The Growth and Tumor Suppressor NORE1A Is a Cytoskeletal Protein That Suppresses Growth by Inhibition of the ERK Pathway *1

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**NORE1A is a growth and tumor suppressor that is inactivated in a variety of cancers. NORE1A has been shown to bind to the active Ras oncoprotein. However, the mechanism of NORE1A-induced growth arrest and tumor suppression remains unknown. Using anchorage-independent growth assays, we mapped the NORE1A effector domain (the minimal region of the protein responsible for its growth-suppressive effects) to the fragment containing the central and Ras association domains of NORE1A (amino acids 191–363). Expression of the NORE1A effector domain in A549 lung adenocarcinoma cells resulted in the selective inhibition of signal transduction through the ERK pathway. The full-length NORE1A (416 amino acids) and its fragments capable of growth suppression were localized to centrosomes and microtubules in normal and transformed human cells in a Ras-independent manner. A mutant that was deficient in binding to centrosomes and microtubules was also deficient in inducing cell cycle arrest. This suggests that cytoskeletal localization is required for growth-suppressive effects of NORE1A. Ras binding function was required for growth-suppressive effects of the full-length NORE1A but not for the growth-suppressive effects of the effector domain. Our studies suggest that association of NORE1A with cytoskeletal elements is essential for NORE1A-induced growth suppression and that the ERK pathway is a target for NORE1A growth-suppressive activities.**

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**Ras is the most common dominant oncogene found in human tumors, especially in those of epithelial origin (1). Ras family consists of three ubiquitously expressed 21-kDa proteins (H-, Ki- and N-Ras) localized at the inner surface of the plasma membrane, and each is capable of guanine nucleotide binding. If GDP is bound to Ras, the protein is inactive; exchange of GDP for GTP activates Ras (2, 3). Ras effectors, proteins which bind to Ras-GTP with a much higher affinity than to Ras-GDP, are thought to be responsible for transmitting Ras signals downstream (4).**

**Nore1 (novel ras effector 1) was identified in a yeast two-hybrid screen as a putative Ras effector. Bacterially expressed Nore1 directly interacts with Ras in vitro in a GTP-dependent manner; in KB cells, endogenous NORE1A associates with endogenous Ras upon serum stimulation (5). The full-length NORE1A cDNA encodes a 47-kDa basic protein, which contains a proline-rich region at its N terminus followed by a putative diacylglycerol/phorbol ester-binding domain (Fig. 1A). The Ras binding domain is located near the C terminus. The human NORE1A gene encodes several isoforms, of which NORE1A and NORE1B are the most prominent (6).**

**NORE1A is the founding member of a small gene family that includes RASSF1 to -4 and RASSF6 (6–9). Each of these genes encodes one or more polypeptides that contain a canonical “Ras association” (RA) domain in their carboxy-terminal segment. RASSF1A (Ras association domain family 1, isoform A), the closest NORE1A relative, was discovered as a tumor suppressor gene located on human chromosome 3p21 in a segment that is often deleted in SCLC, in many NSCLC, and in breast, renal, head and neck, and other cancers (7, 10, 11) (for reviews, see Refs. 10 and 12). Recently, it was suggested that RASSF2 and RASSF4 may have tumor suppressor capabilities (13–18).**

Data obtained by several laboratories strongly suggest that the longest splice isoform of the NORE1 gene, NORE1A, is a growth and tumor suppressor. Most normal tissues, including lung, express NORE1A; however, its expression is lost in several cancer cell lines, including lung carcinoma A549 (6). Hesson et al. (19) showed by methylation-specific PCR that the NORE1A promoter region Cpg islands are hypermethylated in 18% of NSCLC, 10% of SCLC, 40% of breast, 17% of colorectal, and 33% of kidney tumor cell lines. In the same study, 24% of primary NSCLC DNA specimens displayed NORE1A promoter methylation, whereas methylation was not detected in NORE1A from the corresponding normal lung tissue DNA. Another group demonstrated that NORE1A promoter hypermethylation is confined to lung tumors with a wild type Ki-Ras, suggesting that both Ki-Ras and NORE1A may act in the same pathway (20). Vos et al. (21) found that expression of NORE1A protein was lost in 100% of NSCLC and 57% of SCLC cell lines tested and was severely reduced or absent in 80% of primary small and non-small cell lung cancers tested.

**NORE1A was identified as a breakpoint-spanning gene in a familial renal clear cell carcinoma chromosomal translocation. NORE1A expression was down-regulated in all RCC cell lines tested; the NORE1A**

2 The abbreviations used are: RA, Ras association; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; HA, hemagglutinin; FLAG, peptide tag DYKDDDDK; RCC, renal cell carcinoma; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; GIP, green fluorescent protein; HrGFP, R. reniformis green fluorescent protein; HBE3-KT, human bronchial epithelial cells immortalized by retroviral transduction of cyclin-dependent kinase 4 and telomerase catalytic subunit; HBE3-KTR, HBE3-KT cells containing constitutively active Ki-Ras allele; DAPI, 4’,6-diamidino-2-phenylindole; BSA, bovine serum albumin; aa, amino acids.
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promoter was methylated in 32% of sporadic RCC tumors. Restoration of expression of NORE1A in two human kidney cell lines induced growth arrest (22). Aoyama et al. (23) showed, by quantitative reverse transcription-PCR, that expression of NORE1A in NSCLC and melanoma cell lines is very low in comparison with nontransformed matching controls. Re-expression of NORE1A in NSCLC cell line A549 causes a decrease in the number of cells in the S phase of the cell cycle and a reciprocal increase of the fraction of cells in the G1 phase of the cell cycle (23).

However, despite the loss of NORE1A expression in many human cancers, molecular mechanism(s) of the growth and tumor suppression by NORE1A are not known. Specifically, the importance of binding to Ras and related GTPases for the growth-suppressive activity of NORE1A is unclear. The yeast two-hybrid data showed that NORE1A is capable of binding Ras and related GTPases directly (24). Vos et al. (21) showed that the rat ortholog of NORE1 is growth-inhibitory in several cell lines, including A549, NIH 3T3, 293, and 293T in a Ras-dependent manner. However, Aoyama et al. (23) found that the antiproliferative activity of NORE1A is independent of the presence of an activated Ras oncogene or the ability of NORE1A to bind Ras. The effect of NORE1A expression on intracellular signal transduction is not known. The subcellular localization of the endogenous NORE1A protein has not been determined.

In this paper, we characterize the NORE1A effector domain (i.e. the minimal region of the protein responsible for its growth-suppressive effects). We found that expression of this domain selectively suppresses ERK activity in A549 cells. We also found that endogenous NORE1A and its effector domain are targeted to the elements of microtubular cytoskeleton, and this ability is essential for the growth suppression properties of NORE1A.

MATERIALS AND METHODS

Plasmids—Plasmids encoding FLAG or HA epitope-tagged proteins were described in Ref. 25 or prepared using the standard molecular biology techniques (26). Coding sequence of Renilla reniformis green fluorescent protein (hrGFP, Stratagene, La Jolla, CA) was inserted into the pEAK12-MMP retroviral backbone (27). Coding sequences of NORE1A, its mutants, and RASSF1A were subcloned in-frame downstream of the hrGFP moiety. All constructs were sequenced to ensure that they were correct.

Cell Lines, Transfection, and Retrovirus Production—A549 and 293 cells (ATCC, Manassas, VA) were cultivated and transfected as described previously (25). LNCaP cells (a gift of Dr. M. Weber, University of Virginia) were cultivated in T-Medium (Invitrogen) supplemented with 10% fetal bovine serum in 5% CO2 atmosphere at 37 °C. HBEC3-KT and HBEC3-KTR cells were propagated as described in Ref. 28. Primary human small airway epithelial cells (BioWhittaker, Walkersville, MD) were cultivated according to the manufacturer’s instructions. Retroviral supernatants were produced essentially as described in Ref. 27, using the appropriate growth media for target cells and diluted or with a 2-fold dilution for infection at 15–20% target cell confluence.

Immunoprecipitation and Western Blot Analysis—These procedures were performed as described previously (25). Anti-ERK antibody was from Upstate Biotechnology, Inc. (Charlottesville, VA); anti-doubly phosphorylated ERK antibody was from Promega (Madison, WI); and anti-phosphorylated MEK, anti-MEK1/2, anti-Akt, and anti-phospho-Ser473 Akt antibodies were from Cell Signaling (Beverly, MA). The monoclonal antibody 10F10 (available from Upstate Biotechnology) was raised in our laboratory using human NORE1A 119–416 as the antigen. The antibody reacts with NORE1A epitope located within amino acids 119–191 of the human NORE1A protein. Rabbit antibodies against NORE1 were described previously (5). Anti-centrin monoclonal antibody 20H5 was a gift of Dr. J. Salisbury (Mayo Clinic, Rochester, MN).

Flow Cytometry and Anchorage-independent Assays—For flow cytometry, cells were plated in 6-well plates at 3 × 104/well and after 18–24 h were infected in triplicate with retroviruses. 72 h later, cells were fixed with 1% formaldehyde in phosphate-buffered saline, stained with 0.05 mg/ml propidium iodide, and analyzed by FACSVantage (BD Biosciences). Cell cycle distributions of GFP-positive cells were determined by ModFIT software. For the anchorage-independent growth assays, cells were infected with retroviruses encoding hrGFP or hrGFP fusion proteins and plated in 6-well plates in 0.35% agar at 3 × 104 (A549) and 5 × 104 (LNCaP) cells/plate 48 h after infection. Digital images were taken 15 (LNCaP) or 25–28 (A549) days after plating at × 10 magnification under UV illumination using the GFP filter set with a Retiga EX, camera connected to the LEICA DMI2E2 inverted microscope and captured using IPLab 3.6.5a software (Scanalytics, Rockville, MD). The number of green colonies expressing GFP alone or GFP-NORE1A fusions and their area in 20–30 randomly pictured viewfields for each experimental point were determined using IPLab 3.6.5a.

Immunofluorescent Microscopy—Cells were grown in Lab-Tek chamber slides (Nunc, Rochester, NY) and, if required, infected with retroviruses 18–24 h after plating. 36–48 h later, cells were fixed in 100% methanol at −20 °C for 5 min, washed three times in phosphate-buffered saline, and stained with the indicated antibody diluted in 1 mg/ml BSA for 1.5–2 h at room temperature. After 3–4 washes in Tris-buffered saline containing 0.1% BSA and 0.05% Tween 20, cells were stained with a secondary antibody diluted in 1 mg/ml BSA for 45 min at room temperature. Slides were washed as described above and mounted using Vectashield medium containing DAPI. Digital images were taken at 100× magnification as described above using appropriate filter sets. For some experiments, cells were fixed in 3.7% formaldehyde in phosphate-buffered saline for 10 min at 37 °C, extensively washed in phosphate-buffered saline, permeabilized, and blocked in 0.1% Triton X-100 containing 1 mg/ml BSA for 30 min at room temperature and stained with indicated antibodies described above. Both methods of fixation gave the same results.

Tubulin-binding Assays—Purified bovine brain tubulin (Cytoskeleton, Denver, CO) was polymerized into microtubules in 80 mM PIPES, pH 7.1, 1 mM MgCl2, 1 mM EGTA, 20 μM taxol for 20 min at 35 °C. For in vitro cosedimentation assay, 10 μg of microtubules was mixed with 10 μl of A549 cell lysate, which expressed GFP-NORE1A, or 293 lysate, which expressed FLAG-NORE1A or its mutants, or FLAG-NORE1A protein that was expressed in 293 cells, purified on FLAG-agarose, and eluted with FLAG peptide. After incubation for 30 min at room temperature, microtubules were pelleted by centrifugation at 50,000 × g for 1 h at room temperature, and supernatant was collected and adjusted to 1× SDS gel loading buffer with its 4× stock. The pellet was dissolved in 1× SDS gel loading buffer and adjusted to the same volume as supernatant. Equal volumes of supernatant and pellet were separated by SDS gel and analyzed by Western blotting.

Centrosome Isolation—Isolation of centrosomes was performed as described in Ref. 29.

RESULTS

Expression of NORE1A Protein in Normal and Tumor Human Cells—We characterized expression of NORE1A in several normal and tumor human cell lines, using a monoclonal antibody raised in our laboratory.
Fig. 1B shows that immortalized human HBEC3-KT cells display on a Western blot a single immunoreactive band or close doublet of the expected molecular mass, ~46 kDa. In contrast, most tumor cell lines tested, including non-small cell lung cancer A549 (Fig. 1B), NCI-H23, H358, H157, renal carcinoma CaKi-1 (data not shown), and prostate carcinoma LNCaP (Fig. 1B), lack NORE1A expression.

The Effect of NORE1A on Cell Cycle Distribution and Soft Agar Growth—For characterization of the biological properties of NORE1A, we fused the coding sequence of NORE1A with the C terminus of Renilla green fluorescent protein (hrGFP) to generate hrGFP-NORE1A. Retroviral vectors containing hrGFP-NORE1A or hrGFP alone were introduced in tumor cells, and cell cycle distribution of hrGFP-expressing cells was determined by flow cytometry.

In A549 cells growing as a monolayer on plastic support, NORE1A re-expression usually induced a small increase, on average 5%, of cells in the G1 population (Table 1). In other cell lines, such as LNCaP, CaKi-1, H460, and H358, NORE1A expression did not induce any statistically significant changes in cell cycle distribution (Supplemental Fig. 1B and C, and data not shown).

Importantly, in our hands, the re-expression of the closest relative to NORE1A, the RASSF1A protein, fused to hrGFP similarly to the way we re-expressed NORE1A, in the A549 cell line, resulted in a significant (2–3-fold over GFP) increase in the G1/M cell content with the concomitant decrease in G2 and S phases of the cell cycle and growth suppression (Supplemental Fig. 1A).

Cells expressing NORE1A grew at the same rate as parental cells, whereas RASSF1A expression significantly suppressed cell growth. We did not observe any NORE1A-expressing cells with a DNA content less than G1/G2, suggesting that NORE1A does not induce apoptosis (data not shown).

The anchorage-independent growth of tumor cells correlates best with the in vivo tumor-forming capabilities (30). We employed this assay to examine the effect of NORE1A on anchorage-independent growth of A549 and LNCaP cells. GFP fusion of NORE1A or hrGFP alone was introduced into cells by retrovirus-mediated gene transfer. Individual cells and cell colonies in soft agar that express recombiant proteins were identified by fluorescent microscopy with a GFP filter set. As shown in Table 2 and supplemental Fig. 1D, NORE1A efficiently suppressed the formation of large colonies in both A549 and LNCaP cell lines.

Characterization of NORE1A Domains Responsible for Growth Suppression—We used the anchorage-independent growth assay to determine the domains of the NORE1A molecule that are required for its growth-suppressive activity. For this purpose, a set of NORE1A truncations (Fig. 1A), fused in-frame with the C terminus of hrGFP, or hrGFP alone was introduced in A549 and LNCaP cells by retrovirus-mediated gene transfer, and the colony-forming ability of cells expressing recombinant proteins was evaluated.

Table 2 shows that deletion of the last 53 amino acids of NORE1A, representing the SARAH domain that binds MST kinases (25), has only a small effect on NORE1A ability to suppress anchorage-independent growth in both A549 and LNCaP cells. Thus, determinants responsible for inhibition of anchorage-independent growth are located in the first 363 amino acids of the protein. We next tested the growth-suppressive capabilities of the N-terminal half of this segment, representing the NORE1A proline-rich and zinc finger domains (aa 1–191), and the C-terminal half, consisting of the Central and RA domains (aa 191–363). As shown in Fig. 1C, NORE1A 1–191 was deficient in suppression of soft agar growth, whereas NORE1A 191–363 efficiently suppressed growth in soft agar. In the case of A549 cells, NORE1A 191–363 was more efficient in growth suppression than wild type NORE1A. Thus, NORE1A growth-suppressive functions are determined by amino acids 191–363 in two cell types, A549 lung adenocarcinoma and LNCaP prostate carcinoma. In our subsequent studies, we focused on the effect of NORE1A on A549 cells, since the potential NORE1A involvement in lung carcinogenesis using this cell line is well described (19–21).
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TABLE 1
Cell cycle distribution of A549 cells expressing GFP, NORE1A, and its truncation mutants

A549 cells were infected in triplicate with recombinant retroviruses coding for hrGFP or hrGFP fusions as indicated, fixed, and stained with propidium iodide and analyzed as described under “Materials and Methods.” The untagged NORE1A protein, expressed from a retroviral backbone carrying IRES-GFP, also failed to induce changes in cell cycle distribution (data not shown). The percentage of GFP-positive cells in each phase of the cell cycle ± S.D. and the number of experiments in parentheses are shown.

| Construct                | G1     | S        | G2/M    |
|--------------------------|--------|----------|---------|
| GFP alone                | 54.8 ± 1.5 (4) | 32.9 ± 1.3 | 12.6 ± 0.6 |
| GFP-NORE1A               | 60.1 ± 1.8 (4)* | 27.9 ± 1.5 | 12 ± 0.4 |
| GFP-NORE1A 1–191         | 57.1 ± 2.3 (4) | 31.3 ± 2.1 | 11.8 ± 1.1 |
| GFP-NORE1A 1–363         | 57.6 ± 0.9 (2) | 30 ± 0.1  | 11.4 ± 0.9 |
| GFP-NORE1A 191–363       | 75.5 ± 3.3 (3)* | 15.8 ± 1.2 | 8.7 ± 2.5 |
| GFP-NORE1A 118–363       | 67.2 ± 1.6 (3)* | 23 ± 1.2  | 9.7 ± 1.3 |
| GFP-NORE1A 191–416       | 60.9 ± 1.2 (3) | 18.1 ± 3.2 | 21 ± 4.4 |

* p = 0.012 versus GFP by Student’s t test.
* * p = 0.001 versus GFP-NORE1A by Student’s t test.
* * * p = 0.007 versus GFP by Student’s t test.

TABLE 2
The effect of wild type NORE1A and its mutants deficient in binding to Ras or MST kinases on soft agar colony formation of A549 and LNCaP cells

A549 and LNCaP cells were infected with the retroviruses as indicated and plated in soft agar. Images were taken and analyzed as described under “Materials and Methods.” The untagged NORE1A protein, expressed from a retroviral backbone carrying IRES-GFP, also failed to induce changes in cell cycle distribution similar to wild type NORE1A (Table 2). In addition, in most A549 cells, NORE1A decorated cytoplasmic fibers positive for α-tubulin, a microtubular marker (Fig. 2A, bottom row). In mitotic A549 cells, GFP-NORE1A was localized to the centrosomes and mitotic spindle (supplemental Fig. 2A). The GFP-NORE1A expressed in A549 cells, was cofractionated with centrosomes, in a manner very similar to the endogenous NORE1A (supplemental Fig. 2B). To confirm the ability of NORE1A to bind microtubules, we performed an in vitro microtubule cosedimentation assay. Extracts of A549 cells expressing GFP-NORE1A were incubated with taxol-polymerized microtubules, the microtubules were pelleted and NORE1A distribution in the pellet, and supernatant was examined by Western blotting. Fig. 2B shows that most of NORE1A was associated with the microtubular pellet. Similar results were obtained with extracts of 293 cells containing FLAG-NORE1A. However, if purified NORE1A rather than cell extracts from NORE1A-expressing cells was presented to polymerized microtubules, no interaction of NORE1A with microtubules was detected, suggesting the interaction may be occurring indirectly (Fig. 2C). Thus, NORE1A is localized to the elements of microtubular cytoskeleton in normal and tumor cells.

Next we investigated the domains of NORE1A responsible for its subcellular localization. NORE1A 1–363, which lacks the MST binding domain, displayed both cytoskeletal and nuclear localization (supplemental Fig. 2A). The N-terminal NORE1A fragments, amino acids 1–191 and 1–268, were localized almost exclusively to the nuclei, showing little or no localization with cytoskeletal elements (Fig. 3, fourth row, and supplemental Fig. 2A).

In contrast, the NORE1A effector domain, aa 191–363, was targeted to centrosomes and microtubules (Fig. 3, third row, and supplemental Fig. 3). Other fragments that contain the NORE1A effector domain were also targeted to centrosomes and microtubules, displaying colocalization with tubulin in immunofluorescent microscopy and binding to polymerized microtubules in the in vitro cosedimentation assay. (Fig. 3 and supplemental Fig. 3, A and B). Thus, the ability to localize to cytoskeletal structures correlates with growth-suppressive properties of NORE1A truncation mutants.

In summary, NORE1A 1–363 is capable of binding cytoskeletal components, but NORE1A 1–268 is not. This suggests that determinants encoded by the Ras association domain are required for cytoskeletal localization of NORE1A. The only function suggested so far for the RA

Next, we tested the effect of the expression of NORE1A truncation mutants on the cell cycle distribution of A549 cells in monolayer. The NORE1A 1–363 and 1–191 truncations induced small changes in the A549 cell cycle distribution, similar to wild type NORE1A (Table 1). In contrast, the expression of NORE1A 191–363 fragment induced a profound arrest in the G2 phase of the cell cycle with the reciprocal decrease in S and G1/M populations. NORE1A 118–363 was less potent in the HBEC3-KT cells revealed that the NORE1A was cofractionated with centrosomal marker (Fig. 2).

We reasoned that NORE1A could either be a resident centrosomal protein or be transported along microtubules and localized to the centrosomes en route. To discriminate between these possibilities, we treated HBEC3-KT cells with 5 μM nocodazole for 4 h to disrupt the microtubule network. The nocodazole treatment did not change subcellular localization of NORE1A, suggesting that NORE1A is a resident centrosomal protein (Fig. 2A, third row). The centrosomal localization of NORE1A was not changed in the presence of activated Ki-Ras in the HBEC3-KTR cells (Fig. 2A, fourth row).

In A549 lung adenocarcinoma cells and in primary human small airway epithelial cells, hrGFP-NORE1A, introduced in cells by retrovirus-mediated gene transfer, was also localized to centrosomes (supplemental Fig. 2A). In addition, in most A549 cells, NORE1A decorated cytoplasmic fibers positive for α-tubulin, a microtubular marker (Fig. 2A, bottom row). In mitotic A549 cells, GFP-NORE1A was localized to the centrosomes and mitotic spindle (supplemental Fig. 2A). The GFP-NORE1A expressed in A549 cells, was cofractionated with centrosomes, in a manner very similar to the endogenous NORE1A (supplemental Fig. 2B). To confirm the ability of NORE1A to bind microtubules, we performed an in vitro microtubule cosedimentation assay. Extracts of A549 cells expressing GFP-NORE1A were incubated with taxol-polymerized microtubules, the microtubules were pelleted and NORE1A distribution in the pellet, and supernatant was examined by Western blotting. Fig. 2B shows that most of NORE1A was associated with the microtubular pellet. Similar results were obtained with extracts of 293 cells containing FLAG-NORE1A. However, if purified NORE1A rather than cell extracts from NORE1A-expressing cells was presented to polymerized microtubules, no interaction of NORE1A with microtubules was detected, suggesting the interaction may be occurring indirectly (Fig. 2C). Thus, NORE1A is localized to the elements of microtubular cytoskeleton in normal and tumor cells.

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In summary, NORE1A 1–363 is capable of binding cytoskeletal components, but NORE1A 1–268 is not. This suggests that determinants encoded by the Ras association domain are required for cytoskeletal localization of NORE1A. The only function suggested so far for the RA...
FIGURE 2. Subcellular localization of wild type NORE1A and interaction of NORE1A with polymerized microtubules. A, human bronchial epithelial cells (HBEC3-KT (top three rows) or HBEC3-KTR (fourth row)) were plated on multiwell coverslips, fixed 3–4 days after, and stained for NORE1A with 10F10 monoclonal antibody followed by GTU-88 anti-γ-tubulin antibody (Sigma) labeled with Alexa 488 with a Zenon Kit (Invitrogen) and DAPI (blue). Cells shown in the third row were incubated with 5 μM nocodazole (Sigma) for 4 h at 37 °C. Bottom row, A549 cells were infected with retroviruses coding for hrGFP-tagged NORE1A. Cells were fixed 40 h postinfection, stained, and imaged as described under “Materials and Methods.” Staining was performed with DM-1-A anti-α-tubulin antibody (Sigma) followed by Rhodamine RedX anti-mouse IgG (red) and DAPI (blue). For each row, NORE1A and tubulin images and a superimposed image containing an additional image of nuclei stained with DAPI are shown. The arrows indicate centrosomes. Bar, 10 μm. B and C, purified tubulin was polymerized into microtubules (+MT) or left unpolymerized (--MT), and equal amounts of it were mixed with A549 cell lysate expressing GFP-NORE1A (B) or 293 lysates, which express FLAG-NORE1A or FLAG-NORE1A protein that was expressed in 293 cells, purified on FLAG-agarose, and eluted with FLAG peptide (C). After incubation for 30 min at room temperature, microtubules were pelleted, and equal amounts of supernatant and pellet were analyzed by Western blotting with anti-NORE1A (B) or FLAG (C) antibody (top). Bottom, the distribution of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a cytoplasmic marker, is shown. D, endogenous NORE1A cofractionates with centrosomes in HBEC3-KT. Centrosomes were isolated as described in Ref. 29. Equal aliquots of dissolved centrosomal pellet from each fraction was subjected to Western blotting (WB) for NORE1A (top) and a centrosomal marker, γ-tubulin (bottom). Fraction 1 was at the top of the gradient. Representative experiments are shown.
We tested whether this function is necessary for NORE1A subcellular localization by using its Ras association domain mutant K306E,F307A, which is unable to bind Ras (Fig. 4A). This mutant is expressed at a level comparable with wild type NORE1A, both in 293 (Fig. 4A) and A549 cells (Fig. 4B and data not shown). As Fig. 4B displays, the K306E,F307A mutant in a NORE1A 191–363 fragment has the same cytoskeletal localization as NORE1A 191–363 without the RA domain mutation. Thus, the Ras association domain has function(s) other than Ras binding, such as to determine the proper subcellular localization of NORE1A. This latter function is independent of Ras binding.

However, the RA domain is not the only structural determinant of NORE1A that is responsible for cytoskeletal localization. As shown in Fig. 3, bottom row, the C-terminal part of NORE1A, aa 254–416, consisting of RA and MST-binding domains, is strictly cytoplasmic, suggesting that both RA and central domains of NORE1A are required for its cytoskeletal localization.

To determine whether Ras binding is important for the growth-suppressive function of NORE1A, we employed the NORE1A RA domain mutant, K306E,F307A, deficient in binding to Ras (Fig. 4A). As shown in Table 2, the NORE1A K306E,F307A mutant of the full-length NORE1A was deficient in suppression of soft agar growth. However, when K306E,F307A mutation was introduced in the NORE1A 191–363 fragment, it did not interfere with its ability to induce A549 cell cycle arrest in monolayer culture (Fig. 4C) or soft agar growth (Fig. 1C). Thus, the Ras binding function of NORE1A is required for growth suppression of the full length protein but not its effector domain, amino acids 191–363.

The Association with Cytoskeletal Elements Is Essential for NORE1A Growth-suppressive Function—In summary, the data described are consistent with the hypothesis that the association of NORE1A with centrosomes and microtubules is required for its growth-suppressive capabilities. We sought to test this hypothesis by investigating...
growth-suppressive capabilities of a NORE1A mutant defective in binding to these cytoskeletal components. For this purpose, we deleted amino acids 226–253 in the NORE1A central domain. In the closest NORE1A homologue, RASSF1A, similar deletions made the protein defective in both microtubule binding and growth suppression (31, 32). In A549 cells, the NORE1A 191–363 fragment containing an aa 226–253 deletion has little or no ability to localize to centrosomes and microtubules and is also deficient in inducing G1 cell cycle arrest (Fig. 4, B and D). This suggests that interaction with cytoskeletal elements is required for growth suppression by the NORE1 effector domain.

Expression of NORE1A Effector Domain Inhibits ERK Activity—We sought to determine the mechanism of NORE1A 191–363 growth inhibition using A549 cells as a model. A549 cells contain active Ras; therefore, several signal transduction pathways that are downstream of Ras might be activated. Examination of two major pathways involved in cell proliferation and cell survival, Raf/Mek/Erk and PI3K/Akt, revealed that they are indeed activated in A549 cells (Fig. 5, A–C). Expression of full-length NORE1A had no effect on the activity of either of these pathways (Fig. 5, A and B, lane 1). In contrast, when the NORE1A 191–363 growth-inhibitory fragment was expressed in A549 cells, it potently inhibited activation of ERK1 and ERK2 without any apparent change in Akt activation (Fig. 5, A and B, lane 2). The NORE1A 191–363 containing the mutation in the Ras association domain K306E,F307A was also capable of suppressing ERK activity in A549 cells (supplementary Fig. 4).

For the closest NORE1A homologue, RASSF1A, a naturally occurring RA domain mutant, R257Q, was described that is defective in growth suppression (31). Interestingly, NORE1A has a glutamine residue in the analogous position, Gln331; we replaced it with arginine to create NORE1A Q331R. This mutant expressed well in 293 and A549 cells (Fig. 4, A and C) and was capable of binding to Ras (Fig. 4A). However, in A549 cells, the NORE1A 191–363 fragment containing the Q331R mutation has little or no ability to induce G1 cell cycle arrest (Fig. 4C) and suppress ERK activity (Fig. 5A, lane 3). Similarly, the NORE1A 191–363 fragment containing an aa 226–253 deletion deficient in inducing G1 cell cycle arrest was also deficient in suppressing ERK activity (supplemental Fig. 4).
MAP/ERK kinases 1 and 2 (MEK1/2) are the only upstream activators of mammalian ERK known to date (33, 34). We tested the effect of expression of NORE1A 191–363 on MEK activity in A549 cells using the antibodies specific for the doubly phosphorylated, active MEK isoform. As shown in Fig. 5C, the active MEK is much less abundant in cells expressing NORE1A 191–363, whereas the expression of wild type NORE1A or the NORE1A 191–363 Q331R mutant or GFP has no apparent effect on MEK activity. This suggests that the NORE1A effector domain blocks MEK activation in A549 cells. Thus, the ERK pathway appears to be the target for the NORE1A effector domain, amino acids 191–363, in A549 lung adenocarcinoma cells.

**DISCUSSION**

To our knowledge, this study provides the first detailed structure-functional characterization of the NORE1A tumor suppressor. Our data suggest that central and Ras association domains represent a NORE1A effector domain that is responsible for growth-suppressive capabilities, subcellular localization to the microtubular cytoskeleton, and inhibition of signaling through the ERK pathway.

In our studies, wild type NORE1A induced minor changes in cell cycle distribution of A549 cells growing in monolayer, usually an increase of 5% in the G1 population (Table 1), although in some experiments, it was closer to 10% (Fig. 4D). Recently, Aoyama et al. reported that NORE1A induced an ~10% G1 increase in A549 cells (23). In several other cell lines, such as LNCaP, NCI-H23, H157, H358, and CaKi-1, NORE1A induced little or no changes in cell cycle distribution. Expression of GFP-NORE1A did not have apparent toxicity and was maintained in cells growing on plastic for at least 1 month (data not shown). In contrast, the closest NORE1A relative, RASSF1A tumor suppressor, in our studies and those by others (35) induced strong G1/M cell cycle arrest in A549 cells (supplemental Fig. 1). The effect of NORE1A on A549 is distinctly different from that observed with RASSF1A and might reflect differences in mechanisms of action of the two proteins.

Despite the relatively weak effect of NORE1A on cell cycle distribution in monolayer cultures, it potently suppresses soft agar growth of A549 and LNCaP cells (Table 2). The ability to inhibit anchorage-independent growth is a measure that correlates best with tumor suppression in vivo (30). Thus, in studies of NORE1A and possibly other tumor suppressors of the NORE/RASSF family, one should interpret effects (or absence of effects) of expression of these proteins on the cell cycle distribution with caution and use other more stringent tests to evaluate the tumor suppressor function. In addition, our data suggest that wild type NORE1A may exert growth-suppressive effects only on cells that have lost contact with the substrate.

In our studies, NORE1A growth-inhibitory effects were seen in tumor cells with both wild type (LNCaP) and mutationally activated Ras (A549) (36, 37). This suggests that NORE1A growth-suppressive effects might not be restricted to tumors with mutated Ras allele, as was previously suggested (25).

Using the soft agar assay with NORE1A truncated mutants, we determined that the NORE1A structural determinants essential for growth suppression are located within amino acids 191–363 (i.e. central and Ras association domains of the protein). Thus, the proline-rich, zinc finger, and MST-binding SARAH domains of NORE1A are dispensable for the growth-suppressive effects. The data on MST involvement are in agreement with the results obtained by a different assay, suppression of the colony-forming ability of A549 cells on a plastic surface (23). Since soft agar and colony-forming assays measure different aspects of the transformed phenotype, MST kinases do not appear to be required for the NORE1A growth-suppressive effects, at least in A549 and LNCaP cells. However, MST kinase may be necessary at other points of the NORE1A life cycle, such as attenuation of growth-suppressive signals at conditions of mitogenic stimulation of substrate-attached cells. Our data suggest that the NORE1A 191–363 fragment induces more pronounced G1 cell cycle arrest of A549 cells compared with the NORE1A 191–416 fragment that also contains the MST-binding domain. This argues in favor of a different function for the MST-binding domain (Table 1). The two N-terminal NORE1A domains, proline-rich (aa 1–118) and zinc finger (aa 119–167), appear to be involved in attenuation of the growth-inhibitory effects, since their deletion enhances both the ability of NORE1A to induce G1 cell cycle arrest of A549 cells and suppress anchorage-independent growth. It is possible that conformational changes of these domains, for example triggered by post-translational

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**FIGURE 5.** The effect of wild type (W) NORE1A and its mutants on ERK and Akt kinase activity in A549 cells. A, A549 cells were infected with retroviruses coding for hrGFP or hrGFP fusions as indicated and lysed with electrophoresis sample buffer. Lysates were analyzed by Western blotting (WB) for ERK phosphorylation status (top) and ERK expression (middle) as described under “Material and Methods.” Bottom, expression of NORE1A or its fragments, assayed by rabbit anti-NORE1A polyclonal antibodies. B, same lysates were probed for the Akt phosphorylation status (top) and Akt expression (bottom). C, same lysates were probed for the MEK phosphorylation status (upper panel) and MEK expression (lower panel). A representative experiment is shown.
modifications or binding to an additional regulatory protein(s), regulate effector domain activity.

We determined that Ras binding is required for full-length NORE1A to induce growth suppression in soft agar. Recently, Aoyama et al. suggested that the ability of NORE1A to suppress colony formation in monolayer is independent of NORE1A ability to bind Ras. The colony formation in monolayer and anchorage-independent assays measures different characteristics of the transformed phenotype: the ability of a single cell to form a colony on a plastic surface versus the ability of a single cell to form a colony without surface attachment. The anchorage-independent growth is considered to be a better measure of the transformed phenotype than the colony-forming ability on plastic (30). Recently, it has been shown that normal immortalized human bronchial cells are able to form colonies on plastic but are unable to grow in soft agar (28). Thus, the difference between our results and those of Aoyama et al. could be explained by differences in test systems used for evaluation of growth-suppressive capabilities of NORE1A Ras binding mutants.

However, in contrast with full-length NORE1A, the Ras binding function was not required for the growth suppression induced by the NORE1A effector domain, amino acids 191–363 (Fig. 1C). An attractive explanation for this difference might be that Ras binding is required for NORE1A activation (e.g. removal of an inhibitory effect of the N-terminal proline-rich and zinc finger or C-terminal SARAH domains on the NORE1A effector domain). A population of microtubule-bound Ki-Ras could play a role in NORE1A activation (38). If the NORE1A effector domain is expressed by itself, it no longer needs Ras for activation. However, the presence of active Ras is not sufficient to activate growth-suppressive functions of full-length NORE1A, since in A549 cells that have mutationally activated Ki-Ras, full-length NORE1A induce little or no growth arrest in monolayer (Table 1 and Fig. 4C).

Our data demonstrate for the first time that NORE1A is located to centrosomes in normal human cells (primary small airway epithelial cells and established HBEC3-KT), cells that express activated Ki-Ras and possess some characteristics of the transformed phenotype (HBEC3-KTR) and tumor cells (lung adenocarcinoma A549). This suggests that NORE1A centrosomal localization in cells growing in monolayer is independent of the presence of an activated Ras oncogene and the transformed phenotype.

In A549 cells, exogenous NORE1A, in addition to centrosomes, is targeted to microtubules at a low or moderate expression level. Moreover, in the *in vitro* microtubule co-sedimentation assay, NORE1A displays a potent ability to interact with microtubules. This suggests that NORE1A is capable of associating with microtubules within the cell in certain conditions. NORE1A may be transported on microtubules to or from centrosomes during some stage(s) of its life cycle, for example after synthesis of NORE1A protein. Alternatively, the interaction of NORE1A with microtubules could be specific for tumor cells, such as A549. If this is the case, such interaction might be important for NORE1A growth and tumor-suppressive function. Our finding that association of NORE1A with microtubules is indirect (Fig. 2C) suggests that it might be regulated. We are currently addressing the question of the importance of NORE1A-microtubular interactions.

The closest NORE1A relative, RASSF1A, was found on centrosomes and the spindle during mitosis, and a role for RASSF1A in the regulation of the stability of mitotic cyclins and the timing of mitotic progression has been described (35). Since we also found NORE1A on centrosomes and the spindle in mitosis, it may also participate in the regulation of mitosis.

Experiments with deletion and point mutants suggest that both the central and RA domains are required for cytoskeletal localization of NORE1A. Studies of RASSF1A protein demonstrate that its microtubule association depends on a stretch of amino acids 119–288, which include the RA and central domains of this protein (32, 39, 40). Thus, NORE1A and RASSF1A possibly share some common structural determinants required for microtubular and centrosomal localization within their central and RA domains. The mutation of amino acids 363Δ226–253 that abolishes Ras binding to NORE1A does not prevent targeting of the NORE1A 191–363 fragment to microtubules (Fig. 4B). Thus, the RA domain in NORE1A and RASSF1A clearly has functions other than Ras binding, such as to determine microtubular localization.

Our findings that the NORE1A effector domain mutant, aa 191–363Δ226–253, is deficient both in binding to microtubules and centrosomes and inducing G1 cell cycle arrest in A549 cells in comparison with NORE1A 191–363 (Fig. 4, B and D) strongly suggests that association with components of microtubule cytoskeleton is required for NORE1A-induced growth suppression. Currently, the reason for this is unknown. One explanation could be that NORE1A is capable of binding to its effector(s), essential for growth suppression, only on the microtubule cytoskeleton. Alternatively, activated NORE1A might change microtubular dynamics, resulting in growth suppression.

In an attempt to elucidate the molecular mechanism of NORE1A action, we discovered that the expression of the NORE1A effector domain, amino acids 191–363, suppress activation of the ERK pathway in A549 cells. The ERK pathway in this cell type is constitutively active, most likely as a result of the presence of an activated Ki-Ras allele. The association with the microtubular cytoskeleton appears to be required for suppression of the ERK pathway, since the NORE1A effector domain mutant 191–363Δ226–253, that does not localize to the cytoskeleton, was also deficient in suppression of the ERK activity. The NORE1A effector domain blocks the ERK pathway at the level of ERK activators, MEK1 or MEK2 protein kinases, or higher. Importantly, the NORE1A effector domain does not interfere with the activity of the Akt protein kinase, which is downstream of PI-3 kinase, another Ras effector (Fig. 5B). We also do not observe inhibition by NORE1A 191–363 of the activity of two other MAP kinases, c-Jun N-terminal kinase and p38 (data not shown). In addition, since the NORE1A effector domain mutant that is deficient in Ras binding, 191–363 K306E,F307A, retains the ability of suppressing ERK activity, the growth-inhibitory effect cannot be explained by titration of Ras by the effector domain of NORE1A (supplemental Fig. 4). Thus, the action of NORE1A effector domain appears to be selective toward the ERK pathway.

Based on our results, we hypothesize that NORE1A associates with cytoskeletal structures, centrosomes and microtubules, in normal and transformed cells. This association is required for its growth- and tumor-suppressive activities. The growth suppression requires NORE1A activation by binding to activated Ras or Ras-like GTPases. However, the presence of active Ras by itself is not sufficient for growth suppression, other input(s) are also required. Upon activation, NORE1A induces growth and tumor suppression through inhibition of signal transduction through the ERK pathway. This finding may have implications for the development of novel therapeutics for cancer treatment.

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REFERENCES

1. Bos, J. L. (1989) Cancer Res. 49, 4682–4689
2. Egan, S. E., and Weinberg, R. A. (1993) Nature 365, 781–783
3. Shields, J. M., Pruitt, K., McFall, A., Shaub, A., and Der, C. J. (2000) Trends Cell Biol. 10, 147–154
4. Marshall, C. J. (1996) Curr. Opin. Cell Biol. 8, 197–204
5. Vavvas, D., Elam, C., Dallol, A., Clark, G. J., and Latif, F. (2003) Oncogene 22, 2713–2720
6. Dammann, R., Lia, Q., Yang, J., Dallol, A., and Pfeifer, G. P. (2005) Cancer Res. 65, 3497–3508
7. Hesson, L., Bieche, I., Kreis, D., Cricenti, E., Hoang-Xuan, K., Maher, E. R., and Latif, F. (2004) Oncogene 23, 2408–2419
8. Chow, L. S., Lo, K. W., Kwong, J., Wong, A. Y., and Huang, D. P. (2004) Oncol. Rep. 11, 781–787
9. Eckfeld, K., Hesson, L., Vos, M. D., Bieche, I., and Clark, G. J. (2004) Cancer Res. 64, 8688–8693
10. Vos, M. D., Ellis, C. A., Ulku, A. S., Taylor, B. J., and Clark, G. J. (2003) J. Biol. Chem. 278, 28045–28051
11. Levy, P., Vidaud, D., Leroy, K., Laurendeau, I., Wechsler, J., Bolasco, G., Farait, B., Wolkenstein, P., Vidaud, M., and Bieche, I. (2004) Mol. Cancer 3, 20
12. Hesson, L. B., Wilson, R., Morton, D., Adams, C., Walker, M., Maher, E. R., and Latif, F. (2005) Oncogene 24, 3987–3994
13. Hesson, L., Dallol, A., Minna, J. D., Maher, E. R., and Latif, F. (2003) Oncogene 22, 947–954
14. Hsu, L. C., and White, R. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12983–12988
15. Cifone, M. A. (1982) Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
16. VanOmmen, G. J., and DiMaio, J. M. (2001) Hum. Genet. 12, 150–156
17. Hull, J., Rowlands, K., Lockhart, E., Sharland, M., Moore, C., Hanchard, N., and Ferretti, L. (2001) Mamm. Genome 12, 150–156
18. Dammann, R., Jin, S. G., Zhang, X. F., Avruch, J., and Pfeifer, G. P. (2002) J. Biol. Chem. 277, 5439–5442
19. Vos, M. D., Martinez, A., Ellis, C. A., Vallecorsa, T., and Clark, G. J. (2003) J. Biol. Chem. 278, 21938–21943
20. Chen, J., Lui, W. O., Vos, M. D., Clark, G. J., Takahashi, M., Schoumans, J., Khoo, S. K., Petillo, D., Lavery, T., Sugimura, J., Astuti, D., Zhang, C., Kagawa, S., Maher, E. R., Larsson, C., Alberts, A. S., Kanayama, H. O., and Teh, B. T. (2003) Cancer Cell 4, 405–413
21. Aoyama, Y., Avruch, J., and Zhang, X. F. (2004) Oncogene 23, 3426–3433
22. Ortiz-Vega, S., Khokhlatchev, A., Nedwidek, M., Zhang, X. F., Dammann, R., Pfeifer, G. P., and Avruch, J. (2002) Oncogene 21, 1381–1390
23. Khokhlatchev, A., Rabiszadeh, S., Xavier, R., Nedwidek, M., Chen, T., Zhang, X. F., Seed, B., and Avruch, J. (2002) Curr. Biol. 12, 253–265
24. Ortiz-Vega, S., Khokhlatchev, A., Nedwidek, M., Zhang, X. F., Seed, B., and Avruch, J. (2002) Curr. Biol. 12, 253–265
25. Vavvas, D., Li, X., Avruch, J., and Zhang, X. F. (2005) Cancer Res. 65, 3497–3508
26. Song, M. S., Song, S. J., Ayad, N. G., Chang, J. S., Lee, J. H., Hong, H. K., Lee, H., Choi, Y., Kurie, J. M., DiMaio, J. M., Milchgrub, S., Smith, A. L., Souza, R. F., Gilbery, L., Zhang, X., Gandia, K., Vaughan, M. B., Wright, W. E., Gazdar, A. F., Shay, J. W., and Minna, J. D. (2004) Cancer Res. 64, 9027–9034
27. Liu, L., Tommasi, S., Lee, D. H., Dammann, R., and Pfeifer, G. P. (2003) Mol. Cancer 2, 4112–4116
28. Ramirez, R. D., Shrivastava, S., Girardi, L., Sato, M., Kim, Y., Pollack, J., Peyton, M., Zou, Y., Kurie, J. M., DiMaio, J. M., Milchgrub, S., Smith, A. L., Souza, R. F., Gilbery, L., Zhang, X., Gandia, K., Vaughan, M. B., Wright, W. E., Gazdar, A. F., Shay, J. W., and Minna, J. D. (2004) Cancer Res. 64, 9027–9034
29. Rong, R., Jin, W., Zhang, J., Sheikh, M. S., and Huang, Y. (2004) Oncogene 23, 8216–8230
30. Cifone, M. A. (1982) Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
31. Dallol, A., Agathanggelou, A., Fenton, S. L., Ahmed-Choudhury, J., Hesson, L., Vos, M. D., Clark, G. J., Downward, J., Maher, E. R., and Latif, F. (2004) Cancer Res. 64, 4112–4116
32. Raman, M., and Cobb, M. H. (2003) J. Biol. 13, R886–R888
33. Seidel, C., and Russell, D. W. (2001) Nat. Cell Biol. 3, 891–896
34. Zhang, X. F., Dammann, R., and Pfeifer, G. P. (2002) J. Biol. Chem. 277, 5439–5442
35. Ramirez, R. D., Shrivastava, S., Girardi, L., Sato, M., Kim, Y., Pollack, J., Peyton, M., Zou, Y., Kurie, J. M., DiMaio, J. M., Milchgrub, S., Smith, A. L., Souza, R. F., Gilbery, L., Zhang, X., Gandia, K., Vaughan, M. B., Wright, W. E., Gazdar, A. F., Shay, J. W., and Minna, J. D. (2004) Cancer Res. 64, 9027–9034
36. Rong, R., Jin, W., Zhang, J., Sheikh, M. S., and Huang, Y. (2004) Oncogene 23, 8216–8230
37. Raman, M., and Cobb, M. H. (2003) J. Biol. 13, R886–R888
38. Hsu, L. C., and White, R. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12983–12988
39. Song, M. S., Song, S. J., Ayad, N. G., Chang, J. S., Lee, J. H., Hong, H. K., Lee, H., Choi, N., Kim, J., Kim, H., Kim, J. W., Choi, E. J., Kirschner, M. W., and Lim, D. S. (2004) Nat. Cell Biol. 6, 129–137
40. Valenzuela, D. M., and Groffen, J. (1986) Nucleic Acids Res. 14, 843–852
41. Pergolizzi, R. G., Kreis, W., Rottach, C., Susin, M., and Brosme, J. D. (1993) Cancer Invest. 11, 25–32
42. Thissen, A. J., Gross, J. M., Subramanian, K., Meyer, T., and Casey, P. J. (1997) J. Biol. Chem. 272, 30362–30370
43. Liu, L., Tommasi, S., Lee, D. H., Dammann, R., and Pfeifer, G. P. (2003) Oncogene 22, 8125–8136
44. Vos, M. D., Martinez, A., Elam, C., Dallol, A., Taylor, B. J., Latif, F., and Clark, G. J. (2004) Cancer Res. 64, 4244–4250