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# Caspase-mediated Specific Cleavage of Human Histone Deacetylase 4*[S]

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**Fang Liu‡§, Melissa Dowling‡§, Xiang-Jiao Yang¶, and Gary D. Kao‡‖**

*From the ‡Department of Radiation Oncology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104 and the ¶Molecular Oncology Group, Department of Medicine, McGill University Health Center, Montréal, Quebec H3A 1A1, Canada*

**Histone deacetylase 4 (HDAC4) is a class II HDAC implicated in controlling gene expression important for diverse cellular functions, but little is known about how its expression and stability are regulated. We report here that this deacetylase is unusually unstable, with a half-life of less than 8 h. Consistent with the instability of HDAC4 protein, its mRNA was also highly unstable (with a half-life of less than 4 h). The degradation of HDAC4 could be accelerated by exposure of cells to ultraviolet irradiation. HDAC4 degradation was not dependent on proteasome or CRM1-mediated export activity but instead was caspase-dependent and was detectable in diverse human cancer lines. Of two potential caspase consensus motifs in HDAC4, both lying within a region containing proline-, glutamic acid-, serine-, and threonine-rich (PEST) sequences, we identified, by site-directed mutagenesis, Asp-289 as the prime cleavage site. Notably, this residue is not conserved among other class IIa members, HDAC5, -7, and -9. Finally, the induced expression of caspase-cleavable HDAC4 led to markedly increased apoptosis. These results therefore unexpectedly link the regulation of HDAC4 protein stability to caspases, enzymes that are important for controlling cell death and differentiation.**

Histone deacetylases (HDACs)[1] have been increasingly implicated in mediating diverse fundamental cellular activities. Based on sequence homology with their yeast orthologs, mammalian HDACs have been divided into three classes. Class I HDACs include HDAC1, -2, -3, -7, -8, and -11, whereas class II HDACs contain HDAC4, -5, -6, -7, -9, and -10 (for recent reviews, see Refs. 1–4). Among class II, HDAC4, -5, -7, and -9 form a subclass known as class IIa, whereas HDAC6 and -10 constitute class IIb. A third class of mammalian HDACs includes the Sir2-like proteins Sirt1–7 (5, 6). The most well characterized function of HDACs is the deacetylation of core histones, which in turn leads to the compaction of nucleosomes to repress gene transcription. HDACs have also been implicated in the deacetylation of nonhistone targets. For example, HDAC6 regulates the deacetylation of tubulin, which may in turn promote cell motility (7–9).

Despite the increasing repertoire of cellular activities that have been found to involve HDACs, relatively little is known regarding mechanisms regulating their expression. For example, HDAC1 binding to the CCAAT/enhancer-binding protein $\alpha$-promoter increased upon treatment of cells with a proteasome inhibitor, but the protein levels were not directly assessed (10). HDAC5 and HDAC6 are ubiquitinated, but it is unclear how their stability is regulated (11). Interestingly, HDAC1 and HDAC4 undergo sumoylation, a post-translational modification that is reminiscent of ubiquitination but does not appear to regulate protein degradation (12, 13).

To assess how the levels of HDAC4 and other HDACs might be controlled, we measured their protein stability following the inhibition of *de novo* synthesis. HDAC4 was found to be exceptionally unstable, with a half-life of less than 8 h, far less stable than HDAC1, -2, -3, and -6. Analysis of truncation mutants of HDAC4 revealed that the instability was mediated by a proline-, glutamic acid-, serine-, and threonine-rich (PEST)-containing region that also contains two potential caspase cleavage sites. Surprisingly, the degradation of HDAC4 was neither proteasome-dependent nor inhibited by blocking active nuclear export but was blocked by the inhibition of caspase activity. Ultraviolet irradiation greatly accelerated the caspase-dependent degradation of HDAC4 protein. In addition, Asp-289 appeared to be essential for caspase cleavage and is not conserved among HDAC5, -7, and -9. The induced expression of caspase-cleavable HDAC4 led to increased apoptosis of HeLa cells when compared with induced expression of HDAC4 mutated at the caspase cleavage site. Together, these novel findings link the differential regulation of class IIa HDAC proteins to components of the intracellular apoptotic machinery.

## EXPERIMENTAL PROCEDURES

*Cell Culture, Reagents, and Treatments*—All cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA), and grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 15% fetal bovine serum at 37 °C in 5% $CO_2$. Actinomycin, cycloheximide, lactacystin, ALLN, and MG-132 were from Sigma, leptomycin-B was from Bio-Source, and the cell-permeable caspase inhibitors DEVD-CHO (inhibitor of caspase-3) and YVAD-CHO (inhibitor of caspase-1) were from either Calbiochem or Bio-Source (Camarillo, CA), and both were used at 20 $\mu$M final concentration. All inhibitors were prepared as concentrated stock solutions in $Me_2SO$. Caffeine, hydroxyurea, okadaic acid, and aphidicolin were all obtained from Sigma and used at the following final concentrations: caffeine, 2 mM; hydroxyurea, 2 mM; okadaic acid, 100 $\mu$M; and aphidicolin, 2 $\mu$g/ml. UV was per-

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[S] The on-line version of this article (available at http://www.jbc.org) contains two supplementary figures pertinent to HDAC4.

§ Both authors contributed equally to this work.

‖ To whom correspondence should be addressed: Dept. of Radiation Oncology, University of Pennsylvania School of Medicine, John Morgan 180 H, Philadelphia, PA 19104. Tel.: 215-573-5503; Fax: 215-898-0090; E-mail: Kao@xrt.upenn.edu.

[1] The abbreviations used are: HDAC, histone deacetylase; PEST, proline-, glutamic acid-, serine-, and threonine-rich; LMB, leptomycin B; GFP, green fluorescent protein.

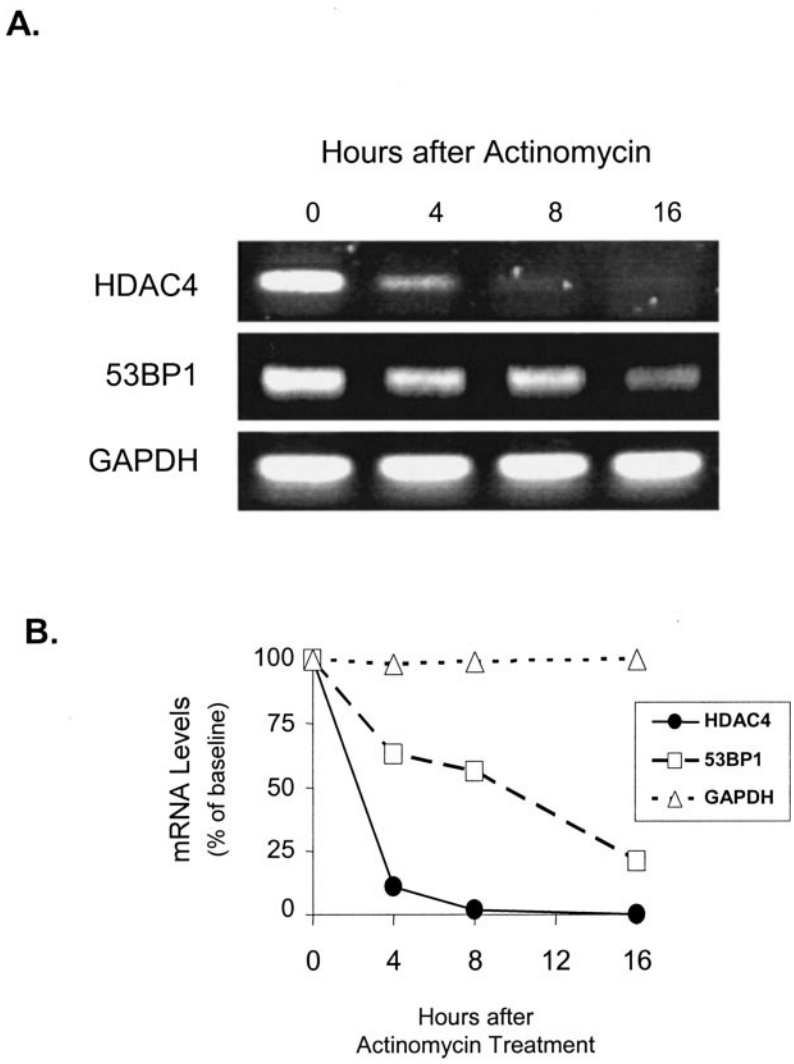


FIG. 1. **Instability of HDAC4 mRNA.** *A*, RT-PCR was performed on total mRNA isolated from HeLa cells at the times indicated after treatment with actinomycin to inhibit *de novo* transcription. RT-PCR was performed in parallel under identical conditions using primer pairs targeting HDAC4, or as controls, 53BP1 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The final products were separated via electrophoresis in ethidium bromide-labeled agarose and photographed under UV illumination. Each RT-PCR reaction yielded a single band as shown. *B*, the concentrations of mRNA as shown in *A* was quantified via spectrophotometry, normalized to pre-actinomycin levels, and plotted as shown.

formed with a GS UV Linker (Bio-Rad), whereas $\gamma$ irradiation (ionizing irradiation) was delivered using a high dose rate cesium irradiator (12.84 grays/min). Mock-treated control cells were handled in similar manners except that no drugs were used.

*Analytic Methods*—Cell lysates were prepared via scraping on ice and pelleting at 4 °C followed by resuspension in Laemmli buffer and sonication. For immunoblotting, samples (10 $\mu$g/lane) were boiled for 5 min and separated via SDS-PAGE and then transferred to nitrocellulose membranes. After transfer, the membranes were blocked with 5% nonfat milk in phosphate-buffered saline and then probed with the indicated primary antibodies followed by the appropriate secondary antibodies conjugated with horseradish peroxidase. Anti-HDAC4 polyclonal antibodies were purified as described previously (14). Specifically, these antibodies were generated against either the SalI-HindIII fragment of HDAC4 (when translated, encompasses 601 amino acids in the N-terminal portion of the protein) or the larger SalI-XhoI fragment of HDAC4 (when translated, spans 784 amino acids starting from the N-terminal portion of the protein). Anti-HDAC2 antibodies were from Biomol, anti-HDAC1 was from Santa Cruz Biotechnology, and anti-HDAC3 and anti-HDAC6 were from Cell Signaling. Washes were performed with phosphate-buffered saline with 0.1% Tween. Finally, after probing with primary and secondary antibodies, the membranes were exposed to film after enhanced chemiluminescence (ECL) (Amersham Biosciences). Densitometry of immunoblots was performed on images obtained under nonsaturated conditions and quantitated with NIH Image 1.54 software.

For pulse labeling experiments, 10 $\mu$Ci/ml [$^{35}$S]methionine (Amersham Biosciences cell labeling grade) was added to the cellular medium for 0.5–4 h. Cells were lysed as above, and cytoplasmic extracts were run by SDS-PAGE. For autoradiography, gels were then dried and exposed to x-ray film.

Cell viability and death were assessed by direct visualization of cell morphology, trypan blue exclusion, Hoescht 33342 vital staining, and flow-assisted cytometric analysis of cells with sub-$G_1$ DNA content. These methods showed good general agreement. Viable cells were defined as those excluding trypan blue, with uncondensed chromatin or mitotic chromosomes as visualized by vital staining and with $G_1$ or greater DNA content.

*Reverse-transcriptase (RT)-PCR*—Endogenous mRNA was isolated using TRIzol reagent (Invitrogen) as per the manufacturer's instructions, and assessed via RT-PCR. The Titan One Tube RT-PCR system (Roche Applied Science) was used with the following primers: HDAC4, 5′-CAA GAA CAA GGA GAA GGG CAA AG-3′, 5′-GGA GAA CTC TGG TCA AGG GAA CTG-3′; 53BP1, 5′-AGG TGG GTG TTC TTT GGC TTC C-3′, 5′-TTG GTG TTG AGG CTT GTG GTG ATA C-3′; glyceraldehyde-3-phosphate dehydrogenase, 5′-CAA CTT TGG TAT CGT GGA AGG ACT C-3′, 5′-AGG GAT GAT GTT CTG GAG AGC C-3′. Reactions for all targeted mRNAs were performed under similar conditions, with comparatively identical results under a range of cycle times and numbers (specific details regarding the PCR parameters used are available upon request).

*Site-directed Mutagenesis*—Expression plasmids encoding HDAC4 mutated in potential caspase cleavage sites were derived from constructs that have been described previously (15, 16). The respective point mutations were generated with the QuikChange site-directed mutagenesis kit(Stratagene). For the HDAC4 (D237E) mutation, the oligonucleotide primer pair used was 5′-TGT ACG ACG CCA AAG A*G*G ACT TCC CTC TTA GG-3′ and 5′-AAG AGG GAA GTC *C*TC TTT GGC GTC GTA CAT TCC CAG-3′. For the HDAC4 (D289E) mutation, the oligonucleotide primer pair used was 5′-AGC GTC CGT TGG ATG TCA CAG A*A*T CCG CGT GCA G-3′ and 5′-CTG CAC GCG GA*T* TCT GTG ACA TCC AAC GGA CGC T-3′. Direct DNA sequencing was repeatedly performed to confirm the engineered mutations. As described previously, green fluorescent protein (GFP) constructs were derived from

pEGFP-C2 (Clontech). Cells were transfected with plasmids expressing GFP fusion proteins using LipofectAMINE 2000 reagent (Invitrogen).

*Construction of Doxycycline-inducible HDAC4 Expression Vectors*—cDNA encoding amino acids 1–1061 of human HDAC4 or 1–1061 mutated at Asp-289 (D289E) was subcloned into the mammalian expression plasmid pTRE-Tight (Clontech) to generate pTRE HDAC4 1–1061 or pTRE HDAC4 D289E. Nucleotide sequences of the resulting constructs were confirmed by DNA sequencing. Cells transfected with these constructs were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% Tet-On™ approved fetal bovine serum (Clontech) and in the presence of 100 $\mu$g/ml G418 and 50 $\mu$g/ml hygromycin B (Roche Applied Science) and then induced with doxycycline (1 $\mu$g/ml) as described by the manufacturer.

## RESULTS

*Stability of HDAC4 mRNA*—We had found previously that HDAC4 protein levels could be efficiently knocked down by RNA interference (14). In preparatory studies, we discovered that HDAC4 mRNA was extremely unstable. Cells treated with actinomycin D to inhibit *de novo* RNA synthesis were harvested at serial time points and assessed for HDAC4, 53BP1, or glyceraldehyde-3-phosphate dehydrogenase mRNA (Fig. 1, *A* and *B*). HDAC4 mRNA levels was substantially decreased within hours and was virtually undetectable by 8 h. HDAC4 mRNA therefore appeared to have a half-life of 4 h or less. In comparison, under the same conditions, 53BP1 mRNA levels were moderately decreased at 4–8 h but still detectable at 16 h, whereas glyceraldehyde-3-phosphate dehydrogenase mRNA was essentially unchanged throughout the experiment.

*Stability of HDAC4 Protein*—The instability of HDAC4 mRNA suggested that HDAC4 protein might be also be unstable. To assess protein stability, cells with cycloheximide to inhibit *de novo* protein synthesis were harvested at serial time points following treatment and assessed for protein levels. In accord with the short half-life of the mRNA, HDAC4 protein levels were appreciably decreased within hours and were virtually undetectable by 12 h after the addition of cycloheximide (Fig. 2, *A* and *B*). By contrast, levels of HDAC1, -2, -3, and -6 remained virtually unchanged.

To formally exclude the possibility that the instability of HDAC4 was unique to HeLa cells, we repeated to assess its levels in PA1 ovarian cancer and HCT116 colorectal cancer cells after mock or cycloheximide treatment. HDAC4 protein levels varied in these cell lines before treatment (Fig. 2*C*, *lanes 1*, *3*, and *5*) but decreased considerably after exposure to cycloheximide in all cases (compares *lanes 1*, *3*, and *5* with *lanes 2*, *4*, and *6*, respectively). These results suggest that although there are cell type-specific differences in HDAC4 expression levels and its rate of degradation, instability is a general characteristic of this protein.

*HDAC4 Stability Is Not Proteasome- or CRM1-dependent but Dependent on Caspase Activity*—Our initial studies assessing the instability of HDAC4 were confirmed with both commercial anti-HDAC4 peptide antibodies (results not shown) as well as polyclonal antibodies that we generated against the N-terminal 601-amino-acid residues (Fig. 2). We noted, however, that the peptide antibodies lacked sensitivity when compared with the antibodies directed against a larger portion of the protein, requiring 20–100 times higher concentrations of antibody to generate equivalent signal. To optimize the sensitivity of our antibodies, we generated a second series of antibodies against the N-terminal 784-amino-acid residues and which further improved sensitivity for detecting HDAC4. Furthermore, when we used these antibodies to probe immunoblots derived from cycloheximide-treated cells, we consistently detected, in addition to the full-length 140-kDa HDAC4 protein, a second band of 90 kDa (Fig. 3). Interestingly, as full-length HDAC4 underwent degradation in the presence of cycloheximide, the intensity of the 90-kDa band increased, suggesting that this band represents a cleaved product.

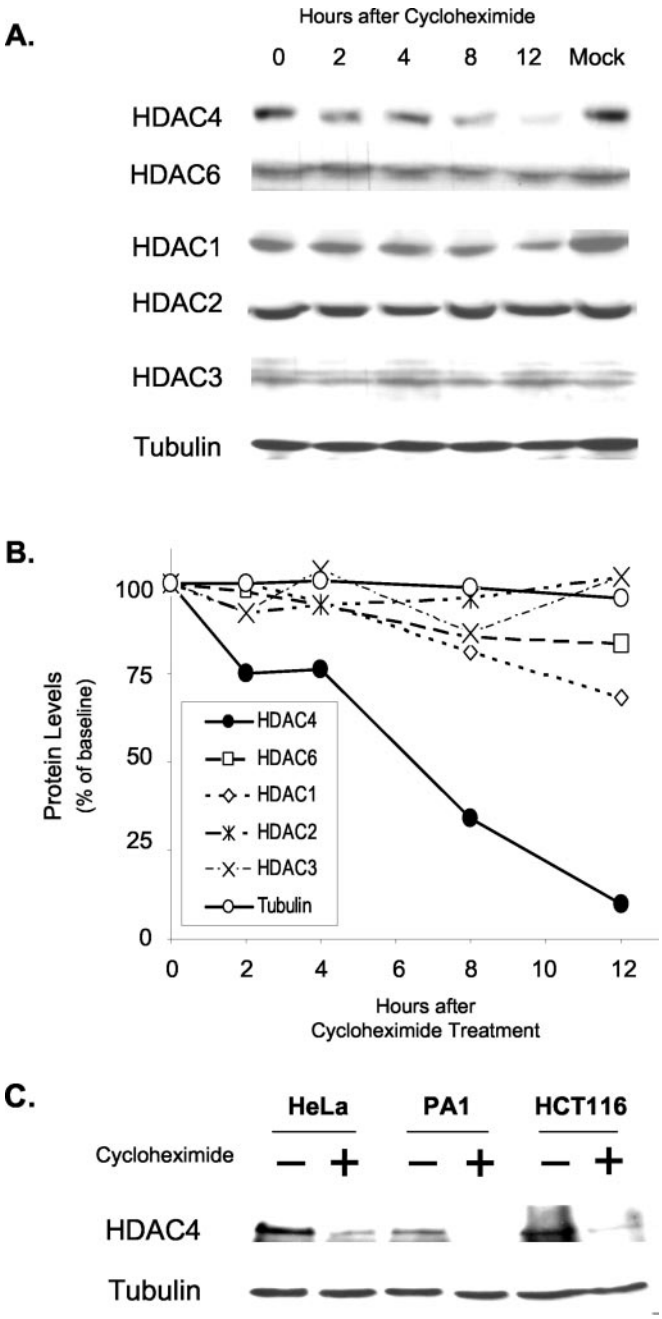


FIG. 2. **Instability of HDAC4 protein.** *A*, the instability of HDAC4 protein is not a general property of Class I and II HDACs. HeLa cells treated with cycloheximide (final concentration 25 ng/ml) were harvested at serial time points as indicated, and the total cell lysates were separated via SDS-PAGE, transferred to nitrocellulose, and immunoblotted for the indicated proteins. *B*, expression levels of each respective protein in the blots in *A*. were quantified via densitometry and plotted as shown. *C*, HeLa, PA1 or HCT116 cells were treated with or without cycloheximide and harvested 12 h later, and total cell lysates were separated via SDS-PAGE and immunoblotted for HDAC4 or $\alpha$-tubulin, PA1

Using these anti-HDAC4 antibodies, we probed cells that were treated with cycloheximide alone or concurrently with lactacystin, a cell-permeable and irreversible inhibitor of proteasome-mediated proteolysis. The cells were harvested at serial time points for immunoblotting. The degradation of HDAC4 did not display any difference in the presence or absence of the proteasome inhibitor (Fig. 3*A*). Treatment with the proteasome-inhibitor MG-132 (also known as Z-Leu-Leu-Leu-

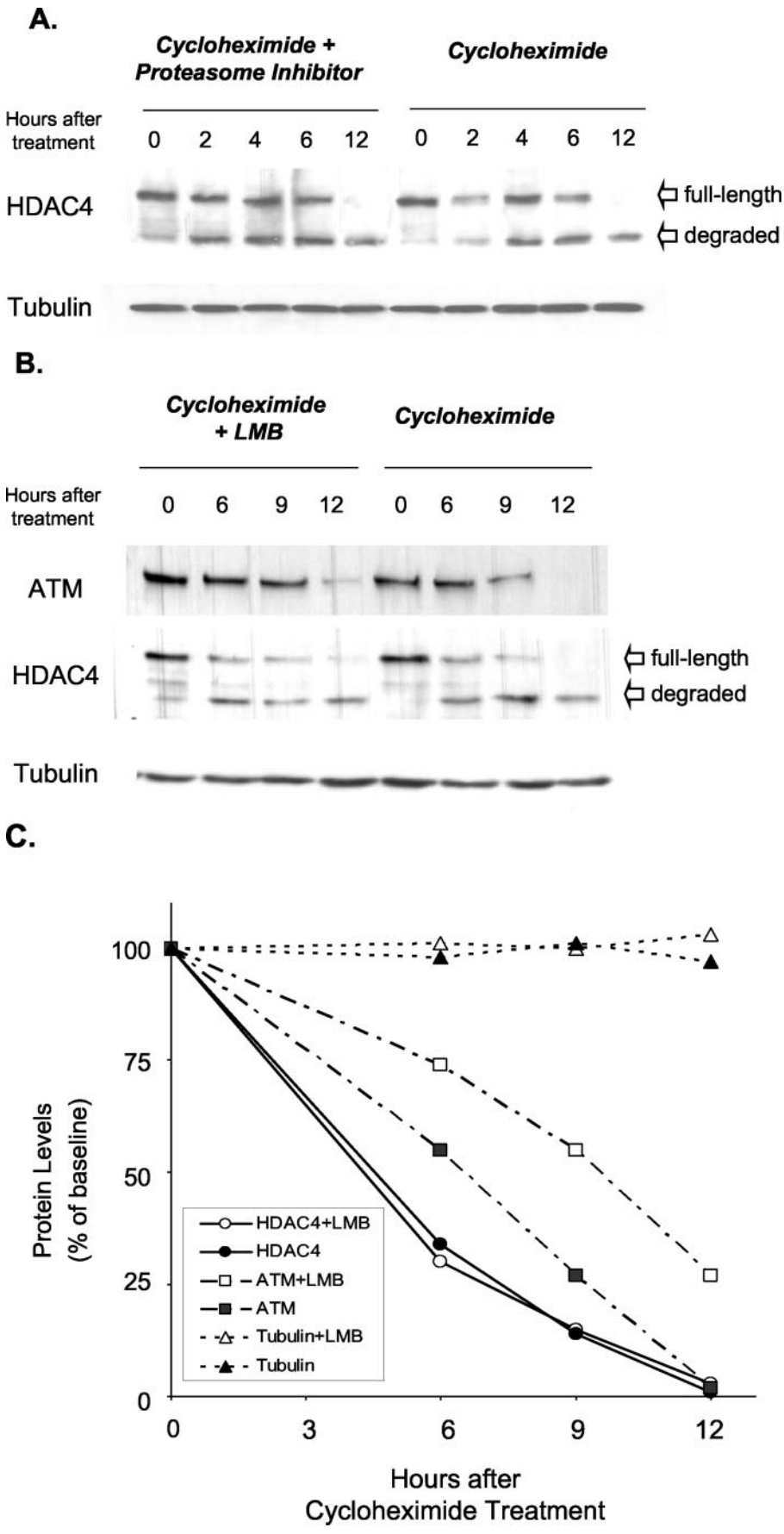


FIG. 3. **Proteasome- or CRM1-inhibition does not impede HDAC4 protein degradation.** *A*, HeLa cells were pretreated with the irreversible proteasome inhibitor lactacystin (10 $\mu$M final concentration) for 30 min followed by treatment with cycloheximide. Cells were then harvested at serial time points, and total cell lysates were immunoblotted using antibodies directed against the N-terminal 784 residues of HDAC4) or $\alpha$-tubulin. *B*, HeLa cells were pretreated with LMB (25 ng/ml final concentration) for 30 min followed by mock treatment or treatment with cycloheximide. Cells were then harvested at serial time points, and total cell lysates were probed as in *A* for HDAC4 or $\alpha$-tubulin. *ATM*, ataxia-telangiectasia mutated. *C*, the levels of each respective protein and treatment in the blots in *B* were quantified via densitometry and plotted as shown.

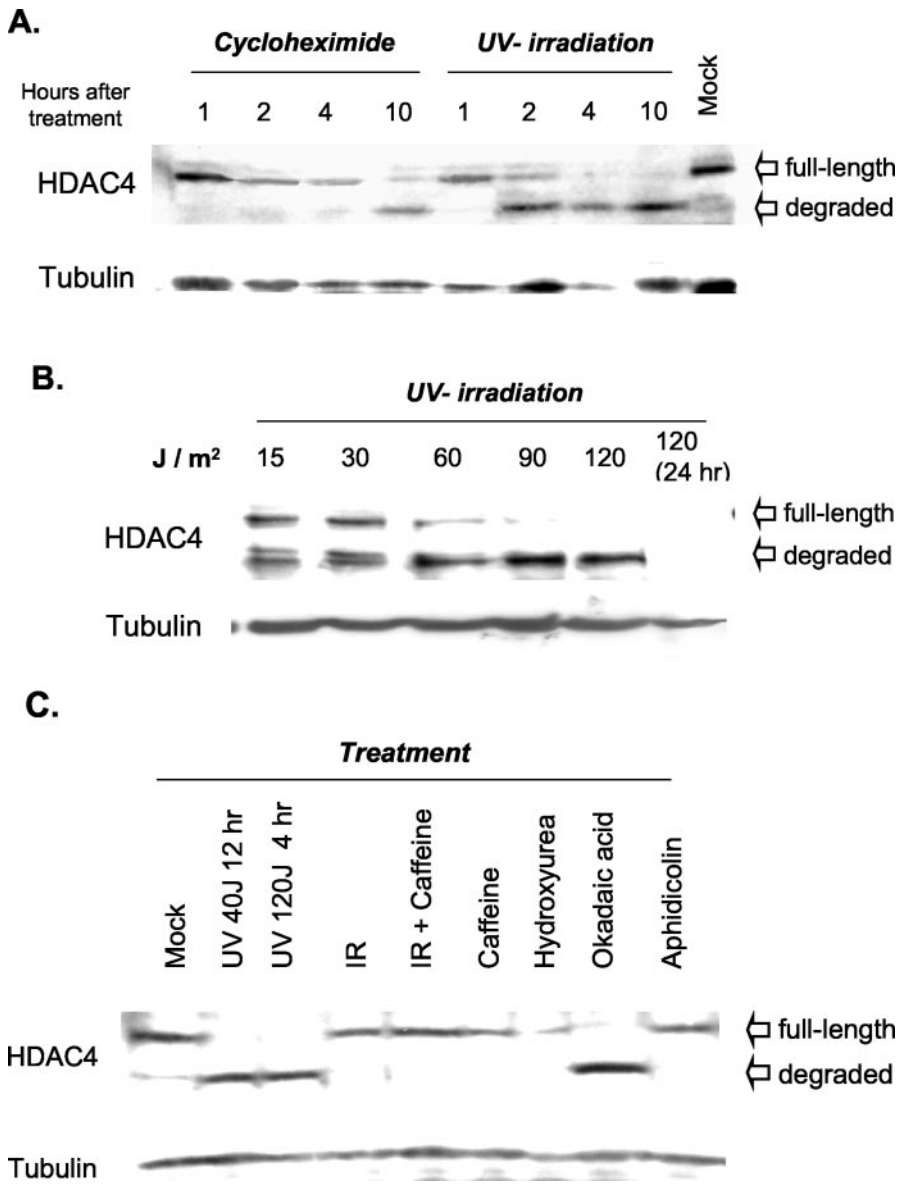


FIG. 4. **HDAC4 degradation is accelerated by UV irradiation.** *A*, HeLa cells were treated with cycloheximide or UV-irradiated (120 J/m$^2$) and harvested at the time points indicated following treatment, and total cell lysates were separated by SDS-PAGE and subsequently immunoblotted for HDAC4 or tubulin. Mock-treated cells (*Mock*) serve as a control. UV rapidly diminished the amount of full-length HDAC4, which was accompanied by an increase in the lower molecular weight degraded form. *B*, cells were UV-irradiated with the indicated doses of UV, harvested 10 h later, and immunoblotted for HDAC4 and tubulin as in *A*. A parallel plate of cells was irradiated at the highest UV dose but not harvested until 24 h after treatment. *C*, cells were mock-treated or treated with UV (60 J/m$^2$ and then harvested 12 h, or 120 J/m$^2$ and then harvested 4 h after irradiation), 5 grays of ionizing irradiation (*IR*) alone or with caffeine, caffeine alone, hydroxyurea, okadaic acid, or aphidicolin. In all instances, unless noted otherwise, cells were then harvested 12 h after treatment. Total cell lysates were then immunoblotted for HDAC4 and tubulin as in *A* and *B*.

al), ALLN (also known as calpain inhibitor I or MG101), and *N*-Acetyl-L-leucyl-L-leucyl-L-methioninal (calpain inhibitor II) all likewise had no clear effect on the degradation (results not shown).

We next investigated whether HDAC4 degradation might be determined by CRM1-mediated mechanisms. We treated cells again with cycloheximide, either alone or concurrently with leptomycin B (LMB), a specific inhibitor of CRM1-mediated nuclear export. In cell lysates that were harvested sequentially after treatment, we found that HDAC4 degradation did not appear to be significantly different in the presence or absence of LMB (Fig. 3, *B* and *C*). HDAC4 underwent substantial degradation by 6 h after cycloheximide treatment and was virtually undetectable by 12 h both in the presence and in the absence of LMB. In contrast to the lack of effect on HDAC4 degradation, LMB delayed the degradation of the product of the ataxia-telangiectasia mutated (*ATM*) gene, which, in the presence of LMB, was still detectable 12 h after the addition of cycloheximide (Fig. 3, *B* and *C*).

*Ultraviolet Irradiation Accelerates HDAC4 Degradation*—The efficient degradation of HDAC4 noted after exposure to cycloheximide suggested that cells might contain continuously active proteolytic mechanisms that are especially unmasked when *de novo* synthesis is blocked, a response that might be induced by environmental stimuli or stress, such as ultraviolet irradiation. We treated cells with ultraviolet irradiation or cycloheximide and harvested cells at successive time points. Whereas both treatments led to the complete degradation of HDAC4, the rate of degradation was faster after UV irradiation. The degradation of HDAC4 was complete by 4 h after 120 J/m$^2$ UV, whereas at this time after cycloheximide, 50% of HDAC4 remained. HDAC4 underwent accelerated degradation after UV in a dose-dependent manner as lower doses of UV led to decreased levels of HDAC4 degradation (Fig. 4*B*). A parallel plate was UV-irradiated at the highest dose but harvested at a later time point. Interestingly, this showed that with additional time, the amount of the lower molecular weight band of HDAC4 diminishes as well, suggesting that this band may represent an intermediate degradation product that after high UV doses ultimately undergoes full degradation.

We sought to investigate further conditions to determine whether these led to HDAC4 degradation. Ionizing radiation is generally thought to induce single- and double-strand DNA damage through free radical formation. In HeLa cells, ionizing irradiation generally does not induce apoptosis, unless sensitized by caffeine (15). Hydroxyurea and aphidicolin, through

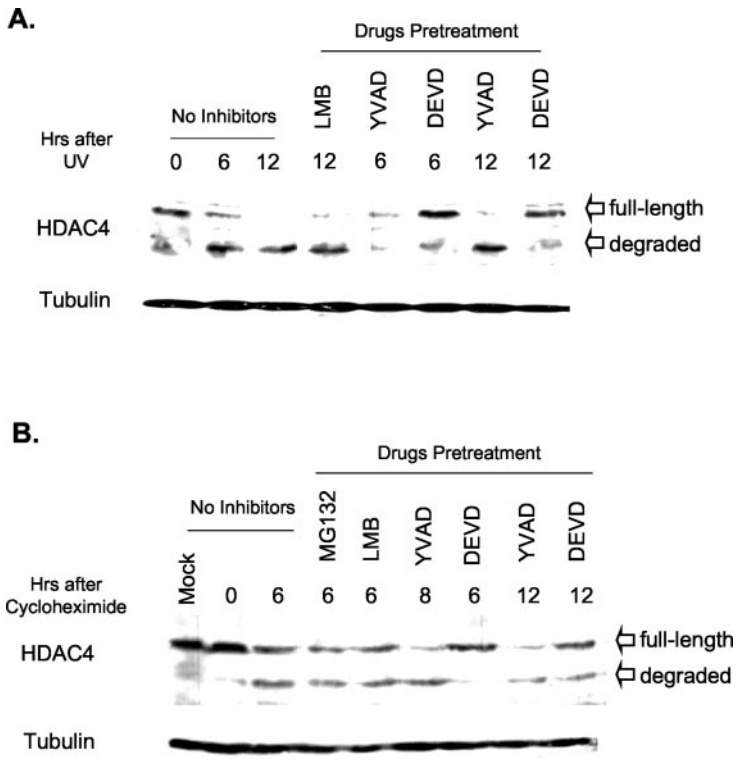


FIG. 5. **HDAC4 is stabilized by inhibition of caspase-3.** *A*, cells were pretreated with the indicated inhibitors (or mock-pretreated) followed by UV irradiation (120 J/m$^2$). LMB was used as an inhibitor of CRM1. The cells were then harvested at the times indicated after treatment, and total cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for HDAC4 or α-tubulin. Abbreviations are as follows: *YVAD*, YVAD-CHO, cell-permeable inhibitor of caspase 1; *DEVD*, DEVD-CHO, cell-permeable inhibitor of caspase-3. *B*, cells pretreated as in *A* but then treated with cycloheximide were harvested at the times indicated, and total cell lysates were probed for HDAC4 and α-tubulin. Cells mock-treated and not exposed to cycloheximide (*Mock*) and cells pretreated with the proteasome inhibitor MG132 were included as additional controls.

the inhibition of ribonucleotide reductase (resulting in depletion of intracellular dNTP precursors) and DNA polymerase-α, respectively, both block DNA replication, and under continuous exposure, they will eventually lead to the death of cells (16, 17). Okadaic acid is considered a phosphatase 1 and -2A inhibitor and induces apoptosis in a variety of cell types, likely due to caspase activation (18, 19). We found that under these treatment conditions, both UV and okadaic acid lead to the degradation of HDAC4, whereas the others had no such effect (Fig. 4*C*).

To further investigate whether HDAC4 degradation might be caspase-dependent, we treated cells with UV (Fig. 5*A*) or cycloheximide (Fig. 5*B*) in the presence or absence of pretreatment with cell-permeable inhibitors of caspase 1 (YVAD-CHO) or caspase 3 (DEVD-CHO). After either cycloheximide or UV treatment, the inhibition of caspase 3 decreased the degradation of HDAC4, whereas the inhibition of caspase 1 was ineffective. As additional controls, under identical experimental conditions, pretreatment with inhibitors of proteasome (MG132) or CRM1 (LMB) did not affect HDAC4 degradation, consistent with the results shown in Fig. 3.

Finally, to verify that the decrease in HDAC4 was not due to a global repression of protein synthesis due to the doses of UV used, we pulse-labeled cells with [$^{35}$S]methionine immediately after UV irradiation. After a brief pulse-chase, the cells were lysed, and autoradiography was performed on the total cell extracts. At doses of UV irradiation sufficient to result in the virtually complete degradation of HDAC4, little discernable difference in overall methionine incorporation could be detected (Supplemental Fig. 1).

*Identification of Potential PEST Sequences in the Unstable Region of HDAC4*—Having found that HDAC4 stability did not appear to be affected by proteasome inhibitors or inhibitors of CRM1 export, we investigated whether the stability of different portions of the HDAC4 might vary. We therefore assessed the respective stabilities of portions of HDAC4 fused to GFP to distinguish from endogenous protein (20). We selected constructs spanning the HDAC4, but lacking the C-terminal nuclear export sequence, to avoid the variable of potentially different nuclear export between constructs (Fig. 6). These constructs were expressed in cells, and protein levels were assessed serially after the addition of cycloheximide. We detected striking differences in protein stability between different regions of HDAC4. The extreme N-terminal fragment, spanning amino acids 1–208, was stable throughout the experiment, as was a C-terminal fragment containing a large portion of the deacetylase catalytic domain (amino acids 621–1040). In contrast, during the same time points after cycloheximide, fragments that include amino acids 1–326, 1–669, 1–1061, and 206–1040 underwent complete or near complete degradation. Amino acids common to these unstable constructs and not contained in the stable constructs therefore included residues from 209 to 574.

The stability of a number of proteins that undergo high turnover has been found to be mediated by proline-, glutamic acid-, serine-, and threonine-rich sequences within the protein (21). These so-called PEST sequences mediate the increased degradation of the protein through as yet not fully characterized mechanisms that do not always appear to require proteasome activity (22). We searched for the presence of PEST sequences within HDAC4 by utilizing a web-based algorithm maintained by EMBnet Austria (www.at.embnet.org/embnet/tools/bio/PESTfind/) with which we identified two potential PEST sequences (Fig. 5, *PEST 1* and *PEST 2*). Interestingly, the region that appears to mediate HDAC4 instability encompasses both of these PEST-containing regions.

*Potential Caspase Recognition/Cleavage Sites in HDAC4*—To begin to assess whether caspase inhibition stabilizes HDAC4 because the protein serves as a caspase substrate, rather than because of indirect effects mediated through other proteins, we searched the sequence of HDAC4 for potential caspase cleavage sites. We identified two potential caspase-recognition sites, 234–237 (DAKD) and 286–89 (DVTD) (Fig. 7). To assess whether these represent actual cleavage sites, we mutated the distal aspartic acid at either site to glutamic acid and expressed the GFP-fused mutants in HeLa cells. Similar to

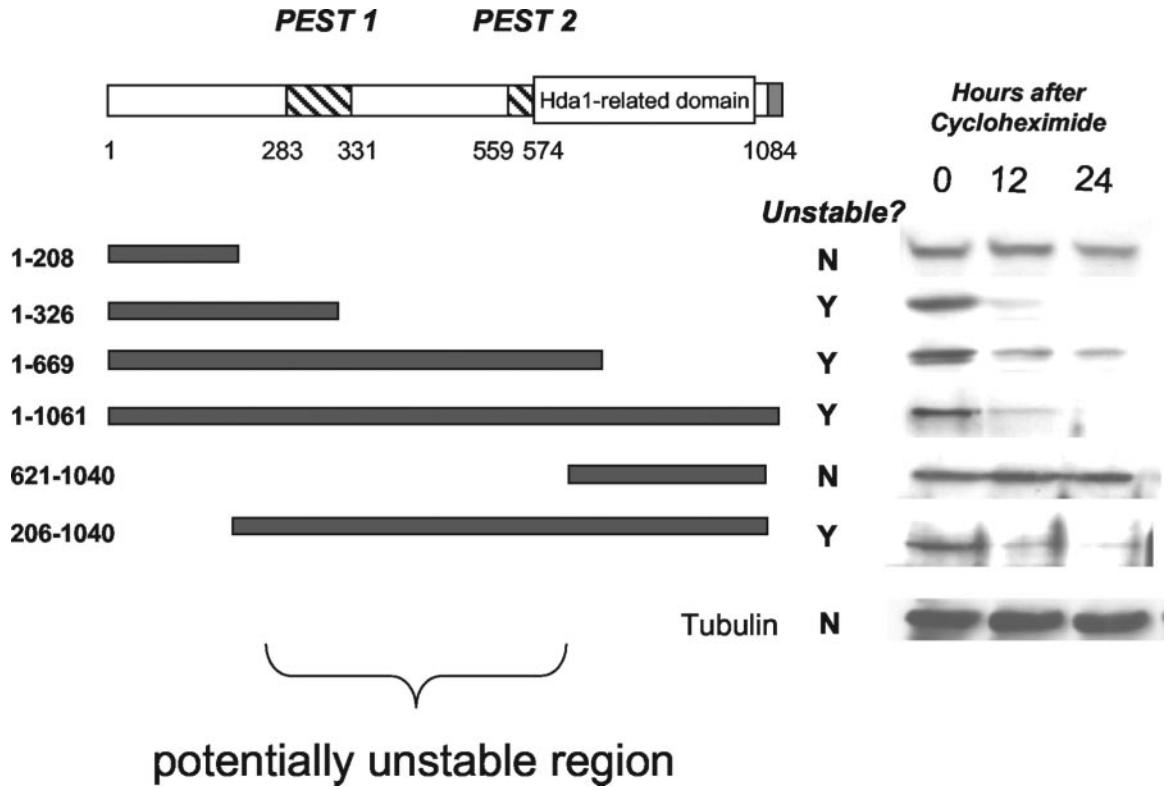


FIG. 6. **The stability of different portions of HDAC4 protein varies.** The indicated portion of HDAC4 was fused to GFP, and each construct was then expressed in cells followed by exposure to cycloheximide. Parallel samples were harvested at the times indicated, and total cell lysates were separated by SDS-PAGE and immunoblotted for GFP. A representative experimental sample probed for α-tubulin is shown as a control. Unstable constructs were defined as those undergoing complete or near complete (>90%) degradation by 12 h after cycloheximide. The common area of HDAC4 shared by unstable mutants is indicated. Potential PEST sequence regions within the protein are indicated in the *hatched boxes* of the protein, whereas the *shaded box* at the extreme C-terminal end of of HDAC4 indicates the nuclear export sequence (Wang and Yang (25)).

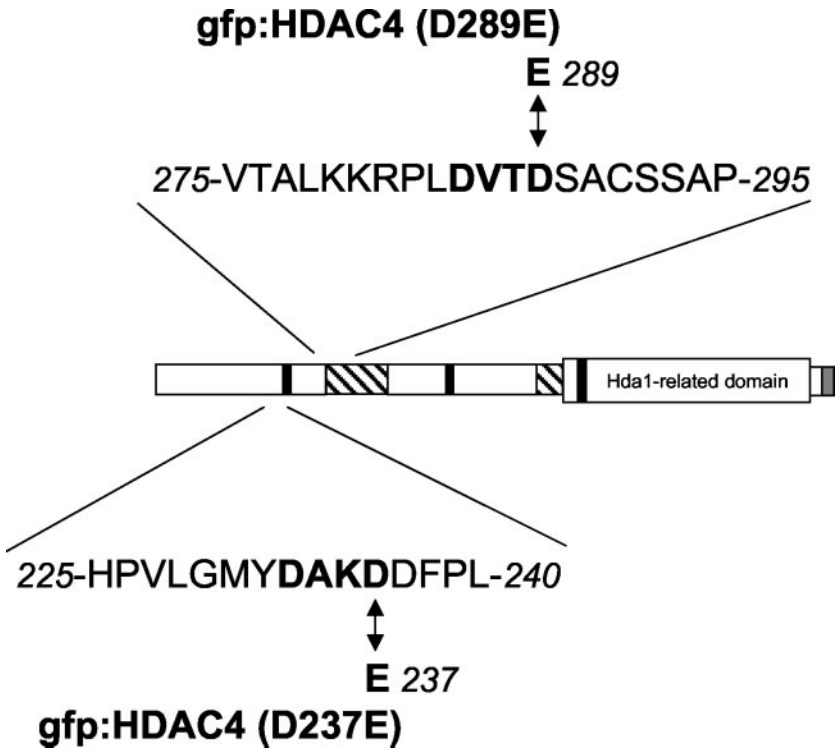


FIG. 7. **Identification of potential caspase cleavage sites in HDAC4.** Analysis of the amino acid sequence of HDAC4 identified two potential caspase-recognition/cleavage sites, as indicated. The second aspartic acid (Asp-237 and Asp-289) was mutated to glutamic acid, resulting in the point mutants D237E and D289E, which were expressed as GFP fusion proteins. The relationship of the potential caspase-cleavage sites to 14-3-3 binding sites (*vertical black bars*) and the PEST sequences (*hatched boxes*) is also shown.

the endogenous HDAC4, and consistent with the results shown in Fig. 5, the unmutated protein (gfp:HDAC4 1–1061) underwent rapid degradation in the presence of cycloheximide, as did the protein mutated at aspartic acid 237 (gfp:HDAC4 D237E). In contrast, the degradation of the protein mutated at aspartic acid 289 (gfp:HDAC4 D289E) was reduced (Fig. 8, *A* and *B*).

Binding of HDAC4 by the 14-3-3 family of proteins has been found to influence its cytoplasmic localization (23–26). Consist-

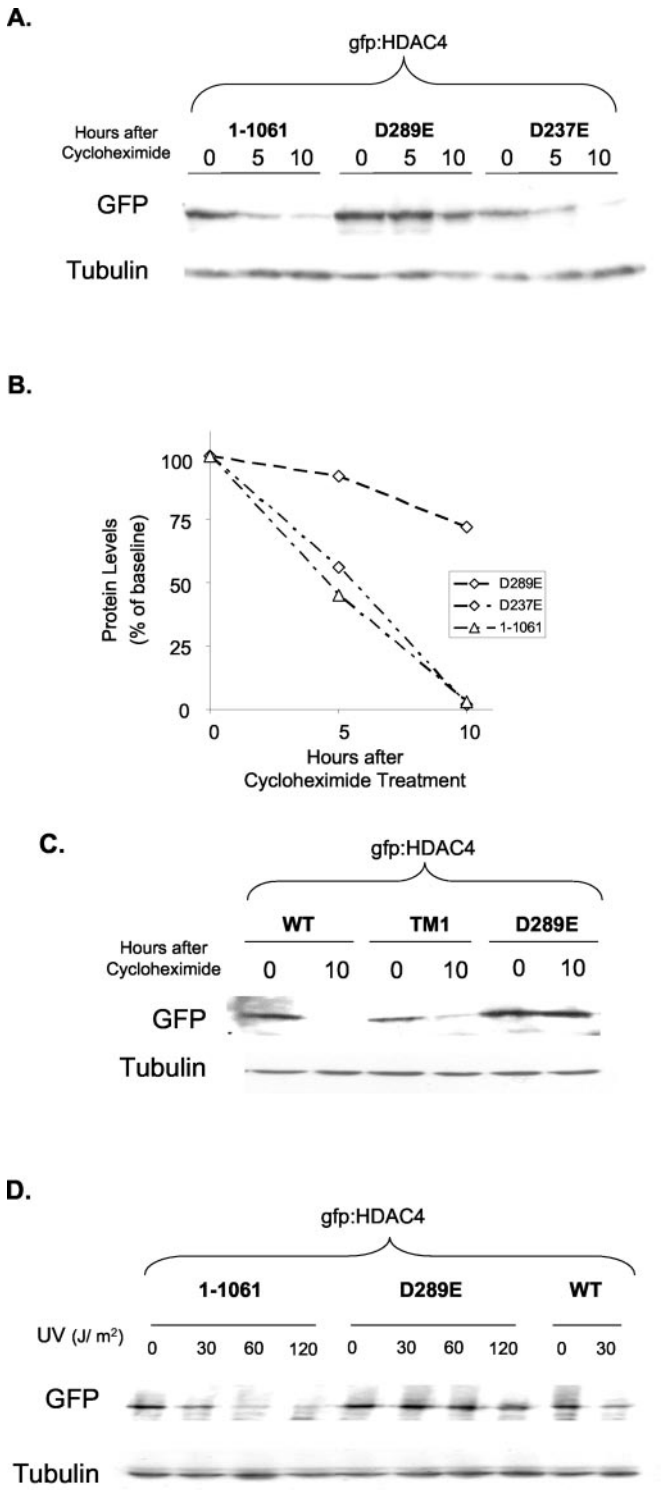


FIG. 8. **Targeted mutation of Asp-289 stabilizes HDAC4.** *A*, mutation of a potential caspase 3 cleavage site stabilizes HDAC4. HeLa cells expressing gfp:HDAC4 (1–1061), which lacks the C-terminal nuclear export sequence, or HDAC4 (1–1061) point mutants with either of the potential caspase cleavage sites (Fig. 7, gfp:HDAC4 (D289E) or (D237E)), were treated with cycloheximide and harvested at the indicated times after treatment. Total cell lysates were separated by SDS-PAGE and immunoblotted with anti-GFP and α-tubulin antibody. *B*, levels of the GFP-fused HDAC4 proteins in A were quantified via densitometry and plotted as shown. *C*, mutation of 14-3-3-binding sites does not impede HDAC4 degradation. Full-length HDAC4 (gfp:HDAC4 wild type (*WT*)), the full-length HDAC4 mutant with three 14-3-3 binding sites mutated (gfp:HDAC4 (TM1) (Ref. 25), and the HDAC4 point mutant in the caspase cleavage site of the 1–1061 background (gfp:HDAC4 (D289E)) were expressed as GFP fusion proteins in HeLa cells. These cells were treated with cycloheximide, harvested at the indicated times, and immunoblotted for with anti-GFP and α-tubulin antibody. *D*, the mutant D289E is resistant to degradation induced by UV. 1–1061, 1–1061 point mutant D289E, and wild type (full-length HDAC4) were expressed as GFP fusion protein in HeLa cells. These cells were then irradiated with UV at the indicated doses and harvested 10 h after the treatment. Total cell lysates were separated by SDS-PAGE and immunoblotted for detection of HDAC4 and α-tubulin.

ent with this notion, full-length HDAC4 triply mutated at the three 14-3-3 binding sites (Fig. 7, gfp:HDAC4 TM1) is no longer retained in the cytoplasm but becomes predominantly nuclear. However, both the full-length protein (gfp:HDAC4 1–1084) as well as the protein triply mutated at 14-3-3 consensus sites underwent similar degradation after cycloheximide, in contrast to the more stable D289E mutant (Fig. 8*C*). These results therefore suggest that binding to 14-3-3 is not a prerequisite of HDAC4 degradation. Furthermore, both full-length HDAC4 and HDAC4 lacking the nuclear export sequence readily underwent degradation in the presence of cycloheximide.

Finally, we assessed the stability after UV of full-length gfp:HDAC4 1–1084, the 1–1061 fragment, and the D289E mutant expressed in cells (Fig. 8*D*). Of these, only the D289E mutant was stable after UV. Together, these results strongly suggest that HDAC4 undergoes caspase 3-mediated cleavage at aspartic acid 289 after exposure to cycloheximide or UV irradiation and does not require interaction with the 14-3-3 family of proteins.

*Induced Expression of Caspase-cleavable HDAC4 Protein Leads to Increased Apoptosis*—An important question is whether HDAC4 is an active participant or an indirect terminal result of cellular death. After treatment with cycloheximide and UV, HDAC4 was rapidly degraded, and the disappearance of the full-length protein occurred long before changes in cell morphology and cell death were evident (Fig. 4 and Supplemental Fig. 2). Consequently, the time course of HDAC4 degradation suggests that HDAC4 cleavage is an early event that precedes clear evidence of cell death. To more rigorously address this issue, we transfected HeLa cells with cDNA encoding either cleavable or noncleavable HDAC4 protein under the control of a doxycycline-responsive promoter. Expression of cleavable HDAC4 resulted in a greater degree of cell death than either the noncleavable form or control cells (Fig. 9), suggesting that when overexpressed, the cleavage products of HDAC4 may contribute to the killing of cells. Interestingly, cleavage of HDAC4 at Asp-289 results in an N-terminal fragment that contains both the nuclear localization signal and the MEF2-binding region (25), suggesting that repression of transcription in the nucleus might be a possible mechanism preceding and contributing to cell death.

## DISCUSSION

We have shown herein that among the deacetylases tested, HDAC4 is unusually unstable. The instability is unmasked by inhibiting *de novo* protein synthesis, suggesting that there are mechanisms that readily degrade HDAC4 *in vivo*. The degradation of HDAC4 could be accelerated by ultraviolet irradiation and appeared to be mediated at least in part by the caspase 3 pathway. Induced expression of cleavable HDAC4 in turn led to increased cell death. These results together therefore link the regulation of the expression of HDAC4, and potentially other class IIa HDACs, to the caspase-based apoptotic machinery.

Why is HDAC4 so unstable? HDAC4 has been linked to the transcriptional repression, including its effects on the MEF2 family of transcription factors (27–30). HDAC4 binds directly to MEF2 and has been identified as a component of corepressor complexes (31–34). Consequently, the instability of HDAC4 may potentially enable cells to rapidly modulate gene expres-

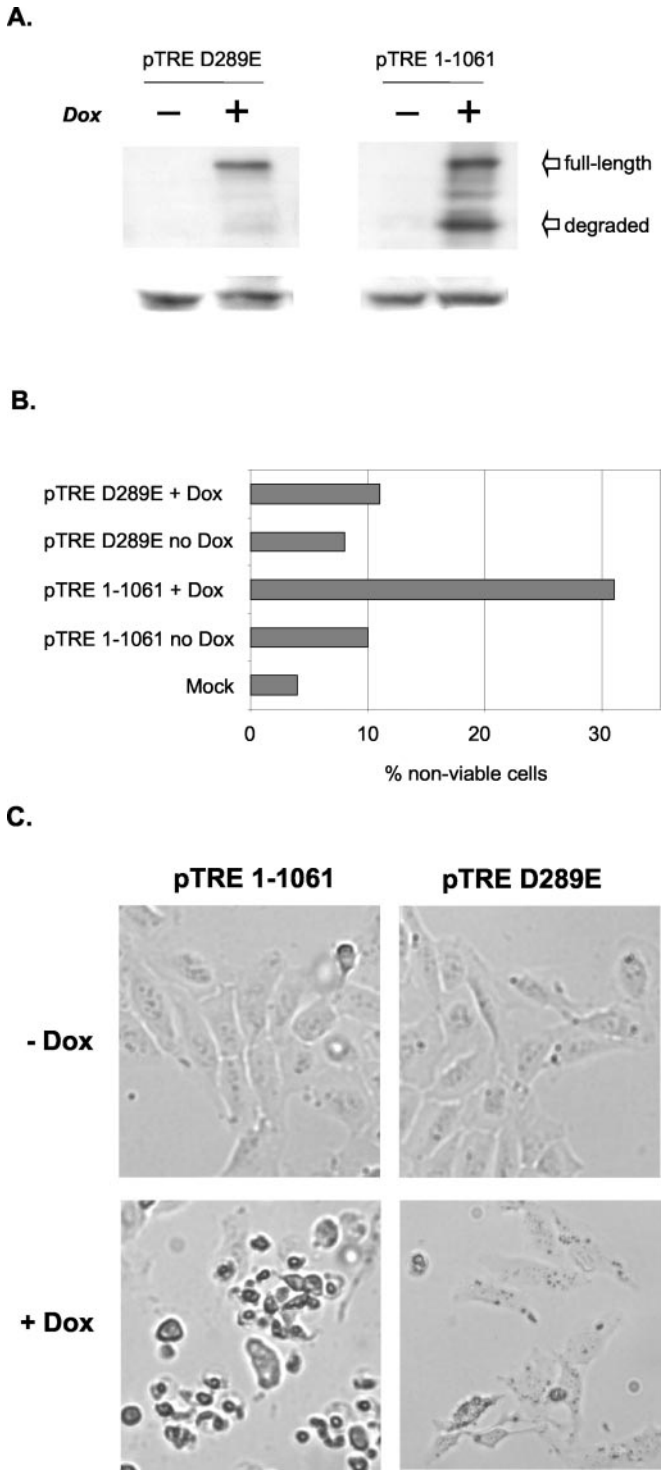


FIG. 9. **Induced expression of caspase-cleavable HDAC4 promotes cell death.** pTet-On™ HeLa cells were transfected with pTRE-TIGHT HDAC4 D289E or HDAC4 1–1061 plasmids, encoding respectively either for HDAC4 mutated at the caspase cleavage site (noncleavable) or for HDAC4 that remains caspase-cleavable. Six hours after transfection, doxycycline (*Dox*) or media was added to induce protein expression. Cells were harvested and assessed for protein (*A*) or cell viability (*B* and *C*) 24 h later as described under "Experimental Procedures." Doxycycline induced strong overexpression of HDAC4 protein. *A*, total cell lysates were probed for HDAC4 or tubulin. Endogenous HDAC4 protein is detectable in uninduced cells with longer film exposure times. Induced overexpression of HDAC4 leads to increased nonviable cells. *B*, nonviable cells were counted as a percentage of all cells and then plotted as showed. *C*, representative phase-contrast images of cells uninduced (− *Dox*) or induced (+ *Dox*) for noncleavable (*HDAC4 D289E*) or caspase-cleavable HDAC4 (*HDAC4 1–1061*).

sion or repression, such as in response to differentiation signals, or to environmental insults such as ultraviolet irradiation. The marked instability of HDAC4 mRNA may further ensure that HDAC4 expression is maximally inhibited under cytotoxic stress. Whereas UV-irradiation likely has diverse intracellular effects involving a multiplicity of pathways, the induction of protein degradation does not appear to be a common response. We are aware of only one other protein, BRCA2, which has been reported to undergo UV-induced degradation (35) prior to apoptosis. Interestingly, in contrast to HDAC4, cycloheximide actually *prevented* BRCA2 degradation after UV, whereas the inhibition of caspase activity had no effect on its degradation. The degradation of BRCA2 and HDAC4 after UV is therefore likely to be mediated by distinct mechanisms, but together may lead to unrepaired DNA damage, and if not reversed, may contribute to cell death.

The identification of caspase-3 as the likely protease involved in the degradation of HDAC4 is especially intriguing as this caspase has been implicated in the degradation of MEF2 family members (36, 37). The smaller cleavage products resulting from caspase-mediated cleavage of HDAC4 at Asp-289 would encompass the MEF2 binding site as well as THE nuclear localization signal (both located in the N-terminal portion of HDAC4) and consequently might retain or even augment the ability to repress MEF2 (41, 42). Whereas the mechanism(s) by which the degradation of MEF2 precipitates, or contributes to, apoptosis remains to be fully elucidated (38–40), cleavage of HDAC4 and MEF2 family members by caspase 3 may suggest that they are regulated by a common or overlapping pathway, thereby contributing to or determining cell death. In select contexts, in which MEF2 has alternatively been linked with induction of apoptosis (44), the activity of caspase 3 and resultant cleavage of HDAC4 would be of great interest. Finally, we note that caspase-mediated cleavage of full-length proteins to generate bioactive cleavage products that independently contribute to cell death has been previously described in a number of other systems (45, 46). In addition to cell death, caspases play important roles in regulating cell differentiation. For example, targeted deletion of the mouse caspase 3 gene impaired myotube/myofiber formation and expression of muscle-specific proteins (43). This is also consistent with the potential regulation of HDAC4 and MEF2 function during muscle differentiation by caspases.

This work raises additional questions that merit further investigation. It is interesting that although caspase 3 is considered to be localized to the cytoplasm (47, 48), mutation of the 14-3-3 binding sites that mediate cytoplasmic localization does not impede the degradation of full-length HDAC4 or even the degradation of portions of the protein that lack the cytoplasmic retention signal and that are primarily localized to the nucleus. It may be possible that small amounts of caspase 3 sufficient to cause HDAC4 degradation gain entry into the nucleus, especially after UV damage. Alternatively, the degradation of HDAC4 might be maximal during mitosis, when nuclear and cytoplasmic contents of the cell mix. The homology between the N-terminal domain of HDAC4 and that of HDAC5, -7, or -9 raises the question of whether the activities of these other class IIa HDACs may be influenced by HDAC4 cleavage. It also remains an open question whether caspase-mediated mechanisms are involved in regulating other class IIa HDACs. Although Asp-289 is not conserved in HDAC5, -7, and -9, a potential caspase site can be identified in HDAC9 (residues 211–214: DAKD), suggesting differential pathways of regulation. While this manuscript was under review, Paroni *et al.* (49) reported similar results about the caspase-dependent processing of HDAC4.

In conclusion, these results together have uncovered a novel regulatory mechanism for HDACs. These enzymes are molecular targets for pharmacologic compounds potentially of therapeutic value for human disease, so this novel regulatory mechanism is not only significant for understanding how HDACs are regulated *in vivo* but is also relevant toward improving the efficacy of such compounds.

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