Identification of Nore1 as a Potential Ras Effector*

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The small GTP-binding protein Ras is pivotal in transmitting growth and differentiation signals downstream of cell surface receptors. Many observations have indicated that Ras transmits signals from cell surface receptors into multiple pathways via direct interaction with different effectors in mammalian cells. We have identified a novel potential Ras effector or target named Nore1. Nore1 has no significant sequence similarity to known mammalian proteins and lacks an identifiable catalytic domain, but contains sequence motifs that predict DAG, PE binding and SH3 domain binding. We show that Nore1 directly interacts with Ras in vitro in a GTP-dependent manner, and the interaction requires an intact Ras effector domain. Nore1 becomes associated with Ras in situ following activation of epidermal growth factor receptor in COS-7 and in KB cells.

The small GTP-binding protein Ras (Ha-, Ki-, and N-Ras) plays a central role in transmitting proliferative and differentiation signals downstream of cell surface receptors in mammalian cells. Ras has been demonstrated to relay signals from receptor tyrosine kinases (1), (e.g. EGFR (2) receptor), non-tyrosine kinase receptors (2) (e.g. T cell antigen receptor), and heterotrimeric G protein-coupled receptors (3). The understanding of the biochemical mechanism by which Ras transmits signals in higher eucaryotic cells has been greatly clarified in recent years. Ras is located at the inner surface of the plasma membrane; activation of cell surface receptors promotes the exchange of Ras-GDP for GTP, thereby converting Ras to the active state. This activation results from GTP-induced conformational change, wherein two discrete Ras segments, called switch I (or the effector domain loop aa 32–40) and switch II (aa 60–72) exhibit a significant replacement as compared with the GDP-bound state. This conformational change renders Ras able to interact effectively with its downstream effectors or targets (4). The first Ras effectors in mammalian cells to be identified are the protein kinases of the Raf family. GTP-bound Ras directly binds Raf primarily through an interaction between the switch I region and amino-terminal segment on Raf (amino acids 50–150). The ability of Raf to bind to Ras in a GTP-dependent manner, in vitro and in situ, is the cardinal biochemical evidence in support of Raf's role as a direct effector of Ras (5). The Raf-MEK-Erk pathway is the best characterized Ras effector pathway and is required for transformation of rodent fibroblasts by oncogenic Ras (6). However, in recent years, many observations have indicated that Ras transmits signals into multiple effector pathways. For instance, constitutively active Ras and Raf both transform NIH3T3 fibroblasts, but only constitutively active Raf, but not Raf, can transform rat intestinal epithelial cells (RIE-1), thus pathways besides the Raf-MEK-Erk pathway need to be activated to transform RIE-1 cells (7). Similarly, in PC-12 cells, activated Raf induces the expression of only a subset of genes which can be induced by oncogenic Ras or nerve growth factor (8). An elegant study demonstrated that in Hela cells and NIH3T3 fibroblasts, the increase in Ras-GTP charging achieved immediately after release from mitosis is much less than a second phase of Ras activation that occurred some 5 h later, in mid-G1. Interestingly, only the first phase of Ras activation was accompanied by Erk activation, whereas the latter, much stronger Ras activation occurred without significant Erk activation (9). The biologic significance of Ras activation in mid-G1 phase, and the nature of the effectors recruited by activated Ras at that time is entirely unknown.

Following on the discovery of Raf as the initial Ras effector in higher eucaryotic cells, a number of candidate Ras effectors have been proposed based on the ability of these polypeptides to bind to Ras through its effector loop, and in a GTP-dependent fashion, including PI 3-kinase, members of the Raf-GDS family, Rin 1, AF-6, diacylglycerol kinases, PKC-ζ, MEKK1, etc. The standing of these polypeptides as candidate Ras effectors has been reviewed (10, 11). We used the yeast two-hybrid system to look for novel proteins that directly interact with Ras. We describe here the identification of a potential new Ras effector, which we have named Nore1.

EXPERIMENTAL PROCEDURES

Two-hybrid Screen—A cDNA encoding V12-Ha-Ras deleted of the initial two amino acids was subcloned into vector pAS-CYH-II carboxy-terminal to the Gal-4 DNA binding domain to form the bait construct pAS-Ras. 100 μg of cDNA made from a mouse T cell library constructed in the Gal-4 DNA activation domain vector pACT was transformed into the yeasts expressing pAS-Ras, and the transformants were plated on His-Leu-Trp+ selection plates. After 8 days, 20 large colonies appeared. X-gal filter assay was performed for all the colonies and all showed strong blue color.

cDNA Cloning of Nore1—The 2.5-kb cDNA encoding Nore1 from the initial two-hybrid screen was labeled with [α-32P]dCTP and used to screen a cDNA library made from mouse brain (CLONTECH’s mouse brain 5’ -stretch plus cDNA library in λgt 10 vector, catalog number ML 3000a). A positive clone, which contains a 3-kb insert, was isolated.

Tissue and Cell Line Western Blot—Sprague-Dawley rats (65 g) were starved overnight, anesthetized with pentobarbital, and tissues were excised in the following order: gastrocnemius, testis, spleen, kidney, liver, lung, and heart. Brain was excised from other intact anesthetized animals after decapitation. Cell lines were grown to 80–90% confluence before harvesting. Both tissues and cell lines were disrupted and extracted in radiimmune precipitation buffer.

Detection of Ras/Nore1 Binding in Vitro—Purified, procarcyotic re-
combinant c-Ha-Ras (2.5 mg/ml) was loaded with GTP•S (2 mM) or GDP•S (2 mM) at 37 °C for 15 min in the buffer containing 50 mM Tris-HCl, pH 7.5, 7.5 mM EDTA, 2.5 mM MgCl₂, 0.5 mg/ml bovine serum albumin, 1 mM dithiothreitol. Various amounts of GTP•S- or GDP•S-loaded Ras proteins were mixed with purified procaryotic recombinant GST-Nore1 (188–413). Subsequent steps were essentially the same as described previously (12).

### Detection of Ras-Nore1 Association in COS-7 Transient Expression System

COS-7 cells were plated at a density of 1.2 million/10-cm dish and transfected 24 h later with 7 μg of phc2B-GST-GST-Nore1 using the DEAE-dextran method. 48 h later, cells were starved for 24 h and subsequently were stimulated with 100 ng/ml EGF for various times. Cells were extracted in lysis buffer (30 mM HEPES, pH 7.4, 1% Triton X-100, 20 mM NaF, 20 mM KCl, 2 mM EDTA, 7.5 mM MgCl₂, 14 mM β-mercaptoethanol, and a mixture of protease inhibitors). Lysates were freeze-thawed once and subsequently were stimulated with anti-HA antibodies and protein A-G-Sepharose beads for 3–4 h at 4 °C and then washed extensively with lysis buffer. The washed beads were eluted in SDS sample buffer and the extracted proteins subjected to SDS-PAGE, transferred on PVDF membranes, and probed using the antibodies indicated. Bound antibodies were visualized using ECL.

### Antibody Production

GST-Nore1-(188–413) was used to immunize New Zealand White rabbits. The antiserum was first depleted of GST-reacting antibodies by repeated incubation with immobilized GST. The GST-depleted antiserum was then affinity-purified using immobilized Nore1-(188–413) on a PVDF membrane.

### RESULTS AND DISCUSSION

A yeast two-hybrid screen was carried out to identify potential new Ras effectors in mammalian cells. One million yeast transformants coexpressing a V12 Ras bait plasmid and a cDNA library prepared from activated mouse T cells were screened. Twenty strong positives were obtained, which showed both interaction-dependent growth on selective media and interaction-dependent expression of Lac-Z activity. DNA sequencing revealed that 18 of the 20 positives were either mouse A-Raf or c-Raf-1. Two positive clones both encoded a novel Ras effector. Although the sequence indicated that an open reading frame of a 2.5-kb cDNA representing a new gene, which was named Nore1 (novel Ras effector). Although the sequence indicated that an incomplete open reading frame had been recovered, we made use of the yeast two-hybrid system to examine the specificity of the interaction of Nore1 with two Ras-related proteins Rap1b and RaIa, and two well defined Ras effector domain mutants, Ras 12V34,38A or Ras 12V38N, which are defective in binding known Ras effectors like Raf (14). Rap1b and RaIa belong to the Ras subfamily of small GTP-binding proteins (13); Rap1 (A and B) has identical sequence to Ras in the region corresponding to the Ras effector domain (aa 32–40) and binds to several previously identified Ras effectors like Raf, PI 3-kinase and RaI-GDS, whereas RaIa does not bind to these polypeptides. Nore1 interacts with wild type Ras but not with Ras 12V34,38A and Ras 12V38N. Nore1 also interact with Rap1b, but not with RaIa (Table 1). Thus, the interactions of Nore1 with these Ras-related proteins parallels closely the pattern exhibited by other well established Ras effectors.

The 2.5-kb Nore1 cDNA insert was used as the hybridization probe to isolate the entire cDNA from a mouse brain cDNA library. A 3018-base pair cDNA was isolated. The cDNA sequence around the first ATG matches the Kozak consensus sequence for a translational start. The open reading frame from this methionine includes 413 amino acids. The DAG_Pe binding domain and PX-PX motifs are underlined. B, alignment of Nore1 with C. elegans gene product T24F1.3. The predicted RA (Ras/Rap association) domain (16) in T24F1.3 is underlined (aa 396–496). The GCG command BESTFIT was used to create the alignment. GAP creation and extension penalties were 4 and 2, respectively.

![Figure 1](image-url)

**FIG. 1.** Predicted protein sequence of Nore1 and alignment with C. elegans gene product T24F1.3. A, the predicted protein sequence of Nore1. The open reading frame contains 413 amino acids. The DAG_Pe binding domain and PX-PX motifs are underlined. B, alignment of Nore1 with C. elegans gene product T24F1.3. The predicted RA (Ras/Rap association) domain (16) in T24F1.3 is underlined (aa 396–496). The GCG command BESTFIT was used to create the alignment. GAP creation and extension penalties were 4 and 2, respectively.
A single immunoreactive band at 46 kDa is seen in a brain extract, which is in agreement with the predicted size of the polypeptide encoded by Nore1 cDNA isolated from the mouse brain library. A similar 46-kDa band is also seen in other tissues, including lung and testis. In addition, however, prominent immunoreactive bands at other molecular masses are seen in most tissues, and some tissues lack a 46-kDa band entirely (e.g., skeletal muscle, heart, spleen, and liver). All tissues but brain show a major 65-kDa band, and two bands around 55 kDa are also seen in lung, spleen, testis, and liver. The 65- and 55-kDa bands may represent isoforms of Nore1, the existence of which is suggested by the partial cDNAs isolated from a variety of cDNA libraries (data not shown). Alternatively, these bands may reflect polypeptides unrelated to Nore1, except for the presence of sequence epitopes recognized by the polyclonal antibodies to Nore1. The anti-Nore1 antibody also immunoblotted a single polypeptide in an extract prepared from C. elegans. This band is approximately 74 kDa, as compared with the molecular mass of T24F1.3 gene product of 69.1 kDa. The murine brain Nore1 cDNA was tagged at the Nore1 amino terminus with an HA epitope and expressed transiently in COS cells. As seen in Fig. 2C, HA-Nore1 shows the expected size of 46 kDa by immunoblot with anti-Nore1 antibodies. Extracts prepared from several cell lines were subjected to Nore1 immunoblot; of the cell lines examined, only BC3H1, a vascular smooth muscle-like line derived from a radiation-induced murine brain tumor, shows a single band at 46 kDa. A band of similar size is seen in several other cell lines, including RIE-1 (rat intestinal epithelial), MCF-7 (human breast cancer), HEK 293 (human embryonic kidney), and KB (human oral carcinoma); however, immunoreactive polypeptides of 55 kDa (RIE-1, MCF-7, HEK 293, and KB) and 65 kDa (RIE-1, HEK 293, and KB), are as or more abundant in these cell lines, and some lines show only bands other than the 46-kDa polypeptide (e.g., Huh-7, 40 kDa; L6, 55 kDa). We preabsorbed the affinity-purified anti-Nore1 antibodies with an excess amount of recombinant Nore1-(188–413) for 1 h and used this preabsorbed antibodies to probe the blots used in Fig. 2B and C, and we did not see the predominant bands at 46, 55, and 66 kDa, suggesting that these bands in both figures are probably specific.

A GST-Nore1-(188–413) fusion protein (corresponding to the Nore1 polypeptide encoded in the initial cDNA isolate) was expressed and purified from Escherichia coli. Procaryotic recombinant c-Ha-Ras was loaded with GTP \( \gamma \) or GDP \( \gamma \)S, and various amounts were mixed with a fixed amount of GST-Nore1-(188–413) or GST as control. After incubation at 30 °C for 20 min, GST or GST fusion proteins and any associated proteins were recovered by addition of glutathione-Sepharose beads. The beads were washed and eluted into SDS sample buffer; proteins were separated by SDS-PAGE, transferred to PVDF membrane, and probed for Ras using a monoclonal antibody, 12CA5. The washed immunoprecipitates were eluted with glutathione-Sepharose beads.

We then attempted to detect an interaction between Nore1 and Ras in mammalian cells and to determine whether this binding was dependent on Ras activation in situ. COS-7 cells were cotransfected with plasmids encoding GST-Nore1 and HA-tagged c-Ha-Ras. Forty-eight hours later, cells were serum-starved for 24 h and then stimulated with EGF or TPA for various times, extracted into buffer containing Triton X-100, and HA-Ras was recovered using the anti-HA monoclonal antibody, 12CA5. The washed immunoprecipitates were eluted.
Nore1, a Novel Ras Effector

**Fig. 3.** Specific, GTP-dependent interaction of purified recombinant Nore1 and Ras polypeptides in vitro. V12-Ras protein purified from bacterial expression was loaded with either GTPyS or GDPβS. The loaded Ras proteins were incubated with GST or GST-Nore1(188–412). Glutathione-Sepharose beads were used to pull down the Ras-Nore1(188–412) complexes. Ras protein was detected using the pan-Ras antibody-2 (Oncogene Science) in the Western blot shown above.

into SDS sample buffer and separated by SDS-PAGE, transferred to PVDF membrane, and probed with affinity-purified anti-GST polyclonal antibodies. As seen in Fig. 4A, GST-Nore1 was specifically pulled down with HA-c-Ha-Ras, but only after the cells were treated with EGF or TPA; the expression of HA-c-Ha-Ras and of GST-Nore1 was uniform throughout. Thus, Nore1 is not detectably associated with Ras in serum-starved COS cells; however, within 5 min after stimulation by EGF (or TPA), Nore1 associates specifically with Ras; this association diminishes by 15 min after EGF addition and is largely reversed by 40 min, probably reflecting the down-regulation of Ras activation after EGF treatment.

We next attempted to detect an in situ association between endogenous Ras and endogenous Nore1, under conditions where the levels of the two polypeptides are not increased artificially by transient overexpression. We chose to examine the human oral carcinoma cell line KB, because Nore1 expression is readily detectable, and these cells express substantial numbers of EGF receptors. KB cells grown to 80% confluence were serum-starved for 24 h and then treated with EGF for various times. Triton X-100-soluble cell lysates were subjected to immunoprecipitation using the monoclonal anti-Ras antibody, Y13-238, which are known to enable isolation of Ras-Raf complexes. The Ras immunoprecipitates were washed extensively with the lysis buffer, eluted into SDS sample buffer and subjected to SDS-PAGE, transferred to PVDF membrane, and immunoblotted with the affinity-purified polyclonal anti-Nore1 antibodies. As shown in Fig. 4B, although equal amounts of endogenous Ras were recovered in all samples, the Ras immunoprecipitates contain immunoreactive Nore1 only after treatment of the cells with EGF. The time course of Ras-Nore1 association after EGF treatment in KB cells is more sustained than that observed in COS-7 cells. This may reflect different time course of down-regulation of Ras activation in those cells. Interestingly, only the 46-kDa (and not the equally abundant 55-kDa) immunoreactive Nore1 polypeptide is recovered with c-Ras.

In summary, we have identified Nore1, a potential new Ras effector or target, using the yeast two-hybrid screen with Ras as bait. We show that Nore1 can bind Ras directly in vitro using purified recombinant Ras and Nore1 polypeptides. The Ras/Nore1 association in vitro depends strongly on Ras being in the GTP-bound form. We show that with yeast two-hybrid assay, Nore1 interacts with Ras 12V but not two transformation defective effector loop mutants, Ras 12V343A and Ras 12V38N. This profile of interaction with Ras is identical to that exhibited by known and potential Ras effectors, including Raf, PI 3-kinase, Raf GDS, Rin1, and AF-6. We also show that the Ras/Nore1 association occurs in vivo following EGF and TPA activation of Ras in COS-7 cells overexpressing Ras and Nore1. Finally, it is clear that a stimulus-dependent association of endogenous Ras and Nore1 occurs following EGF receptor activation in KB cells. To our knowledge, Nore1 is the only other candidate mammalian Ras effector, other than Raf, wherein the endogenous polypeptide has been demonstrated to associate with Ras in vivo following receptor activation. Taken together, these properties indicate that Nore1 is very likely to be a physiologic Ras effector.

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