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Induction of apoptosis by NORE1A in a manner dependent on its nuclear export

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Abstract

The RASSF family proteins were identified as tumor suppressors in a variety of human cancers, and evidenced distinct subcellular localization patterns among their subfamilies and isoforms. In this study, we showed that NORE1A was exported actively via its nuclear export signal (NES) in the C-terminus (residues 372–379). Substitutions of three lysine residues of NORE1A NES to alanines (L372, 376, 379A) showed its localization to the dot structures of the nucleus, which was similar to the NORE1A localizations observed after the administration to cells of Leptomycin B, a nuclear export inhibitor. The NORE1A NES mutant inhibited caspase-mediated apoptosis, whereas wild-type NORE1A induced caspase-3 activation. Furthermore, the NORE1A NES mutant did not co-localize with GFP-MST1, the direct downstream target of NORE1A. These results show that the nuclear export of NORE1A via NES is involved in the NORE1A-mediated induction of apoptosis.

Keywords: NORE1A; Nuclear localization signal; Nuclear export signal; Nucleocytoplasmic shuttling; MST1; Cell death signaling pathway

Ras performs crucial functions in signaling pathways associated with tumor progression and immune response. The specificity of Ras molecules in signal cascades is modulated via downstream RAS effectors in a variety of cellular processes [1]. Recently, six RASSF family members and their isoforms were identified as novel Ras effectors, and were shown to encode for Ras-binding proteins [2–4]. Among them, NORE1/RASSF5 has been identified as a major RASSF family isoform, together with RASSF1, and both evidence alternative splicing variants with distinct functions in tumor progression, apoptosis, and immune response. NORE1A was originally identified in a yeast two-hybrid screen as a putative Ras effector [5], and NORE1B is an alternatively spliced isoform [6]. Both harbor an RA (Ras association) and SARAH (Sav/Rassf/Hpo) domain in the C-terminal region, but NORE1A harbors sequence motifs that evidence SH3 domain binding and diacylglycerol/phorbol ester binding in the N-terminal region [6]. Unlike other alternative splicing forms, the difference in the N-terminal domains between NORE1A and NORE1B is indicative of distinct subcellular localizations of NORE1 isoforms, in a manner dependent on the presence of NLS [7]. NORE1A is constitutively expressed in normal tissues, although this isoform is markedly inactivated in human cancer tissues via hypermethylation within the promoter regions, whereas NORE1B is predominantly expressed in lymphoid tissues, and functions in the immune system and in Ras signaling in response to T cell receptor activation [1]. Thus, the isoforms of the RASSF family may evidence distinct localizations and may perform diverse functions according to the presence or absence of the functional domains located in the N- or C-terminus.

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The nucleocytoplasmic shuttling of particular proteins constitutes an important phenomenon in the transport of functional proteins into specific cellular compartments. The nuclear import and export of proteins is mediated by specific amino acid sequences, which are referred to as NLS and NES, respectively [8]. These two opposite signals are involved in the trafficking of the protein either into the nucleus or the cytoplasm, and are known to determine the interactional network of particular proteins within the subcellular compartment. Recently, several lines of evidence uncovered in studies of the RASSF family have suggested that the functions of RASSF proteins are controlled via nucleocytoplasmic shuttling [7,9]. For example, RASSF1C is released from promyelocytic leukemia-nuclear bodies (PML-NBs) via DNA damage, and then translocated to the cytoplasmic microtubules via the degradation of Daxx, subsequently inducing the activation of SAPK/JNK [9]. As an additional piece of evidence for the nucleocytoplasmic shuttling of the RASSF family, it was previously reported that the NLS of NORE1/RASSF5 regulates the inhibition of cell growth in A549 cells [7]. However, the exact subcellular localization and function in the nucleocytoplasmic shuttling of RASSF family proteins remains to be clearly elucidated.

In the current study, we identified NORE1A as a nucleocytoplasmic shuttling protein. We also identified the NES of NORE1A, which is well-conserved among members of the RASSF family and orthologues of NORE1A. Additionally, we demonstrated that the cytoplasmic NORE1A, which is exported actively via C-terminal NES, performs an important function in the progression of the NORE1A-mediated induction of apoptosis.

Materials and methods

Cell culture and transfection. Cos-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in addition to 100 μg/ml of streptomycin and 100 U/ml of penicillin (Gibco-BRL, Life-Technology) under 5% CO_2 at 37 °C. In all cases, the cells were transfected with Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's instructions.

Plasmid construction. Mouse Nore1A and Mst1 cDNA, and human Nore1B were acquired from mouse and human heart mRNA (Clontech) by PCR amplification, respectively. The green fluorescent protein fusion and Hc-Red fusion forms of Nore1A were constructed by the digestion of the PCR-amplified Nore1A cDNA with EcoRI and SalI, followed by cloning into EGFP- and Hc-Red-C2 vector (Clontech). pCMV-Flag Nore1A was generated by insertion of the EcoRI–SalI DNA fragment derived from wild-type GFP-NORE1. The NES mutation of EGFP-NORE1A (L372A, L376A and L379A) was generated by QuickChange site-directed mutagenesis kit (Stratagene). The expression plasmid encoding for nuclear GFP (the EGFP coding region followed by three copies of SV40 NLS) was generously provided by YH Kim (Digital Biotech, Korea). The two copies of NORE1A NES were inserted into the NotI and XhoI sites of the nGFP expression plasmid in order to generate a plasmid encoding for nGFP-2X NORE1A NES. The NES oligonucleotide sequence was as follows. 5′-GGCCGCAGAACTCCAGAACTTT TTAA CTATCCTGGAACTCCAGAACTTTTTAACTATCCTGGAAC-3′. All constructs were confirmed by the digestion of plasmids with restriction enzymes and DNA sequencing analyses.

Immunofluorescence analysis. Cos-7 cells were seeded in 24-well plates containing round coverglasses and transfected with each of the expression plasmids. Thirty-six hours after transfection, the cells were washed three times in PBS and fixed for 15 min in 4% paraformaldehyde. After fixation, the cells were washed twice in PBS and were covered with anti-fade mounting medium containing DAPI (Molecular Probes). The localizations of the proteins were assessed with an Olympus microscope with a 400× objective. The Flag-NORE1A-expressing cells were probed with anti-Flag-M2 monoclonal antibody (Sigma) followed by incubation with Alexa 488-conjugated anti-mouse secondary antibody (Molecular Probes). Each staining was visualized with an Olympus fluorescence microscope with a 400X objective.

Apoptosis assays. A pCaspase3-sensor system (Clontech) was utilized to monitor the activation of caspase3 and the induction of apoptosis. The pCaspase3-sensor expression plasmid encodes for a fusion protein composed of four polypeptide components. The N-terminal MEKK NES is followed by a PARP domain which harbors the cleavage site via activated caspase3. The PARP polypeptide is followed by nuclear EYFP which harbors SV40 NLS at the C-terminus of EYFP. The fusion protein is localized into the cytoplasm due to its N-terminal NES, which is dominant over the C-terminal NLS. In the presence of active caspase-3, the nuclear export signal is cleaved off, and the nuclear localization signal promotes the transport of nuclear EYFP into the nucleus. In this study, the cells were transfected with 100 ng of pCaspase3 sensor together with expression plasmid for either RFP-NORE1A wild-type or NES mutant. In order to assess apoptotic induction, EYFP localization and the shrinkage of the nuclei of the cells expressing either wild-type RFP-NORE1A or RFP-NORE1A NES mutant were assessed using an Olympus fluorescence microscope at 36 h post-transfection.

Cell fraction and Western blot. Cells were washed twice with phosphate-buffered saline, collected by centrifugation, and fractionated by nuclear and cytoplasmic extraction reagent (Pierce) according to the instruction of manufacturer. Nuclear and cytoplasmic extracts were separated on a 10% SDS–polyacrylamide gel, and transferred to PVDF membrane. Subsequently, these membranes were incubated for overnight at room temperature with anti-Flag-M2 monoclonal antibody (Sigma), anti-Histone H2B (Upstaste), anti-MEK1 (Santa Cruz) in 5% nonfat milk in Tris-buffered saline/0.1% Tween 20 and were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Calbiochem). Finally, protein bands were visualized using SuperSignal West Pico kit (Pierce).

Results

NORE1A is a nucleocytoplasmic shuttling protein

Ras effector NORE1/RASSF5 is expressed as two isoforms of NORE1A and NORE1B via alternative splicing (Fig. 1A). It was recently demonstrated that NORE1A harbors an arginine-rich NLS within the N-terminal region spanning the 51th to 100th amino acid residues, and the NLS is required for the entry of NORE1 into the nucleus and nucleolus [7]. NORE1B is the N-terminal truncated form of NORE1A and has no NLS located within the N-terminus of NORE1A. As NORE1 isoforms harbor NLS differentially, we attempted to determine whether the function of NORE1 isoforms depends on its subcellular localization. To this end, we evaluated the localization of GFP-NORE1A and GFP-NORE1B in Cos-7 cells. We demonstrated that GFP-NORE1A is present in both the nucleus and the cytoplasm, whereas GFP-NORE1B was localized only to the cytoplasm (Fig. 1B). GFP-NORE1A was associated with a variety of subcellular structures or organelles,

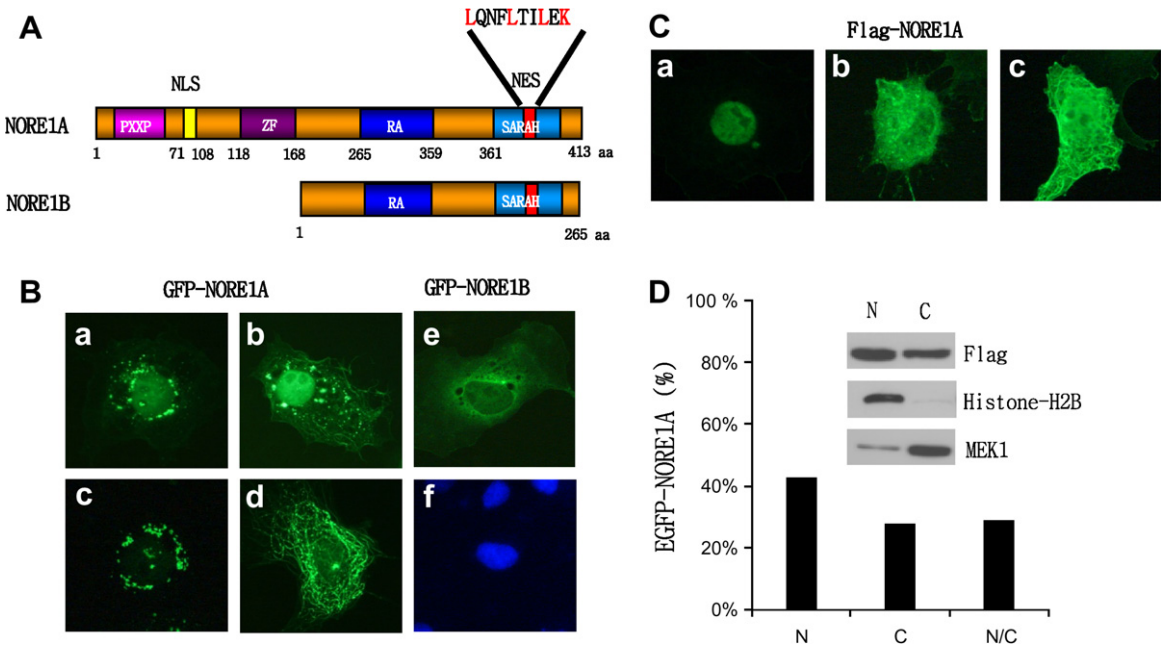

Fig. 1. Nucleocytoplasmic shuttling of NORE1A. (A) Schematics of NORE1 isoforms of NORE1A and NORE1B. NLS is located in the N-terminus of NORE1A, but not NORE1B. The NES characterized in this study is located in the C-terminus of both NORE1A and NORE1B. PXXP, proline-rich motifs; ZF, zingerfinger motif; RA, Ras-associated domain; SARAH, Sav/Rassf/Hpo domain (B) Subcellular localization of EGFP-NORE1A and -NORE1B in Cos-7 cells. Typical localization images of EGFP-NORE1A and -NORE1B following transfection into Cos-7 cells were acquired with a fluorescence microscope. (C) Immunocytochemistry for Flag-tagged NORE1A expressed in Cos-7 cells. The subcellular localizations of Flag-NORE1A were determined via the indirect immunostaining of cells transfected with Flag-NORE1A using anti-Flag antibody. (D) The numbers of Cos-7 cells expressing Flag-NORE1A either in the cytoplasm only, in the nucleus only or in the nucleocytoplasm were counted in three independent arbitrary regions of the fluorescence microscopic image with a 100× objective. The percentages of cells showing each of the localization patterns are depicted on the graph. The distribution of Flag-NORE1A was biochemically analyzed using the nuclear fraction and cytoplasmic fraction of cell lysates via Western blot analysis with anti-Flag antibody, anti-Histone-H2B, and anti-MEK1 (inset). N and C indicate the nucleus and the cytoplasm, respectively.

including the microtubules, mitochondria, perinucleus, nucleoli, and nucleus (Fig. 1B). In an effort to verify the patterns of NORE1A localization, we also assessed the localizations of Fag-tagged NORE1A. As is shown in Fig. 1C, Flag-NORE1A was present in both the nucleus and the cytoplasm (Fig. 1C), and the percentage of cells evidencing the localizations of the nucleus only, cytoplasm only, and both nucleus and cytoplasm were 42%, 28%, and 30%, respectively. Consistently, the results of our Western analyses of the nuclear and cytoplasmic fractions of the cell lysates showed that NORE1A was equally distributed throughout the nucleus and cytoplasm (Fig. 1D). These results indicate that NORE1A may be a nucleocytoplasmic shuttling protein, and that its subcellular localizations are regulated depending on the cellular conditions.

In order to determine whether the dynamic cytoplasmic localization of NORE1A is caused by incomplete NLS function or active export via NES after nuclear translocation, domain analysis was conducted with GFP-NORE1A. Either N-terminal or C-terminal deleted NORE1A was fused to the C-terminus of EGFP (Fig. 2A), and each localization of the GFP-NORE1A deletion mutants was observed after transfection into Cos-7 cells. N-terminal deleted mutants and C-terminal deleted mutants were localized into the cytoplasm and nucleus, respectively (Fig. 2B). In order to determine whether the cytoplasmic localizations of N-terminal deletion mutants were attributable to either incomplete NLS function or active protein export via NES, cells expressing each of the GFP-NORE1A deletion mutants were administered with Leptomycin B (LMB), an inhibitor of CRM-dependent protein export. LMB administration resulted in the nuclear localizations of NORE1A N-terminal deletion mutants. These results strongly indicate that there active NES might be present in the C-terminus of NORE1A.

NORE1A has a conserved NES in the C-terminus region

One consensus sequence for leucine-rich NES was detected at residues 372L to 379L of NORE1A, and the sequences are well-conserved among the RASSF family and NORE1/RASSF5 orthologues (Fig. 3A). In order to determine whether the putative NES functions as an export sequence of NORE1A, cellular localization of GFP-NORE1A NES mutant was observed following the substitutions of all the L372, L376, L379 to alanines (Fig. 3B). Interestingly, the GFP-NORE1A NES mutant was localized to the punctate structures of the nucleus, which is reminiscent of the localization of GFP-NORE1A after the administration of LMB (Fig. 3C). In order to determine whether the function of NORE1A NES is transferable to other proteins, and to verify the function of NORE1A

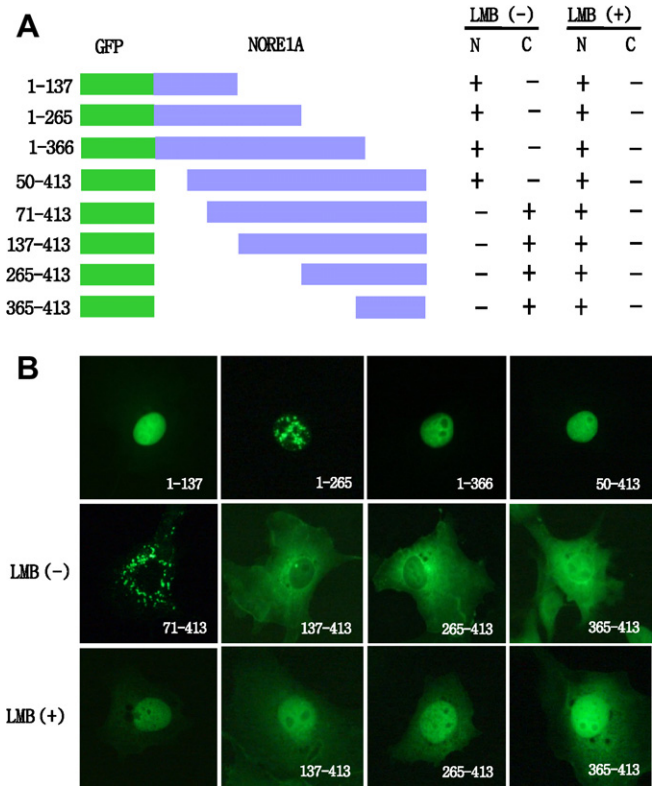

Fig. 2. Schematics of EGFP-NORE1A deletion mutants and their subcellular localizations in the absence or presence of LMB, an inhibitor of CRM-dependent nuclear export. (A) A schematic diagram of the EGFP-NORE1A deletion mutants utilized in this study. The localization of each mutant in both the presence and absence of LMB is summarized at the right of the figure. N and C indicate the nucleus and the cytoplasm, respectively. (B) A typical image of each deletion mutant is shown (upper and middle panels). The cells transfected with EGFP-NORE1A deletion mutants were treated with LMB (20 ng/ml) for 12 h and the subcellular localization of EGFP-NORE1A deletion mutants was assessed via fluorescence microscopy (lower panel).

NES in a heterologous system, two copies of NORE1A NES were fused downstream of nuclear GFP, after which we determined its localization. As shown in Fig. 3D, nuclear GFP was exported via fusion with the two copies of NORE1A NES. Collectively, these results indicate that NORE1A harbors both NLS and NES in the N-terminus and C-terminus, respectively, and its cellular localizations might be regulated via protein interactions and/or cellular environmental cues.

NORE1A induces MST1-mediated apoptosis in a manner dependent on its nuclear export

The NORE1-MST1 complex is a novel Ras effector unit which mediates the apoptotic functions of Ki-RasG12V [4]. In order to gain insight into the relationship between the cellular compartmentalization of NORE1A and its cellular function, NORE1A-mediated apoptosis was explored with regard to whether the induction of apoptosis depends on its localization. To this end, pCaspase3-Sensor system (Clontech) was utilized, in which nuclear EYFP was fused to the artificial domains including both the caspase3 cleavage acceptor sites of PARP and NES of MAPKK in order to monitor the nuclear localization of EYFP after the cleavage of a synthetic fusion protein by caspase-3 under apoptotic conditions. The caspase3-sensor expression plasmid was transfected into Cos-7 cells together with either wild-type NORE1A or NES mutant and EYFP localization was assessed via fluorescence microscopy. The caspase-sensor (PARP cleavage domain-EYFP fusion protein) alone was localized to the cytoplasm as the result of its N-terminal NES. The co-expression of NORE1A wild-type with the caspase3-sensor, however, was shown to result in the translocation of EYFP into the nucleus and the shrinkage of the nucleus, a typical apoptotic cell phenotype. Meanwhile, the co-expressed NORE1A NES mutant was not observed to colocalize with the caspase3-sensor and did not induce EYFP translocation (Fig. 4A). These results indicate that the induction of apoptosis by NORE1A is dependent on its cytoplasmic localization. We then assessed the effects of NORE1A NES mutation on apoptotic induction mediated by MST1, a direct downstream target of NORE1 after Ras signaling. The activation of MST1 kinase after co-expression with either wild-type NORE1A or its NES mutant could be monitored by the nuclear condensation of MST1-expressing cells and its translocation into the nucleus, where MST1 phosphorylates a variety of target proteins, including histone 2B, thereby inducing nuclear condensation. The co-expression of wild-type NORE1A resulted in nuclear shrinkage and its co-localization with wild-type NORE1A in the apoptotic nucleus, whereas the NORE1A NES mutant neither colocalized with GFP-MST1 nor induced the nuclear condensation of MST1-expressing cells. Taken together, the cytoplasmic localization of NORE1A via C-terminal NES is crucial for the induction of apoptosis via the NORE1A-MST1 complex.

Discussion

NORE1A/RASSF5 is a founding member of the RASSF family proteins, which have recently been recognized as tumor suppressors, due to their frequent loss of expression in a variety of human tumors. The representative cellular functions of NORE1A include both the inhibition of cell growth and the induction of apoptosis. These functions of NORE1A have yet to be elucidated clearly in terms of upstream signaling cues and the network of interacting proteins for each function. Herein, we provided evidence that NORE1A is a nucleocytoplasmic shuttling protein, and harbors NLS and NES in the N-terminus and C-terminus of NORE1A, respectively. NORE1A was exported actively via its NES, which is quite well-conserved among the RASSF family members and orthologues (Fig. 3A), thereby suggesting that the intracellular distribution of proteins constitutes another factor for the regulation of RASSF family proteins by internal or external

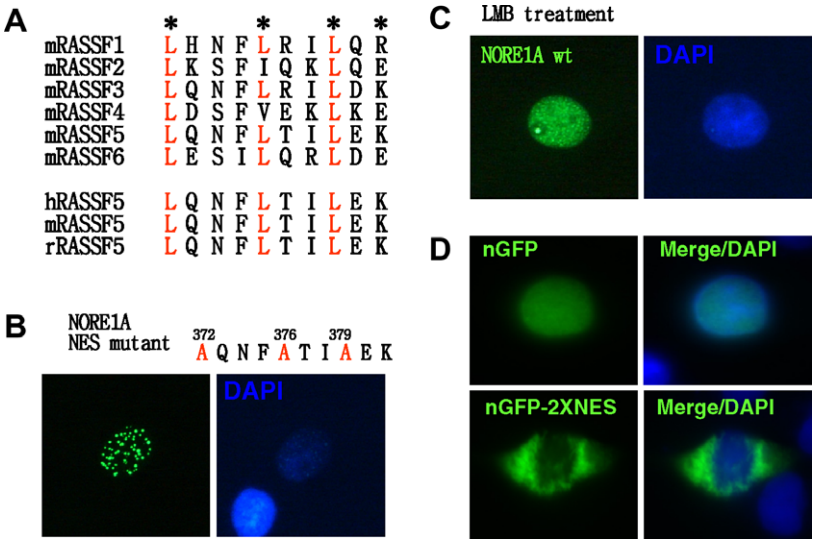

Fig. 3. Characterization of NES of NORE1A. (A) Comparison of NES in the mouse RASSF family and RASSF5/NORE1A orthologues. hRASSF5, mRASSF5, and rRASSF5 indicate the RASSF5 protein originating from humans, mice, and rats, respectively. The asterisk indicates the typical residues for leucine-rich NES (LxxxLxxLxL, x represents any amino acid) and the conserved leucine residues are displayed in red color. (B) Subcellular localization of EGFP-NORE1A NES mutant (L372A, L376A, L379A). Note that substitutions of NES lysine residues resulted in localizations of the NORE1A mutant to the dot structures of the nucleus. (C) The effects of LMB treatment (20 ng/ml) on the subcellular distribution of EGFP-NORE1A. Cos-7 cells expressing EGFP-NORE1A were treated with LMB, and its localizations were assessed via fluorescence microscopy. DAPI staining indicates the location of the nucleus. (D) Characterization of NORE1A NES in a heterologous system. The expression plasmids encoding for either nuclear GFP (three copies of SV40 NLS was fused to GFP) or nGFP-2XNES (two copies of NORE1A NES were fused to nuclear GFP) were transfected into Cos-7 cells, and their localizations were assessed via fluorescence microscopy. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

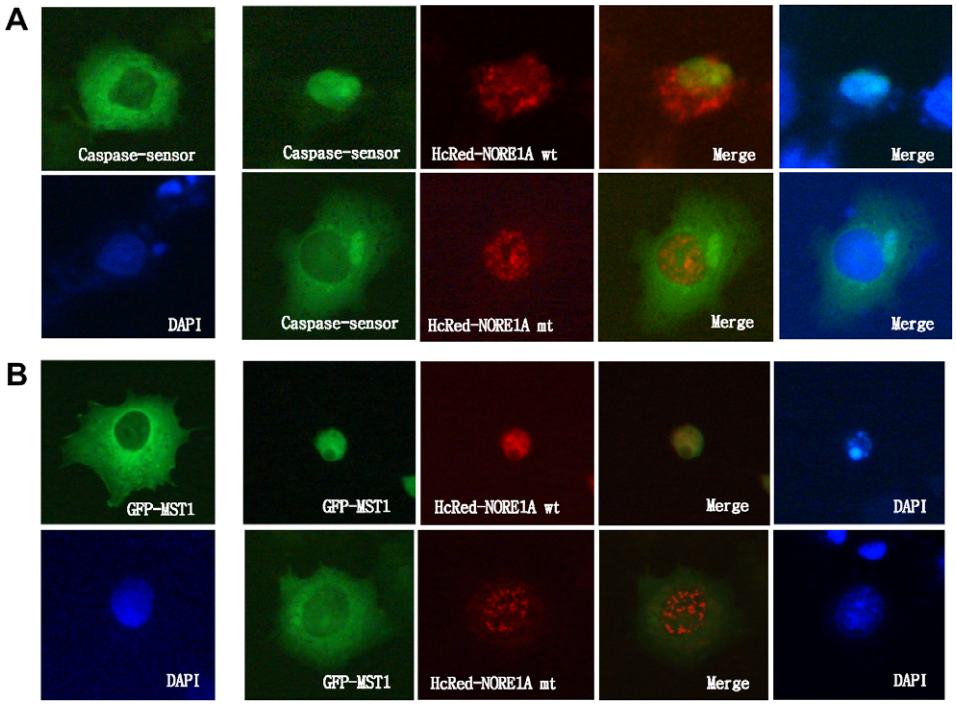

Fig. 4. NORE1A induces apoptosis in a cytoplasmic localization-dependent manner. (A) Cos-7 cells were transfected with pCaspase3-sensor vector together with either Hc-Red-NORE1A wild-type or NES mutant. The green (EYFP) and red signals (RFP-NORE1A) were acquired with EYFP and RFP fluorescence, respectively. DAPI staining indicates the location of the nucleus. Note that the nuclei of cells expressing wild-type NORE1A were condensed, whereas this was not the case in the cells expressing mutant NORE1A NES. (B) Cos-7 cells were transfected with GFP-MST1 together with either Hc-Red-NORE1A wild-type or NES mutant. The green (GFP-MST1) and red signals (RFP-NORE1A) were obtained with GFP and RFP fluorescence, respectively. DAPI staining shows the location of the nucleus. Note that the nuclei of cells expressing NORE1A wild-type were condensed, whereas that was not the case in the NORE1A NES mutant-expressing cells. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

cellular signaling inputs. Recently, Kumari et al. reported that NORE1/RASSF5 is translocated into the nucleus and nucleolus via arginine-rich nuclear targeting signals [7]. In addition, Kitagawa et al. showed that RASSF1C is released into the microtubules from the Daxx-RASSF1C complex of PML-nuclear bodies when Daxx is degraded via DNA damage [9]. The sequences of NORE1A NLS are not well-conserved in other RASSF family members, and have no similarity to any other NLSs, including lysine-rich SV40 NLS. These data indicate that the distinct localization among RASSF proteins may perform different functions in Ras-mediated pathways, in addition to the Ras-independent pathways. For example, RASSF1C forms a complex with Daxx within the PML-nuclear bodies and is relocalized into the cytoplasm as a response to genotoxic stress [9]. RASSF1C does not harbor NLS in its N-terminus, but NES is well-conserved within its C-terminus, which is similar to NORE1A NES. It is conceivable that the NES of RASSF1C may be involved in the relocalization of RASSF1C into the cytoplasm after Daxx degradation. Thus, we concluded that RASSF proteins may associate with other proteins in a manner more dependent on NES than on NLS in nucleocytoplasmic shuttling.

Although NORE1A is a downstream effector of Ras, some functions of NORE1 occur independently of Ras signaling [10]. Aoyama *et al*. reported that NORE1A induced an inhibition of growth independent of Ras and MST1/2 activation [10], and nuclear NORE1A induced growth inhibition in A549 cells, but not for the NORE1A NLS mutant [7]. We also observed that the NORE1A NES mutant induced an inhibition of growth in cells that were transiently transfected with the NORE1A NES mutant as well as wild-type NORE1A in colony formation assays using HeLa cells (data not shown). In the case of apoptosis induction, however, MST1 was localized into the cytoplasm and thus was not colocalized with the NORE1A NES mutant, whereas the wild-type NORE1A induced apoptosis in conjunction with co-expressed MST1 in cytoplasm before forming the nuclear condensation (Fig. 4B). Hwang et al. previously demonstrated that the MST1 SARAH domain interacts with its homologous domain of RASSF1 and NORE1A/Rassf5 via the formation of a heterodimer which mediates the apoptotic process [11]. Collectively, our results show that both the SARAH domain of NORE1 and NES embedded in the SARAH domain may be required for heterodimer formation with MST1 and the cytoplasmic distribution of NORE1A for colocalization with MST1, respectively, in the RAS-NORE1-MST1 mediated induction of apoptosis. These results indicate that NORE1A-induced growth inhibition is exerted by nuclear NORE1A and the induction of apoptosis is mediated by cytoplasmic NORE1A via CRM-dependent nuclear export.

In this study, we demonstrated that NORE1A was localized to diverse subcellular compartments, including the nucleus, nucleolus, microtubules, mitochondria, and ER. Therefore, further elucidation of NORE1 motifs for trafficking into specific compartments and interaction with binding partners would provide insights into the variety of NORE1 functions in manners both dependent and independent on Ras signaling activation.

Acknowledgments

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