Antiapoptotic Protein Partners Fortilin and MCL1 Independently Protect Cells from 5-Fluorouracil-induced Cytotoxicity*

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Fortilin, a potent 172-amino acid antiapoptotic polypeptide (Li, F., Zhang, D., and Fujise, K. (2001) J. Biol. Chem. 276, 47542–47549), binds MCL1, a protein of the antiapoptotic Bcl-2 family. The fortilin-MCL1 interaction stabilizes and increases the half-life of fortin but not necessarily of MCL1 (Zhang, D., Li, F., Weidner, D., Mnjoyan, Z. H., and Fujise, K. (2002) J. Biol. Chem. 277, 37430–37438). It is not known to what extent each protein depends on the other for its apoptotic activity. Here, we present evidence that fortin and MCL1 are capable of functioning as antiapoptotic proteins independently of each other. Using a robust small interfering RNA (siRNA)-mediated gene silencing system developed in our laboratory, we analyzed the cytotoxic effects of fortin and MCL1 together and apart in U2OS cell lines exposed to 5-fluorouracil (5-FU) in both mononclonal and polyclonal cell populations. When MCL1 was silenced by MCL1-targeted siRNA, fortin was still able to protect cells from 5-FU-induced cytotoxicity in a dose-dependent manner. Conversely, when fortin was silenced by fortin-targeted siRNA, MCL1 was also able to protect cells from 5-FU-induced cytotoxicity in a dose-dependent manner. Together, these data clearly suggest that fortin and MCL1 can exert their cytoprotective activities independently of each other. The silencing of fortin and MCL1 did not qualitatively change the subcellular localization of MCL1 and fortin, respectively. The biological significance of fortin-MCL1 interaction may be that it increases cellular resistance to apoptosis by allowing MCL1, an independently antiapoptotic protein, to stabilize another independently antiapoptotic protein, fortin.

Fortilin is a 172-amino acid polypeptide that was originally identified by yeast two-hybrid library screening as a molecule that specifically interacted with MCL1, a protein of the antiapoptotic Bcl-2 family (1). Fortilin is also known as translationally controlled tumor protein (2, 3). Early analyses of fortin in our laboratory revealed that its amino acid sequence is highly evolutionarily conserved; that fortin is ubiquitous in normal tissues, especially in liver and kidney; and that it localizes in both the nucleus and cytosol. In addition, we found that its overexpression prevents HeLa and U2OS cells from undergoing etoposide-induced apoptosis and that antisense depletion of fortin can induce MCF-7 cells to die spontaneously. Taken together, these findings have established fortin as a unique antiapoptotic protein.

Because the amino acid sequence of fortin does not resemble that of either Bcl-2 family proteins or IAPs (inhibitor of apoptosis proteins) and because fortin specifically interacts with MCL1, an antiapoptotic Bcl-2 family protein, we first hypothesized that the antiapoptotic function of fortin is mediated through MCL1. Intriguingly, we found that fortin interacted only with MCL1, not with other Bcl-2 family proteins, suggesting that fortin might be an MCL1-specific cofactor in the regulation of apoptosis (4). At that time, we devised our first-generation small interfering RNA (siRNA) system, in which we could specifically and effectively knock down MCL1 or fortin expression in vivo. Using this system, we unexpectedly found that MCL1 depletion by siRNA targeting MCL1 (siRNA\textsubscript{MCL1}) drastically reduced the intracellular concentration of fortin, whereas the siRNA targeting fortin (siRNA\textsubscript{fortin})-mediated depletion of fortin did not affect the intracellular concentration of MCL1 (4). Further investigation revealed that siRNA\textsubscript{MCL1} did not affect the amount of fortin transcripts in the cell in a real-time quantitative reverse transcription-PCR assay and that siRNA\textsubscript{fortin}-induced MCL1 silencing drastically shortened the half-life of fortin protein in a pulse-chase assay. Finally, a fortin point mutant that failed to interact with MCL1 (fortin\textsubscript{R21A}) was degraded far more quickly than was wild-type fortin in vivo. These data suggested that MCL1, through its binding to fortin, stabilizes fortin and that the turnover of MCL1 is not affected by fortin (4).

We then asked whether these protein partners, fortin and MCL1, could function as antiapoptotic proteins in the absence of each other. We first attempted to address the question by generating cell lines that could stably express fortin and fortin\textsubscript{R21A}. Unfortunately, it was not possible to generate cells that would stably and robustly express fortin\textsubscript{R21A}, most likely because of the rapid degradation of fortin\textsubscript{R21A}. We then modified the siRNA system further, which eventually allowed us to knock down MCL1 and fortin expression for extended periods of time (up to 120 h). This second-generation siRNA system

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‡¶§ The abbreviations used are: siRNA, small interfering RNA; 5-FU, 5-fluorouracil; HA, influenza virus hemagglutinin; ANOVA, analysis of variance.
which we used in the current work, is unique in that the MCL1-targeting siRNA represented a mixture of four different siRNAs, directed against four different regions of MCL1 mRNA. The same was true for siRNA targeted against fortilin. This system, together with cells stably overexpressing wild-type fortilin or MCL1, finally provided us with a tool to evaluate whether MCL1 and fortilin could function antiapoptotically in the absence of each other. The data presented here suggest that they can.

EXPERIMENTAL PROCEDURES

Cells, Cell Lines, and Culture Conditions—U2OS cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics.

Small Interfering RNA System—Small interfering RNAs (5, 6) were synthesized by Dharmacon Research, Inc. (Lafayette, CO). The siRNA_fortilin actually consisted of a mixture of four siRNA duplexes targeting four different regions of fortilin mRNA, namely, GAGATGTTCTCCGACACATCA, CCGAGTTACCGAAAGCACA, GGAGATCAGGCAGGGTT, and GTTACCGAAACGACATA. Similarly, the siRNA against MCL1 (siRNA_MCL1) actually consisted of a mixture of four siRNA duplexes targeting four different regions of MCL1 mRNA, namely, AAGGCGAATGCTGTAATCA, TCAACAGACTTCCTCTGTAAAG, GGGAGATGATGACCATGTATTTT, and GGGAGATGCTGAGTTACAA. The siRNA against luciferase, a nonmammalian protein from Photinus pyralis (American firefly), was used as a control. All procedures were performed in an RNase-free environment as previously described in detail by us (4). Briefly, the transfection of cells with siRNA duplexes was performed using TransIT-TKO transfection kits (Mirus Corp., Madison, WI), at a final concentration of 1.1%. To minimize the cytotoxicity of the reagent itself, cells were washed once with PBS, and media were changed 6 h after transfection.

Western Blot Analysis of Cell Lysates—Cells were harvested by the direct addition of SDS gel loading buffer (1, 4). Lysate samples were collected and incubated at 45 °C for 1 h. The genomic DNA in the lysate was sheared by passing the lysate through 27-gauge needles five times. Whole cell extracts, harvested from cells transfected into radioimmune precipitation assay buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, and protease inhibitors (Complete Protease Inhibitor Mixture Tablets; Roche Applied Science)), followed by the determination of protein concentrations using BCA methods (Bio-Rad). Exactly 10 μg of total proteins were resolved by 12% SDS-PAGE and subjected to Western blot analyses. The successful purification of nuclear and cytosolic fractions was confirmed by Western blot analyses using anti-histone H1 (clone AE-4; Santa Cruz Biotechnology) and anti-α-tubulin (Sigma) antibodies.

RESULTS

A Robust siRNA System for Knockdown of MCL1 and Fortilin Has Been Developed—To determine the feasibility of creating a microenvironment in which MCL1, fortilin, or both can be selectively depleted, we generated siRNAs against MCL1 (siRNA_MCL1) and fortilin (siRNA_Fortilin). We then tested these siRNAs for their ability to knock down expression of the target proteins. When U2OS cells were transfected with siRNA_MCL1, MCL1 expression was knocked down to an undetectable level within 12 h (Fig. 1A, top panel). Similarly, siRNA_Fortilin was able to knock down fortilin expression, although it took up to 48 h to do so (Fig. 1A, bottom panel). Higher siRNA_fortilin concentrations did not shorten the time required for complete silencing (data not shown). Treatment of cells with TransIT (Mirus Corp.) alone did not affect the intracellular levels of MCL1 and fortilin (Fig. 1B, N versus T). Surprisingly, once knocked down, MCL1 and fortilin expression remained undetectable for as long as 120 h. In addition, in dose-response experiments, we found that 25 nM siRNA_MCL1 and 25 nM siRNA_Fortilin were sufficient to completely knock down MCL1 and fortilin, respectively (Fig. 1B). Furthermore, we found that it was possible to silence both MCL1 and fortilin at the same time by introducing both siRNA_MCL1 and siRNA_Fortilin into the cells simultaneously (Fig. 1C). With 25 nM each of siRNA_MCL1 and siRNA_Fortilin, both MCL1 and fortilin were silenced within 24 h (Fig. 1C). These data suggested that it is possible to quickly and persistently silence MCL1 and/or fortilin expression in U2OS cells by a single transfection with siRNA_MCL1 and/or siRNA_Fortilin. The concentrations of siRNA needed to achieve silencing in our system were substantially lower than those described previously by us (4) and by others (6).

Depletion of Fortilin or MCL1 Increases the Susceptibility of Cells to 5-FU-induced Cell Death, Whereas Overexpression of Fortilin or MCL1 Protects Cells from 5-FU-induced Cell Death—We also developed a system in which we could evaluate the viability of cells treated with siRNAs. First, U2OS cells were treated with 25 nM siRNA, incubated for 48 h, and then incubated with 1 μM 5-FU for another 24 h. Then, cells were subjected to trypan blue assay for the assessment of cell viability. As shown in Fig. 2A, silencing of fortilin and MCL1 was associated with a significantly greater rate of 5-FU-induced cell death than was treatment with irrelevant siRNA_Luciferase.
silencing of MCL1 and fortilin, respectively (Fig. 3). We assessed the intracellular localization of fortilin and MCL1, as evidenced by the depletion of intracellular fortilin and MCL1 would change the cellular localization of MCL1 and fortilin, respectively. Before fortin silencing, MCL1 localized predominantly in the cytosol and somewhat in the nucleus (Fig. 3B, lanes 1 and 4, MCL1), consistent with the immunostaining data reported above. Upon fortin silencing, this pattern of cytosolic predominance persisted (Fig. 3B, lanes 3 and 6, MCL1). Before its silencing, fortin localized somewhat more in the cytosol than in the nucleus in this system (Fig. 3B, lanes 1 and 4, Fortin). Upon MCL1 silencing, this pattern again persisted (Fig. 3B, lanes 2 and 5, MCL1). Together, these data suggested that the depletion of MCL1 and fortin by siRNA does not change the intracellular localization of their protein partners, namely, fortin and MCL1, respectively.

Fortin Prevents Cells from Undergoing Cell Death in the Absence of Its Protein Partner, MCL1—Next, we further characterized U2OSFortilin-8, one of the clones of U2OS cells overexpressing fortin, using U2OSEmpty-1 as control. Trypan blue assay showed that U2OSFortilin-8 cells were significantly more resistant to 5-FU-induced cell death than were U2OSEmpty-1 cells (Fig. 4A, p < 0.005 by ANOVA).

We then evaluated our first principal hypothesis, namely, that fortin was capable of blocking cell death even in the absence of MCL1. Specifically, we transfected U2OSEmpty-1 and U2OSFortilin-8 cells with siRNAFortilin, siRNA MCL1, or siRNA Luciferase; challenged the cells with 1 mM 5-FU for 24 h; and finally determined their viability using the trypan blue assay. Consistent with data reported above (Figs. 2B and 4A), fortin-overexpressing cells (U2OSFortilin-8) were more resistant to 5-FU-induced cell death than were control cells (U2OSEmpty-1) (Fig. 4B, U2OSFortilin-8 (lane 6) versus U2OSEmpty-1 (lane 5): 33.4 ± 0.6% versus 54.5 ± 0.4%, p < 0.005 by two-sample t test). When native fortin expression was knocked down by siRNAFortilin in U2OSEmpty-1 cells (Fig. 4B, lane 4 versus lane 5, Fortin), the loss of native fortin was associated with an increase in cell susceptibility to 5-FU treatment (no fortin (lane 4) versus native fortin (lane 5) in U2OSEmpty-1 cells: 61.3 ± 1.1% versus 54.5 ± 0.4%, p < 0.005 by two-sample t test). Thus, intracellular fortin levels consistently and significantly correlated with the degree of cell survival (Fig. 4B, no fortin (lane 4) versus native fortin (lane 5) versus native plus overexpressed fortin (lane 6), p < 0.005 by two-sample t test).

Next, we fractionated the lysate from cells that had been treated with either siRNA MCL1, siRNA Fortilin, or siRNA Luciferase into cytosolic and nuclear fractions. As shown in Fig. 3B, Western blot analyses revealed that α-tubulin was detectable only in the cytosolic fraction, and histone H1 was detectable only in the nuclear fraction, suggesting that the cytosolic and nuclear fractions did not cross-contaminate each other. In addition, the treatment with siRNA MCL1 depleted MCL1 in both the nucleus and cytosol (Fig. 3B, lanes 3 and 6). Similarly, treatment with siRNAFortilin depleted fortilin in both the nucleus and cytosol (Fig. 3B, lanes 2 and 5). In this system, we asked whether the depletion of fortilin and MCL1 changed the predominant intracellular localization of MCL1 and fortilin, respectively. Before fortin silencing, MCL1 localized predominantly in the cytosol and somewhat in the nucleus (Fig. 3B, lanes 1 and 4, MCL1), consistent with the immunostaining data reported above. Upon fortin silencing, this pattern of cytosolic predominance persisted (Fig. 3B, lanes 3 and 6, MCL1). Before its silencing, fortilin localized somewhat more in the cytosol than in the nucleus in this system (Fig. 3B, lanes 1 and 4, Fortin). Upon MCL1 silencing, this pattern again persisted (Fig. 3B, lanes 2 and 5, MCL1). Together, these data suggested that the depletion of MCL1 and fortilin by siRNA does not change the intracellular localization of their protein partners, namely, fortilin and MCL1, respectively.

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We then evaluated our first principal hypothesis, namely, that fortilin was capable of blocking cell death even in the absence of MCL1. Specifically, we transfected U2OSEmpty-1 and U2OSFortilin-8 cells with siRNAFortilin, siRNA MCL1, or siRNA Luciferase; challenged the cells with 1 mM 5-FU for 24 h; and finally determined their viability using the trypan blue assay. Consistent with data reported above (Figs. 2B and 4A), fortilin-overexpressing cells (U2OSFortilin-8) were more resistant to 5-FU-induced cell death than were control cells (U2OSEmpty-1) (Fig. 4B, U2OSFortilin-8 (lane 6) versus U2OSEmpty-1 (lane 5): 33.4 ± 0.6% versus 54.5 ± 0.4%, p < 0.005 by two-sample t test). When native fortilin expression was knocked down by siRNAFortilin in U2OSEmpty-1 cells (Fig. 4B, Western blot, lane 4 versus lane 5, Fortin), the loss of native fortilin was associated with an increase in cell susceptibility to 5-FU treatment (no fortilin (lane 4) versus native fortilin (lane 5) in U2OSEmpty-1 cells: 61.3 ± 1.1% versus 54.5 ± 0.4%, p < 0.005 by two-sample t test). Thus, intracellular fortilin levels consistently and significantly correlated with the degree of cell survival (Fig. 4B, no fortilin (lane 4) versus native fortilin (lane 5) versus native plus overexpressed fortilin (lane 6), p < 0.005 by two-sample t test)
Fortilin-MCL1 Interaction in Apoptosis

Fortilin and MCL1, two proteins involved in the regulation of apoptosis, were studied in the context of 5-fluorouracil (5-FU)-induced cytotoxicity in U2OS cells. Fortilin and MCL1 gene silencing was associated with increased 5-FU-induced cytotoxicity in U2OS cells depleted of fortilin or MCL1 (Fig. 4B, Western blot, Fig. 5A). Importantly, the total amount of siRNA introduced into these cells was kept constant by the addition of siRNA_{Luciferase}, an irrelevant control siRNA. As shown in Fig. 4B, the introduction of siRNA_{MCL1} into the cells caused the intracellular level of native MCL1 to decrease to levels undetectable by Western blot analyses (Fig. 4B, Western blot, MCL1, lanes 1–3). In cases of double knockdown, Western blot analysis showed no significant signals of MCL1 or fortilin proteins (Fig. 4B, Western blot and Fortilin, lane 1). Strikingly, as assessed by trypan blue assay, the increasing intracellular concentration of fortilin was associated with increasing resistance to 5-FU-induced cell death even in the absence of MCL1 (Fig. 4B, no fortilin (lane 1) versus native fortilin (lane 2) versus native plus overexpressed fortilin (lane 3): 76.8 ± 0.7% versus 64.7 ± 0.5% versus 47.4 ± 0.1%, p < 0.001 by ANOVA). In other words, fortilin was equally capable of protecting cells from 5-FU-induced cytotoxicity in both the presence and absence of MCL1 and did so in a dose-dependent fashion (Fig. 4B, lanes 4–6 versus lanes 1–3). Together, these data suggested that fortilin does not require the presence of MCL1 to exert its antiapoptotic activity.

MCL1 Prevents Cells from Undergoing Cell Death in the Absence of Its Protein Partner, Fortilin—Having established that fortilin does not require MCL1 in order to be antiapoptotic, we then asked whether the reverse was true. We first characterized U2OS_{MCL1-6}, one of the clones of U2OS cells overexpressing MCL1, using U2OS_{Empty-1} as a control. Trypan blue assay showed that U2OS_{MCL1-6} cells were significantly more resistant to 5-FU-induced cell death than were U2OS_{Empty-1} cells (Fig. 5A, p < 0.01 by ANOVA, when comparing U2OS_{Empty-1} and U2OS_{MCL1-6} cells).

We then evaluated our second principal hypothesis, namely, that MCL1 is capable of blocking cell death even in the absence of fortilin, using the reagents and systems we had already characterized (Figs. 1, 2, and 5A). Specifically, we transfected U2OS_{Empty-1} and U2OS_{MCL1-6} cells with siRNA_{Fortilin}, siRNA_{MCL1} or siRNA_{Luciferase}; challenged the cells with 1 mM 5-FU for 24 h; and then determined their viability using the trypan blue assay. In the presence of native fortilin, the higher the intracellular levels of MCL1 were, the lower the susceptibility of cells to 5-FU-induced cell death was (Fig. 5B, no MCL1 (lane 4) versus native MCL1 (lane 5) versus native plus overexpressed MCL1 (lane 6), 60.1 ± 2.1% versus 40.5 ± 1.7% versus 29.0 ± 0.4%, p < 0.01 by ANOVA). In other words, MCL1 protected U2OS cells from 5-FU-induced cytotoxicity in a dose-dependent fashion in the presence of native fortilin.

We then set out to determine whether this dose dependence would hold in the absence of fortilin in the cells by treating U2OS_{MCL1-6} and U2OS_{Empty-1} cells with siRNA_{Fortilin} to silence fortilin. In some cases, both MCL1 and fortilin were knocked down simultaneously by cotransfection of siRNA_{Fortilin} and siRNA_{MCL1} as optimized above (Fig. 1C). As shown in Fig. 5B, the introduction of siRNA_{Fortilin} into the cells caused the intracellular level of fortilin to fall to undetectable levels (Fig. 5B, Western blot, Forilin, lanes 1–3). In cases of double knockdown, Western blot analysis showed no significant signals for either MCL1 or fortilin protein (Fig. 5B, Western blot, MCL1 and Fortilin, lane 1). Strikingly, the increasing intracellular concentration of MCL1 in these cells was associated with increasing resistance to 5-FU-induced cell death in the absence of fortilin (Fig. 4B, no MCL1 (lane 1) versus native MCL1 (lane 2) versus native plus overexpressed MCL1 (lane 3), 74.6 ± 3.3% versus 60.2 ± 7.4% versus 32.9 ± 0.9%, p < 0.01 by ANOVA). In other words, MCL1 was equally capable of protecting cells from 5-FU-induced cytotoxicity in both the presence and absence of fortilin and did so in a dose-dependent fashion (Fig. 5B, lanes 4–6 versus lanes 1–3). Together, these data suggested that MCL1 does not require the presence of fortilin to protect U2OS cells from apoptosis.

**Fig. 2.** MCL1 and fortilin prevent 5-FU-induced cell death, as shown by siRNA gene knockout and overexpression assay. A. Increased 5-FU-induced cytotoxicity in U2OS cells depleted of fortilin or MCL1. Cells were transfected with siRNA_{Luciferase}, siRNA_{MCL1}, or siRNA_{Fortilin}; incubated for 24 h; challenged with 1 mM 5-FU; incubated for 28 h; and subjected to trypan blue assay. After counting, cells were subjected to Western blot analysis with anti-MCL1, anti-fortilin, and anti-actin antibodies. B. Protection of U2OS cells overexpressing fortilin or MCL1 from 5-FU-induced cytotoxicity. U2OS cells were stably transfected and cloned to establish clones harboring empty plasmids (U2OS_empty; E1 and E2) or overexpressing fortilin (U2OS_{Fortilin}, F8 and F9) or MCL1 (U2OS_{MCL1}, M1 and M6). Clones were evaluated by Western blot analysis with anti-MCL1, anti-fortilin, and anti-actin antibodies. Two clones each from all the appropriate clones were randomly selected (E1, E2, F8, F9, M1, and M6), propagated, and challenged with 5-FU for 48 h, and subjected to trypan blue assay. Overexpression of fortilin and MCL1 was associated with significantly less 5-FU-induced cell death. *p < 0.005 in comparison with U2OS cells harboring empty plasmids. siRNA_{Fortilin}, siRNA against luciferase, an irrelevant nonmammalian protein (control); Trypan Blue, trypan blue exclusion assay.

In other words, the presence of native MCL1, the higher the intracellular levels of fortilin were, the lower the susceptibility of cells to 5-FU-induced cell death was (Fig. 4B, lanes 4–6). In still other words, fortilin had a dose-dependent anticytotoxic effect on 5-FU-challenged U2OS cells.
cells from 5-FU-induced cell death.

In Polyclonal Cell Populations, Fortilin Prevents Cells from Undergoing Cell Death in the Absence of Its Protein Partner, MCL1, and Vice Versa—To make certain that the independent cytoprotective effects of protein partners fortilin and MCL1 represented in Figs. 4 and 5 did not originate from the selection process associated with the establishment of monoclonal cell populations, we generated polyclonal populations of U2OS cells that stably expressed fortilin (U2OSFortilin-poly) or MCL1 (U2OSMCL1-poly). Western blot analyses showed that U2OSFortilin-poly and U2OSMCL1-poly cells robustly overexpressed fortilin and MCL1, respectively (Figs. 6A and 7A).

We then transfected U2OSFortilin-poly cells with 25 nM siRNAFortilin and with varying amounts of siRNAMCL1 and siRNALuciferase, which was added to keep the total amount of siRNAs constant; challenged the cells with 1 mM 5-FU for 24 h; and then determined their viability using the trypan blue assay. With 25 nM siRNALuciferase, there was no detectable MCL1 in the cells (Fig. 6B). In this setting, increasing the amount of siRNAFortilin caused the intracellular fortilin concentration to decrease drastically. The reduction of intracellular fortilin was associated with the increase in susceptibility of the cells to 5-FU-induced cell death (p < 0.0001 by ANOVA). In summary, fortilin protected U2OS cells from 5-FU-induced cytotoxicity in a dose-dependent fashion in the absence of MCL1. These data again suggest that fortilin does not require the presence of MCL1 to protect U2OS cells from 5-FU-induced cell death.

Finally, we transfected U2OSMCL1-poly cells with 25 nM siRNAMCL1 and with varying amounts of siRNAFortilin and siRNALuciferase, which was added to keep the total amount of siRNAs constant; challenged the cells with 1 mM 5-FU for 24 h, and then determined their viability using the trypan blue assay. With 25 nM siRNAFortilin, there was no detectable MCL1 in the cells (Fig. 7B). In this setting, increasing the amount of siRNAMCL1 caused the intracellular MCL1 concentration to decrease drastically. The reduction of intracellular MCL1 was associated with the increase in susceptibility of the cells to 5-FU-induced cell death (p < 0.0001 by ANOVA). In summary, MCL1 protected U2OS cells from 5-FU-induced cytotoxicity in...
strikingly, in the absence of MCL1 (lanes 1), siRNA MCL1 and siRNA luciferase (lanes 2 and 3), siRNA fortilin and siRNA luciferase (lane 4), or siRNA luciferase alone (lanes 5 and 6). Western blot analyses showed that both U2OS Empty-1 and U2OS Fortilin-8 cells expressed no fortilin after siRNA Fortilin treatment (lane 1). In the presence of MCL1 (lanes 4–6), higher intracellular fortilin levels were associated with lower cytotoxicity as assessed by trypan blue assay. Strikingly, in the absence of MCL1 (lanes 1–3), higher intracellular fortilin levels were still associated with lower cytotoxicity as assessed by trypan blue assay. ++++, p < 0.001 by ANOVA.

not been any reports of such heterodimerization between a Bcl-2 family protein (in this case MCL1) and a non-Bcl-2 family antiapoptotic protein (in this case fortilin) or reports that such heterodimerization would stabilize dimerizing protein(s). Taken together with the fact that MCL1 is an inducible molecule (18, 19), it is likely that fortilin-MCL1 interaction represents a unique cellular mechanism for quickly creating an antiapoptotic microenvironment protective against certain noxious extracellular conditions.

The siRNA system (5, 6) we used in the current work was a powerful tool for investigating the functional dependence between fortilin and MCL1 proteins. In the siRNA system that we have developed, fortilin and MCL1 messages are targeted by multiple kinds of siRNA duplexes. This knockdown strategy, while requiring much lower concentrations, results in much longer silencing of MCL1 and fortilin genes. Consequently, the strategy has allowed us to attain higher cell viability after transfection without compromising the silencing efficiency.

By using this strategy of siRNA-mediated gene silencing to test the independence of MCL1 and fortilin antiapoptotic func-
showed that siRNA MCL1-treated U2OS Fortilin-poly cells expressed no dependent nature of fortilin’s cytoprotection. Western blot analyses
using monoclonal populations of U2OS cells (Fig. 4). Difference in cell death was significant at p < 0.0001 by ANOVA.

In immunocytochemical and subcellular fractionation experiments using the siRNA system described above, we showed that the intracellular localization of fortilin and MCL1 remained the same in the absence of fortilin and MCL1, respectively. First, regardless of the presence of fortilin, MCL1 was present to a greater extent in the cytosol and to a lesser extent in the nucleus. Second, regardless of the presence of MCL1, fortilin was shown by immunostaining to predominate in the nucleus but shown by subcellular fractionation to predominate in the cytosol. Despite this apparent discrepancy, it still holds true that the patterns of subcellular localizations of fortilin and MCL1 did not differ depending on the presence of their protein partners. Taken together, these data suggest that it is unlikely that fortilin-MCL1 interaction regulates the subcellular localization of fortilin and MCL1. The fact that fortilin and MCL1 can be located in both the nucleus and the cytosol suggests that fortilin and MCL1 are shuttle molecules, like p53 (20), mdm2 (21), β-catenin (22), and many components of the phosphatidylinositol 3-kinase pathway such as the insulin receptor (23), insulin receptor substrates (24), phosphatidylinositol 3-kinase (25), and protein kinase B (26), moving back and forth from the nucleus in response to changes in the cellular microenvironment.

In the previous report, we used a standard pulse-chase assay to show that MCL1 stabilizes fortilin and that the lack of MCL1 leads to the destabilization of fortilin, which is sometimes true that the patterns of subcellular localizations of fortilin and MCL1 did not differ depending on the presence of their protein partners. Taken together, these data suggest that it is unlikely that fortilin-MCL1 interaction regulates the subcellular localization of fortilin and MCL1. The fact that fortilin and MCL1 can be located in both the nucleus and the cytosol suggests that fortilin and MCL1 are shuttle molecules, like p53 (20), mdm2 (21), β-catenin (22), and many components of the phosphatidylinositol 3-kinase pathway such as the insulin receptor (23), insulin receptor substrates (24), phosphatidylinositol 3-kinase (25), and protein kinase B (26), moving back and forth from the nucleus in response to changes in the cellular microenvironment.
Fortilin-MCL1 Interaction in Apoptosis

24 h to silence fortlin in the presence of MCL1 (Fig. 1C). Fortilin was capable of knocking down fortlin within 24 h in the absence of MCL1 (Fig. 1C), whereas siRNA for fortlin took more than 24 h to silence fortlin in the presence of MCL1 (Fig. 1A, bottom panel). It is not entirely clear why fortlin signals occasionally persisted upon the silencing of MCL1, as seen in the Western blot analyses (Figs. 1, A and B). It is possible that fortlin expression was up-regulated by unknown transcriptional factors, thus masking the destabilization of fortlin attributable to the lack of MCL1. The transcriptional regulation of fortlin appears to be very complex (27), and further investigation is needed to evaluate the role of various pathways in it.

The current work places fortlin in a new class of antiapoptotic molecule. Fortilin is not a cofactor of antiapoptotic MCL1, augmenting its function. However, this does not rule out the possible presence of an apoptosis executioner protein that is inhibited by fortlin or an antiapoptotic molecule other than MCL1 that is stimulated by fortlin. The amino acid sequence of fortlin does not resemble that of either the Bcl-2 family or the inhibitor of apoptosis proteins (IAPs) (1). The exact mechanism of action of fortlin as an antiapoptotic molecule is unknown. Thaw et al. (28) have uncovered a structural similarity between fortlin and Mss4 (mammalian suppressor of Sec4). Mss4 is a guanyl nucleotide exchange factor, which facilitates GDP release from and subsequent GTP binding to a subset of the Rab GTPases (29). GTP-bound Rab GTPases function as active forms and recruit effector molecules, such as coiled-coil proteins involved in membrane tethering and docking, enzymes, or cytoskeleton-associated proteins (30). Recently, Cans et al. (31) reported that fortlin interacts with translation elongation factor eEF1A and with its guanyl nucleotide exchange factor, eEF1Bγ. Intriguingly, despite its homology to guanyl nucleotide exchange factor, fortlin exhibited guanine nucleotide dissociation inhibitor activity, stabilizing the GDP (inactive) form of eEF1A (31). The up-regulation of eEF1A is reported to be associated with oxidative stress-induced apoptosis (32). Although it is possible that fortlin binds to and keeps eEF1A inactive when eEF1A is up-regulated by apoptotic stimuli, further investigation is needed to define the role of fortlin-eEF1A interaction in the regulation of apoptosis.

The role of fortlin in tumorigenesis has been established. Tuynnyder et al. (33) performed differential gene expression analyses in which they compared aggressive cancer cell lines with nonaggressive, or reverted, cancer cell lines. Fortilin showed the most striking up-regulation (up to 124-fold on the transcriptional level) in aggressive cancer cell lines. Fortilin overexpression also reduced the sensitivity of cancer cell lines to chemotherapeutic agents such as etoposide (1) and 5-FU (Figs. 2 and 4). On the contrary, the depletion of intracellular fortlin was associated with the spontaneous death of MCF7 cells (1), poly(ADP-ribose) polymerase cleavage in U937 cells (33), and increased susceptibility to 5-FU in U2OS cells (Figs. 2, 4, and 5). Further dissection of the mechanism of the antiapoptotic activity of fortlin will be important if fortlin is to be developed as a novel target of cancer therapy. The siRNA systems we have described here will be a highly useful tool for such investigations.

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