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NM23-H2 involves in negative regulation of Diva and Bcl2L10 in apoptosis signaling

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Abstract

The Bcl-2 family members are evolutionally conserved and crucial regulators of apoptosis. Diva (Boo), an ortholog of Bcl2L10 or Bcl-B, is a member of the Bcl-2 family that has contradictory functions in apoptosis. To understand the signaling mechanisms of Diva, we searched for proteins that interact with Diva using the yeast two-hybrid system. We identified a nucleoside diphosphate kinase isoform, NM23-H2. Here, we show that Diva bound to NM23-H2 in cells in which the transmembrane domain of Diva was required, and both proteins were colocalized in cytoplasm. Of interest, Diva protein level was significantly down-regulated by NM23-H2 as knock down of NM23-H2 restored Diva expression. Overexpression of NM23-H2 induced apoptosis, and the depletion of NM23-H2 led to the increase of Diva's apoptotic activity. Thus, these results indicate the existence of a previously undiscovered mechanism by which NM23-H2 involves in the regulation of Diva-mediated apoptosis.

Keywords: Diva; Bcl2L10; Boo; Bcl-B; NM23-H2; NME; Apoptosis

Apoptosis, one type of programmed cell death, is critical for embryo development and for homeostasis of normal cells and plays an important role in the pathogenesis of a wide variety of diseases including cancer [1]. Evolutionally conserved members of the Bcl-2 family of proteins are central regulators of apoptosis from virus to human [2,3]. Mouse Diva (Boo) was initially cloned by two separate groups who reported contradictory observations; one group reported Diva was pro-apoptotic [4], while the other described it as an anti-apoptotic member of the Bcl-2 family [5]. In adult mouse, the expression of Diva is restricted to ovary and testis [4,5]. Diva forms a ternary complex with apoptosis-activating factor-1 (Apaf-1) and caspase-9, components of the apoptosome along with cytochrome *c* [5,6]. The closest human homolog of Diva was later identified and designated human Boo/Diva, Bcl2L10 or Bcl-B [7–9]. Bcl2L10 is also known to function as either a pro- or anti-apoptotic Bcl-2 family member, but is present in a wide variety of human tissues [7,8]. Because of their low sequence homology, differential expression patterns, and disparate binding partners of Diva and Bcl2L10, it has been disputed whether Bcl2L10 is the human ortholog of mouse Diva.

Using the yeast two-hybrid screening system, we identified a novel Diva-binding protein, NM23-H2 (Non Metastatic cells H2), also known as NME2, NM23B and NDKB, which is an isoform of multifunctional proteins involved in a variety of cellular activities including proliferation, development, adhesion, and differentiation

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[10]. NM23 proteins, also known as nucleoside diphosphate kinases (NDPK), are a family of highly conserved proteins found in eukaryotes [11] and are implicated in tumorigenesis as suppressors of tumor metastasis [12] and transcriptional activators of the oncogene, *c-myc* [13]. However, only limited studies that investigate the role of NM23 proteins in apoptosis signaling are available, and these describe inconsistent effects.

In the present study, we describe NM23-H2 as a specific binding protein of Diva and Bcl2L10, identified a new biological function for NM23-H2 in apoptosis, and discussed the implication of NM23-H2 protein in the regulation of Diva and Bcl2L10.

Materials and methods

Plasmid construction. The full-length human NM23-H2 (BC002476) sequence was amplified using PCR. The primers used were human-NM23-H2-F (5′-CTAGGATCCTATGGCCAACCTGGAGCGC) and human-NM23-H2-F (5′-CTACTCGAGAATTCTTATTCATAGACCCAGTC). The PCR product was cloned into hemagglutinin (HA) epitope-tagged pcDNA3 (Invitrogen, Carlsbad, CA). DNA fragment encoding rat NM23-H2 was PCR amplified using primers, rat-NM23-H2-F (5′-ACGACT AGTGGATCCCATGGCCAACCTCGAGCGT) and rat-NM23-H2-R (5′-CTACTCGAGAATTCC-TACTCATACACCCAGTC). Mouse pcDNA3 Diva was kindly provided by Dr. Nunez (Department of Pathology and Comprehensive Cancer Center, The University of Michigan Medical School, Ann Arbor, MI). The open-reading frame of Bcl2L10 (Accession No.: BC104442) was PCR amplified using primers, Bcl2L10-F (5′-CTAGGATCCAATGGTTGACCAGTTGCGG) and Bcl2L10-R (5′-CTAGAATTCTCATAATAATCGT-GTCCA). N-terminus Flag-tagged Bcl2L10 was amplified using PCR. The primers used were Bcl2L10-R and Flag-Bcl2L10-F (5′-CTAGGATCCATGGACTACAAAGACGACGAC GACAAAGTTGACCAGTTGCGGGG). C-terminus-truncated Flag-Bcl2L10 mutants, Bcl2L10-%ΔTM and Bcl2L10-ΔTM/BH2, which lack either the transmembrane (TM) domain or TM and BH2 domains, respectively, were amplified by PCR using primers: Flag-Bcl2L10-F and Bcl2L10-ΔTM-R (5′-CTACTGCAGCTCGAGTCATGGAAAGGGGG TCCTGAA) for Bcl2L10-ΔTM and Flag-Bcl2L10-F and Bcl2L10-ΔTM/BH2-R (5′-CTACTGCAGCTCGAG-TCACTCCAGCAGCGTCCCTG C) for Bcl2L10-ΔTM/BH2. The BH3 domain mutant of Bcl2L10, in which seven amino acids were changed to alanine, was constructed by PCR. The primers used were BH3-1 (5′-CTAGGATCCATGGACTACAAAGAC GACGACGACAAAGTTGACCAGTTGCGGGAG), BH3-2 (5′-GGG CGTGGATGGCGCCGGCTC), BH3-3 (5′-GAGCCGGCGCCATC CACGCCCGCAGCAGCAGCAGCAGCAGCGGCCGCCAGGT TACGG), and BH3-4 (5′-CTAGAATTCTCATAATAATC-GTGT CCA).

Yeast two-hybrid screening. The open reading frame of mouse Diva cDNA was fused in frame with the GAL4-binding domain (BD) in the pGBT9 yeast shuttle vector (Clontech, Palo Alto, CA). This plasmid was used to screen 1.5 million transformants harboring GAL4-activation domain (AD)-tagged rat ovarian fusion cDNA library to identify Diva-interacting proteins. Yeast cells were sequentially transformed with pGBT9 Diva and cDNAs from the ovarian library by the lithium acetate method, and colonies were selected on tryptophan-, leucine-, histidine-deficient plates that contained 30 mM 3-amino-1,2,4-triazole (Sigma, St. Louis, MO). Plasmids were isolated from positive clones, transformed into KC18 *Escherichia coli* cells (Clontech) for DNA sequencing.

Mammalian cell culture. SK-OV3 (ATCC, Manassas, VA), were cultured in RPMI 1640 (Gibco, Carlsbad, CA), and 293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco). All media contained 10% heat-inactivated Fetal Bovine Serum (FBS) (Gibco) and 1% penicillin–streptomycin (Gibco).

Cell viability assay. SK-OV3 cells were seeded at a density of 2.5×10^4 per well in 48-well plates. Twenty-four hours later, cells were transfected with a total of 100 ng DNA, including 10 ng of pEGFP-N1 (Clontech) for detecting transfected cells, using Lipofectamine 2000 (Invitrogen). 293T cells (1×10^5) were plated in 48-well plates and transfected with a total of 100 ng DNA. For each well, the same amount of total DNA was transfected. At 24 h post-transfection, GFP-positive cells were counted under the fluorescence microscope after adding Trypan blue (Sigma). Data were expressed as the percentage (mean ± SD) of viable cells compared with the control group that received empty vector.

Flow cytometry analysis of Annexin V-positive cells. HeLa cells (1.0×10^6) were cultured in 6-cm culture dishes for 24 h, transfected with total of 4 μg of various pcDNA3 plasmid constructs, harvested with 0.5 mM EDTA (Sigma). Binding buffer (10 mM Hepes at pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) was added to the cells, and an aliquot was transferred to a new tube containing FITC Annexin V (BD Pharmingen, San Diego, CA). Propidium iodide (PI; BD Pharmingen) was added to the cells before starting FACS (BD, Franklin Lakes, NJ) analysis.

Establishment of Nm23-H2 knock down cell line. Short hairpin RNA (shRNA) sequences of human NM23-H2 were obtained from RNAi Codex (http://codex.cshl.edu/). The upper and lower strand oligomers (5′-GATCCCCCGCCTCGTGGCCATGAAGTTTCAAGAGAACTTCAT GGCCACGAGGCGTTTTTGGAAA and 5′-AGCTTTTCCAAAAA CGCC TCGTGGCCATGCAGTTTCAAGAGAACTTCATGGCCACG AGGCGGGG, respectively) were annealed and cloned into pSuper puro shRNA according to the manufacturer's instructions (Oligoengine, Seattle, WA). The 293T cells were plated at a density of 1.5×10^6 cells per 60-mm dish and transfected with 4 μg of pSuper puro hNM23-H2 plasmid using Lipofectamine 2000. At 24 h post-transfection, the transfected cells were cultured in DMEM containing 3 μg/ml of puromycin (Sigma). Knock down of hNM23-H2 was confirmed by Western blotting with anti-NM23 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunoprecipitation and Western blot analysis. 293T cells (1.3×10^6) were plated onto 6-cm culture dishes. After 24 h, the cells were transfected with 4 μg of plasmid DNA using Lipofectamine 2000. Cell lysates were prepared with TNE buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl at pH 8.0, 1% NP-40) in the presence of 10% proteinase inhibitor cocktail (Sigma). For quantitative protein analysis, a standard curve was established with BSA solution (Pierce, Rockford, IL), and equal amounts of protein were loaded on SDS–PAGE gels. Western blot analysis was performed as described previously [14]. Flag-tagged Bcl2L10 and its mutant proteins were detected with anti-Flag M2 monoclonal antibody (Sigma). Diva protein was detected with anti-Diva polyclonal antibodies (Santa Cruz Biotechnology), and NM23-H2 proteins were detected with anti-NM23-H2 polyclonal and anti-HA monoclonal antibodies (Sigma). Membranes were incubated with horseradish peroxidase-conjugated anti-rabbit, anti-mouse, or anti-goat IgG secondary antibodies before visualization by enhanced chemiluminescence (Welgene, Seoul, Korea), and proteins were detected by the LAS image program (Fuji, New York, NY). Immunoprecipitation experiments were performed as previously described [14].

Immunofluorescence analysis. HeLa cells were seeded at a density of 2.5×10^4 cells per well in 24-well plates. After 24 h, cells were transfected with 500 ng of plasmids. Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde (Sigma), permeabilized with 0.2% Triton X-100 (Sigma), and incubated with blocking buffer (PBS containing 2% FBS and 0.01% NaN_3). Cells were then incubated with antibodies in 0.1% Tween-20 in PBS. To detect Flag-Bcl2L10 and NM23-H2, anti-Flag monoclonal and anti-NM23-H2 polyclonal antibodies were used, respectively. Cells were incubated with secondary antibodies, Alexa Fluor 555 goat anti-mouse IgG or 488 goat anti-rabbit IgG (Invitrogen). Fluorescence was detected by confocal microscopy (Bio-Rad, Hercules, CA).

Results

Both Diva and Bcl2L10 interact with endogenous NM23-H2

To confirm *in vivo* interaction of Diva and NM23-H2 in mammalian cells, we performed immunoprecipitation experiments. As shown in Fig. 1A, both endogenous and overexpressed NM23-H2 proteins were co-immunoprecipitated by antibodies to Diva. Diva was also efficiently co-immunoprecipitated with endogenously and ectopically expressed NM23-H2 proteins pulled down by anti-NM23-H2 antibodies (Fig. 1B). Endogenous Diva protein was not detectable with the anti-Diva antibodies.

To further test whether this association also occurs between human Diva (Bcl2L10) and human NM23-H2, 293T cells were transfected with constructs encoding

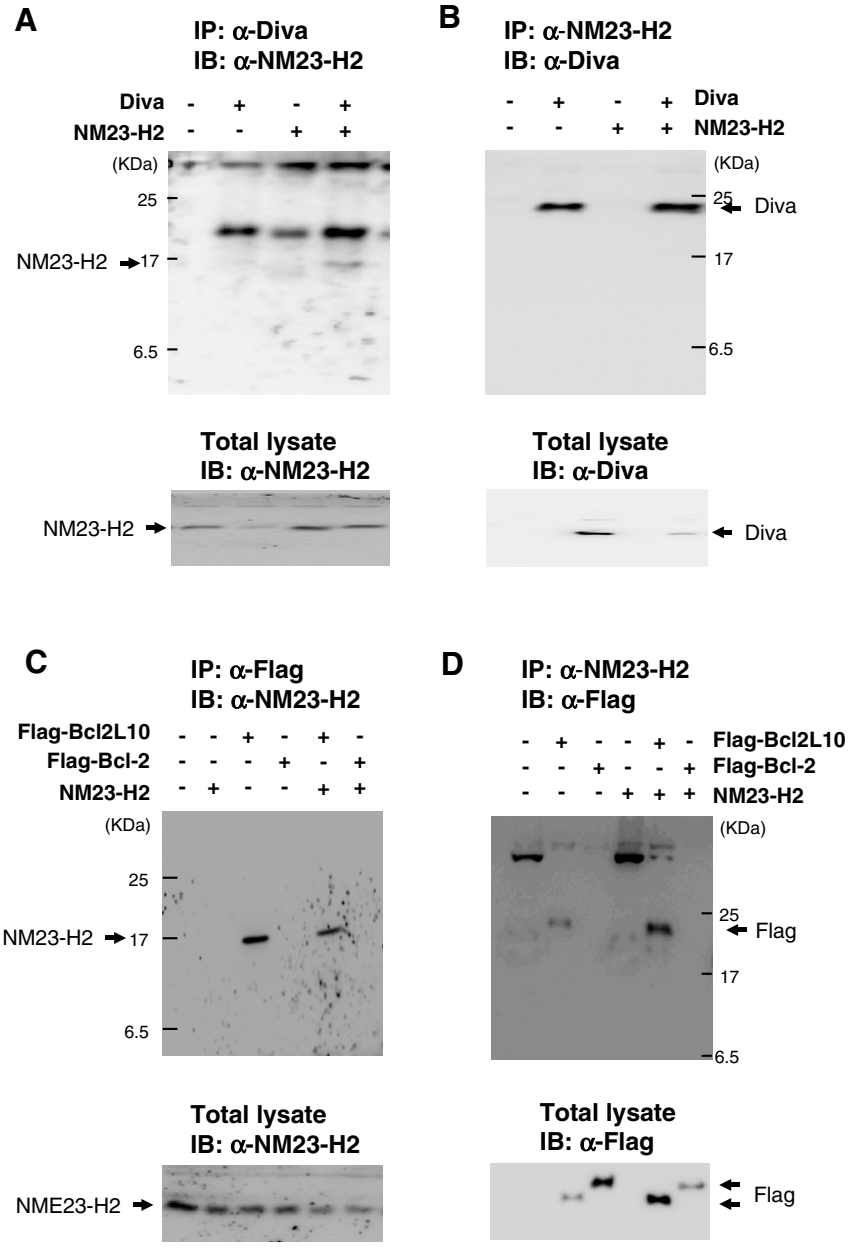

Fig. 1. Diva and Bcl-2L10 interact with endogenous and overexpressed NM23-H2 *in vivo*. (A) 293T cells were transfected with expression plasmids for Diva, rat NM23-H2 or both; control cells received empty vector. The cell lysates were incubated with anti-Diva antibodies, and the immunoprecipitates were immunoblotted with anti-NM23-H2 antibodies. Aliquots of total cell lysates were immunoblotted with anti-NM23-H2 antibodies. (B) The same lysates prepared in (A) were immunoprecipitated with anti-NM23-H2 antibodies and detected using anti-Diva antibodies. Both endogenous and overexpressed NM23-H2 protein efficiently bound Diva in 293T cells. (C) The cells were transfected with DNA construct encoding Flag-tagged Bcl2L10, Bcl-2 or human NM23-H2. Cell lysates were immunoprecipitated with anti-Flag M2 antibody. Protein interactions were determined by immunoblotting with anti-NM23-H2 antibodies. Bcl2L10 pulled down endogenous and overexpressed NM23-H2, whereas Bcl-2 did not. (D) Portions of the same lysates used in (C) were immunoprecipitated with anti-NM23-H2 antibodies, and the immunoprecipitates were detected with M2 antibody against the Flag epitope. Both endogenous and overexpressed NM23-H2 protein efficiently bound Diva in 293T cells.

Flag-Bcl2L10, Flag-Bcl-2, and/or NM23-H2. Both endogenous and overexpressed NM23-H2 was detected in a complex with Flag-Bcl2L10, as determined by immunoprecipitation with M2 antibody against the Flag-epitope and Western blotting with anti-NM23-H2 antibodies (Fig. 1C). Conversely, immunoprecipitation of the same lysates with anti-NM23-H2 antibodies pulled down Flag-Bcl2L10 (Fig. 1D). In contrast, Flag-Bcl-2 failed to co-precipitate with NM23-H2 (Fig. 1C and D), suggesting that interaction with NM23-H2 is a specific biological event for Diva and Bcl2L10 among the Bcl-2 family members. These results provide direct evidence that NM23-H2 is a binding partner of both Diva and Bcl2L10 *in vivo*.

Transmembrane domain of Bcl2L10 is required for its binding to NM23-H2

To determine the domain of Bcl2L10 responsible for its interaction with NM23-H2 proteins, Bcl2L10 mutants were generated, and their ability of binding to NM23-H2 was tested by immunoprecipitation. Bcl2L10 is comprised of BH4, 3, 1, 2, and TM domains as shown in Fig. 2A. Bcl2L10 mutant proteins that lack either the TM domain (Bcl2L10 ΔTM) or the BH2 along with the TM domain (Bcl2L10 ΔTM/BH2) failed to immunoprecipitate with NM23-H2, whereas full-length Bcl2L10 was efficiently pulled down with anti-NM23-H2 antibodies (Fig. 2A), suggesting that the TM domain of Bcl2L10 is required for its association with NM23-H2 protein. We were unable to detect expression of Bcl2L10 BH3 mutant from total cell lysate.

Both NM23-H2 and Bcl2L10 co-localize in the cytoplasm

To investigate the intracellular distribution of NM23-H2 and Bcl2L10, HeLa cells were overexpressed with respective plasmids and immunostained using anti-NM23-H2 and anti-Flag antibodies. Confocal microscopic analyses showed that Bcl2L10 and NM23-H2 proteins were mainly detected in the cytoplasmic region and their merge image confirmed their co-localization (Fig. 2B). When both proteins were co-expressed in cells, they showed a distinct punctuate pattern which possibly implies their association in the intracellular organelle(s). In addition, the Bcl2L10 mutant (Bcl2L10 ΔTM), which was devoid of TM and did not interact with NM23-H2, was overexpressed with NM23-H2. In contrast to the full-length Bcl2L10 protein, Bcl2L10 ΔTM displayed rather dispersed expression pattern throughout the cells (Fig. 2C), suggesting the TM is important for its proper intracellular membrane targeting. Furthermore, when the mutant Bcl2L10 (ΔTM) was expressed, NM23-H2 was detected in both nucleus and cytoplasm, as others reported the localizations after NM23-H2 expression [15]. Thus, our immunostaining data suggest that NM23-H2 and Bcl2L10 co-localize in the cytoplasmic region through their physical interaction.

NM23-H2 down-regulates Bcl2L10 expression

Transfection of increasing concentrations of Diva expression plasmid augmented the expression of Diva protein as determined by immunoblotting with anti-Diva antibodies (Supplementary Fig. 1A). However, when cells were co-transfected with NM23-H2 along with Diva, cells expressed significantly lower levels of Diva compared to cells that were not overexpressing NM23-H2 (Supplementary Fig. 1A; upper panel, lanes 2–6 vs. 8–12). Furthermore, we confirmed that the down-regulation of Diva protein is mediated by NM23-H2 through the use of NM23-H2 knock down cells. Silencing of NM23-H2 was evident as shown by Western blot analysis in Supplementary Fig. 1A (middle panel, lanes 13–18). When Diva was overexpressed in the NM23-H2 knock-downed cells, the expression levels of Diva were not decreased but unaltered (Supplementary Fig. 1A; upper panel, lanes 14–18 vs. 2–6 and 8–12), indicating that NM23-H2 is responsible for down-regulating Diva protein levels in the cells.

Because we also observed a tendency for the down-regulation of NM23-H2 when Diva is present, the effects of Bcl2L10 on NM23-H2 protein levels were further investigated. Transfection of increasing amounts of HA-tagged NM23-H2 yielded higher expression of NM23-H2 as evident by Western blot analysis using anti-HA antibody (Supplementary Fig. 1B; upper panel, lanes 2–5). However, in the presence of Bcl2L10, NM23-H2 protein levels were attenuated (Supplementary Fig. 1B; upper panel, lanes 2–5 vs. 7–10), and increasing expression of Diva also decreased the levels of both endogenous and overexpressed NM23-H2 (Supplementary Fig. 1A; middle panel, lanes 1–12). Together, these data suggest that Diva (Bcl2L10) and NM23-H2 reciprocally affect each other's protein level; however, the inhibitory effect of NM23-H2 on Diva was more prominent.

Diva, Bcl2L10, and NM23-H2 induce apoptosis, and NM23-H2 silencing increases Diva-induced apoptosis

To assess the biological role of Bcl2L10 and NM23-H2 in apoptosis, we performed cell viability assays and FACS analysis. Transfection of increasing amounts Diva or Bcl2L10 expression plasmids into SK-OV3 cells induced concentration-dependent cell death with similar trends (Supplementary Fig. 2A and B). Overexpression of rat or human NM23-H2 protein also resulted in the reduced cell viability, but to a lesser extent than Diva or Bcl2L10 (Supplementary Fig. 2A and B).

To further analyze Bcl2L10- and NM23-H2-mediated cell death activities, transfected HeLa cells were stained with Annexin V and PI and analyzed by flow cytometry. Overexpression of either Bcl2L10 or NM23-H2 increased the Annexin V-positive and PI-negative cell population (Supplementary Fig. 2C), confirming that the Bcl2L10- and NM23-H2-mediated cell death events indeed involve apoptotic cell death. Coexpression of Bcl2L10 and

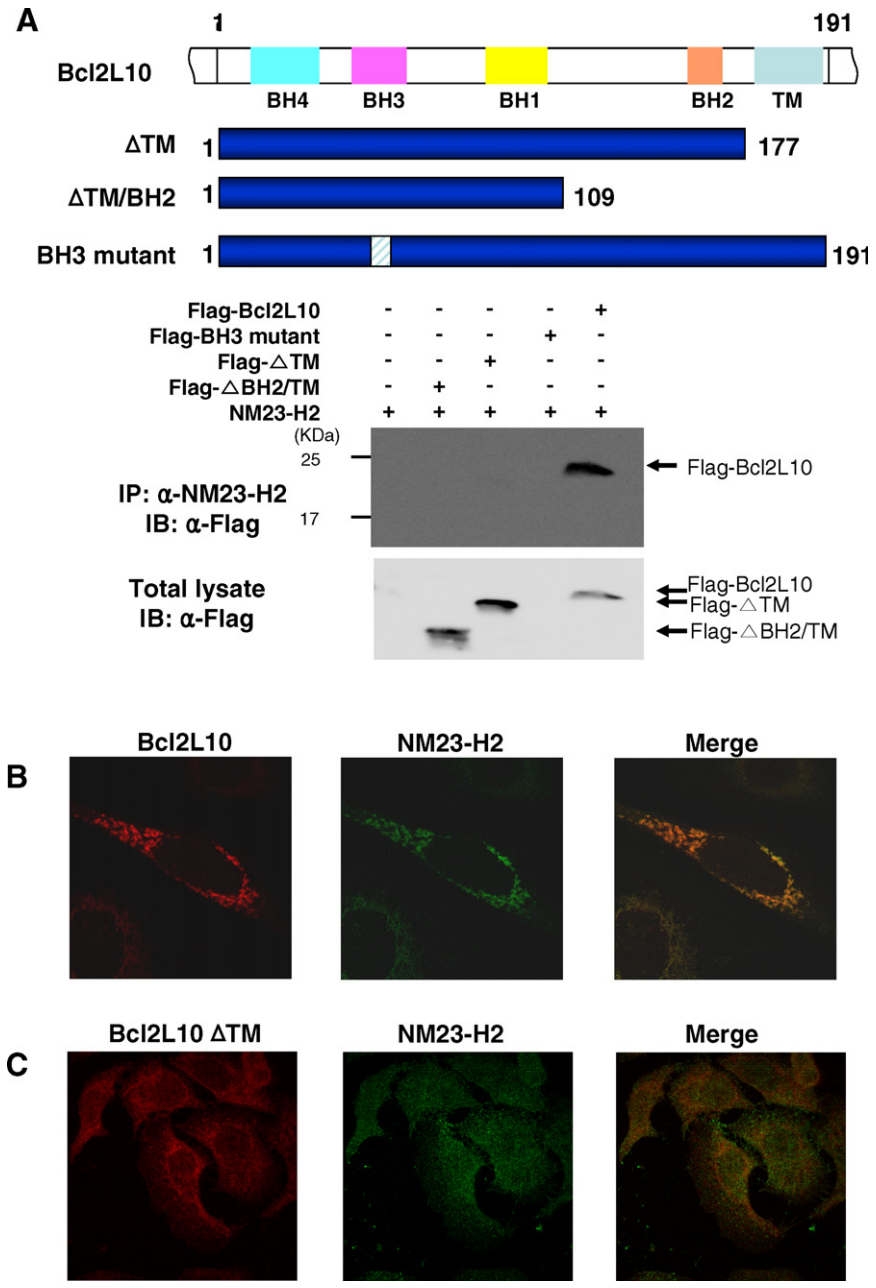

Fig. 2. Bcl2L10 and NM23-H2 co-localize involving transmembrane domain of Bcl2L10. (A) 293T cells were transfected with plasmids expressing Bcl2L10 or its mutants (Bcl2L10 ΔTM and ΔTM/BH2) together with DNA encoding hNM23-H2. Cell lysates were prepared and incubated with anti-NM23-H2 antibodies. Immunoprecipitates were Western blotted using M2 antibody. Total cell lysates were immunoblotted with M2 antibody to determine expression of Flag-tagged Bcl2L10 and its mutants. (B) Co-localization of Flag-tagged Bcl2L10 and NM23-H2 were tested in HeLa cells transfected with plasmids encoding Bcl2L10 or NM23-H2. The cells were stained with M2 and anti-NM23-H2 antibodies. (C) Bcl2L10 mutant (Bcl2L10 ΔTM) that lacks interaction with NM23-H2 was overexpressed, and the localization of NM23-H2 was determined as in (B).

NM23-H2 reduced Annexin V-positive cell population compared to expression of either Bcl2L10 or NM23-H2 alone (Supplementary Fig. 2C).

Next, to investigate the functional consequences of the association between Bcl2L10 and NM23-H2, cell viability assays were performed in 293T cells depleted for NM23-H2. NM23-H2 knock-downed 293T cells expressed little NM23-H2 as determined by Western blotting with anti-NM23-H2 antibodies (Supplementary Fig. 2D). Transfection of increasing amounts of plasmid encoding Bcl2L10 into 293T cells induced cell death as in SK-OV-3 and HeLa cells, and this proapoptotic effect was further increased in the cells deficient of NM23-H2 (Supplementary Fig. 2D).

Discussion

We observed that Diva/Bcl2L10 functions as a pro-apoptotic protein in human cell lines, including SK-OV3, 293T, and HeLa cells, and identified NM23-H2, a multi-

functional protein, as an interacting protein of Diva and Bcl2L10.

Evidence from the current study suggests that Bcl2L10 is the human ortholog of Diva as both Bcl2L10 and Diva interacted with endogenous NM23-H2 protein but Bcl-2 did not. Similar to mouse Diva, human Bcl2L10 has also been reported to behave as a pro- and anti-apoptotic protein [7–9]. In the present study, we observed that Bcl2L10 promotes cell death with comparable potency to Diva in various cells. Therefore, the roles of Diva and Bcl2L10 seem depend on the cellular context and conditions.

NDPK family proteins are becoming recognized as multifunctional regulatory proteins rather than as simple kinases that transfer a γ-phosphate from nucleoside triphosphate to diphosphate as initially described [10]. Although the NM23 family proteins were known to be involved in cellular proliferation, apoptosis, differentiation, and invasion, the underlying mechanisms by which NM23 proteins function in various physiological and pathological conditions are still unclear [16]. We demonstrated that NM23-H2 induces apoptosis as assessed by cell viability assays and Annexin-V binding (Supplementary Fig. 2). In addition, this study provides an evidence for a direct mechanistic link of NM23-H2 protein in apoptosis signaling, as NM23-H2 binds to and involves in the regulation of Diva, a member of an essential family that controls the fate of cell survival and death.

NM23-H2 protein was found in both nuclear and cytoplasmic regions in the presence of the mutant Diva devoid of physical interaction with NM23-H2 (Fig. 2B and C). Interestingly, we observed that, upon Diva overexpression, NM23-H2 largely localized in the cytoplasm where it co-localized with Diva in a particulate form. Therefore, regulatory effects of NM23-H2 on Diva likely take place in the cytoplasm rather than in the nucleus. Diva resides in the membranes of intracellular organelles [5] and can translocates from endoplasmic reticulum (ER) to mitochondria upon apoptotic stimulus [8]. Thus, our results confirm that Diva and NM23-H2 co-localize in the cytoplasm, which implies that the protective role of NM23-H2 in Diva-induced cell death is not likely a consequence of a NM23-H2 function that takes place in the nucleus.

Although overexpression of either Bcl2L10/Diva or NM23-H2 alone induced apoptosis, coexpression of Bcl2L10 with NM23-H2 did not further increase cell death, but rather attenuated the apoptotic activities of each (Supplementary Fig. 2). In addition, Bcl2L10-induced cell death effect was greater in NM23-H2 knock-downed cells (Supplementary Fig. 2D). Interestingly, immunoblot analysis showed that coexpression of NM23-H2 resulted in significantly lower levels of Diva, whereas NM23-H2 knock down completely restored Diva expression (Supplementary Fig. 1). Therefore, the modulatory effect of NM23-H2 on Diva's activity may be related to its ability to down-regulate Diva expression. In addition, the fact that the expression level of NM23-H2 is also moderately decreased upon forced expression of Diva implies that Diva and NM23-H2 may reciprocally regulate each other's function. In light of this, studies to explore the effects of Diva on the anti-metastatic activity of NM23-H2 are of interest.

In summary, this study provides a direct connection between NM23-H2 and apoptosis signaling through Diva and Bcl2L10, members of Bcl-2 family. At the same time, the data suggest that NM23-H2 is implicated in the regulation of Diva and Bcl2L10 proteins in apoptosis pathways.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.05.090.

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