1ΔN54) or 79 amino acids (MCL-1ΔN79) was partially impaired. A similar effect was observed when the C-terminal transmembrane domain was deleted (MCL-1ΔTM). The increased cyt c release observed for the MCL-1 mutants, as compared with wild-type MCL-1, was not a consequence of their lower expression as they were all expressed at similar or higher levels (MCL-1ΔN79) as compared with full-length MCL-1 (Fig. 1B). Similarly, stable expression of MCL-1ΔN79 in HeLa cells, despite being expressed at higher levels than the wild-type protein (Fig. 1C), only partially prevented cyt c release and caspase activation, as compared with the full-length protein, after UV irradiation (Fig. 1, D and E).

MCL-1 is a protein with a short half-life, and its degradation after UV irradiation is required for subsequent induction of apoptosis (9). A recent report indicating that deletion of the first 20 or so amino acids of MCL-1 led to an increased stability of the protein (21) as well as the greater expression of MCL-1ΔN79 (Fig. 1) suggested that MCL-1ΔN79 could have an altered half-life. To test whether the impaired activity of MCL-1ΔN79 is due to an altered half-life, the degradation rate of the different MCL-1 constructs was tested after CHX treatment and UV irradiation. The half-life of overexpressed MCL-1, as determined using CHX, was very short (about 60 min) and was not influenced by the ΔN79 deletion (Fig. 2A). Similarly, UV irradiation of HeLa cells provoked the rapid degradation of both MCL-1 and MCL-1ΔN79, with similar kinetics (Fig. 2A).

The caspase-cleaved form of BID (tBID) promotes apoptosis by directly activating BAX and BAK at the mitochondria (22, 23). Importantly, apoptosis induced after infection of HeLa cells with an adenovirus expressing tBID (Ad tBID) occurs in the absence of MCL-1 degradation (Fig. 2B). In this context full-length MCL-1, but not MCL-1ΔN79, could reduce significantly tBID-induced cyt c release (Fig. 2C). Altogether, these results suggest that the altered anti-apoptotic activity of MCL-1ΔN79 is not a consequence of altered sensitivity to proteasomal degradation.

MCL-1 prevents apoptosis at least in part by sequestering pro-apoptotic BAK (24). Although complete deletion of the N-terminal portion of MCL-1 does not affect binding to BH3-only proteins (13), the effect of the N79 deletion on the interaction between BAK and MCL-1 was tested. Because full-length MCL-1 and BAK interacted very weakly in CHAPS-containing buffer (data not shown), the experiment was carried in Triton X-100-containing buffer as a measure of the capacity of these proteins to interact. Wild-type MCL-1 and MCL-1ΔN79 were transiently transfected into HeLa cells along with FLAG-BAK, followed by immunoprecipitation with an anti-FLAG antibody. As shown in Fig. 2D, FLAG-BAK immunoprecipitated both MCL-1 constructs with a similar efficiency, indicat-
The First 79 Amino Acids of MCL-1 Are Required for Its Mitochondrial Localization

MCL-1 localizes to the mitochondria where it can block apoptosis by interacting with pro-apoptotic BAK (24, 25). Because deletion of the C-terminal transmembrane domain of MCL-1 had a similar effect on its anti-apoptotic activity than the ΔN79 deletion, we analyzed the subcellular localization of the different MCL-1 constructs. This was tested in transiently transfected HeLa cells using a MCL-1-specific antibody, as the levels of endogenous MCL-1 in HeLa cells are below detection levels by immunofluorescence (see the untransfected cells which do not show MCL-1 staining in Fig. 3A). As shown in Fig. 3A, full-length MCL-1 co-localized with the mitochondrial protein TOM20. In contrast, MCL-1ΔN79 localized to the cytosol in a majority of the cells, although a weak association with mitochondria could still be observed (Fig. 3A, V5-MCL-ΔN79 is depicted; similar results were obtained in absence of the tag (not shown)). As expected, deletion of the C-terminal transmembrane domain, which is required to target BCL-2 homologues to intracellular membranes (5), showed no mitochondrial localization (MCL-1ΔTM; Fig. 3A).

To determine whether the N-terminal 79 amino acids of MCL-1 act as a mitochondrial targeting sequence, this domain was expressed as a GFP fusion protein (Nt-MCL-GFP). As shown in Fig. 3B, Nt-MCL-GFP localized to the cytosol. Similar results were obtained when Nt-MCL was tagged with a V5 epitope at its C terminus (not shown). This suggests that although required for the proper mitochondrial localization of MCL-1, this domain is not sufficient.

Nt-MCL Enhances the Mitochondrial Localization of MCL-1—The number of cells demonstrating mitochondrial localization of MCL-1 was quantified for each construct. Only cells in which most of MCL-1 co-localized with TOM20 (as for MCL-1 in Fig. 3A) were counted as mitochondrial. Although full-length MCL-1 was mitochondrial in most cells, MCL-1ΔN79 co-localized with TOM20 in only 15% of the cells (Fig. 4A). As expected, there was no association of MCL-1ΔTM with mitochondria (Fig. 4A). The effect of the expression of Nt-MCL on the localization of MCL-1 was then tested. HeLa cells stably expressing a V5-tagged version of Nt-MCL (Nt-MCL-V5) were transiently transfected with the different constructs, examined by immunofluorescence using a MCL-1-specific antibody, and scored for mitochondrial localization.
drial localization of the MCL-1 constructs. As shown in Fig. 4A, Nt-MCL-V5 significantly increased the mitochondrial localization of both full-length MCL-1 and MCL-1ΔN79 but had no effect on MCL-1ΔTM, consistent with the requirement of the transmembrane domain for mitochondrial targeting.

MCL-1 is synthesized on cytosolic ribosomes before being imported into mitochondria. If the cytosolic localization of MCL-1ΔN79 is the consequence of a slower rate of import, it should nevertheless accumulate at the mitochondria given enough time. Therefore, by preventing synthesis of new MCL-1ΔN79 using CHX, it should be possible to observe its accumulation at the mitochondria. As shown in Fig. 4B, the number of cells with mitochondrial MCL-1ΔN79 did increase in a time-dependent manner after the inhibition of protein synthesis, whereas the localization of MCL-1 and MCL-1ΔTM was not affected. In addition, Nt-MCL-V5 did increase the rate at which cells with mitochondrial MCL-1ΔN79 appeared (Fig. 4C) but had no effect on the half-life of MCL-1ΔN79 under these conditions (Fig. 4D). These results indicate that mitochondrial targeting is less efficient in MCL-1ΔN79 and that the presence of Nt-MCL can at least partially compensate for this defect.

Although the bulk of endogenous MCL-1 is found associated with mitochondria, some can also be found in the cytosol or nucleus under some conditions (9, 11, 12). To test whether Nt-MCL can enhance the mitochondrial localization of endogenous MCL-1, HeLa cells stably expressing vector alone or Nt-MCL-V5 were fractionated, and the heavy membrane-containing mitochondria were isolated (19). The presence of Nt-MCL resulted in an increase in the amount of MCL-1 associated with heavy membrane (HM; Fig. 5, A and B), suggesting that Nt-MCL stimulates the association between MCL-1 and mitochondria. However, there was also a small increase (~20%) in the total amount of MCL-1 in Nt-MCL-expressing cells (Fig. 5, A and B) that may partially account for the increase in mitochondrial MCL-1. Therefore, to better establish the role of Nt-MCL in promoting the association between MCL-1 and mitochondria, we looked at its insertion into mitochondria. MCL-1, like other anti-apoptotic BCL-2 homologues, is predicted to insert into the outer mitochondrial membrane through its C-terminal transmembrane domain and, thus, be resistant to extraction by 0.1 M Na₂CO₃. As previously reported (25), only a fraction of MCL-1 was resistant to alkaline extraction, and in contrast with total mitochondrial MCL-1, the amount of alkaline-resistant MCL-1 did not increase in the cells expressing Nt-MCL (Fig. 6, A and B). This suggests that Nt-MCL promotes the association of MCL-1 with mitochondria but not its insertion into the membrane. Again, the effects of Nt-MCL were not the consequence of an altered stability of MCL-1, as the degradation of endogenous MCL-1 after CHX or UV was the same in the presence or absence of Nt-MCL-V5, whereas the addition of the proteasome inhibitor MG132 could delay both CHX- and UV-induced degradation of endogenous MCL-1 (Fig. 5C).

Nt-MCL Promotes Cell Survival—The anti-apoptotic function of MCL-1 is dependent on its association with BAK at the mitochondria (24). The increase in mitochondrial MCL-1 caused by the expression of Nt-MCL should, therefore, promote cell survival after apoptosis induction. To test this hypothesis, HeLa cells stably expressing Nt-MCL-V5 were irra-
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Several approaches were used to test whether Nt-MCL requires endogenous MCL-1 for its function. NOXA is a BH3-only protein that specifically binds to MCL-1 and A1 to inactivate it, resulting in a greater sensitivity to apoptosis (18, 24, 26). Because A1 is absent from HeLa cells (24), an adenovirus-expressing human NOXA (Ad NOXA) was used to selectively inhibit MCL-1 in these cells. Two hours after UV irradiation, HeLa cells do not yet show signs of apoptosis such as cyt c release (Fig. 7A). However, infection of these cells with Ad NOXA, although insufficient to cause cyt c release on its own, sensitized them to UV-induced cytochrome c release, which could be prevented by overexpression of anti-apoptotic BCL-2 (Fig. 7A). Expression of Nt-MCL in this context did not provide any protection (Fig. 7A). The levels of MCL-1 protein were also knocked down using siRNA (Fig. 7B). As with Ad NOXA, loss of
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MCL-1 expression resulted in sensitization of the cells to UV-induced cytochrome c release that was not blocked by Nt-MCL (Fig. 7B). Of note, knocking down a different anti-apoptotic BCL-2 homologue, BCL-X, did sensitize cells to UV-induced apoptosis, but Nt-MCL could still inhibit cytochrome c release in this context (Fig. 7B), indicating that the absence of protection by Nt-MCL is not due to a general sensitization of the cells to apoptosis. Altogether, these results suggest that Nt-MCL specifically requires the presence of full-length MCL-1 to block apoptosis.

To test the effect of Nt-MCL on the anti-apoptotic activity of the various MCL-1 mutants independent of endogenous MCL-1, we used MCL-1 null MEFs (MCL-1Δ17). These cells were transfected with the various constructs, and the effect of Nt-MCL-GFP on their survival was tested. Infection with Ad tBID was used as an apoptotic inducer for these experiments because it allows dissociating its anti-apoptotic activity from possible consequence of its rapid turnover. As expected, MCL-1 null MEFs were more sensitive than their wild-type counterparts (Fig. 7C). In addition, although Nt-MCL-GFP could protect the wild-type cells from tBID-induced cyt c release, it had no effect on MCL-1 null cells (Fig. 7C), consistent with the Noxa and siRNA experiments (Fig. 7, A and B). Similar to HeLa cells, MCL-1 did prevent cyt c release in MCL-1 null MEFs transfected with GFP alone, whereas MCL-1ΔN79 and MCL-1ΔTM were much less efficient (Fig. 7D). The effect of Nt-MCL-GFP was then tested. As shown in Fig. 7C, Nt-MCL-GFP prevented cyt c release in MCL-1 null MEFs expressing MCL-1ΔN79, but not MCL-1ΔTM, consistent with ability to target MCL-1ΔN79 but not MCL-1ΔTM to mitochondria. Of note, Nt-MCL-GFP did not increase the expression of the MCL-1 constructs in the MEFs (Fig. 7E). In fact, despite MCL-1ΔN79 being more efficient at preventing cytochrome c release in the presence of Nt-MCL-GFP, its expression was lower. These results suggest that Nt-MCL prevents apoptosis by targeting MCL-1 to mitochondria rather than by increasing MCL-1 expression.

The N Terminus of MCL-1 Regulates Its Nuclear Function—MCL-1 has been reported to localize to the nucleus under certain circumstances, where it can slow down the cell cycle by interacting with PCNA and CDK1 (11, 12). Although only a small amount of nuclear MCL-1 can be detected in transfected HeLa cells, both MCL-1ΔN79 and MCL-1ΔTM showed significant nuclear localization (Fig. 8A; the nuclear localization of the mutants can also be observed in Fig. 3A). We then tested whether, by regulating the localization of MCL-1, the N terminus of MCL-1 could negatively regulate its anti-proliferative activity. HeLa cells were transfected with various MCL-1 constructs, and proliferation was assessed using BrdUrd incorporation. As previously reported, expression of MCL-1 in HeLa cells did reduce their proliferation rate (Fig. 8B). Both MCL-1ΔN79 and MCL-1ΔTM showed a more robust inhibition of BrdUrd incorporation than MCL-1. On the other hand, more cells expressing Nt-MCL-GFP than control GFP incorporated BrdUrd into their DNA. Again, the effect did not correlate with expression levels of MCL-1, as cells expressing Nt-MCL-GFP had more MCL-1 than cells expressing GFP alone (Fig. 8C). Altogether, these results are consistent with a role for the N terminus of MCL-1 in promoting the association of MCL-1 with mitochondria, at the expense of its nuclear localization.

DISCUSSION

MCL-1 is a highly regulated BCL-2 homologue, presumably because of its requisite role in development and maintenance of homeostasis (6, 7, 27). Its expression is tightly regulated by cytokines and other growth factors (28). In addition, MCL-1 is a short-lived protein as a consequence of its targeting for proteasomal degradation by the BH3-containing E3-ligase Mule (29, 30). However, other regulatory mechanisms are likely to play a role in MCL-1 turnover as it is still degraded in the absence of Mule, albeit at a slower rate (30). For example, both the BH3-only protein NOXA and phosphorylation by glycogen synthase kinase 3 promote the degradation of MCL-1, the latter through the E3-ligase β-TRCP (15, 16, 24). The glycogen synthase kinase 3 phosphorylation site is located within the N-terminal half of MCL-1, within the PEST domain, suggesting that this domain has an important regulatory function. In addition, deletion of first 17 amino acids of MCL-1 have been recently
FIGURE 7. Nt-MCL regulates apoptosis by controlling the localization of MCL-1. A, HeLa cells stably expressing Nt-MCL or vector alone were either mock-infected or infected with 10 pfu/cell Ad NOXA for 24 h. Alternatively, cells were transiently transfected with a vector expressing BCL-2 and GFP (to detect transfected cells). Cells were then irradiated with 200 mJ/cm² UV in the presence of 50 μM zVAD-FMK, fixed after 2 h, and analyzed for cytochrome c release by immunofluorescence. B, HeLa cells stably expressing Nt-MCL or vector alone were transfected with the indicated siRNAs. After 24 h, the cells were treated and analyzed as in A. Shown are the results of four independent experiments ± S.D. *, p ≤ 0.01. The left panel shows the expression levels of MCL-1, BCL-X, and the p85 subunit of PI3K (as a loading control). C, MCL-1 null MEFs were transfected with the indicated MCL-1 constructs and either GFP or Nt-MCL-GFP. Cells were then infected with Ad tBID (200 pfu/cell) and fixed 8 h later. GFP-positive cells were analyzed for cytochrome c release by immunofluorescence. Shown are the results of three independent experiments ± S.D. *, p < 0.01. WT, wild type. D, expression levels of the MCL-1 constructs in MCL-1 null MEFs. MCL-1 null MEFs were transfected with the indicated constructs and analyzed by Western blot for MCL-1 expression. Because mouse (endogenous) and human (constructs) MCL-1 are not recognized by the same antibody, the membrane was probed simultaneously for both an antibody against mouse MCL-1 (Rockland) and human MCL-1 (S-19). The blot shown is one exposure of the same blot, although they are shown separately to clearly mark the fact that the proteins are recognized by separate antibodies. E, effect of Nt-MCL-GFP on the expression of the MCL-1 constructs in MCL-1 null MEFs. MCL-1 null MEFs were transfected with the indicated MCL-1 constructs and either GFP or Nt-MCL-GFP and analyzed for the presence of MCL-1 using a human MCL-1-specific antibody (S-19). The blot was reprobed with an anti-actin antibody as a loading control.
suggested to stabilize MCL-1 (21), and although deletion of the first 79 amino acids of MCL-1 did not significantly increase its half-life in this study, the increased expression levels of the mutant were consistent with such a role. Here we show that the N terminus of MCL-1 also plays an important regulatory role in controlling the mitochondrial localization of MCL-1.

All anti-apoptotic BCL-2 homologues, including MCL-1, possess a C-terminal hydrophobic segment that targets them to intracellular membranes such as endoplasmic reticulum or mitochondria (4, 5). Information contained within this sequence and the flanking positive charges is believed to be sufficient to target these proteins to specific organelles (5). In fact, deletion of the C-terminal transmembrane domain of MCL-1 disrupts its interaction with mitochondria (MCL-1ΔTM; Figs. 3 and 4), indicating that the transmembrane domain is essential for mitochondrial targeting. However, because deletion of the first N-terminal 79 amino acids of MCL-1 (MCL-1ΔN79) also leads to a predominantly cytosolic localization of MCL-1, this region is likely to carry additional information required for the proper localization of MCL-1. The expression of the N-terminal region (Nt-MCL), although itself cytosolic, can partly revert the cytosolic localization of MCL-1ΔN79 (but not MCL-1ΔTM), suggesting that this domain is not a classical signal sequence. It might, however, modulate the interaction between MCL-1 and other co-factors required for its translocation to the mitochondria. For example, the mitochondrial localization of BCL-2 and BCL-X₇ has previously been linked to their association with the FK506-binding protein FKBP38 (31).

The activity of anti-apoptotic BCL-2 homologues is dependent on their association with intracellular membranes such as the endoplasmic reticulum and mitochondria. It is, therefore, not surprising that disruption of their association with these organelles would at least partially impair their anti-apoptotic activity. One of the main targets of MCL-1 is pro-apoptotic BAK, which is localized at the surface of the mitochondria (24), making it especially important for MCL-1 to associate with mitochondria to promote survival. Indeed, disruption of MCL-1 interaction with mitochondria by deleting either its N terminus (MCL-1ΔN79) or C-terminal transmembrane domain (MCL-1ΔTM) impaired its anti-apoptotic function. This loss of function could be dissociated from effects on the turnover of MCL-1 since MCL-1ΔN79 is at least as stable as MCL-1, expressed at higher levels, and the effects were still observed when apoptosis was induced with Ad tBID, which does not cause the degradation of MCL-1. Conversely, Nt-MCL promoted both the association of MCL-1 with mitochondria and cell survival after induction of apoptosis with both UV and Ad tBID, in a fashion that is dependent on the presence of MCL-1. The effects of Nt-MCL could also be dissociated from the effects on MCL-1 expression as it promoted survival in both HeLa and MCL-1 null MEFs despite having opposite effects on the expression levels of MCL-1 and MCL-1ΔN79 in the different settings. Of note, the increased mitochondrial MCL-1 in Nt-MCL-expressing cells was not reflected in the amount of MCL-1 inserted into the outer mitochondrial membrane (alkali-resistant). Because Nt-MCL-expressing cells are nonetheless more resistant to apoptosis, this could indicate that the alkali-sensitive MCL-1 constitutes the active fraction of the protein, similar to BCL-W, which undergoes a change from an active alkali-sensitive form to inactive alkali-resistant form after induction of apoptosis (32).

Several anti-apoptotic BCL-2 homologues possess an anti-proliferative activity in addition to their well characterized anti-apoptotic function (for review, see Ref. 33). Although BCL-2 and BCL-X₇ affect G₂/M transition, MCL-1 acts at the S/G₂ checkpoint. This has been proposed to be through the interaction between MCL-1 and either PCNA or CDK1 (11, 12). Of note, both these interactions take place in the nucleus, whereas
most of MCL-1 is associated with mitochondria in healthy cells, thus providing a possible mechanism to regulate the anti-proliferative function of MCL-1. The N terminus of MCL-1 indeed seems to regulate the balance between these two seemingly opposite roles of MCL-1. Specifically, expression of the first 79 amino acids of MCL-1 promoted the association of MCL-1 with mitochondria at the expense of its nuclear anti-proliferative function (Fig. 8). Interestingly, proteolytic cleavage of the N terminus of MCL-1 (around amino acid 17) was recently shown to occur in normally growing cells (21). Although this cleavage was shown to enhance the stability of MCL-1, it could also potentially regulate the anti-apoptotic (mitochondrial) versus anti-proliferative (nuclear) activities of MCL-1. It is also tempting to speculate that phosphorylation of serine 64 by CDK1 (34) enhances the stability of MCL-1, it could also potentially regulate the anti-apoptotic (mitochondrial) versus anti-proliferative function of MCL-1. The N terminus of MCL-1 indeed seems to regulate the balance between these two activities. In any case, the studies presented here have revealed a novel and important function for the N terminus of MCL-1 in the regulation of this important protein.

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