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This project aims to devise strategies to antagonize the promitogenic action of Ras and thereby suppress the transforming activity of the Erb2 oncogene found in 70% of human breast adenocarcinomas. We have focussed on the identification and characterization of proteins that interact with Rap-1 and Ras through their effector loop in GTP-dependent fashion. We have carried out an extensive two-hybrid screening for proteins that bind to Ras and Rap 1. This has yielded the known Ras-Rap partners and a novel set of noncatalytic polypeptides which have provided the main focus of our efforts. The first of these to be characterized was the polypeptide known as AF-6; this binds more avidly to Rap 1-GTP than to Ras-GTP. Of greater interest is the polypeptide NORE-1, which binds to Ras-GTP at its carboxyterminus and encodes a centrally located zinc finger, and an aminoterminal proline rich domain. High affinity antibodies to NORE-1 cross-react with a family of polypeptides, some of which bind to Ras-GTP *in vivo* after growth factor stimulation. Recently, a candidate tumor suppressor locus on chromosome 3p21 (frequently deleted in Breast Cancer) was identified as a protein RDA32, a 271AA polypeptide that is 55% identical to NORE1. Ongoing work is focussed on the biologic activation of the NORE-1 family of Ras effectors and their possible role in the negative regulation of cell growth.

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Introduction:

Breast cancer is believed to arise from a multistep process involving multiple somatic mutations resulting in the generation of oncogenes and loss of suppressor genes. The process is superimposed on an initial genotype that may contain predisposing mutations (e.g. BRCA1) and is influenced strongly by ovarian steroid hormones (1).

The most common dominant oncogenes found in breast cancer arise from activating mutations in the Erb B2 receptor tyrosine kinase (RTK) resulting in a potent continuous promitogenic signal (1, 2). In all cellular systems examined, the mitogenic capacity of RTKs requires the recruitment of the Ras GTPase (3, 4, 5). Ras itself when mutated to a constitutively active (GTPase deficient) form is a very potent oncogene. Such Ras mutations are very unusual in breast cancer (6), but cRas is frequently overexpressed (2), and this phenotype, when it occurs concomitant with Erb B2 activation, a mutation that occurs in 70% of breast cancers, is associated with a particularly poor prognosis (2).

The overall goal of the studies proposed is to better understand the biochemical mechanism by which RTKs, acting through Ras, promote growth in the breast epithelial cell, and to identify strategies that can be used to antagonize the promitogenic signal conveyed by Ras.

Approximately 4 years ago, work from this and several other laboratories (reviewed in 7) uncovered the first direct evidence as to the biochemical mechanism by which activated Ras promotes cell growth. We showed that the active, GTP bound form of Ras bound directly to regulatory domain of the protein (Ser/Thr) kinase protooncogene, cRaf-1; in fact all three members of the Raf subfamily (ARaf and BRaf) exhibit this interaction. Moreover, introduction of activated, Ras independent forms of Raf into fibroblasts led to transformation. Earlier work from our lab had shown that a major substrate of the Raf protein kinase is the dual specificity kinase known as MAPK kinase or MEK (8), which is the immediate activator of the MAP kinase. In fact, several groups showed that expression of constitutively activated forms of MEK was sufficient to give transformation of murine fibroblasts.

These results suggested that the ability of Ras to bind Raf, recruit it to the plasma membrane and initiate its activation provided a sufficient explanation for Ras' potent transforming action, and strategies that interfered with the Ras/Raf interaction were likely to be antimitogenic.

Body:

One plausible strategy that provided the initial focus for the present proposal was suggested by the properties of the Ras-related small GTPase, Rap-1. Rap-1 is about 50% identical

to Ras in amino acid sequence, and completely identical over the amino acids corresponding to the so-called Ras effector domain, (Ras residues 32-44) (reviewed in 9). Rap-1A was first isolated as a cDNA capable of causing reversion of the morphologic and growth phenotype of V12 Ras transformed fibroblasts (9). The ability of Rap-1 overexpression to revert Ras transformation, together with the identity of the Rap effector domain to that of Ras suggested that Rap-1 might bind Ras' mitogenic effectors, and sequester them in an inactive state. In fact we were able to show that Rap-1 is capable of binding c-Raf in a yeast expression system, in a manner similar to Ras (7). These findings led us to propose that Rap-1, which was known to be expressed in breast, might be recruited to serve as an endogenous Ras antagonist. We therefore proposed to:

1. Create antibody and cDNA reagents necessary for the study of Rap-1 in normal and malignant breast epithelia.

2. Characterize the regulation of the Raf-MAP Kinase pathway in normal and malignant breast epithelia.

3. Examine the effects of Rap-1 overexpression on Ras-directed signal transduction and cell growth.

4. Examine the regulation of Rap-1 activity by extracellular agonists.

5. Determine the nature of the major Rap-1 targets and their relation to Ras action.

6. Examine the control of Rap-1 gene and polypeptide expression in normal and malignant breast tissue.

As described in the 1st years progress report, we had created a satisfactory polyclonal anti Rap-1 anti serum (Task1) and initiated studies on the regulation of Rap-1 *in situ* and its interaction with Raf-1 (Tasks 2, 4). In addition, we had initiated an expression cloning effort to isolate the major Rap-1-associated cellular proteins that might account for Rap-1's anti Ras action, and other of Rap-1's cellular effects. This latter effort (Task5) proved to be unusually productive, and led to the isolation of several sets of novel candidate Rap-1 and Ras interacting proteins. Two of these sets comprised polypeptides whose sequence encoded motifs that gave clear indication of their catalytic function. One set, of course, were the Raf family kinases, whose binding to Rap-1 had been observed by us, and whose role in Ras signaling is well established. A second set of Rap-1 interactors consisted of four distinct polypeptides which each encoded a domain homologous to the catalytic domain of guanyl nucleotide exchange proteins for small GTPases of the Ras superfamily. These four proteins included:

1. cDNAs identical to those previously reported as encoding a Ral specific exchanger (Ral GDS) (10-13).

2. cDNAs sharing about 60% identity to Ral GDS, reported by several groups as Ral GDS-like (RGL) (11).

3. cDNA encoding a protein about 30% identical to Ral GDS and RGL, which was recently reported as RLF (14).

4. One novel, as yet unreported polypeptide which contains a GDS catalytic domain approximately 30% identical to several functionally characterized GDS enzymes, including the Ras specific in m-SOS, the Rap-1 specific C3G, etc.

In addition to these two categories of catalytic polypeptides, we recovered 3 other categories of cDNA encoding polypeptides which lacked catalytic domains, although several encoded protein domains known to be important in protein-protein or protein-lipid interaction, such as a zinc finger domain, a pleckstrin homology domain, ankyrin (ANK) repeats, etc.

Each of these polypeptides, like Raf, interacted both with Rap-1 and Ras in a yeast expression system, in an effector domain-dependent way, indicating that the bound preferentially to the GTP charged forms of the GTPases, and like Raf, were candidate effector molecules. At this point we were faced with the choice of devoting effort primarily to the further characterization of these new candidates (Task5) or proceeding with the descriptive studies of Rap-1 overexpression and regulation (Tasks 2 and 3).

Our decision was strongly influenced by emerging reports from a number of laboratories which indicated that multiple Ras-activated pathways in addition to Raf appeared be required for the transforming action of Ras in many cell backgrounds, (15, 16). Moreover, activated Raf was not capable of transforming a variety of epithelial cell lines that were potently transformed by V12 Ras, including the MCF-10A human breast epithelial cell line (17). This result indicated that although Raf remained an important effector of Ras, other mitogenic effectors, remained to be identified, including elements that were especially important in breast and other epithelial lines (e.g. colonic epithelia, etc.). We therefore elected to focus our effort on the characterization of the novel candidate Ras and Rap-1 effectors we had cloned during the initial period.

We first characterized the polypeptide known as AF-6. AF-6 was originally cloned as one of the multiple 3' fusion partners of ALL-1, a gene involved in the chromosome 11q 23 translocations present in 5% of acute myeloid and lymphoblastic leukemia, and in the majority of infant leukemias (19). The human AF-6 gene encodes a 1612 amino acid protein; northern blot shows that the AF-6 gene is widely expressed in adult mouse tissues. The expression level is highest in brain, liver and kidney, lowest in spleen (data not shown). The two AF-6 cDNA fragments (0.6kb) isolated from the yeast two-hybrid screen as Rap-1 and encoded a fragment corresponding to amino acids 28-228 of human AF-6. This AF-6 fragment also interacted with H-Ras in the two-hybrid assay, but not with RalA or cRaf-1. A point mutation in the Ras effector domain (H-Ras 38A) abolished the interaction between Ras and AF-6. These results strongly

suggest that the common effector domain region in both Rap1b and H-Ras is required for interaction with AF-6.

We characterized the binding of AF-6 (28-228) to Rap-1 *in vitro,* and compared the interaction of AF-6 (28-228) with Ras and Rap-1. A purified prokaryotic recombinant GST-AF-6 (28-228) fusion protein was incubated with various amounts of Rap-1b, charged with either GTP-γ- S or GDP-βS. Rap-1b forms a complex with GST-AF-6 (28-228), but not with GST, demonstrating that the interaction between Rap-1b and AF-6 is direct and specific. Moreover, at every Rap-1b concentration examined, GST-AF-6 binds much more GTPγS-Rap-1b than GDPβS-Rap-1b, demonstrating that GST-AF-6(28-228) has a strong preference for GTP bound Rap-1 (Fig. 2a). GST-AF-6(28-228) also binds GTPγS Ras directly, and with a higher affinity than GDPβS Ras (Fig 2b).

The relative affinity of AF-6 (28-228) for Rap-1b and Ras was examined, and compared with the relative binding of Raf (1-257) to Rap-1 and Ras, using the same GTPase polypeptides and methods. In these experiments, a tracer amount of ^{32}P-γ-GTP Ras 61L was mixed with various amounts of GTP-γ-S-Rap-1b or GTP-γS-Ras proteins and a fixed amount of GST, GST-Raf (1-257) or GST-AF-6 (28-228) was then added. The GST fusion proteins (and the associated proteins) were recovered by addition of glutathione sepharose beads. The ^{32}P-γGTP radioactivity remaining bound to the washed beads was determined and, after subtraction of the ^{32}P-γ-GTP bound to GST alone, plotted against the concentration of the unlabeled competitor Rap-1 or Ras protein added (Fig. 3). GTP-γ–S-Ras-displaces ^{32}PγGTP- Ras from GST-Raf (1-257) more effectively than does GTP-γ–S-Rap-1b (IC_{50} 20 μM vs 47 μM); thus GTP-γ–S-H-Ras has a higher affinity for Raf (1-257) than does GTP-γ–S-Rap-1b, as previously reported. By contrast, the binding of ^{32}PγGTP- Ras to GST-AF-6 (28-228) is displaced more effectively by GTP-γ–S-Rap-1b than by GTP-γ–S-H-Ras, (IC_{50} 8.4 μM vs 15.2 μM), indicating that GTP-γ–S-Rap1b has a higher affinity toward AF-6(28-228) than does GTP-γ–S-Ras.

Thus the aminoterminal region of AF-6 demonstrates a specific, direct interaction with both Rap-1b and Ras. The interaction requires an intact effector domain and is GTP dependent. The AF-6 aminoterminus binds more avidly to Rap-1b-GTP than to Ras-GTP, whereas the cRaf-1 aminoterminus exhibits a higher affinity for Ras-GTP than for Rap-1-GTP. Thus, although Raf and AF-6 can each bind to Ras and Rap-1, the higher affinity of AF-6 (and, as previously reported, Ral-GEF for Rap-1 over Ras suggests strongly that AF-6 and Ral-GEF are regulated primarily by Rap-1 *in situ,* whereas Raf-1 is primarily a Ras effector.

As our studies of AF-6 progressed, Kaibuchi and colleagues (20) reported the isolation of AF-6 from brain extracts by GTP-Ras affinity chromatography. These workers went on to show

that AF-6 was localized to sites of cell-cell contact, and interacted directly with the tight junction protein ZO-1 through the (AF-6) Ras binding domains, and that this interaction was disrupted V12 Ras (21) and this phenomenon may underlie the ability of V12 Ras to disrupt cell-cell contacts.

We next turned our effort to the characterization of a 51kDa protein, which we ultimately name NORE 1, which was isolated multiple library screens using both Ras and Rap 1 as bait

The NORE 1 open reading frame from this methionine includes 413 amino acids, yielding a highly basic polypeptide (PI=9.41) with a predicted molecular weight of 46.4 KD (22). One obvious structural feature is the presence of a cystein-histidine rich segment typical of a diacylglycerol/.phorbol ester (DAG_PE) binding site. NORE 1 also has a proline rich region in its aminoterminal region, with five PXXP sequences, which are possible SHE3 domain binding sites.

A polyclonal antibody was raised against a carboxyterminal fragment of NORE 1 and purified by affinity chromatography using the recombinant antigen. Immunoblot of extracts prepared from different rat tissue show a single immunoreactive band at 46 KD in a brain extract, which is in agreement with the predicted size of the polypeptide encoded by NORE 1 cDNA isolated from the mouse brain library. A similar 46 KD band was also seen in other tissues including lung and testes. In addition, however, prominent immuno-reactive to bands at other molecular weights are seen in most tissues, and some tissues lack a 46 KD band entirely (e.g. skeletal muscle, heart, spleen and liver). All tissues but brain, show a major 65 KD band, and two bands around 55 KD are also seen in lung, spleen, testes and liver. The 65 and 55 KD bands may represent isoforms of NORE 1, the existence of which is suggested by partial cDNAs isolated from a variety of cDNA libraries. Alternatively, these bands may reflect polypeptides unrelated to NORE 1, except for the presence of sequence epitopes recognized by the polyclonal antibodies to NORE 1. The murine brain NORE-1 cDNA was tagged at the
NORE 1 aminoterminus with an HA epitope and expressed transiently in COS cells. HA-NORE 1 shows the expected size of 46 KD by immunoblot with anti-NORE 1 antibodies. Extracts prepared from several cell lines were subjected to NORE 1 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 KD. 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 KD (RIE-1, MCF-7, HEK 293 and KB) and 65 KD (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 KD polypeptide (e.g., Huh-7, 40 KD, L6, 55KD).

A GST-NORE 1 fusion protein (corresponding to the NORE 1 polypeptide encoded in the initial cDNA isolate) was expressed and purified from *E. Coli.* Prokaryotic recombinant c-H-Ras was loaded with GTP-γ-S or GDP-β-S and various amounts were mixed with a fixed amount of GST-NORE 1 or GST as control. After incubation at 30^0 C for 20 minutes, 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 anti-Ras antibody. GST-NORE 1, but not GST bound Ras and considerably more Ras-GTP-γ-S is bound than Ras-GDP-β-S. These results establish that the effector loop-dependent interaction between NORE 1 and Ras identified by two-hybrid techniques reflects the direct binding of the two proteins, and that the binding between NORE 1 and Ras is GTP-dependent.

We next attempted to detect and *in situ* association between endogenous Ras and endogenous NORE, under conditions where the levels of two polypeptides are not increased artificially by transient overexpression. We chose to examine the human oral carcinoma cell line KB because NORE 1 expression is readily detectable and these cells express substantial numbers of EGF receptors. KB cells grown to 80% confluence were serum starved for 24 hours, and then treated with EGF for various times. Triton X-100 soluble cell lysates were subjected to immunoprecipitation using the monoclonal anti-Ras antibodies, 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, transfer to PVDF membrane and immunoblotted with the affinity purified polyclonal anti-NORE 1 antibodies. Although equal amounts of endogenous Ras was recovered in all samples, the Ras immunoprecipitates contain immunoreactive NORE 1 only after treatment of the cells with EGF.

Recent work has focussed on the elucidation of the biologic function of NORE 1. Based on the presumption that NORE 1 is serving as an "adaptor" we have screened a brain cDNA library by the two-hybrid method seeking proteins other than Ras which interact with NORE 1. The major partners retrieved are the microtubule- associated protein 1 b, and spectrin, suggesting that NORE 1 might serve as a Ras-regulated bridge between the microtuble network and the actin cytoskeleton. Recently, NORE 1 was reported to bind to the protein MSS4, which is thought to be an exchange protein (23) or chaperone (24) for the Rab family of GTPases. Thus, NORE 1 might be a Ras-regulated element in membrane traffic and targeting.

The most intriguing development is the recent Genbank submission of a 270 amino acid polypeptide called RDA32, whose sequence is 55% identical (72% similar) to NORE 1, especially over the region corresponding to the NORE 1 Ras binding domain. RDA32 is encoded on chromosome 3p21.3, a region thought to encode a putative tumor suppressor which is frequently

deleted in breast cancers and small cell lung cancers (25). This raises the possibility that NORE 1 and its family members are concerned with the negative regulation of cell proliferation.

Conclusion:

The goal of this work was to identify the mechanisms employed by Ras in promoting the transformation of epithelial cells, and to identify strategies for interfering with Ras' proliferative actions.

The initial focus was on the role of the Rap 1 protein, a putative Ras inhibitor, in breast cancer. Because of the descriptive and ultimately inconclusive nature of this effort, we turned to a search for new Ras/Rap 1 effectors which could either collaborate with Raf in the transformation of epithelial cells or might negatively regulate growth.

After an initial characterization of the protein AF-6, a Ras-regulated component of tight cell-cell contacts at junctions, we focussed effort on the first of a new family on noncatlytic Ras-GTP binding proteins, which we named NORE 1. The 51kDa isoform of NORE 1 is one member of a large family of immunochemically-related polypeptides that bind to Ras only after receptor tyrosine kinase activation *in vivo*. The nature of the Ras-regulated functions downstream of NORE 1 remain unclear, but the recent identification of RDA32 , a protein over 70% similar to NORE 1 in sequence, as a candidate tumor suppressor that is frequently lost in Breast Cancer, points to the possibility that NORE 1 and its homologs participate in the negative regulation of cell growth in response to Ras activation. This provides the basis for entirely new avenues for the control of cellular proliferation in Breast Cancer, and other Ras-dependent tumors.

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Communication

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.* EGF[1] 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 displacement 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 Ras, 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-G_1. 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-G_1 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 Ral-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 last four amino acids was subcloned into vector pAS-CYH-II carboxyl-termianl 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 out 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-^{32}P]dCTP and used to screen a cDNA library made from mouse brain (CLONTECH's mouse brain 5′-stretch plus cDNA library in λ-gt 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 gown to 80–90% confluence before harvesting. Both tissues and cell lines were disrupted and extracted in radioimmune precipitation buffer.

Detection of Ras/Nore1 Binding in Vitro—Purified, procaryotic re-

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[1] The abbreviations used are: EGF, epidermal growth factor; aa, amino acid(s); PI, phosphatidlyinositol; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; kb, kilobase pair(s); GTPγS, guanosine 5′-*O*-(thiotriphosphate); GDPβS, guanyl-5′-yl thiophosphate; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; HA, hemagglutinin; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DAG, diacylglycerol.

TABLE I
Two-hybrid interactions of Nore1 with Ras mutants and Ras-related proteins

Y190 was transformed with the indicated constructs and plated on Leu$^-$, Trp$^-$ selection plates. X-gal filter assay was performed on day 4 after the transformation.

Coexpression of GAL4 DNA binding domain Nore1-(188–413) fusion with GAL4 activation domain fused to:					
v-Ras	c-Rap1b	Ras[Δ34A38]	Ras[N38]	RalA	Raf-1
Blue	Blue	White	White	White	White

combinant c-Ha-Ras (2.5 mg/ml) was loaded with GTPγS (2 mM) or GDPβS (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 $MgCl_2$ 0.5 mg/ml bovine serum albumin, 1 mM dithiothreitol. Various amounts of GTPγS- or GDPβS-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 pMT2-HA-c-Ha-Ras or empty vector and 12 μg of pEBG-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 β-glycerophosphate, 2 mM $NaPP_i$, 1 mM orthovanadate, 20 mM NaF, 20 mM KCl, 2 mM EGTA, 3 mM EDTA, 7.5 mM $MgCl_2$, 14 mM β-mercaptoethanol, and a mixture of protease inhibitors). Lysates were freeze-thawed once and spun at 17,000 × *g* for 20 min. Supernatants were incubated 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.

Detection of Ras-Nore1 Association in KB Cells—KB cells were grown to 80% confluence, starved of serum for 24 h, and subsequently stimulated with EGF (100 ng/ml) for various time. Lysates were prepared as for the COS-7 cells, and Ras was immunoprecipitated with anti-Ras antibody (Y13-238) and protein A-G-Sepharose beads. Subsequent steps were similar to those used in the COS-7 experiment.

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 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 RalA, and two well defined Ras effector domain mutants, Ras 12VΔ34,38A or Ras 12V38N, which are defective in binding known Ras effectors like Raf (12). Rap1b and RalA 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 Ral-GDS, whereas RalA does not bind to these polypeptides. Nore1 interacts with wild type Ras but not with Ras 12VΔ34,38A and Ras 12V38N. Nore1 also interact with Rap1b, but not with RalA (Table I). Thus, the interactions of Nore1 with these Ras-related proteins parallels closely the pattern exhibited by other well established Ras effectors.

A

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  1 MASPAIGQRP YPLLLDPEPP RYLQSLGGTE PPPPARPRRC IPTALIPAAG
 51 ASEDRGGRRS GRRDPEPTPR DCRHARPVRP GLQPRLRLRP GSHRPRDVRS
101 IFEQPQDPRV LAERGEGHRF VELALRGGPG WCDLCGREVL RQALRCANCK
151 FTCHSECRSL IQLDCRQKGG PALDRRSPGS TLTPTLNQNV CKAVEETQHP
201 PTIQEIKQKI DSYNSREKHC LGMKLSEDGT YTGFIKVHLK LRRPVTVPAG
251 SGPSPSMDAI KEVNPAATTD KRTSFYLPLD AIKQLHISST TTVSEVIQGL
301 LKKFMVVDNP QKFALFKRIH KDGQVLFQKL SIADYPLYLR LLAGPDTDVL
351 SFVLKENETG EVEWDAFSIP ELQNFLTILE KEEQDKIHQL QKKYNKFRQK
401 LEEALRESQG KPG
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B

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Nore1      112 AERGEGHRFVELALRGGPGWCDLCGR...EVLRQALRCANCKFTCHSECR 158
T24F1.3    159 ANKINNHSFKTHSLL.HPTWCDKCGDFIWGILKEALKCEHCNYTCHARCR 207

Nore1      159 SLIQLDCRQKG.....................G....PAL..... 173
T24F1.3    208 DLVTLDCRSPGSSLASSTEFDSIYPQLDGTLGTIPKGLILPPAMSSSTGS 257

Nore1      174 DR.....RSPG.STLTPTLN.QN...VCKA...VE.....ETQHPP.... 201
T24F1.3    258 DKENGNGNSAGISAENPIFSVKNSFTLPKSFSPVDSLRTKEPSAPPESRY 307

Nore1      202 .TIQ.............EIKQ.....KIDSYNSREKHCLGMK..LSE 227
T24F1.3    308 ATLRVVERYVKEDTPFEWTDEYKEMDLERKIHSYNSLAK...GMEITLHE 354

Nore1      228 DG.TYTGFIKVHLKLRRPVTVPAGSGPSPSM.DAIKEV.NPAATTDKR.. 272
T24F1.3    355 DGVNFGGHIHVNMNLSRPISVVQGVIP.PTVYDVVNTAKSTAKTTSLRTI 403

Nore1      273 TSFYLPLDAIKQLHISSTTTVSEVIQGLLKKFMVVDNPQKFALF..KRIH 320
T24F1.3    404 TSFFLPRNTAKVINIDSKTTARKMIVTLLKKFRVADNPRKFALVECEQIT 453

Nore1      321 KDGQ.VLFQKLS.IAD..YPLYLRL.LAGPDTDVLSFVLKENETGEVEWD 365
T24F1.3    454 DEATCTLNRKLTRISDDACPLKVVLNWQSPHCG.RALVLQENDTGDILWD 502

Nore1      366 AFSIPELQNFLTILEKEEQDKIHQLQKKYNKFRQKLEEALRESQG      410
T24F1.3    503 AFEIPELENFLRILGMEEKQYVFQTQQKYQQYRYHLDAELRQ.RG      546
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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 PXXP 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.

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, as shown in Fig. 1*A*, yielding a highly basic polypeptide (pI = 9.41) with a predicted molecular mass of 46.4 kDa. One obvious structural feature of Nore1 is the presence of a cysteine-histidine-rich segment typical of a diacylglycerol/phorbol ester (DAG_PE) binding site (14) (aa 118–165, H-X_{13}-C-X_2-C-X_{10}-C-X_2-C-X_4-H-X_2-C-X_7-C). Nore1 also has a proline-rich region in its amino-terminal region, with five PXXP sequences (aa 17–20, PEPP; aa 31–34. PPPP; aa 34–37, PARP; aa 77–80, PVRP; and aa 105–108, PQDP), which are possible SH3 domain binding sites (15). A search of the GeneBank™ using BLASTP command found that a *Caenorhabditis elegans* gene product called T24F1.3 has significant homology to Nore1. Fig. 1*B* shows the sequence similarity; the most significant homology between the two proteins is in their carboxyl-terminal regions. T24F1.3 has been suggested previously to contain a Ras/Rap association domain (RA

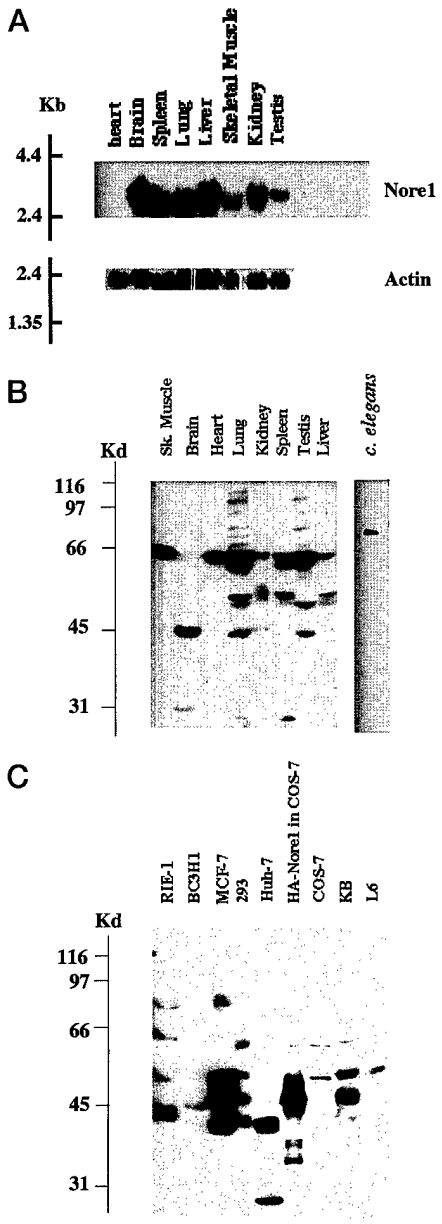

FIG. 2. **Expression of Nore1 mRNA in mouse tissues and immunoreactive Nore1 polypeptides in rat tissues and cell lines.** *A*, expression of Nore1 mRNA in mouse tissues. A 220-base pair cDNA fragment (nucleotides 90–310) was labeled with [^{32}P]dCTP by random priming method and used for probing Nore1 mRNA from various mouse tissues. The mouse multiple tissue blot was purchased from CLONTECH. *B*, protein expression of Nore1 in rat tissues. *C*, protein expression of Nore1 in cell lines. Cell lysates were all prepared in radioimmune precipitation buffer. Affinity-purified anti-Nore1 antibodies were used to probe the membrane. Bands were visualized by the ECL method.

domain) (16), located at aa 396–496. This domain is within the region most closely related in sequence to Nore1.

Nore1 mRNA abundance and complexity in murine tissues was examined by Northern blot (Fig. 2*A*). A single mRNA generally about 3.1 kb was detected in most mouse tissues, although some size variation is noted. The highest levels are observed in brain, liver, and spleen, with barely detectable levels in heart.

A polyclonal antibody was raised against a carboxyl-terminal fragment of Nore1 (aa 188–413) and purified by affinity chromatography using the recombinant antigen. Immunoblot of extracts prepared from different rat tissue is shown in Fig. 2*B*. 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. 2*C*. 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. 2, *B* 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 GTPγS or GDPβS, 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 anti-Ras antibody. GST -Nore1-(188–413), but not GST binds Ras, and considerably more Ras-GTPγS is bound than Ras-GDP-β-S (Fig. 3). These results establish that the effector loop-dependent interaction between Nore1 and Ras identified by two-hybrid techniques reflects the direct binding of the two proteins and that the binding between Nore1 and Ras is GTP-dependent.

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

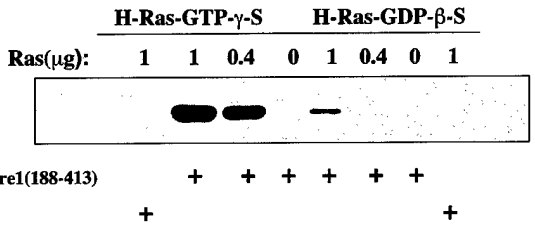

FIG. 3. **Specific, GTP-dependent interaction of purified recombinant Nore1 and Ras polypeptides *in vitro*.** V12-Ras protein purified from bacterial expression was loaded with either GTPγS or GDPβS. The loaded Ras proteins were incubated with GST or GST-Nore1-(188–413). Glutathione-Sepharose beads were used to pull down the Ras-Nore1-(188–413) 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. 4*A*, 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 immunobloted with the affinity-purified polyclonal anti-Nore1 antibodies. As shown in Fig. 4*B*, 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 12VΔ34,38A 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, Ral GDS, Rin1, and AF-6. We also show that the Ras/Nore1 association occurs *in vivo* following EGF and TPA

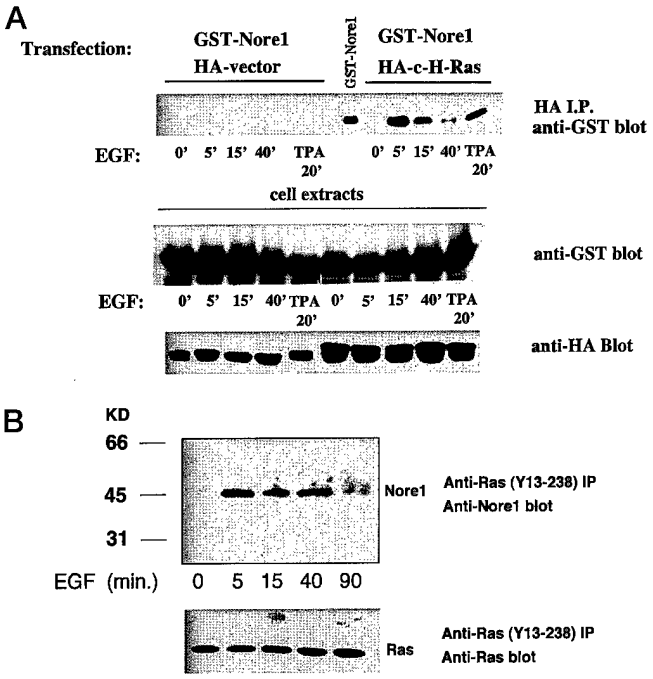

FIG. 4. **EGF and TPA stimulated association of Nore1 with Ras in transfected COS-7 cells and in nontransfected KB cells.** *A*, EGF and TPA stimulated association of Nore1 with Ras in transfected COS-7 cells. pEBG-Nore1 together with PMT2-HA or PMT2-HA-c-Ha-Ras were transiently expressed in COS-7 cells. Transfected cells were first serum-starved for 24 h and then stimulated with EGF (100 ng/ml) or TPA (100 nM) for the time indicated. Monoclonal anti-hemagglutinin antibodies were used to immunoprecipitate HA-c-Ha-Ras or HA alone. *B*, EGF stimulated association of endogenous Nore1 with endogenous Ras in KB cells. Confluent KB cells were serum-starved for 24 h and then stimulated with EGF (100 ng/ml) for various time. Triton X-100-soluble cell extracts were prepared, and the moloclonal anti-Ras antibody Y13-238 were used to immunoprecipitate Ras proteins.

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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Item 11:

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Publications:

1. Vavvas, D., L, Xin., Avruch, J., Zhang, X-F. Identification of NORE-1 as a potential Ras effector. *J. Biol. Chem.* **273**:5439-5442, 1998.

Meeting Abstract:

1. Identification of Novel Effectors of the Ras and Rap-1 GTPases

   Joseph Avruch, Amelia Marti, Zhijun Luo, Demetrios Vavvas and Xian-feng Zhang

Personnel:

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2. Xian-feng Zhang
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4. Amelia Marti Del Mora
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