Characterization of Fortilin, a Novel Antiapoptotic Protein*

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Apoptosis is meticulously controlled in living organisms. Its dysregulation has been shown to play a key role in a number of human diseases, including neoplastic, cardiovascular, and degenerative disorders. Bcl-2 family member proteins and inhibitors of apoptosis proteins are two major negative regulators of apoptosis. We report here the characterization of novel antiapoptotic protein, fortilin, which we identified through yeast two-hybrid library screening. Sequence analysis of fortilin revealed it to be a 172-amino acid polypeptide highly conserved from mammals to plants. Fortilin is structurally unrelated to either Bcl-2 family member proteins or inhibitors of apoptosis proteins. Northern blot analysis showed the fortilin message to be ubiquitous in normal tissue but especially abundant in the liver, kidney, and small intestine. Western blot analysis using anti-fortilin antibody showed more extensive expression in cancerous cell lines (H1299, MCF-7, and A549) than in cell lines derived from normal tissue (HEK293). Immunocytochemistry using HeLa cells transiently expressing FLAG-tagged fortilin and immunohistochemistry using human breast ductal carcinoma tissue and anti-fortilin antibody both showed that fortilin is predominantly localized in the nucleus. Functionally, the transient overexpression of fortilin in HeLa cells protected them from undergoing etoposide-induced apoptosis. Consistently, U2OS cells stably expressing fortilin protected the cells from cell death induced by etoposide over various concentrations and durations of exposure. In addition, fortilin overexpression inhibited caspase-3-like activity as assessed by the cleavage of fluorogenic substrate benzyloxycarbonyl-DEVD-7-amido-4-(trifluoromethyl)coumarin. Furthermore, the antisense depletion of fortilin from breast cancer cell line MCF-7 was associated with massive cell death. These data suggest that fortilin represents a novel antiapoptotic protein involved in cell survival and apoptosis regulation.

Apoptosis represents a highly efficient and extremely sophisticated system for removing cells from the surrounding microenvironment (1–8). As deadly as it may be, apoptosis is essential for the elimination of aberrant cells and the survival of the living organism as a whole. It is not surprising, therefore, that apoptosis is meticulously controlled. Among major regulatory cellular mechanisms described for other systems, ever increasing numbers of them are also being shown to be involved in the regulation of apoptosis. These include (a) proteolytic processing and activation by co-factors (3, 9, 10); (b) covalent modification such as phosphorylation (11, 12), S-nitrosylation (13), thioester bond formation (14), and ubiquitination (15, 16); and (c) compartmentalization (10, 17, 18) of pro- and antiapoptotic molecules.

In addition, there exist molecules specifically designed to inhibit the various stages of apoptosis. Bcl-2 family member proteins and inhibitor of apoptosis proteins (IAPs)1 represent two major negative regulators of apoptosis. First, Bcl-2 inhibits the release of cytochrome c from mitochondria in the presence of various proapoptotic stimuli (19). Also, it has been shown that Bcl-xL binds apoptotic protease activating factor-1, thereby interfering with its recruitment of caspase-9 (20). In addition, Bcl-2 heterodimerizes with Bax, a proapoptotic protein, thereby preventing Bax from undergoing the conformational changes required for the formation of cytotoxic pores in the mitochondrial membrane (21). Furthermore, Bcl-2 can associate with Raf-1, a protein kinase that phosphorylates and inactivates the proapoptotic protein BAD (22). The inhibitor of apoptosis proteins (IAPs), originally identified as a family of proteins in baculovirus that inhibited the apoptotic response of insect cells to viral infection (23), represent a group of antiapoptotic proteins structurally distinct from Bcl-2 family proteins, consisting of two N-terminal repeats (baculovirus IAP repeats) and a C-terminal RING finger domain. Several human IAPs have been described, including survivin (24), X-linked inhibitor of apoptosis protein (25), neuronal apoptosis inhibitor protein (26), and others. Antiapoptotic function of IAPs appears to be mediated through direct binding and inhibition of effector caspases (caspases 3, 7, and 9) (25, 27–30).

In order to explore the apoptosis regulatory mechanism related to Bcl-2 family member proteins, we searched for proteins that interacted with myeloid cell leukemia protein-1 (MCL1), a Bcl-2 homologue, using the yeast two-hybrid system. The amino acid sequence of one positive clone that specifically interacted with MCL1 was identical to that of human translationally controlled tumor protein (31–33), the function of which was unknown. Analysis of the promoter region of this gene has shown the presence of the binding sites for multiple transcription factors, implying that this gene, like other genes, is under transcriptional control (34). We then discovered that this mol-

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1 The abbreviations used are: IAP, inhibitor of apoptosis protein; MCL1, myeloid cell leukemia protein-1; LacZ, β-galactosidase; PAGE, polyacrylamide gel electrophoresis; DAPI, 4,6-diamidino-2-phenylindole; LDH, lactate dehydrogenase; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate; AFC, 7-amido-4-(trifluoromethyl) coumarin.
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eucleotide. In addition, we found that it prevented cells from undergoing apoptosis. On the basis of these observations, it is now designated fortilin (from fortis, meaning strong and robust in Latin). In the study reported here, we focused on characterizing the specific function of fortilin as an antiapoptotic protein. The data we present indicate that fortilin is a novel nuclear antiapoptotic protein, structurally distinct from both Bcl-2 and IAP family antiapoptotic proteins.

MATERIALS AND METHODS

Cell Lines and Culture Conditions—The ML1a cell line was maintained in RPMI1640 medium supplemented with 10% fetal calf serum (Mediatech, Herndon, VA) and antibiotics. The A543, U2OS, HeLa, 293, MCF-7, and NIH 3T3 cell lines were maintained in Dulbecco’s modified Eagle’s medium (Mediatech) supplemented with 10% fetal calf serum and antibiotics.

Molecular Cloning—The cDNA fragments of full-length fortin, p21, β-galactosidase (β LacZ), Bcl-xl, and MCL1 were obtained by standard polymerase chain reaction techniques, using appropriate primer sets, and were ligated in frame to the appropriate bacterial, yeast, and mammalian expression vectors. In all cases, the authenticity of cloned constructs was confirmed by automated deoxyxynucleotide sequencing (SeqWright Co., Houston, TX).

Yeast Two-hybrid Library Screening and Sequence Analysis—Full-length MCL1 was cloned into pAS2.1 (CLONTECH, Palo Alto, Calif), a vector that encodes the GAL4 DNA-binding domain, and used as bait. A human fetus liver library was screened, according to the manufacturer’s instructions (CLONTECH) and as described previously (36, 37). The full-length human fortin sequence was aligned to those of other species by the Omega nucleic acid and a protein analysis program (Genetics Computer Group, Madison, WI). The hydropathic score was determined with the same program according to the methods of Goldman, Engelberg, and Stetc (GES) (38) and von Heijne (39).

Northern Blotting—Northern blotting was performed using multiple-tissue Northern cDNA (or GenHunter, Carlsbad, California), or with pcDNA6 vector encoding wild-type fortin, by using FuGENE6 (Roche Molecular Biochemicals) according to the appropriate filter sets. Cells that emitted red fluorescence were evaluated for nuclear morphology. Condensed small nuclei or fragmented nuclei were counted as apoptotic cells. The apoptotic index was calculated as follows: (number of red-fluorescing cells with apoptotic nuclei)/number of total red-fluorescing cells counted) × 100. All experiments were performed in duplicate.

Generation and Characterization of U2OS Cells Stably Expressing Fortilin—U2OS cells were transfected with empty pcDNA6, a mammalian expression vector containing a blasticidin resistance gene (Invitrogen, Carlsbad, California), or with pcDNA6 vector encoding wild-type fortin, by using FuGENE6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Transfected cells were selected for ~3 weeks and characterized by Western blot analysis. For the cytotoxicity assay, U2OS cells stably expressing wild-type fortin (U2OS.F) and U2OS cells stably harboring empty pcDNA6 (U2OS.E) were seeded in a 96-well plate in quadruplicate. For the investigation of dose response, cells were challenged with various concentrations of etoposide (0–20 μg/ml) for 48 h. For the time course study, cells were challenged with 5 μg/ml of etoposide and harvested after various incubation periods (0–96 h). In both cases, the cell media were assayed for the lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells, using a cytotoxicity detection kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. The LDH activity index was calculated as follows: (LDH activity in the medium − background LDH activity)/LDH activity in the medium of cells lysed by 1% Triton X-100 − background LDH activity) × 100.

Assay of Caspase-3-like Activity—Approximately 2 × 10^6 U2OS stable transfectants, either expressing fortin (U2OS.F) or harboring empty expression vector (U2OS.E), both of which have been characterized...
ized above, were challenged either with Me2SO (vehicle) or with etoposide at a final concentration of 10 μM for 48 h. Caspase-3-like activity was then determined as previously described (13). In brief, cytosolic proteins were extracted in hypotonic cell lysis buffer (25 mM HEPES, pH 7.5, 5 mM MgCl2, 5 mM EDTA, 5 mM dithiothreitol, 0.05% phenylmethylsulfonyl fluoride; all from Sigma) by three cycles of freezing and thawing. The protein concentration of samples was determined by using a Bio-Rad Bradford protein assay kit (Bio-Rad). Ten micrograms of cytosolic extracts were added to caspase assay buffer (312.5 mM HEPES, pH 7.5, 31.25% sucrose, 0.3125% CHAPS) with benzyloxycarbonyl-DEVD-7-amido-4-(trifluoromethyl)coumarin as substrates (Calbiochem). Release of 7-amido-4-(trifluoromethyl)coumarin (AFC) was quantified, after 2 h of incubation at 37 °C (or, in the case of time course experiment, every 10 min), using a Fluoroskan system (Thermo-Labsystems, Helsinki, Finland) set to an excitation value of 355 nm and emission value of 525 nm. The results were expressed as relative fluorescence units/μg of protein.

Assay of Antisense-treated MCF-7 Cell Survival—For the Western blot analysis to evaluate intracellular fortilin concentration with antisense treatment, 1 × 10^5 MCF-7 cells, a malignant breast ductal carcinoma cell line, were seeded on a six-well plate. The next day, benzyloxycarbonyl-DEVD-fluoromethylketone (Kamiya Biomedical Company, Seattle, WA), a caspase-3 inhibitor, was added to the medium at the concentration of 100 μM. Twenty-four hours after the addition of the caspase inhibitor, cells were transfected with pFLAG-antisense fortilin by FuGENE6 (Roche Molecular Biochemicals). Cells were harvested immediately after the transfection and 8 h after the transfection by the direct addition of radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS), supplemented with phenylmethylsulfonyl fluoride and aprotinin. Exactly 10 μg of protein extracts were then resolved by SDS-PAGE and subjected to Western blot analysis using anti-actin (Chemicon, Temecula, CA) and anti-fortilin antibodies with appropriate secondary antibodies conjugated to horseradish peroxidase (Southern Biotechnology Associates, Inc.). The rest of the immunoprobing was performed as previously described (36). For the cell survival assay, MCF-7 cells were seeded onto 24-well plates in triplicate. The next day, cells were transfected by FuGENE6 (Roche Molecular Biochemicals) with either pFLAG-antisense fortilin or empty pFLAG.
vector, with pFLAG-LacZ used as a transfection survival marker. Cells were harvested 12, 24, 36, 48, or 72 h after the transfection and assayed for β-galactosidase activity with the Galacto-Light Plus Assay Kit (Tropix, Bedford, MA). The loss of exogenous β-galactosidase activity in the assay reflected the death and loss of transfected cells, while the remaining exogenous β-galactosidase activity represented the survival and retention of transfected cells. The survival index of the antisense-treated cells was calculated as follows: (β-galactosidase activity of control cells at the same time point)/(β-galactosidase activity of control cells at the same time point) × 100.

Statistical Analysis—Dunnett’s or two-sample t tests were used to evaluate the difference in apoptotic and survival indices among cells transfected with different plasmids. To evaluate linear trends between the amount of plasmids used for the transfection and apoptotic indices, analysis of variance regression analysis was employed. A p value of less than 0.05 was considered to be statistically significant. For Dunnett’s t test, the confidence intervals were 0.95, and the α value was 0.05.

RESULTS

The fortilin cDNA encodes a 172-amino acid polypeptide with no significant homology to any known proteins, in whole or in part. Fortilin is highly conserved not only in mammalian species but also in nonmammalian species (Fig. 1A). The degree of conservation is unusually high in fortilin; human and mouse Bcl-2 are 72% identical, while human and mouse fortilin are 95% identical. Fortilin does not contain typical nuclear localization signals (41), or a hydrophobic transmembrane anchor or signal sequence (Fig. 1A and B). Overall, fortilin is a hydrophilic protein whose central portion (designated domain 2) is most hydrophilic (Fig. 1B). In addition, fortilin contains two conserved cysteine residues, one in domain 1 (28th amino acid; Fig. 1A) and the other at the very C-terminal end (172nd amino acid; Fig. 1A). It has not been studied whether these cysteine residues are engaged in a disulfide bond formation. Fortilin also contains a potential glycosylation site at Asn51-X-Ser53, which is highly conserved. The glycosylation status of fortilin has not been previously investigated and awaits elucidation.

Northern blot analysis using 32P-labeled human fortilin cDNA as probe revealed the ubiquity of fortilin in normal human tissues (Fig. 2). Fortilin signals were especially abundant in the liver, kidney, small intestine, skeletal muscle, and testis. Northern blot analysis using 32P-labeled human fortilin cDNA as probe revealed the ubiquity of fortilin in normal human tissues (Fig. 2). Fortilin signals were especially abundant in the liver, kidney, small intestine, skeletal muscle, and testis. The presence of a weaker band at 1.2 kilobase pairs, just above the fortilin 1.0-kilobase pair band, may represent the message of a fortilin-like molecule that remains...
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Fortilin, a novel antiapoptotic protein, was identified by a polyclonal rabbit anti-fortilin antibody raised against amino acids 99–113 of fortilin within domain 2 (Fig. 1B) and was characterized for its ability to bind full-length fortilin (Fig. 3). The anti-fortilin antibody was capable of detecting at least 12.5 ng of His$_6$-fortilin (Fig. 3A). When blocked with a peptide consisting of amino acids 99–113 of fortilin used as antigen, the antibody no longer bound to the fortilin (Fig. 3B). In summary, the rabbit anti-fortilin antibody recognized full-length recombinant fortilin with good sensitivity (Fig. 3A) and specificity (Fig. 3B).

This antibody was then used to evaluate the expression of native fortilin in various cell lines, using SDS-PAGE under denaturing conditions. Immunoblotting with rabbit preimmune serum produced no signal in any cell lines, but the use of the anti-fortilin serum yielded one discrete band at about 28 kDa (Fig. 3C). The calculated size of fortilin is about 19 kDa. Since the recombinant human fortilin, tagged with polyhistidine and expressed in E. coli, exhibited a band at about the same size (Fig. 3C), the discrepancy between the calculated size and apparent size on the immunoblot may have been due to the aberrant migration of fortilin on the gel, although the possibility of covalent modifications cannot be totally eliminated. Consistently, two groups of investigators who expressed fortilin in bacteria reported the size of the recombinant protein on the SDS gel to be 23–25 kDa (42, 43).

The degree of fortilin expression varied significantly among the cell lines tested, being elevated in cancerous cell lines such as H1299 (non-small cell lung cancer), MCF-7 (breast cancer), HeLa (cervical cancer), and A549 (lung adenocarcinoma) and less so in soft tissue tumor cell lines such as ML1a (promyelocytic leukemia) and U2OS (osteosarcoma). Cell lines derived from normal tissues such as NIH-3T3 (mouse fibroblasts) and human embryonic kidney (HEK) 293 expressed very little fortilin (Fig. 3C). Thus, the expression of fortilin appeared to be up-regulated in tumor cell lines, especially those of epithelial origin.

Next, the intracellular localization of fortilin was determined by immunostaining HeLa cells that had been transiently transfected with a pFLAG vector containing the cDNA of p21, β-galactosidase (LacZ), or fortilin. Cells were then fixed, permeabilized, and stained with anti-FLAG and anti-fortilin antibodies for native fortilin expression. Fortilin was predominantly found in the nucleus (Fig. 3C). Moreover, immunostaining of human breast cancer tissue, using anti-fortilin antibody detected mostly in the nucleus and LacZ in the cytosol (Fig. 4A), consistent with previous reports (44, 45). In this system, the FLAG-tagged fortilin was found predominantly in the nucleus (Fig. 4A). Moreover, immunostaining of human breast cancer tissue, using the rabbit anti-fortilin antibody described above (Fig. 3, A–C), showed fortilin to be mainly localized in the nucleus (Fig. 4B). Thus, fortilin was shown to be a predominantly nuclear protein.

Next, the specific function of fortilin was determined. Speculating that fortilin might play a role in apoptosis regulation, we tested whether fortilin prevented cell death induced by etoposide, a chemotherapeutic agent (46). For these experiments, HeLa cells were transiently transfected with a pFLAG vector containing the cDNA of fortilin, Bcl-xL, MCL1, or LacZ and were then challenged with etoposide. Cells expressing the FLAG-tagged proteins were identified by immunostaining with an anti-FLAG antibody. Nuclei were stained by DAPI and examined for apoptotic nuclei. Cells were considered apoptotic when the nucleus was condensed and small or fragmented (47). Consistent with previous reports (48, 49), the overexpression of Bcl-xL and MCL1 prevented HeLa cells from undergoing apoptosis. In this system, fortilin exhibited an antiapoptotic effect comparable with those of MCL1 and Bcl-xL (Fig. 5A). In addition, the greater amounts of the fortilin exerted more prominent antiapoptotic effects in the same system (Fig. 5B). Together, these data suggested that fortilin is an antiapoptotic protein that inhibits etoposide-induced apoptosis in HeLa cells. Because fortilin has no structural homology with either Bcl-2 (50) or IAP (inhibitors of apoptosis) family proteins (51) (Fig. 1A), fortilin may represent a new class of antiapoptotic proteins.

Next, the apoptotic activity of fortilin was confirmed using a different cell line and a different cell death detection system. To this end, U2OS cells stably expressing fortilin (U2OS.F) were generated and compared with control U2OS cells stably possessing empty pcDNA6 vector (U2OS.E) for their ability to withstand etoposide-induced cytotoxicity, using a standard LDH cytotoxicity assay as described under “Materials and Methods.” As shown in Fig. 6A, the stable expression of fortilin in U2OS cells was associated with significantly less cytotoxicity over the wide range of etoposide concentrations (2.5, 5, 10, and 20 μg/ml; for all, p < 0.001) (Fig. 6A). Furthermore, U2OS.F exhibited significantly less cytotoxicity at various time points after the etoposide challenge (48 h, p < 0.05; 72 and 96 h, p < 0.001) (Fig. 6B). Taken together, these results suggested that fortilin is an antiapoptotic protein that prevents etoposide-induced cell death.

**Fig. 4. Intracellular localization of fortilin.** A, transient expression system in HeLa cells. HeLa cells were transfected with mammalian expression vectors encoding either FLAG-epitope-tagged p21, β-galactosidase (LacZ), or fortilin. Cells were then fixed, permeabilized, and stained with anti-FLAG and anti-fortilin antibodies for native fortilin expression. Fortilin was predominantly found in the nucleus (Fig. 3C). B, native fortilin expression in human tissue samples. Tissue sections from a patient with ductal breast carcinoma were stained with rabbit anti-fortilin antibody for native fortilin expression. Fortilin was predominantly found in the nucleus. Purified rabbit IgG was used at the same concentration as the anti-fortilin antibody as control. The magnification was ×200, with the scale bar representing 25 μm.
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To further explore the role of fortin in apoptosis regulation, the status of caspase-3-like activity in fortilin-overexpressing cells upon etoposide challenge was examined. In brief, the caspase-3-like activity of the U2OS cells stably expressing fortilin (U2OS.F) as described above was compared with that of the control U2OS cells (U2OS.E) when challenged with etoposide. As shown in Fig. 7A, in the absence of etoposide challenge (Fig. 7A, Etoposide –), the caspase-3-like-activities of U2OS.E (Fig. 7A, C) and U2OS.F (Fig. 7A, F) were similarly low (158.2 ± 18.0 and 283 ± 22.6 for U2OS.E and U2OS.F, respectively; not statistically significant (NS)) (Fig. 7A). When these cells were challenged with etoposide (Fig. 7A, Etoposide +), however, U2OS.E exhibited a 9.4-fold increase in the activity with the caspase-3-like activity index reaching 1488.1 ± 58.7, while the caspase-3-like activity index of U2OS.F remained at 713.9 ± 62.6 (p < 0.05; Fig. 7A, asterisk). Consistently, the caspase-3-like activities of etoposide-challenged U2OS.E were always higher than, and diverged continuously from, those of U2OS.F during the 2-h period of the kinetics assay (Fig. 7B).

Together, these data suggested that the protection by fortilin against etoposide-induced cytotoxicity, as assessed by LDH assay (Fig. 6, A and B), was at least partially due to the prevention of caspase-3 activation by fortilin, an antiapoptotic molecule.

Next, we tested the antiapoptotic role of fortilin in MCF-7 cells, a malignant human ductal carcinoma cell line. To this end, the effect of fortilin depletion by antisense fortilin treatment on the survival of MCF-7 cells was studied. First, we evaluated the intracellular concentration of fortilin in response to antisense fortilin treatment, using Western blot analysis. As is shown in Fig. 8A, the antisense fortilin treatment, using a pFLAG vector containing the antisense fortilin nucleotide sequence (pFLAG-antisense-fortilin), significantly reduced the intracellular concentration of fortilin. We then transiently transfected MCF-7 cells with either an empty pFLAG vector or pFLAG-antisense-fortilin that we characterized above (Fig. 8A), along with pFLAG-LacZ used as a transfection survival marker. Cells were harvested at various time points and assayed for β-galactosidase activity. MCF-7 cells that had been transfected with the empty pFLAG vector and pFLAG-LacZ accumulated β-galactosidase intracellularly over time, as evidenced by the increase in β-galactosidase activities at 24, 36, 48, and 72 h (Fig. 8B, lower panel, closed bars). In contrast, MCF-7 cells that had been transfected with pFLAG-antisense fortilin and pFLAG-LacZ had significantly less β-galactosidase activity than did the control cells at these time points (Fig. 8B, lower panel, open bars, p < 0.01, for 24, 36, 48, and 72 h). The survival of the antisense-treated cells, calculated as the ratio of β-galactosidase activities of the antisense-treated and control
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FIG. 7. Inhibition of caspase-3-like activity by fortin in etoposide-challenged U2OS cells. A, approximately 2 × 10⁶ U2OS stable transfectants, either expressing fortin (F) or harboring empty expression vectors (C), were challenged either with Me₂SO (vehicle; −) or with etoposide (+) at a final concentration of 10 μg/ml for 48 h. Caspase-3-like activity was then determined by the amount of AFC released, after a 2-h incubation at 37 °C, by the Fluoroskan system (Thermo-Labsystems, Helsinki, Finland) set at an excitation value of 355 nm and emission value of 525 nm. The results (AFC) were expressed as relative fluorescence units (R.F.U./μg of protein (R.F.U./μg protein)). Base-line caspase-3-like activities were not different between control and fortin-expressing cells (NS, not statistically different). Upon etoposide challenge, fortin significantly inhibited the caspase-3-like activity in U2OS cells (asterisk, p < 0.05). B, kinetics of caspase-3-like activities in control and fortin-overexpressing cells. Approximately 2 × 10⁶ U2OS, either expressing fortin (F) or harboring empty expression vectors (C), were challenged with 10 μg/ml of etoposide and subjected to the assay of caspase-3-like activity as described above. The fluorescence signals were measured every 10 min for 2 h. Fortin significantly inhibited the caspase-3-like activity in U2OS cells at all time points (p < 0.05).}

Fortilin, a Novel Antiapoptotic Protein

Fortilin, also known as translationally controlled tumor protein, has been described in literature only sparsely. In fact, human fortin has never been expressed for functional study in eucaryotic cells. Fortilin was originally cloned as a human homologue to the mouse p21 protein (32), which is abundantly expressed in mammalian eucaryotic cells. Fortilin was originally cloned as a human homologue to the mouse p21 protein (32), which is abundantly expressed in mammalian eucaryotic cells. Fortilin was originally cloned as a human homologue to the mouse p21 protein (32), which is abundantly expressed in mammalian eucaryotic cells. Fortilin was originally cloned as a human homologue to the mouse p21 protein (32), which is abundantly expressed in mammalian eucaryotic cells. Fortilin was originally cloned as a human homologue to the mouse p21 protein (32), which is abundantly expressed in mammalian eucaryotic cells.

DISCUSSION

We report here the first functional characterization of fortin as an antiapoptotic protein. We show that fortin is a hydrophilic nuclear protein, which is unusually well conserved across species. Fortin exists in various normal tissues, yet its expression is much higher in cancerous cell lines than in cell lines derived from normal tissues. Strikingly, fortin inhibited etoposide-induced apoptosis in both HeLa cells and U2OS cells. In addition, fortin blocked the caspase-3-like activity in etoposide-challenged U2OS cells. Furthermore, antisense depletion of fortin from MCF-7, a breast cancer cell line, caused massive cell death. Since the amino acid sequence of fortin does not resemble that of Bcl-2 family member proteins or that of IAPs, fortin may represent a new class of apoptosis modulator that may play a role in basic cellular function and, in case of its dysregulation, tumorigenesis.

FIG. 8. The anti-cell death activity of fortin in MCF-7, a human breast ductal carcinoma cell line. A, the reduction of intracellular fortin concentration by antisense fortin treatment. The intracellular concentration of fortin was evaluated in MCL-7 cells that were treated (Antisense, +) and compared with that from control cells (Antisense, −), using Western blot analysis of total cell lysate by anti-actin and anti-fortin antibodies. The fortin signal of treated cells was significantly weaker than that of control cells. B, the induction of spontaneous MCF-7 cell death by antisense fortin treatment. MCF-7 cells were transfected with either pFLAG-antisense fortin or empty pFLAG, along with pFLAG-LacZ used as a transfection survival marker. Cells were harvested at the indicated times, assayed for β-galactosidase, and the survival index was calculated. L.U., light units. Asterisks in the survival curve indicate statistically significant (p < 0.01) reduction in survival rate relative to that at 12 h. Asterisks in the β-galactosidase activity indicate statistically significant (p < 0.01) differences in the activity between antisense-treated cells (open bars) and control cells (closed bars).

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apoptosis (54, 55). In addition, thapsigargin, a Ca\textsuperscript{2+}-ATPase inhibitor that increases intracellular calcium by inhibiting the uptake of calcium into the endoplasmic reticulum, also induces apoptosis in certain cell types (56). Furthermore, the rise in intracellular calcium has been shown to be necessary for DNA degradation in anti-Fas-induced apoptosis of Jurkat cells (57). On the other hand, antiapoptotic proteins have been shown to modulate intracellular calcium levels. For example, Bel-2 inhibits thapsigargin-induced apoptosis in WEHI7.2 cells by blocking the increase of intracellular Ca\textsuperscript{2+} (35). It is possible that fortilin, by binding and scavenging Ca\textsuperscript{2+} released in response to apoptotic stimuli, modulates the intracellular calcium concentration and blocks apoptosis mediated by fluctuating Ca\textsuperscript{2+} concentration. The ability of fortilin to bind Ca\textsuperscript{2+} and its functional significance must be tested in mammalian cells.

Further investigation of fortilin may reveal additional regulatory mechanisms of apoptosis, which is already proving itself to be ever more sophisticated and complex. The anti-fortilin antibody we have characterized here may prove to be a useful reagent for further investigating the role of fortilin in human tumorigenesis and other proliferative diseases.

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