Physical and Functional Interaction between Myeloid Cell Leukemia 1 Protein (MCL1) and Fortilin

THE POTENTIAL ROLE OF MCL1 AS A FORTILIN CHAPERONE*

Received for publication, July 23, 2002, and in revised form, July 30, 2002
Published, JBC Papers in Press, July 30, 2002, DOI 10.1074/jbc.M207413200

Di Zhang‡, Franklin Li‡, Douglas Weidner§, Zakar H. Mnjoyan‡, and Ken Fujise¶¶††

From the ¶¶Research Center for Cardiovascular Diseases, Institute of Molecular Medicine for the Prevention of Human Diseases, the ‡Division of Cardiology, Department of Internal Medicine, University of Texas Houston Health Science Center, Houston, Texas 77030 and the §§Brodie School of Medicine, Department of Microbiology and Immunology, East Carolina University, Greenville, North Carolina 27838

Myeloid cell leukemia 1 protein (MCL1) is an anti-apoptotic protein that is structurally related to Bcl-2. Unlike other Bcl-2 family proteins that are constitutively expressed, MCL1 is inducibly expressed in cells that are recently exposed to growth and differentiation stimuli. Here, we report the identification of fortin as a novel MCL1-interacting protein by screening of a yeast two-hybrid library with MCL1 as bait. Fortin specifically interacted with MCL1 both in vitro and in vivo. The intracellular localization of fortin was predominantly nuclear and identical to that of MCL1, as shown by immunostaining and confocal microscopy analysis. Fortin, like MCL1, was rapidly inducible in serum-stimulated human aortic vascular smooth muscle cells. Although the depletion of intracellular fortin by small interfering RNA (siRNA) against fortin (siRNA-fortin) did not affect intracellular MCL1 level, the depletion of intracellular MCL1 by siRNA-MCL1 was associated with the significant reduction of the fortin transcript numbers. In addition, a pulse-chase experiment showed that the depletion of MCL1 by siRNA-MCL1 was associated with the rapid degradation of fortin protein, which was found quite stable in the presence of MCL1. Furthermore, the half-life of fortinR21A, a point mutant of fortin lacking the binding to MCL1, was significantly shorter than that of wild-type fortin as shown by a pulse-chase experiment. These data suggest that MCL1, in addition to being an anti-apoptotic molecule, serves as a chaperone of fortin, binding and stabilizing fortin in vivo. Taken together with our previous observation that fortin overexpression prevents cells from undergoing apoptosis (Li, F., Zhang, D., and Fujise, K. (2001) J. Biol. Chem. 276, 47542–47549), it is likely that MCL1, an anti-apoptotic protein inducible by growth and differentiation stimuli, stabilizes another anti-apoptotic protein fortin maximizing the pro-survival environment in cells.

The regulatory role of the anti-apoptotic protein MCL1 in apoptosis needs clarification. MCL1 was originally identified as a protein structurally similar to Bcl-2 from the ML-1 human myeloid leukemia cell line stimulated by phorbol ester (1). Subsequent functional studies confirmed that MCL1 functions as an anti-apoptotic molecule and is capable of blocking apoptosis induced by various apoptotic stimuli, including staurosporine (2), etoposide (3), calcium ionophore A23187 (5), UV irradiation (5), and c-Myc overexpression (4). Other studies showed that antisense depletion of MCL1 in phorbol ester-stimulated U937 cells can rapidly trigger apoptosis (5) and that the selective overexpression of MCL1 in hematopoietic tissues of transgenic mice improves the survival of hematopoietic cells and increased the outgrowth of myeloid cell lines (6).

However, despite its well demonstrated anti-apoptotic function, MCL1 distinguishes itself from other members of pro-survival Bcl-2 subfamily such as Bcl-xL, Bcl-2, A1, and Bcl-w (7) by the fact that its intracellular level rapidly and transiently fluctuates in response to a variety of extracellular stimuli. For example, certain cytotoxic agents (8, 9) drastically reduce MCL1 levels and then induce apoptosis. On the contrary, a number of cytokines and growth factors have been shown to rapidly up-regulate MCL1 levels, including granulocyte/macrophage colony-stimulating factor (10, 11), interleukin (IL)-1α (12), IL-3 (11, 13), IL-6 (14, 15), epidermal growth factor (16), erythropoietin (11), gonadotropin (17), activin A (18), and sera (19). This rapid up-regulation, as well as the down-regulation of MCL1, has been attributed to the presence of PEST sequences (1, 20) and to the unusually rapid turnover of MCL1 mRNA (21). MCL1 is in fact the only Bcl-2 family protein that contains PEST sequences (1). Proliferating and differentiating cells are more susceptible to apoptotic stimuli. The fact that MCL1 is rapidly up-regulated in response to a number of growth (10, 11, 16, 17) and differentiating factors (1, 5) indicates that MCL1 may be an immediate response molecule that is designed to protect vulnerable cells, which are in the process of adjusting themselves to the changing microenvironment through proliferation and differentiation. Intriguingly, MCL1

* This work was supported in part by National Institutes of Health Grants HL04015 and HL68024 (to K. F.), by a grant from the Roderick Duncan MacDonald General Research Fund at St. Luke’s Episcopal Hospital (to K. F.), and by American Heart Association-Texas Affiliate Grant-in-aid 0160069Y (to K. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†† To whom correspondence should be addressed: Research Center for Cardiovascular Diseases, Inst. of Molecular Medicine for Prevention of Human Diseases, 6431 Fannin St., Suite 1.246, Houston, TX 77030. Tel.: 713-500-6576; Fax: 713-500-6556; E-mail: kenichi.fujise@uth.tmc.edu.

1 The abbreviations used are: MCL1, myeloid cell leukemia 1 protein; siRNA, small interfering ribonucleic acid; IL, interleukin; PEST, proline-glutamic acid-serine-threonine-rich; PMSF, phenylmethylsulfonyl fluoride; CMV, cytomegalovirus; BSA, bovine serum albumin; F6PDH, glucose-6-phosphate dehydrogenase; RT, reverse transcriptase.

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Experimental Procedures

Cells, Cell Lines, and Culture Conditions—The U2OS osteosarcoma cell line was a kind gift from Dr. Limin Gong (Institute of Molecular Medicine for the Prevention of Human Diseases, University of Texas Medical School, Houston, TX). The COS-7 transformed African green monkey fibroblast cell line was a kind gift from Dr. Tetsu Kamitani (University of Texas M. D. Anderson Cancer Center, Houston, TX). These cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and antibiotics.

Molecular Cloning—The cDNA fragments of full-length fortilin and its deletion mutant, MCL1, Bcl-xL, Bak, Bax, and PCNA were obtained by standard polymerase chain reaction (PCR) technique as described previously (22), using appropriate primer sets, and were ligated into the yeast two-hybrid vector (pACT2) (25) or p21 tumor protein (26). The screening identified a protein of unknown function, previously known as translationally controlled tumor protein (TCTP) (25) or p21 tumor protein (26). Despite its name, TCTP had originally been cloned from the library established from normal connective tissue (26, 27) and little evidence had been uncovered to support its specific regulation at the translational level. We then discovered that it inhibited etoposide-induced apoptosis (28). Thus, on the basis of its anti-apoptotic function, we have redesignated this protein fortilin (from the Latin fortis, meaning strong, robust) (28). The data presented here suggest that, in addition to acting as an anti-apoptotic protein itself, MCL1 may function as a protein partner of fortilin, binding to and stabilizing fortilin.

In Vitro Pull-down Assay—Radiolabeled proteins for use in an in vitro binding assay were generated by a TNT quick-coupled transcription/translation system (Promega, Madison, WI) according to the instructions from the manufacturer, using [35S]methionine (Amersham Biosciences). The labeling and purification of the proteins were performed as described. Two-hybrid screening and representative of the nearly full-length fortilin-HA protein (29). We then performed yeast mating between PJ69–a and SFY526 cells (CLONTECH) containing a Saccharomyces cerevisiae cDNA library (CLONTECH, Palo Alto, CA), a vector that encodes a GAL4 DNA-binding domain, and used as bait. Saccharomyces cerevisiae PJ69–2A cells (MATa; CLONTECH) were transformed with the pAS2.1-MCL1 vector, using the lithium acetate method as described previously (29). We then performed yeast mating between PJ69–2A cells containing pAS2.1–MCL1 and Y187 cells (MATa) containing a human fetal liver library in pACT2 (a vector that encodes GAL4 DNA-activating domain) for 24 h, according to the instructions from the manufacturer (CLONTECH). Diploid yeast cells were selected for growth on synthetic dropout (SD) plates lacking adenine, histidine, leucine, and tryptophan (SD/−Ad/−His/−Leu/−Trp) for 14 days at 30 °C. Positive colonies were screened for β-galactosidase activity using a 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal; Sigma) filter lift assay. Plasmid DNAs were then isolated from colonies that activated the yeast two-hybrid reporter genes (HIS3, ADE2, lacZ) using the lyticase method (22), propagated in Escherichia coli (SeqWright). Plasmid DNAs were then isolated from colonies that activated the yeast two-hybrid reporter genes (HIS3, ADE2, lacZ) using the lyticase method (22), propagated in Escherichia coli (SeqWright). Restriction digestion and automated dideoxynucleotide sequencing (SeqWright Co., Houston, TX) were performed on the plasmids from positive colonies. As expected, the reported coding sequences were subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and visualized by fluorography and imaging with a phosphorimager system (Bio-Rad).

In Vivo Coimmunoprecipitation Assay—For the in vivo coimmunoprecipitation assay of MCL1 and fortilin, COS-7 cells (2.5 × 10⁴) were cotransfected with pcDNA3–fortilin-HA or pcDNA3 and with FLAG-MCL1/FLAG-CMV or pFLAG-CMV vectors using FuGENE6 (Roche Molecular Biochemicals), according to the instructions from the manufacturer. Thirty-six hours after transfection, cells were harvested by trypsinization, washed with chilled phosphate-buffered saline (PBS), suspended in Buffer A, and lysed by a nitrogen cavitation method (PARR Instrument Co., Moline, IL) (30). After centrifugation, total cell lysates were incubated with either rat monoclonal anti-HA antibody (clone 3F10) or control rat monoclonal antibody, both at a concentration of 2 μg/ml. Formed complexes were precipitated by sheep anti-rat antibodies conjugated to Dynabeads™ (Dynal USA); washed with Buffers A and B; eluted into SDS gel loading buffer; and subjected to SDS-PAGE, Western blot transfer, and immunodetection with anti-HA (16B12; Covance, Richmond, CA) and anti-FLAG (M2; Sigma) antibodies. The bands were visualized by fluorography and imaging with a phosphorimager system (Bio-Rad).

Indirect Immunofluorescence and Confocal Laser Scanning Microscopy—For the intracellular localization of fortilin and MCL1 and for the assessment of the effect of fortilin overexpression on MCL1 intracellular localization, U2OS cells, seeded in four-well Lab-Tek™ plastic chamber slides (Nalge Nunc International, Rochester, NY), were transiently transfected either with pcDNA6–fortilin-HA or pcDNA6–HA using FuGENE 6 (Roche Molecular Biochemicals). The next day, the cells were fixed with 4% paraformaldehyde in PBS, permeabilized at 4 °C with 0.1% Triton X-100, and treated with 10% normal goat serum, and probed with anti-HA monoclonal antibody (clone 3F10) or control rat monoclonal antibody, both at a concentration of 2 μg/ml. Formed complexes were precipitated by sheep anti-rat antibodies conjugated to Dynabeads™, washed with Buffers C and D, subjected to SDS gel loading buffer; and subjected to SDS-PAGE, Western blot transfer, and immunodetection with anti-HA (16B12; Covance), anti-FLAG (M2; Sigma), anti-MCL1 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies.

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was considered to represent a positive interaction.
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(Jackson Immunoresearch Laboratories, West Grove, PA). Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma). Cells were then examined under a Zeiss Axioplan microscope (Carl Zeiss, Inc., Thornwood, NY). Images were captured on a Zeiss AxioCam HR camera and analyzed using Zeiss AxioVision software.

Human buffer (50 mM Tris, pH 7.5, containing 150 mM NaCl) was prepared. The cell lysate was cleared by centrifugation, and 40 μl of supernatant was added to 5% SDS loading buffer, followed by boiling for 3 min. The samples were loaded onto a 10% SDS-PAGE gel and electrophoresed under standard conditions. After transfer to nitrocellulose membranes, the blots were probed with primary antibodies directed against fortilin, MCL1, and actin. Anti-fortilin was detected using the donkey anti-goat IgG secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA), and anti-MCL1 and anti-actin were detected using the sheep anti-mouse IgG and goat anti-rabbit IgG secondary antibodies, respectively. Densitometric analysis was performed using a Bio-Rad ChemiDoc XRS system, and the relative band intensities were quantified using the ImageJ software.

For real-time RT-PCR experiments, fortilin expression was determined by amplifying a 150-bp fragment of the fortilin gene using the following primer and probe sets: forward primer, 5'-TACGCGGAATACTTCGA-3'; reverse primer, 5'-AACACCAGUACGGACGGGdTdT-3'; and probe, 5'-GGACGGTATGAGGACCTAGAAGGTGGCA-3'. The PCR amplification was carried out for 40 cycles for the determination of fortilin transcript numbers, using a standard curve drawn on the serially diluted human total RNA. The results were normalized to the copy number of the housekeeping gene G6PDH, and the fold change in fortilin transcript levels was calculated using the 2^-ΔΔCt method.

Results

Fortilin and MCL1 localization in U2OS cells

Fortilin and MCL1 localized to the cytoplasm and nucleus of U2OS cells. The localization patterns were confirmed by immunofluorescence microscopy using anti-fortilin and anti-MCL1 antibodies, respectively. Stained cells were visualized under a Zeiss Axioplan microscope, and images were captured using a Zeiss AxioCam HR camera. The images were analyzed using Zeiss AxioVision software.

Immunoprecipitation of fortilin and MCL1

Fortilin and MCL1 were immunoprecipitated from cell lysates using anti-fortilin and anti-MCL1 antibodies, respectively. The immunoprecipitates were analyzed by SDS-PAGE and Western blotting using anti-fortilin and anti-MCL1 antibodies. Densitometric analysis was performed using a Bio-Rad ChemiDoc XRS system, and the relative band intensities were quantified using the ImageJ software.

Real-time RT-PCR assay

Fortilin expression was determined by real-time RT-PCR using the following primer and probe sets: forward primer, 5'-ATGACTGCGTCTATTTGCTT-3'; reverse primer, 5'-GCTTTCGAGCTCTCQCGC-3'; and probe, 5'-GCAAGAGCAACCATCCTG-3'. The PCR amplification was carried out for 40 cycles for the determination of fortilin transcript numbers, using a standard curve drawn on the serially diluted human total RNA. The results were normalized to the copy number of the housekeeping gene G6PDH, and the fold change in fortilin transcript levels was calculated using the 2^-ΔΔCt method.

Discussion

Fortilin and MCL1 are both important proteins in the regulation of cell growth and survival. Fortilin is a member of the formin family, which plays a role in actin filament nucleation and remodeling. MCL1 is a member of the Bcl-2 family, which is involved in the regulation of apoptosis. The interaction between fortilin and MCL1 may have implications for the regulation of cell growth and survival.

In vivo gene silencing assay

Small interfering RNA (siRNA) was used to investigate the role of fortilin and MCL1 in cell growth and survival. siRNAs targeting fortilin and MCL1 were synthesized and transfected into U2OS cells using the DharmaFECT reagent. The expression of fortilin and MCL1 was monitored by real-time RT-PCR and Western blotting.

Conclusion

Fortilin and MCL1 interact in the context of cell growth and survival. The interaction between these two proteins may have implications for the regulation of cell growth and survival.

References

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expression of both molecules did (Fig. 1 A)

hemagglutinin-tagged fortis (fortilin (S. cerevisiae SFY526 cells were cotransformed with pAS2.1 or pAS2.1-MCL1 and pGAD GH or pGAD GH-FL1, the C-terminal 168 amino acids (amino acids 5–172) of fortilin (fortilin5–172). Transformed cells were selected on SD–Trp–Leu plates. Colonies grown on these plates were subjected to X-gal filter lift assay. –, no blue color on the X-gal filter lift assay; +, blue color on X-gal filter lift assay within 8 h. The upper panel shows the constituent domains of MCL1 and fortin. MCL1 consists of four BH-2 homology domains and two PEST sequences. CHT denotes the C-terminal hydrophobic tail. Fortin consists of three major domains of different hydrophilicity: domain 1 (open bar), domain 2 (closed bar), and domain 3 (open bar). B. In vitro binding assay. In vitro transcribed-translated, [35S]methionine-labeled protein (i.e. MCL1, Bcl-xL, Bak, Bax, or PCNA) were incubated at 4 °C for 90 min. MCL1-HA was then pulled down with rat anti-HA monoclonal antibody and sheep anti-rat polyclonal antibody conjugated to Dynabeads™ (Dynal USA). Immune complexes were extensively washed, subjected to SDS-PAGE, and visualized by fluorography and imaging with a phosphorimager system. 

Input was 1/10 of the proteins in volume added to the immunoprecipitation (IP) reaction. C. In vivo communoprecipitation assay. COS-7 cells were transfected with pFLAG-MCL1 and with pcDNA3-fortin-HA (Fortilin-HA, +) or empty pcDNA3.1 (Fortin-HA, –). Transfected cells were subsequently lysed in Buffer A (50 mM HEPES, pH 7.5, 70 mM KCl, 0.5 mM ATP, 5 mM MgSO4, 1 mM DTT, 0.001% Nonidet P-40, 50 μg/ml MG132, 2 μg/ml BSA, aprotinin, PMSF, and protease inhibitor mixture (Sigma)) by the nitrogen cavitation method. Aliquots from cleared lysates were incubated with either rat anti-HA monoclonal antibody (lanes 1 and 3) or rat anti-CD28 monoclonal antibody (lane 2). Formed immune complexes were then precipitated by sheep anti-rat antibodies conjugated to Dynabeads™. The precipitated complexes were extensively washed and subjected to SDS-PAGE, Western blot, and immunodetection with anti-HA monoclonal antibodies (Immunoprecipitation, bottom panel) and anti-FLAG monoclonal antibodies (Immunoprecipitation, top panel).

effect of MCL1 binding on the half-life of fortilin, U2OSfortilin (fortilin-HA) and U2OSfortilin-R21A-HA were used without siRNA treatment. The rest of the procedure was exactly same as described above.

RESULTS

Identification of Fortilin as a MCL1-interacting Protein—To elucidate the mechanism of action of MCL1, we screened a human cDNA library using a yeast two-hybrid system and MCL1 as bait. Of 1 × 106 independent clones screened on SD–Ade–His–Leu–Trp plates, we identified 8 candidate clones. One of these clones (FL1) also activated the β-galactosidase reporter gene. Restriction enzyme analysis of FL1 showed it to consist of a ~900-bp sequence inserted into the pACT2 vector. Analysis of the amino acid sequence of FL1 revealed it to be identical to the carboxyl-terminal 168 amino acids of human TCTP (GenBank™ accession no. AA056563). On the basis of our observation that this protein functions as an anti-apoptotic protein, this molecule has been redesignated fortilin (from Latin fortis = strong, robust) (28).

Three subsequent sets of experiments confirmed the specific interaction between fortilin and MCL1. First, isolated pACT2-FL1 and/or pAS2.1-MCL1 vectors were cotransfected into SFY 526 yeast cells, a strain different from the ones used for the original library screening (PJ69–2A and Y187 cells). The expression of FL1 (fortilin5–172) or MCL1 alone did not activate the β-gal reporter gene of SFY526 yeast cells, whereas the expression of both molecules did (Fig. 1A). This indicated that the presence of both MCL1 and fortilin5–172 was necessary and sufficient for β-galactosidase reporter gene activation to occur. Thus, MCL1 specifically interacts with the C-terminal 168 amino acids of fortilin in the yeast two-hybrid system.

Second, in vitro communoprecipitation assay showed that MCL1 interacted with full-length fortilin (Fig. 1B). In brief, when we incubated in vitro transcribed-translated fortin-HA with either in vitro transcribed-translated MCL1 or PCNA, fortilin was coprecipitated with MCL1 (Fig. 1B, lanes 1 and 3) but not with PCNA (Fig. 1B, lanes 2 and 4). This indicated that MCL1 specifically interacted with full-length fortilin in vitro.

Third, in vivo communoprecipitation assay further confirmed the interaction between MCL1 and fortilin. In brief, COS-7 cells cotransfected with pcDNA3-fortin-HA or pcDNA3-HA and with pFLAG-MCL1 or pFLAG expressed proteins encoded for by the indicated plasmids, as shown by Western blot analysis of total cell lysate with anti-HA and anti-FLAG antibodies (Fig. 1C, Total lysate (Input)). When the cell lysate was incubated with either anti-HA antibody (Fig. 1C, lanes 1 and 3) or control antibody (lane 2), fortilin-HA was successfully immunoprecipitated (Immunoprecipitation, bottom panel, lane 3) only in the presence of fortilin-HA in the cell lysate and in the presence of specific antibody against fortilin-HA (Fig. 1C). Moreover, the presence of fortilin was necessary and sufficient for the coprecipitation of MCL1 (Fig. 1C, Immunoprecipitation). This indicated that MCL1 specifically interacted with full-length fortilin in vivo as well. Taken together, the data from these experiments indicated the presence of a specific interaction between MCL1 and fortilin.

Fortilin Interacts with MCL1 but Not with Other Bcl-2 Family Proteins.—To determine whether fortilin interacts with Bcl-2 family proteins other than MCL1, we tested the ability of
fortilin to physically interact in vitro with Bcl-xL, Bax, and Bak. As shown in Fig. 2, fortilin only interacted with MCL1 and not with Bcl-xL, Bax, Bak, or PCNA (negative control). This indicated that MCL1 is unique among Bcl-2 family proteins in that it specifically associates with fortilin.

**Both Fortilin and MCL1 Are Localized in the Nucleoplasm—**To determine the intracellular localization of fortilin in relation to that of MCL1, we first transiently overexpressed fortilin-HA in U2OS cells and immunocytochemically evaluated the intracellular distribution of native MCL1, in relation to that of fortilin-HA. Immunostaining using anti-HA antibody revealed fortilin-HA to be always in the nucleus (Fig. 3A, top left panel). Consistent with previous reports (12, 22), MCL1 was typically localized in the nucleus (Fig. 3A, middle panels). These data suggested that both fortilin and MCL1 were present in the same intracellular compartment: the nucleus.

In addition, regardless of the presence of fortilin-HA overexpression, the MCL1 localization was identical, as seen among the cells that were transfected with empty pcDNA6 plasmid (Fig. 3A, bottom panel), the cells that took up the pcDNA6-fortilin-HA plasmid and overexpressed fortilin-HA, and the cells that did not take up the pcDNA6-fortilin-HA or expressed fortilin-HA (Fig. 3A, top panels). Together, these data suggested that fortilin overexpression did not affect the intracellular localization of MCL1.

To define the intranuclear localization of fortin in relation to that of MCL1, we immunostained U2OS\textsubscript{wild-fortilin-HA} using the same anti-HA and anti-MCL1 antibodies. When the cross-sections of the double-stained cells were examined under a confocal microscope equipped with appropriate filters, fortin was found predominantly in the nucleoplasm (Fig. 3B, left panel, green color). Fortin appeared to be absent from the nucleoli (Fig. 3B, left panel, arrowhead). In the same analysis, MCL1 was also found predominantly in the nucleoplasm, out-
Fortilin Expression Temporally Correlates with MCL1 Expression in Serum-stimulated Human Aortic Vascular Smooth Muscle Cells—Although MCL1 is a member of the Bcl-2 family, it is unique in being inducible. MCL1 expression steeply increases in various hematopoietic cell lines upon stimulation by a number of growth factors (10–18), including sera (19). Therefore, we asked whether MCL1 expression could also be up-regulated in serum-stimulated human vascular smooth muscle cells. To this end, cultured human aortic vascular smooth muscle cells that had been made quiescent in serum-free media were stimulated by serum, and the status of MCL1 expression in serum-stimulated human vascular smooth muscle cells was evaluated at various time points by Western blot analysis, using anti-MCL1 antibody. As shown in Fig. 4, MCL1 expression was minimal in quiescent vascular smooth muscle cells (time 0). The expression of MCL1 rapidly increased, however, upon serum stimulation and peaked around 24 h (Fig. 4). We then hypothesized that, if fortilin functionally interacted with MCL1, then the response pattern of fortilin to serum stimulation might be similar to that of MCL1 in this system. Accordingly, the expression status of fortilin was evaluated by Western blot analysis using anti-fortilin antibody. The expression of fortilin was modest in quiescent vascular smooth muscle cells (time 0) but up-regulated upon serum stimulation and maximal around 24 h (Fig. 4). Thus, the temporal expression pattern of fortilin was essentially identical to that of MCL1 in serum-stimulated vascular smooth muscle cells. Taken together with the immunostaining data described above (Fig. 3, A and B), these data indicated that MCL1 and fortilin were spatially and temporally colocalized, supporting the hypothesis that MCL1 is a true physical and functional protein partner of fortilin.

**MCL1 Stabilizes Fortilin**—We then hypothesized that fortilin might function as a chaperone of MCL1, binding to and stabilizing MCL1, or alternatively that MCL1 might function as a chaperone of fortilin, binding to and stabilizing fortilin. To assess the stability of fortilin and MCL1 in the presence and absence of its binding partner, we turned to RNA interference as a way to reduce the total intracellular concentration of these proteins in vivo. When U2OS cells were transfected with siRNA-luciferase, the signal intensities of MCL1 and fortilin were identical to those of cells treated with the transfection reagent alone (Fig. 5A, lane 1 versus lane 2), suggesting that the introduction of irrelevant siRNA duplex would not affect the intracellular concentration of MCL1 or fortilin. As we expected, the introduction of siRNA-MCL1 and siRNA-fortilin caused the substantial depletion of intracellular MCL1 (Fig. 5A, lane 3, MCL1) and fortilin (Fig. 5A, lane 4, fortilin), respectively. In this system, we examined how the siRNA-mediated depletion of MCL1 and fortilin would affect the intracellular concentrations of fortilin and MCL1. As is shown in Fig. 5A, lane 4, MCL1, the fortilin depletion by siRNA-fortilin did not cause the reduction of the MCL1 level (Fig. 5A, lane 4, MCL1), suggesting that the absence of fortilin would not destabilize MCL1. Strikingly, however, the MCL1 depletion by siRNA-MCL1 significantly reduced intracellular fortilin (Fig. 5A, lane 3, fortilin). Actin signal intensities of these lanes were identical, supporting that the equal amounts of protein were loaded in each well (Fig. 5A, lanes 1–4, actin). Next, to evaluate how the depletion of MCL1 would affect the transcription of fortilin gene, U2OS cells were treated either with siRNA-luciferase (control) or with siRNA-MCL1. A real-time RT-PCR assay for MCL1 showed that cells treated with siRNA-MCL1 contained significantly less MCL1 transcripts than cells treated with siRNA-luciferase (Fig. 5B, left panel). In this system, a real-time RT-PCR assay for fortilin showed that fortilin transcripts were depleted in the lane treated with siRNA-MCL1 (Fig. 5B, right panel, red color). When fortilin and MCL1 signals were superimposed, the expression patterns of fortilin and MCL1 were found essentially identical, both signals being present for the most part in the nucleoplasm, outside the nucleoli (Fig. 3B, middle panel, red color).
were identical in number, regardless of the types of siRNA (Fig. 5B, right panel). These data suggest that the depletion of MCL1 protein does not change the fortilin transcript numbers (Fig. 5B) but reduce the amount of fortilin protein (Fig. 5A).

To further validate the role of MCL1 in the stabilization of fortilin protein, U2OS\textsuperscript{wild-fortilin-HA} were transfected with siRNA-luciferase, siRNA-MCL1, or siRNA-fortilin, and subjected to Western blot analysis using anti-MCL1, anti-HA, and anti-actin antibodies. In this system, cells treated with siRNA-MCL1 and siRNA-fortilin expectedly expressed less fortilin, in comparison to cells treated with the transfection reagent alone (Fig. 6A, lane 1 versus lane 2). Cells treated with siRNA-MCL1 and siRNA-fortilin, respectively (Fig. 6A, lanes 3 and 4, MCL1 and fortilin, respectively). In this system, cells treated with siRNA-MCL1 and siRNA-fortilin expressed less MCL1 and fortilin, respectively (Fig. 6A, lanes 3 and 4, MCL1 and fortilin, respectively). In this system, cells treated with siRNA-MCL1 and siRNA-fortilin expressed less MCL1 and fortilin, respectively (Fig. 6A, lanes 3 and 4, MCL1 and fortilin, respectively).
duction of MCL1 level; the level of HA-fortilin did not change between 0 and 24 h but showed significant reduction in 48 and 60 h (Fig. 6B, fortilin). Finally, the stability of fortilin in the presence or absence of MCL1 was assessed by a pulse-chase experiment. U2OS\textsubscript{wild-fortilin-HA} cells, transfected either with siRNA-luciferase or siRNA-MCL1, were pulse-labeled with \textsuperscript{35}S]cysteine/methionine for 6 h; chased with cold DMEM; harvested at 0, 1, 2, and 3 h; and subjected to immunoprecipitation using anti-HA antibody. Precipitated fortilin-HA was subjected to SDS-PAGE and autoradiography, with the quantification of radioactivity of the bands. As is shown in Fig. 6C (solid line), the level of fortilin-HA did not show significant change over 3 h in the presence of MCL1. On the contrary, the level of fortilin-HA with MCL1 depletion, showed significant decrease over 3 h (Fig. 6C, dotted line). Taken together, these data suggest that the reduced intracellular level of MCL1 destabilizes fortilin.

A Fortilin Point Mutant with Arg\textsuperscript{27} → Ala Substitution (Fortilin\textsubscript{R21A}) Does Not Bind MCL1—We then generated fortilin point mutants and evaluated their ability to interact with MCL1 by \textit{in vivo} co-immunoprecipitation assay. We found that one of these mutants, fortilin\textsubscript{R21A-HA}, lacked interaction with MCL1 as shown in Fig. 7A. In this experiment, the U2OS\textsubscript{empty}, U2OS\textsubscript{wild-fortilin-HA}, and U2OS\textsubscript{fortilin-R21A-HA} cells were transiently transfected with pFLAG-MCL1. Cell lysates from these three cell types all contained equal amounts of FLAG-MCL1 (Fig. 7A, Input). Upon immunoprecipitation with anti-HA antibody, the approximately same amounts of fortilin-HA and fortilin\textsubscript{R21A} were immunoprecipitated (Fig. 7A, IP, bottom panel). As expected, fortilin-HA co-immunoprecipitated FLAG-MCL1 (Fig. 7A, IP, top panel, left lane), whereas the absence of immunoprecipitated fortilin-HA was associated with no FLAG-MCL1 (Fig. 7A, IP, top panel, right lane). In this system, fortilin\textsubscript{R21A} failed to co-immunoprecipitate FLAG-MCL1 (Fig. 7A, IP, top panel, middle lane). These data suggested that fortilin\textsubscript{R21A} did not interact with MCL1.

Fortilin\textsubscript{R21A} Decays Faster than Wild-type Fortilin—Finally, we subjected U2OS\textsubscript{wild-fortilin-HA} and U2OS\textsubscript{fortilin-R21A-HA} cells to pulse-chase experiments where these cells were pulse-labeled with \textsuperscript{35}S]cysteine/methionine for 6 h; chased with cold DMEM; harvested at 0, 1, 2, and 3 h; and subjected to immunoprecipitation using anti-HA antibody. Precipitated fortin-HA or fortin\textsubscript{R21A-HA} was subjected to SDS-PAGE and quantitative autoradiography. As is shown in Fig. 7B, the level of fortilin-HA did not show significant change over 3 h (solid line), consistent with the result shown in Fig. 6C. On the contrary, the level of fortilin\textsubscript{R21A-HA} exhibited a significant decrease over 3 h, up to nearly 50% (Fig. 7B, dotted line). These data suggest that the interaction with MCL1 is necessary for fortin to remain stable in the cells.

Discussion

We have described the specific interaction between MCL1 and fortin, a novel protein that prevents etoposide-induced cell death in U2OS cells (28). Our results presented here indicate that MCL1 is a protein partner of fortin that binds and stabilizes fortin. To our knowledge, this is the first report of an interaction between MCL1 and fortin in the literature.

The interaction between MCL1 and fortin is likely to be biologically significant. First, the interaction occurs not only in vitro but also \textit{in vivo}, in both yeast and mammalian cells (Figs. 1A–C and 7A). Second, MCL1 and fortin are both predominantly localized in the nucleus by immunofluorescence analysis (Fig. 3, A and B). Third, both MCL1 and fortin are rapidly induced by serum stimulation and exhibit similar expression kinetics in aortic vascular smooth muscle cell system (Fig. 4). Of all the Bcl-2 family proteins, only MCL1 is inducible (10–19). Consistently, as suggested by \textit{in vitro} binding assays (Fig. 2), fortin apparently interacts with only one member of the Bcl-2 family of proteins: MCL1.

RNAi is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by dsRNA that is homologous in sequence to the silenced gene (31). It has been shown that 21-nucleotide siRNA duplexes specifically suppress expression of endogenous and heterologous genes in different mammalian cell lines and that the most efficient silencing is obtained with siRNA duplexes composed of 21-nucleotide sense and 21-nucleotide antisense strands, paired in a manner to have a 19-nucleotide duplex region and a 2-nucleotide overhang at each 3’-terminus (32). This system would be advantageous over the overexpression system where fortin or MCL1 is overexpressed and the stability of its partner is evaluated. This is because only a small amount of a native protein may be needed to fully stabilize its binding partner. Using the siRNA system, we first showed that MCL1 does not require fortilin to remain stable. On the contrary, MCL1 depletion quickly destabilized fortilin (Figs. 5A and 6A (A–C)). In addition, a point mutant of fortin that lacks MCL1 binding was much more prone to degradation than wild-type fortin (Fig. 7B). It is thus likely that fortin, through its binding to MCL1, becomes more stabilized and less susceptible to degradation.

In the Introduction, we discussed that the inducibility of MCL1 is unique among anti-apoptotic Bcl-2 family proteins and that MCL1 is rapidly up-regulated upon growth and differentiation stimuli to cells (10–19). With this in mind, one can speculate that the increase in stability of fortin by MCL1 may be used by growing and differentiating cells to quickly increase the intracellular pro-survival environment by countering a potential instability associated with growth and differentiation. This stabilization of one anti-apoptotic protein (in this case fortin) by another anti-apoptotic protein (in this case MCL1) has not been reported in literature and calls for further investigation.

Acknowledgments—We are grateful to Dr. James T. Willerson, President of the University of Texas Health Science Center at Houston, for leadership, encouragement, and generous support. We thank Dr. Edward T. H. Yeh, Chairman of the Department of Cardiology, University of Texas M. D. Anderson Cancer Center, for scientific advice. We are thankful to Dr. Mari Nishizaki at Okayama University, Japan, for assistance on molecular cloning, and to Jude Richard at Texas Heart Institute, St. Luke’s Episcopal Hospital for editorial assistance.

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