Regulation of Apoptosis and Cell Cycle Progression by MCL1
DIFFERENTIAL ROLE OF PROLIFERATING CELL NUCLEAR ANTIGEN*

MCL1 (ML1 myeloid cell leukemia 1), a Bcl-2 (B- cell lymphoma-leukemia 2) homologue, is known to function as an anti-apoptotic protein. Here we show in vitro and in vivo that MCL1 interacts with the cell cycle regulator, proliferating cell nuclear antigen (PCNA). This finding prompted us to investigate whether MCL1, in addition to its anti-apoptotic function, has an effect on cell cycle progression. A bromodeoxyuridine uptake assay showed that the overexpression of MCL1 significantly inhibited the cell cycle progression through the S-phase. The S-phase of the cell cycle is also known to be regulated by PCNA. A mutant of MCL1 that lacks PCNA binding (MCL1\textsubscript{AAA}) could not inhibit cell cycle progression as effectively as wild type MCL1. In contrast, MCL1\textsubscript{AAA} retained its anti-apoptotic function in HeLa cells when challenged by Etoposide. In addition, the intracellular localization of MCL1\textsubscript{AAA} was identical to that of wild type MCL1. An in vitro pull-down assay suggested that MCL1 is the only Bcl-2 family protein to interact with PCNA. In fact, MCL1, not other Bcl-2 family proteins, contained the PCNA-binding motif described previously. Taken together, MCL1 is a regulator of both apoptosis and cell cycle progression, and the cell cycle regulatory function of MCL1 is mediated through its interaction with PCNA.

Apoptosis and cell cycle progression are closely linked processes under rigorous control. The integrated molecular mechanism to control apoptosis and cell cycle progression, namely the existence of regulatory molecule(s) that interface between apoptosis and cell cycle progression, has been implicated and extensively investigated.

One such protein participating in the regulation of both apoptosis and cell cycle progression is p53, a tumor suppressor protein. Intriguingly, p53 transcriptionally activates both genes that regulate the S-phase entry, including dihydrofolate reductase (12), thymidine diphosphorylase (14), and p53-inducible genes (5). The p21\textsubscript{WAF1/CIP1} is a dual cell cycle inhibitor, functioning as an inhibitor of cyclin-dependent kinases (CDKs)\(^3\) and of proliferating cell nuclear antigen (PCNA) (1). On the other hand, the activation of bax, noxa, fas, and p53-inducible genes causes cells to undergo apoptosis (2–5). Although the exact mechanism by which p53 preferentially activates genes related to either cell cycle progression or apoptosis induction is unclear, an emerging body of evidence suggests that the phosphorylation of p53 plays a critical role in the selective activation of certain genes by inducing the distinctive conformational change to and modifying the binding site preference of p53 (6).

Another example of molecules that participate both in apoptosis and cell cycle regulation is the E2F (7) family proteins. Transcription factors of the E2F family, composed of E2F-1 to E2F-5, have been suggested to play a key role in the regulation of cell cycle progression (8). Importantly, E2F transcriptionally activates both genes that regulate the S-phase entry, including c-myc (9), cyclin D (10), cyclin E (11), and genes related to DNA synthesis, including dihydrofolate reductase (12), thymidine kinase (13), and DNA polymerase a (10). In addition to its cell cycle regulatory function, E2F-1, one of the E2F family proteins, also functions as an apoptosis regulator. The overexpression of E2F-1 triggers apoptosis. The E2F-1\textsuperscript{-/−} mice exhibit an excess of mature T cells due to a defect in thymocyte apoptosis (7). Taken together, E2F family proteins may function as both apoptosis and cell cycle regulators.

Still another example of the integrated control of apoptosis and cell cycle progression is Survivin, one of the inhibitor of apoptosis protein family members (14). Originally, Survivin was found to prevent cells from undergoing apoptosis upon cytokine deprivation (14). Subsequently, it was shown that Survivin was highly up-regulated in the G2/M-phase of the cell cycle (15). Survivin was found to be associated with microtubules and sustained cell survival during the G2/M-phase (15). Thus, Survivin functions as a cell cycle regulatory protein and as an apoptosis inhibitor.

The above evidence, along with other evidence, suggests that the molecules that regulate apoptosis can participate in the cell cycle regulation and vice versa. It is possible that proteins originally thought to regulate apoptosis may also have a role in cell cycle regulation.

The B-cell lymphoma-leukemia 2 (Bcl-2) protein family represents one of the major groups of apoptosis regulatory proteins, sharing the same structural characteristics (16, 17). At least 15 Bcl-2 family members have been identified in mammalian cells (18). Despite their structural similarities, Bcl-2 family members can either facilitate cell survival (pro-survival Bcl-2 subfamily) or promote cell death (pro-apoptosis Bax and other pro-apoptosis Bcl-2 proteins).
Bcl-2 homologous 3 subfamilies) (18). MCL1 (myeloid cell leukemia 1) is a 37.3-kDa protein originally cloned from a differentiating myeloid leukemia 1 cell line (19). Structurally and functionally, MCL1 belongs to the pro-survival Bcl-2 subfamily (20) that also includes Bcl-xL, Bcl-2, and Bcl-w (18). However, MCL1 possesses two unique features that make it outstanding among the pro-survival Bcl-2 subfamily proteins. First, MCL1 is inducible upon proliferative (21) and differentiating (19) stimuli. Second, the half-life of MCL1 is short (19, 21) most likely because MCL1 contains two PEST sequences (22). These PEST sequences are not present in other Bcl-2 family proteins (19). Interestingly, the PEST sequence is present in a number of cell cycle proteins, including cyclin D1, E2 (23), G2 (24), F (25), I (26) and c-Fos (27).

In order to investigate the mechanism of action and the potentially undiscovered functions of MCL1, we screened a human HeLa cell library with a yeast two-hybrid system using MCL1 as bait. Here we report a specific interaction between MCL1, an anti-apoptotic protein and a cell cycle regulatory protein, PCNA (28). In this report, we propose that MCL1 is not only an anti-apoptotic protein but also a cell cycle regulator and that the cell cycle regulatory function of MCL1 is at least partially mediated through its interaction with PCNA.

EXPERIMENTAL PROCEDURES

Cell Lines—Transformed human embryonic kidney (293T) cells were purchased from the American Type Culture Collection (Manassas, VA). HeLa and U2OS cells (an osteosarcoma cell line) are generous gifts from Dr. Limin Gong (Institute of Molecular Medicine for Prevention of Human Diseases, Houston, TX). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, nonessential amino acids, and antibiotics (Life Technologies, Inc.).

Molecular Cloning—The cDNA fragments of MCL1, PCNA, Survivin, Bcl-xL, Bak, and Bak were obtained by a standard PCR technique (29) using appropriate primer sets. These cDNA fragments were then ligated in-frame to appropriate yeast and mammalian expression vectors. A mutant of MCL1, MCL1[C288], was generated by PCR-based strategies as described previously (30). In all cases, the authenticity of cloned constructs was confirmed by automated dyeoxydeoxynucleotide sequencing (SeqWright Co., Houston, TX).

Yeast Two-hybrid Library Screening—The full-length MCL1 was cloned into pAS2.1 (CLONTECH, Palo Alto, CA), a vector that encodes GAL4 DNA-binding domain, and was used as bait. Saccharomyces cerevisiae X319-2A cells (MATa, CLONTECH) were transformed with pAS2.1-MCL1, using the lithium acetate method (31). We then performed yeast mating between X319-2A cells containing pAS2.1-MCL1 and Y187 cells (MATa) containing human HeLa cell library on pGAD GH (a vector that encodes GAL4 DNA-activating domain) for 27 h, according to the manufacturer’s instructions (31). Diploid yeast cells were selected for growth on synthetic dropout (SD) plates lacking adenine, histidine, leucine, and tryptophan (SD/-Ade/-His/-Leu/-Trp) for 14 days at 30°C. Positive colonies were screened for β-galactosidase activity using a X-gray (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) filter lift assay (31). Plasmid DNAs were then isolated from colonies that activated all three yeast reporter genes (HIS3, ADE2, and lacZ) using the lysis method (31), propagated in Escherichia coli, and analyzed by restriction digest and automated dyeoxydeoxynucleotide sequencing (SeqWright).

Yeast Two-hybrid Assay—S. cerevisiae SFY526 cells (CLONTECH) were co-transformed with pAS2.1 containing full-length MCL1 or empty pAS2.1 and pGAD GH containing M01, the C-terminal half (137th to 261st amino acids) of PCNA or empty pGAD GH. Transformed cells were selected on SD/-Trp/-Leu plates for 7 days and subjected to the X-gray filter lift assay as described above. The blue color that developed within 8 h was considered to represent a positive interaction.

Indirect Immunofluorescence and Confocal Laser Scanning Microscopy—Indirect immunofluorescence staining and confocal laser scanning microscopy was performed as described previously (33). In brief, U2OS cells were seeded in 4-well Lab-Tek® glass chamber slides (Nalge Nunc International, Rochester, NY). The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline, briefly treated with methanol/acetic acid mixture (1:1 v/v) at −20°C, blocked with 10% normal goat serum, and probed with rabbit anti-MCL1 polyclonal antibodies (Santa Cruz Biotechnology). Bound primary antibodies were detected with goat anti-rabbit antibody conjugated to rhodamine Red X (Jackson ImmunoResearch Laboratories, West Grove, PA). Stained cells were analyzed with the Fluoview confocal laser scanning microscope (Olympus, Melville, NY), using the ×60 objective lens. The same analysis was performed to determine the intracellular localizations of overexpressed wild type and MCL1[C288], a mutant MCL1.

Cell Death Assay—The experiment was performed in duplicate. HeLa cells were seeded in 4-well Lab-Tek® chamber slides, trans-
fected either with pFLAG-MCL1, pFLAG-MCL1, or pFLAG-LacZ using FuGENE6 (Roche Molecular Biochemicals), challenged with 5 μg/ml Etoposide for 12 h, and stained for the FLAG epitope using anti-FLAG antibody (M2, Sigma) and anti-mouse IgG conjugated to Rhodamine Red X (Jackson ImmunoResearch Laboratories). The nuclei were stained with DAPI (4,6-diamidino-2-phenylindole, Sigma). Cells were then examined under a Zeiss Axioscope fluorescent microscope (Carl Zeiss Ltd, Herts, UK), using appropriate filter sets. Cells that emitted red fluorescence were evaluated for their nuclear morphology. The condensed or fragmented nuclei were counted as apoptotic. An apoptotic index was then calculated as the number of red cells with apoptotic nuclear morphology divided by the number of total red cells counted.

Bromodeoxyuridine (BrdUrd) Uptake Assay—This experiment was performed in duplicate. Approximately 10,000 HeLa cells were seeded in 4-well Lab-Tek® chamber slides (Nalge Nunc International). The next day, cells were transfected with pFLAG-LacZ, pFLAG-p21Waf1/Cip1, pFLAG-MCL1, or pFLAG-MCL1 using FuGENE6 (Roche Molecular Biochemicals) according to manufacturer’s instructions. Cells were exposed to DNA-FuGENE6 complex for 5 h. Eighteen hours after the media change, cells were pulsed with 50 μM BrdUrd solution for 30 min at 37 °C. Harvested cells were first stained for incorporated BrdUrd, using a BrdUrd staining kit (Roche Molecular Biochemicals). In this procedure, bound anti-BrdUrd antibody was detected by goat anti-mouse Rhodamine Red X antibody (Jackson ImmunoResearch Laboratories). Cells were then stained for FLAG-tagged proteins with rabbit anti-FLAG antibody (Zymed Laboratories Inc., San South Francisco, CA) and goat anti-Rabbit-Cy3 antibody. The nuclei were counterstained with DAPI. The slides were examined under the Zeiss Axioscope fluorescent microscope (Carl Zeiss Ltd.), using appropriate filter sets. At least 150 cells (average = 364) were counted per channel. The BrdUrd incorporation was defined as the number of green cells with red nuclei (FLAG- and BrdUrd-positive) divided by the number of green cells (FLAG-positive).

Statistical Analysis—Statistical analysis was performed using a generalized linear model with Duncan multiple comparison at the significance level of 0.05.

RESULTS

MCL1 Specifically Interacts with PCNA—In order to identify the protein(s) interacting with MCL1, we screened a human HeLa cell cDNA library using the yeast two-hybrid system. Among the 2 × 10^6 independent clones screened, one clone, named “M01,” not only grew on histidine-adenine dropout plates but also activated the β-galactosidase reporter gene by an X-gal colony lift assay. A DNA sequence analysis revealed that the clone M01 represents the C-terminal 124 amino acid residues of human PCNA, which is fused in frame to the GAL4 activation domain (Fig. 1A). The presence of both MCL1 and M01 was necessary and sufficient for β-galactosidase reporter gene to be activated in yeast SFY526 cells (Fig. 1A). Thus, we concluded that MCL1 specifically interacted with the C-terminal half of PCNA in the yeast two-hybrid system.

We then performed an in vitro co-immunoprecipitation assay to test whether MCL1 would interact with the full-length PCNA. Here, we incubated in vitro translated, radiolabeled, influenza hemagglutinin (HA)-tagged PCNA with either in vitro translated, radiolabeled MCL1 or Survivin. Survivin is a member of inhibitor of apoptosis protein family, another anti-apoptotic protein family structurally unrelated to Bcl-2 family proteins (14, 15). As is shown in Fig. 1B, PCNA co-precipitated MCL1 (lanes 1 and 5) but not Survivin (lanes 2 and 6). Moreover, MCL1 could not be precipitated in the absence of PCNA (lanes 3 and 7). Thus, MCL1 specifically interacted with full-length PCNA in vitro.

We proceeded to test if MCL1 would interact with PCNA in mammalian cells in vivo. We transfected human embryonic kidney 293T cells with a plasmid encoding the cDNA of either the EGFP-tagged MCL1 or EGFP alone. As a result, EGFP-MCL1 and EGFP were found plentifully expressed in 293T cells (Fig. 1C, lanes 1 and 2, top panel). On the other hand, PCNA was found abundantly expressed in these cells without forced overexpression (Fig. 1C, lanes 1 and 2, bottom panel). When we immunoprecipitated PCNA in the cell lysate by an anti-PCNA antibody (lanes 3 and 4, bottom panel), only EGFP-MCL1, not EGFP, was co-immunoprecipitated with PCNA (lanes 3 and 4, top panel). When we immunoprecipitated EGFP or EGFP-MCL1 in the cell lysate by anti-EGFP antibody (lanes 5 and 6, top panel), only EGFP-MCL1, but not EGFP, co-immunoprecipitated PCNA (lanes 5 and 6, bottom panel). Thus, MCL1 specifically interacted with PCNA in mammalian cells in vivo.

So far we have shown that MCL1 and PCNA interacted specifically with each other in overexpression systems (Fig. 1, A–C). We wished to evaluate whether this interaction could be demonstrated in a non-overexpression, native system. To this end, the lysate from 2 × 10^7 HeLa cells was incubated with anti-PCNA antibody or with control antibody at the same concentration. As is shown in Fig. 1D, the lysate contained an equal amount of native MCL1 and PCNA as is shown in the bottom two panels (Total Cell Lysate (Input)). When the precipitated immune complexes were probed with anti-PCNA antibody, PCNA was found successfully precipitated by anti-PCNA antibody but not by the control antibody (Fig. 1D, 2nd panel). When the immune complexes were probed with anti-MCL1 antibody, MCL1 was found co-precipitated with PCNA (Fig. 1D, top panel, 1st column). There was no MCL1 precipitated in the absence of PCNA (Fig. 1D, top panel, 2nd column). This result strongly suggests that MCL1-PCNA interaction is biologically significant since it can be demonstrated in native, non-overexpression system as well as an overexpression system.

MCL1 Is Unique Among Bcl-2 Family Member Proteins in Its Ability to Interact Specifically with PCNA—We then evaluated whether PCNA interacted with other Bcl-2 family proteins. By using the same system as the one described in Fig. 1B, we tested the ability of PCNA to co-precipitate other pro- and anti-apoptotic Bcl-2 family proteins including Bcl-xL, Bak, or Bax. As is shown in Fig. 2, PCNA co-precipitated only MCL1 and not Bcl-xL, Bak, or Bax. Thus, MCL1 is an unusual Bcl-2 family protein that is capable of interacting with PCNA, a cell cycle regulatory protein.

MCL1 Is Present in the Nuclei and Cytoplasm—Functionally, PCNA serves as a cofactor to DNA polymerase δ (34) and modulates the function of other nuclear proteins, such as RF-C (Replication Factor- C) and Fen-I (35–37). Previous immunocytochemical studies further support that the functional site of action of PCNA is the nucleus (38). The biochemical evidence that we presented showed the presence of specific interaction between MCL1 and PCNA. We wished to determine the intracellular localization of MCL1. We proceeded to perform immunocytochemical staining of native MCL1 in U2OS cells. The cross-sectional analysis of the stained U2OS cells by confocal microscopy showed that MCL1 was both in the cytosol and nucleus, the nucleus being the predominant location (Fig. 3a). No confocal signals were detected when primary antibodies were omitted (Fig. 3b). Since PCNA is localized in the nucleus (38), the current data suggest that MCL1 is capable of physically interacting with PCNA in the nucleus.

MCL1 Contains a Conserved PCNA-binding Motif, a Mutation of Which Abolishes Its Binding to PCNA—Most of the previously identified PCNA-binding proteins contain a certain conserved amino acid motif (Fig. 4A) (36, 37, 39–45). The motif normally consists of a glutamine residue and a phenylalanine residue separated by 6 amino acid residues (X), QX_5,F. Uniformly, this motif is followed by a region rich in basic amino acids and lysine in particular, which is implicated for sub-
nuclear targeting (Fig. 4A, underlined K) (35). In some PCNA-binding proteins, disruption of this motif is shown to abolish their PCNA binding capacity (35, 40, 41). We evaluated whether MCL1, which was capable of binding PCNA, had the same consensus sequence. The protein sequence analysis of MCL1 and its mouse counterpart EAT revealed that both proteins in fact contained a conserved amino acid motif, i.e., 221QRNHETAF228 sequence followed by lysine-rich regions.
Fig. 1. Specific interaction between MCL1 and PCNA. The binding between in vitro translated, [35S]Met-labeled HA-PCNA and another in vitro translated, [35S]Met-labeled protein (i.e. MCL1, Bcl-xL, Bak, or Bax) was assessed in the system described in Fig. 1B. Only MCL1 (lanes 1 and 6) and not Bcl-xL (lanes 2 and 7), Bak (lanes 3 and 8), or Bax (lanes 4 and 9) was co-immunoprecipitated with PCNA in vitro.

Fig. 2. MCL1 is unique among Bcl-2 family members proteins in its ability to interact with PCNA. The binding between in vitro translated, [35S]Met-labeled HA-PCNA and another in vitro translated, [35S]Met-labeled protein (i.e. MCL1, Bcl-xL, Bak, or Bax) was assessed in the system described in Fig. 1B. Only MCL1 (lanes 1 and 6) and not Bcl-xL (lanes 2 and 7), Bak (lanes 3 and 8), or Bax (lanes 4 and 9) was co-immunoprecipitated with PCNA in vitro.

Fig. 3. MCL1 is present in the nuclei by confocal microscopy in U2OS cells. For the intracellular localization of MCL1, U2OS cells seeded on glass chamber slides were fixed with 4% paraformaldehyde in phosphate-buffered saline, treated with methanol/acetone mixture at −20 °C, and probed either with anti-MCL1 polyclonal antibody (a) or with staining buffer only (negative control; b). Bound antibodies were detected by goat anti-rabbit antibody conjugated to Rhodamine Red X. Stained cells were analyzed with the Olympus Fluoview (×60 objective) confocal laser scanning microscope. Confocal signals are most prominent in the nucleus in U2OS cells. Faint signals were observed in the cytosol.

Interaction between MCL1 and PCNA

by confocal microscopy. We concluded that the mutation introduced within the region of the QX1−6F motif of MCL1 did not alter its intracellular localization. Finally, we tested whether MCL1Δ44A retained anti-apoptotic function like wild type MCL1. To this end, HeLa cells overexpressing FLAG-tagged wild type MCL1, MCL1Δ44A, or β-galactosidase (LacZ) were challenged with etoposide, a topoisomerase II inhibitor, to induce apoptosis. Twelve hours after the challenge, cells were stained with anti-FLAG antibody and DAPI. The cells positively stained with anti-FLAG antibody were examined for their nuclear morphology visualized by DAPI. Strikingly, MCL1Δ44A, a MCL1 mutant that lacks PCNA binding, and the wild type MCL1 equally prevented etoposide-induced apoptosis in HeLa cells (Fig. 4D). This observation is important in several aspects. First, the interaction of MCL1 with PCNA was not necessary for MCL1 to function as an anti-apoptotic protein. Second, the mutation introduced to the PCNA-binding motif of MCL1, 222QRHETAE231, did not abolish the anti-apoptotic function of MCL1. Thus, MCL1 exerts its anti-apoptotic function through a different region than the 222QRHETAE231. Finally, the ability for MCL1 to distribute both in cytosol and nucleus is conferred by different region(s) of MCL1 than the 222QRHETAE228 since intracellular distribution of MCL1Δ44A was similar to that of the wild type as is shown in Fig. 4C.

MCL1 Overexpression Decreases BrdUrd Uptake in HeLa Cells—PCNA was originally isolated as a protein that appeared in elevated levels during the S-phase (34). In addition, the expression of PCNA antisense mRNA in proliferating cells causes the suppression of DNA replication and the cell cycle arrest at the S-phase (46). Moreover, PCNA functions as the processivity factor for DNA polymerase δ (34) and as a stimulatory factor of FEN-1, a protein that is involved in the maturation of an Okazaki fragment during DNA synthesis (37). PCNA-staining patterns co-localize with areas of radioactive thymidine uptake (47) or staining for BrdUrd (48).

Our data showed the presence of specific interaction between MCL1 and PCNA and indicated that the interaction occurs in the nucleus. In addition, we showed above that MCL1-PCNA interaction is not required for the anti-apoptotic function of MCL1. Thus, we speculated that the MCL1-PCNA interaction might contribute to a certain function other than apoptosis regulation. Based on the role of PCNA in the S-phase progression, we hypothesized that MCL1, by binding to PCNA, affects the function of PCNA as an S-phase protein. Accordingly, we evaluated the effect of MCL1 overexpression on DNA synthesis in HeLa cells, using BrdUrd uptake as an indicator of DNA synthesis (Fig. 5, a–c). As is shown in Fig. 5d, 34.1% of the cells transfected with the β-galactosidase gene (lacZ, a control gene) took up BrdUrd during the 30-min pulse-labeling time with BrdUrd. When p21Waf1/Cip1 was overexpressed, none of the cells took up BrdUrd during this time, which was consistent with previous reports. The p21Waf1/Cip1 is shown to bind PCNA, interfering with its interaction with DNA polymerase δ (39). The p21Waf1/Cip1 also binds and inactivates CDKs. Inactivation of CDKs increases the unphosphorylated retinoblastoma gene products, which sequester E2F, a transcriptional factor for the transactivation of S-phase genes (9–11). In this system, MCL1 overexpression was associated with the 63.3% reduction of cells that took up BrdUrd as compared with the control (12.5 ± 1.2% for LacZ, Fig. 5d). Thus, MCL1 is a dual function protein with the anti-apoptotic and S-phase inhibitory functions.

The Lack of PCNA Binding (MCL1Δ44A) Attenuates the Inhibitory Effect of MCL1 on DNA Synthesis—In the same experi-
ment, cells transfected with MCL1\textsubscript{D4A} synthesized 82% more DNA (22.75 ± 2.5%) than cells transfected with wild type MCL1 (12.5 ± 1.2%, significant at 0.05 level; Fig. 5d). Since MCL1\textsubscript{D4A} lacks the capability of binding to PCNA, the relative inefficiency of MCL1\textsubscript{D4A} to suppress DNA synthesis indicates that MCL1 interferes with S-phase DNA synthesis at least partially through its interaction with PCNA. Thus, the interaction of MCL1 with PCNA mediates the cell cycle regulatory function but not the anti-apoptotic function of MCL1. The exact mechanism by which MCL1 interferes with PCNA-dependent DNA synthesis requires further investigation.

**DISCUSSION**

We showed that MCL1 physically and functionally interacts with PCNA (Fig. 1). This MCL1-PCNA interaction is unique because PCNA interacts only with MCL1 and not with other Bcl-2 family member proteins (Fig. 2). The functional significance of the MCL1-PCNA interaction may be that MCL1 interferes with the cell cycle progression through its binding to PCNA (Fig. 5). To our knowledge, the presence of the physical and functional interaction between MCL1 and PCNA has not been reported in literature. The current data also support that
MCL1 is another example of regulatory molecules that interface between apoptosis and cell cycle progression, like p53, E25, and Survivin (see Introduction). Moreover, MCL1 appears to use different regions within the molecule to mediate two distinct functions, anti-apoptosis and cell cycle regulatory functions (Figs. 4 and 5).

PCNA plays a critical role in DNA replication. During DNA replication, PCNA forms a complex with RF-C, a large nuclear complex, which allows PCNA to assemble into a trimer, the functional form of PCNA (28). The RF-C-PCNA complex then binds to the RNA priming site, allowing DNA polymerase δ to bind PCNA and initiate DNA replication (49). In the current study, we demonstrated that the overexpression of MCL1 decreased BrdUrd uptake in a PCNA-dependent fashion (Fig. 5). One of the possible mechanisms to explain this is that MCL1 binds PCNA and prevents PCNA from physically associating with RF-C or with DNA polymerase δ. This can be through simple physical interference or through induced conformational changes within PCNA. The end result of this binding would be the inhibition of DNA synthesis and reduction of BrdUrd uptake, consistent with our findings described in this study. The end result of this binding may explain why p21 Waf1/Cip1 inhibited BrdUrd uptake more completely than MCL1 (Fig. 5d). Although not tested in the current work, it is possible that MCL1 does not have effects on CDKs activities and that transactivation of S-phase genes by E2F, thus blocking the G1-S transition (50). At the same time, p21 Waf1/Cip1 also binds to PCNA and interferes with the binding of PCNA to DNA polymerase δ. Since DNA polymerase δ requires PCNA to synthesize DNA effectively, the presence of p21 Waf1/Cip1 inhibits DNA synthesis, thereby further retardation cell cycle progression (39, 51). The dual actions of p21 Waf1/Cip1 on cell cycle progression may explain why p21 Waf1/Cip1 inhibited BrdUrd uptake more completely than MCL1 (Fig. 5d). Alternatively, it may be that p21 Waf1/Cip1 has other unspecified effects that interfere with DNA synthesis. In our view, the significance of our current data is that we showed MCL1 is a dual function protein with anti-apoptotic and anti-PCNA function; the latter is mediated by its physical interaction with PCNA. In addition, it is likely that MCL1 uses different regions within the molecule to mediate these two
distinct functions. The dual function of MCL1 may be beneficial in cells placed under certain conditions. For example, MCL1 is shown to be transiently up-regulated when cells sustain DNA damage (52). In this instance, up-regulated MCL1 may arrest the cell cycle progression through its interaction with PCNA, thus preventing these cells from replicating altered DNAs. At the same time, MCL1 may exert its anti-apoptotic function and ensure the survival of the cells until DNA repair is completed. Although the biological significance of the interplay between MCL1 and p21Waf1/Cip1, both of which are up-regulated in cells with genetic damage (45, 52), remains to be elucidated, this apparent redundancy may well represent still another safety mechanism by which living organisms increase their chance of survival under diverse environmental stresses.

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REFERENCES

1. Levine, A. J. (1997) Cell 88, 323–331
2. Miyashita, T., and Reed, J. C. (1995) Cell 80, 293–299
3. Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokito, T., Taniguchi, T., and Tanaka, N. (2000) Science 288, 1053–1058
4. Owen-Schaub, L. B., Zhang, W., Cusack, J. C., Angelo, L. S., Santee, S. M., Grana, X., Giordano, A., and Calabretta, B. (1994) Proc. Natl. Acad. Sci. USA 91, 1402–1406
5. Palen, J., Egly, J. M., and Staudt, L. M., Jr., Boguski, M. S., Lashkari, D., Shalon, D., Botstein, D., and Brown, P. O. (1999) Science 283, 83–87
6. Peto, J., and Peto, R. A. (1997) Cell 83, 3256–3260
7. Ambrosini, G., Adida, C., and Altieri, D. C. (1997) Nat. Med. 3, 287–293
8. Weinberg, R. A. (1995) Science 269, 18529–18534
9. Kozopas, K. M., Yang, T., Buchan, H. L., Zhou, P., and Craig, R. W. (1993) Proc. Natl. Acad. Sci. USA 90, 3516–3520
10. Reynolds, J. E., Li, J., Craig, R. W., and Eastman, A. (1996) Exp. Cell Res. 225, 430–436
11. DeGregori, J., Kowalik, T., and Nevis, R. J. (1995) Mol. Cell. Biol. 15, 2415–2424
12. Blake, M. C., and Azikhan, J. C. (1989) Mol. Cell. Biol. 9, 4994–5002
13. Waga, S., and Hubscher, U. (1998) EMBO J. 17, 3786–3795
14. Hirano, T., and Hubscher, U. (1999) EMBO J. 18, 4212–4225
15. Kang, S., and Hubscher, U. (1997) Science 277, 574–578
16. Chuang, L. S., Ian, H. I., Koh, T. W., Ng, H. H., Xu, G., and Li, B. F. (1997) Proc. Natl. Acad. Sci. USA 94, 12863–12868
17. Iyer, V. R., Eisen, M. B., Schuler, G., Moore, T., Lee, J. C. F., Trent, J. M., Staudt, L. M., Hudson, J. Jr., Boguski, M. S., Lashkari, D., Shalon, D., Botstein, D., and Brown, P. O. (1999) Science 283, 83–87
18. DeGregori, J., Kowalik, T., and Nevis, R. J. (1995) Mol. Cell. Biol. 15, 2415–2424
19. Field, S., Tsai, F., Kuo, F., Zubiaga, A., Kaelin, W., Jr., Livingston, D., Orkin, S., and Greenberg, M. (1996) Cell 85, 549–561
20. Li, X., Lieber, M. R., and Burgers, P. M. (1995) Proc. Natl. Acad. Sci. USA 92, 12952–12959
21. Iyer, V. R., Eisen, M. B., Ross, D. T., Schuler, G., Moore, T., Lee, J. C. F., Trent, J. M., Staudt, L. M., Hudson, J. Jr., Boguski, M. S., Lashkari, D., Shalon, D., Botstein, D., and Brown, P. O. (1999) Science 283, 83–87
22. Weinberg, R. A. (1995) Science 269, 18529–18534
23. Adams, J. M., and Cory, S. (1998) Science 281, 1322–1326
24. Prives, C. (1997) J. Cell Biol. 138, 1544–1548
25. Langenfeld, J., Kiyokawa, H., Sekula, D., Boyle, J., and Dmitrovsky, E. (1997) Cancer Res. 57, 515–517
26. Hiebert, S. W., Lipp, M., and Nevins, J. R. (1989) Nature 340, 39465
27. Rajewsky, K., and Holter, S. (1988) Cell 5682–5687
28. Langenfeld, J., Kiyokawa, H., Sekula, D., Boyle, J., and Dmitrovsky, E. (1997) Cancer Res. 57, 515–517
29. Ambrosini, G., Adida, C., and Altieri, D. C. (1997) Nat. Med. 3, 917–921
30. Ambrosini, G., Adida, C., and Altieri, D. C. (1997) Nat. Med. 3, 917–921
31. Yen, E. T. (1997) J. Biol. Chem. 272, 24522–24529
32. Zhang, S. J., Wu, S. M., and Lee, M. Y. (1995) J. Biol. Chem. 270, 22109–22112