ING3 Promotes UV-induced Apoptosis via Fas/Caspase-8 Pathway in Melanoma Cells*

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The novel ING tumor-suppressor family proteins (ING1–5) have been discovered during the past decade and are recognized as the regulators of transcription, cell cycle checkpoints, DNA repair, apoptosis, cellular senescence, angiogenesis, and nuclear phosphoinositide signaling. ING proteins contain a few conserved domains, including plant homeodomain motif, nuclear localization signal, and potential chromatin regulatory domain, suggesting that the ING family proteins may share common biological functions. ING3 has been shown to be induced in p53-mediated transcription, cell cycle control, and apoptosis, possibly by modulating the NuA4 complex histone acetyltransferase activity. Because ING1b and ING2 have been shown to be involved in cellular stress responses such as nucleotide excision repair and apoptosis after UV irradiation, we investigated whether ING3 also mediated UV-induced apoptosis. We found that ING3 expression was rapidly induced by UV irradiation at both mRNA and protein levels. Using the stable clones of melanoma cells overexpressing ING3, we showed that overexpression of ING3 significantly promoted UV-induced apoptosis. Unlike its homologues ING1b and ING2, ING3-increased apoptosis was independent of functional p53. Furthermore, ING3 did not affect the expression of mitochondrial proteins but increased the cleavage of Bid and caspases-8, -9, and -3. Moreover, ING3-mediated apoptosis was blocked by inhibition of caspase-8 or Fas activation. In addition, ING3 up-regulated Fas expression at both mRNA and protein levels. Knock down of ING3 decreased UV-induced apoptosis remarkably. These data indicate that ING3 plays an important role in cellular response to UV irradiation by enhancing UV-induced apoptosis through the activation of Fas/caspase-8 pathway.

Cutaneous malignant melanoma is a life-threatening skin cancer, and the incidence of melanoma has doubled in the last decade (1, 2). Melanoma metastasizes rapidly to other organs, and there is no effective treatment for metastatic melanoma. Even the most established adjuvant immunotherapy using interferon α has encountered the resistance by forming a recombinant trimeric histone acetyltransferase multisubunit complex, ING3 protein is associated with p53 function and can reconstitute robust nucleosomal histone acetyltransferase activity in vitro by forming a recombinant trimeric complex with Tip60 and EPC1 (24). However, unlike other ING genes (ING1, 2, 4, and 5), the ING3 gene locates far from the telomere terminus and is evolutionarily distinct from other family members (13). ING3 does not physically associate with p53, which binds to and is required for the functions of ING1b, ING4, and ING5 (25). However, the exact tumor-suppressive functions of ING3 protein remain to be clarified.

ING1b and ING2 are both DNA damage-inducible genes. ING1b is induced by UV irradiation (26, 27), whereas ING2 is induced by the DNA double strand break-inducing agents etoposide and nocarinos-

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2 The abbreviations used are: NuA4, nucleosome acetyltransferase of histone H4; PI, pro-podium iodide; siRNA, small interfering RNA; RT, reverse transcription; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; UVB, ultraviolet B.
Role of ING3 in UV-induced Apoptosis

In response to UV irradiation, both ING1b and ING2 can promote UV-induced apoptosis in a p53-dependent manner (26–28). They also enhance the p53-mediated repair of UV-damaged DNA (29, 30). However, the role of ING3 in cellular response to UV irradiation is not clear. In this study, we found that ING3 was rapidly induced by UV irradiation in melanoma cells. Overexpression of exogenous ING3 in melanoma cells can dramatically promote UV-induced apoptosis. Interestingly, unlike with its homologues ING1b and ING2, ING3-mediated apoptosis was independent of p53 function. We also demonstrated that ING3 induced Fas expression and promoted UV-induced apoptosis through the Fas/caspase-8 pathway.

EXPERIMENTAL PROCEDURES

Cell Culture and UV Irradiation—The MMRU and MMAN cell lines were kind gifts from Dr. R. Byers, Boston University. The Sk-mel-5 cell line was obtained from the Tissue Bank at NCI, National Institutes of Health. The MEWO, Sk-mel-3, and Sk-mel-24 cell lines were kind gifts from Dr. A. P. Albino (Memorial Sloan-Kettering Cancer Center). MMRU, MMAN, Sk-mel-5, and Sk-mel-24 cells harbor the wild-type p53 gene, but there is no detectable p53 protein in MMAN and the p53 protein in Sk-mel-5 is inactivated (31–34). The MEWO cells carry a mutated p53 gene (35), and the status of p53 in Sk-mel-3 is unknown. All melanoma cell lines were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen), supplemented with 10% fetal bovine serum, 200 U/ml of penicillin, 100 μg/ml of streptomycin, and 25 μg/ml of amphotericin B in a 5% CO2 atmosphere at 37 °C. Normal human epithelial melanocytes were purchased from Clonetics (Walkersville, MD) and fed in melanocyte growth medium (Clonetics) at 37 °C in a 5% CO2 atmosphere. The wild-type p53 and p53-null HCT116 colorectal cancer cells were obtained from American Type Culture Collection (ATCC) and Dr. B. Vogelstein, respectively, and maintained in McCoy’s 5A medium (Invitrogen) supplemented with 10% fetal bovine serum. For UV irradiation, cells were rinsed with PBS and exposed to ultraviolet B (UVB) at 300–320 nm as previously described (29).

siRNA Transfection—At 50–60% confluency, cells were transfected with siRNA using SiLentFect reagent (Bio-Rad) according to the manufacturer’s instruction. The Fas siRNA was purchased from Santa Cruz Biotechnology. The sequences of p53 siRNA (Qiagen) were 5'-GCAGGAACCGGAGGCCCAUdTdT-3' (sense) and 5'-UGGGCCUCGGGUUACAGCdTdT-3' (antisense). The sequences of ING3 siRNA (Ambion, Austin, TX) were 5'-GCUGUAUAAUGCGUGGAAUAAUU-3' (sense) and 5'-UAAUUCAGCCGUAUACUGCU-3' (antisense).

RT-PCR—Total RNA was prepared by TRizol extraction (Invitrogen) and reverse transcribed into cDNA with the SuperScript First-Strand Synthesis System (Invitrogen) according to the manufacturer’s protocol. Hotstart PCR system was performed with Taq DNA polymerase reaction system (Qiagen). The sequences of human ING3 primers were 5'-CAGGCTCTTCTAACTAACAGGC-3' (sense) and 5'-CTTCATCAAAACAGGAC-3' (antisense). The primers for human Fas were 5'-TCTAATGGGGTGCATTCTTC-3' (sense) and 5'-GTGCATACGCTTTTCTCC-3' (antisense). The primers for human glyceraldehyde-3-phosphate dehydrogenase were 5'-CTCATGACGATGTCATGTG-3' (sense) and 5'-CTGTCCACACCTCTTCATGTC-3' (antisense).

Western Blot—Cell pellets were lysed and extracted in 30 μl of triple detergent buffer (50 mm Tris–HCl (pH 8.0), 150 mm NaCl, 0.02% NaN3, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate) containing freshly added protease inhibitors (100 μg/ml of phenylmethylsulfonyl fluoride, 1 μg/ml of aprotinin, 1 μg/ml of leupeptin, 1 μg/ml of peptatin A). The protein concentration was determined by Bradford assay, and Western blot was performed as previously described (24). The primary antisera included antibodies for p53, Bad, Fas, Fas-L, Noxa, actin (Santa Cruz Biotechnology), Bax, Bcl-2, caspase-3, caspase-8, caspase-9 (Cell Signaling, Beverly, MA), Bid (BD Biosciences), ING3 (Abcam, Cambridge, MA), and PARP (Oncogene, Cambridge, MA). The protein expression levels were quantitated with the Quantity One software (Bio-Rad).

FACS Analysis—Cells were collected by trypsinization and pelleted by centrifugation at 500 x g for 5 min. Cell pellets were then suspended in 1 ml of hypotonic fluorochrome buffer (0.1% Triton X-100, 0.1% sodium citrate) containing 25 μg/ml of RNase A and 50 μg/ml of propidium iodide (PI) (Sigma). After being incubated at 4 °C in the dark for 1 h, samples were analyzed by EPICS XL-MCL flow cytometer (Beckman Coulter, Miami, FL) to determine the percentage of subdiploid DNA. Cells in sub-G1 phase were regarded as apoptotic cells. To quantify the cells in apoptosis from necrosis, cells were fixed and stained in Annexin-V-FLUOS staining kit (Roche Applied Science) according to the manufacturer’s protocol. Cells stained by both Annexin-V-FLUOS and PI were regarded as apoptotic.

Hoechst Staining—Cells were rinsed with PBS, fixed in 2% formaldehyde at room temperature for 20 min, and stained with 2.5 μg/ml of Hoechst 33342 (Sigma) in PBS for 5 min. The cells were then washed with PBS, mounted on the slide with glycerol–PBS (1:1), and visualized under a fluorescent microscope (Zeiss, Jena, Germany).

Cell Survival Assay—Cells in 24-well plates at 80% confluency were irradiated with UVB. Twenty-four hours after UV irradiation, cell survival was determined with the sulforhodamine B (S perme) assay as previously described (36). Briefly, after the medium was removed, cells were fixed with 500 μl of 10% trichloroacetic acid for 1 h at 4 °C. The cells were then washed with tap water, air dried, and stained with 500 μl of 0.4% sulforhodamine B (dissolved in 1% acetic acid) for 30 min at room temperature. The cells were destained with 1% acetic acid and air dried. For quantification, the cells were incubated with 500 μl of 10 mm Tris (pH 10.5) on a shaker for 20 min to dissolve the bound dye followed by colorimetric determination at 550 nm for 100-μl aliquots.

Measurement of Caspase-8 Activity—Cells in 100-mm dishes were treated with or without 600 J/m2 UVB and cultured for 5 h before being harvested. Caspase-8 activity was determined using ApoAlert caspase colorimetric assay kits (Clontech, Palo Alto, CA). The protease activity was determined by comparing the absorbance of p-nitroaniline (p-NA) in treated cells with that in untreated cells.

Statistical Analysis—The data were presented as the mean ± S.D. Statistical analyses were performed using Student’s t-test, and p value <0.05 was considered significant.

RESULTS

ING3 is DNA Damage Inducible—To test our hypothesis that ING3 may play an important role in the cellular response to UV irradiation, we analyzed ING3 expression level after UVB irradiation in melanoma cells. We found that the ING3 protein expression was rapidly induced in...
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ING3 Mediates UV-induced Apoptosis Independently of p53—Because both ING1b and ING2, the homologues of ING3, can promote UV-induced apoptosis in a p53-dependent manner (26, 27), we then investigated whether the increased apoptosis by ING3 also required the functional p53 protein. The p53 siRNA was used to knock down p53 expression in ING3 stable clone F5, which harbors the wild-type p53. Western blot analysis showed that the protein levels of p53 and its downstream target Bax were dramatically reduced in both MMRU and F5 cells after siRNA treatment (Fig. 4A, bottom panel). However, knock down of p53 in F5 cells did not cause a reduction of apoptosis after UVB irradiation (Fig. 4A, top panel), suggesting that ING3-mediated apoptosis is p53 independent. Similarly, ING3 overexpression did not have an effect on the expression of p53-targeting mitochondrial proteins Bax, Bcl-2, Bad, and Noxa (Fig. 4B), indicating that ING3 did not directly activate the mitochondrial apoptosis pathway. When HCT116 cells were employed, we found that ING3 promoted both basal and UV-
induced apoptosis in either wild-type p53 or p53-null HCT116 cells (Fig. 4C).

**ING3 Mediates UV-induced Apoptosis via Death Receptor Pathway**—Western blot analysis showed that more caspase-8 was cleaved in F5 cells upon UV irradiation, which activated caspase cascades through the cleavage of Bid (Fig. 5A). The colorimetric assay also suggested higher caspase-8 activity in F5 cells after UV irradiation (Fig. 5B). These data suggested that ING3 may mediate UV-induced apoptosis through the death receptor pathway. To confirm that caspase-8 activation was crucial for ING3-mediated apoptosis under UV stress, we transfected the MMRU and F5 cells with a dominant negative caspase-8 expression vector. Results showed that inhibition of caspase-8 completely abolished the ING3-mediated apoptosis (Fig. 5C). Furthermore, when the activation of caspase-8 was blocked, we did not observe a significant difference in caspase-9 activation between F5 and its parental MMRU cells in response to UV irradiation (Fig. 5D), confirming that caspase-9 was activated by caspase-8 via Bid cleavage in F5 cells.

The Fas death receptor is activated by binding to Fas ligands. Fas death receptors then recruit Fas-associated death domain protein to the plasma membrane, which in turn activates pro-caspase-8, leading to the activation of a cascade of caspasas (37, 38). Studies have shown that Fas death receptor pathway is activated by UV radiation and contributes to UV-induced apoptosis (39, 40). To confirm that Fas death receptor pathway is crucial for ING3-mediated apoptosis under UV stress, MMRU and F5 cells were preincubated at 4 °C for 30 min, which was shown to block the Fas aggregation (41). Our results showed that there was no significant difference in apoptosis rate between MMRU and F5 cells in response to UV irradiation (Fig. 5E). Meanwhile, when Fas expression was knocked down by Fas siRNA, we observed a significant decrease in the apoptosis of F5 cells after UV irradiation (Fig. 5F).

**ING3 Induces Fas Expression**—To investigate how ING3 activated Fas death receptor pathway, we examined whether ING3 could modulate Fas expression. Our data indicated that ING3 induced Fas protein expression at non-stress condition and after UVB irradiation (Fig. 6A). The highest Fas induction by ING3 was observed at 8 h after 600 J/m² UVB irradiation. Moreover, the induction of Fas by ING3 was at the transcriptional level (Fig. 6B). ING3 did not have effect on Fas ligand expression (Fig. 6A).
Knock Down of ING3 Decreases UV-induced Apoptosis—To determine the physiological role of ING3 in UV-induced apoptosis, we knocked down ING3 expression in MMRU cells by ING3 siRNA (Fig. 7A). FACS analysis showed that UV-induced apoptosis in MMRU cells was significantly reduced after ING3 knock down (Fig. 7B).

**DISCUSSION**

Like with its homologues ING1b and ING2, ING3 expression was rapidly induced after DNA damage. This induction by UV irradiation occurred much earlier than the p53 protein accumulation in melanoma cells (Fig. 1A), suggesting that this induction was independent of functional p53. The p53 independency of UV-induced ING3 expression was confirmed in melanoma cell lines with different p53 mutational status and in wild-type p53 and p53-deficient HCT116 cells (Fig. 1, C and D). Although UV can induce G1 or G2 cell cycle arrest (42, 43) and ING3 expression was rapid and transient and the ING3 expression returned to normal level 24 h after UV irradiation.

DNA damage caused by UV and other genotoxic stress activates checkpoint proteins ATM or ATR, which then modulate the cell fate in cell cycle arrest, DNA repair, or apoptosis through activating the downstream mediators, transducers, and effectors like p53, BRCA1, and Chk1/2 (44, 45). Considering that ING3 is a key subunit of the NuA4 histone acetyltransferase complex (24), the inducible ING3 may play an important role in chromatin remodeling in cellular response to UV irradiation. In this study, we for the first time showed that stable overexpression of ING3 can remarkably promote UV-induced apoptosis in melanoma cells. This increased apoptosis was independent of p53 function, although its homologues ING1b and ING2 both enhanced UV-induced apoptosis in p53-dependent manner (28, 29). Because it was reported that ING3 can mediate p53-dependent growth arrest and apoptosis in RKO cells (22), it is likely that ING3 can modulate apoptosis in both a p53-dependent and -independent manner, which may rely on the cell type. The ING family proteins, ING1b, ING2, ING4, and ING5, all induce growth arrest and apoptosis in a p53-dependent manner. Among these ING proteins, ING1b, ING4, and ING5 are physically associated with p53, whereas ING2 and ING3 are not (10). Furthermore, ING1b, ING2, ING4, and ING5 can also activate and stabilize p53 by stimulating the acetylation of p53 at residue Lys-382 and/or Lys-373 (11, 25, and 46). Although ING1b also induced apoptosis in a p53-independent manner, it was possibly through the homologues of p53, p63α and p73α (47). In this study, we did not detect any significant change of p53-responsive proteins, including Bax, Bcl-2, and Noxa, after overexpressing ING3, which further supports the notion that ING3-mediated apoptosis after UV irradiation is p53 independent. This discrepancy of ING3 function from other family members is probably because ING3 is evolutionarily distinct from other family members (13). Both ING1 and ING2 contain a small C-terminal conserved protein-interacting motif that binds a defined subset of peptides and, together with plant homeodomain, binds to phosphatidylinositol monophosphates (13, 48, and 49). ING1b also contains a unique N-terminal PCNA-interacting protein (PIP) domain, through which ING1b promotes UV-induced apoptosis (27). Moreover, there are two distinct insertions of 102 and 54 amino acids located between the potential chromatin regulatory and nuclear localization signals regions of ING3, respectively (13). Therefore, it will be interesting to clarify whether the structural differences between ING3 and ING1b or ING2 are responsible for the different pathways in UV-induced apoptosis.

UV-induced apoptosis can be triggered through both p53-mediated
mitochondrial pathway and death receptor pathways (50). In this study, both Western blot analysis and caspase-8 activity assay indicated that caspase-8 was more activated in ING3 stable clone F5 cells than in parental MMRU cells in response to UV irradiation (Fig. 5, A and B). Moreover, inhibition of caspase-8 activation blocked the cleavage of caspase-9 (Fig. 5D), confirming that ING3 does not directly affect the mitochondrial apoptosis pathway but operates through the cross-talk linked by cleaved Bid. The ING3-mediated apoptosis was abrogated when cells were transfected with dominant negative caspase-8 or preincubated at 4 °C (Fig. 5E) without Fas siRNA treatment significantly reduced ING3-mediated apoptosis (Fig. 5F) and ING3 stable clone F5 had higher Fas mRNA and protein levels in both basal and UV-irradiated conditions (Fig. 6, A and B), Fas death receptor pathways are likely involved in ING3-mediated apoptosis. However, because both the inhibition of caspase-8 function and the blockage of death receptor aggregation can inhibit not only the Fas pathway but all the other death receptor signaling, including tumor necrosis factor (TNF) receptor-1, TNFα-related apoptosis-inducing ligand (TRAIL) receptors, and death receptor-3 (DR-3) signaling (51), it remains possible that other death

FIGURE 5. Blockage of Fas/caspase-8 pathway activation abolished ING3-mediated apoptosis. A, MMRU and F5 cells were collected for Western blot analysis of caspase-8 and Bid 24 h after 600 J/m² UVB irradiation. Fl and Ad stand for floating and adherent cells, respectively. B, MMRU and F5 cells were harvested for the determination of caspase-8 activity using colorimetric assay 24 h after 600 J/m² UVB irradiation. The fold induction of caspase-8 activity was determined by comparing to MMRU cells. C and D, MMRU and F5 cells transfected with either pcDNA3 vector or dominant negative caspase-8 plasmids were irradiated with 600 J/m² UVB and harvested for PI staining and FACS analysis or Western blot analysis 24 h after UV irradiation. E, MMRU and F5 cells were preincubated at 4 °C for 30 min before exposure to 600 J/m² UVB. Twenty-four hours later, cells were harvested for PI staining and FACS analysis. F, MMRU and F5 cells were transfected with control or Fas siRNA for 24 h and then irradiated with 600 J/m² UVB. Cells were harvested for PI staining and FACS analysis 24 h after UV irradiation. The bottom panel shows Fas knockdown by Western blot analysis. Asterisks indicate statistical significance (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

FIGURE 6. ING3 induced Fas expression. MMRU and F5 cells were irradiated with 600 J/m² UVB and harvested at the indicated times for Western blot (A) or RT-PCR (B) analyses. Glyceraldehyde-3-phosphate dehydrogenase and actin were used as loading control for PCR and Western blot analysis, respectively.

FIGURE 7. Knock down of ING3 inhibited UV-induced apoptosis. MMRU cells were transfected with control or ING3 siRNA and harvested for Western blot analysis of ING3 expression (A) or irradiated with 600 J/m² UVB followed by PI staining and FACS analysis (B). Asterisks indicate statistical significance(**, p < 0.01).
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ING3 promotes UV-induced apoptosis in human epidermal cells (20). ING3 expression is induced by UV irradiation and is correlated with the induction of Fas expression (20). ING3 protein is mainly localized in the nucleus and can form a complex with Fas (20). ING3 regulates UV-induced apoptosis through modulating Fas expression (20).

ING3 is a member of the ING (INhibitor of Growth) family of proteins, which includes ING1, ING2, and ING3. ING3 has been shown to interact with Fas and to regulate Fas-mediated apoptosis (20).

ING3 is a nuclear protein and is mainly localized in the nucleus. ING3 interacts with Fas and regulates Fas-mediated apoptosis (20).

ING3 expression is induced by UV irradiation and is correlated with the induction of Fas expression (20).

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