The Dominant Negative Ras Mutant, N17Ras, Can Inhibit Signaling Independently of Blocking Ras Activation*

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Ras plays an important role in a variety of cellular functions, including growth, differentiation, and oncogenic transformation. For instance, Ras participates in the activation of Raf, which phosphorylates and activates mitogen-activated protein kinase kinase (MEK), which then phosphorylates and activates extracellular signal-regulated kinase (ERK), a mitogen-activated protein (MAP) kinase. Activation of MAP kinase appears to be essential for propagating a wide variety of extracellular signals from the plasma membrane to the nucleus. N17Ras, a GDP-bound dominant negative mutant, is used widely as an interfering mutant to assess Ras function in vivo. Surprisingly, we observed that expression of N17Ras inhibited the activity and phosphorylation of Elk-1, a physiological substrate of MAP kinases, in response to phorbol myristate acetate. The activity and phosphorylation of the MAP kinase hemagglutinin epitope (HA)-ERK1 were not affected by N17Ras in response to the same stimulus. Additionally, expression of N17Ras, but not L61S186Ras, a GTP-bound interfering mutant, inhibited MEK-induced Elk-1 phosphorylation, suggesting that inhibition of Elk-1 may be unique to GDP-bound Ras mutants. Finally, we observed that V12Ras-induced focus formation in NIH3T3 cells is inhibited by coexpression of GDP-bound Ras mutants, such as N17, A15, and N17N69. Therefore, N17Ras and V12 Ras may be codominant with respect to Elk-1 activation and cellular transformation. These results indicate that N17Ras appears to have at least two distinguishable functions: interference with endogenous Ras activation and inhibition of Elk-1 and transformation. Furthermore, our data imply the possibility that GDP-bound Ras, like N17Ras, may have a direct role in signal transduction.

Ras family GTPases cycle between inactive GDP- and active GTP-bound states. Oncogenic activation stabilizes Ras in a GTP-bound form, which is therefore constitutively active. Approximately 30% of all human tumors contain activating mutations in one of three Ha-ras genes (H, K, and N-ras) (1, 2). Expression of active Ras mutants in established cell lines can lead to cellular transformation, and the same mutants cooperate with the Myc oncoprotein to transform primary cells, demonstrating a key role for Ras in cellular transformation (3).

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Genetic studies in Drosophila and Caenorhabditis elegans have established that Ras plays critical roles in several developmental events, including photoreceptor differentiation and vulval development (2). Furthermore, microinjection of neutralizing Ras antibodies or expression of dominant negative Ras mutants demonstrated that Ras function is required for cell proliferation in response to serum and growth factors (4, 5).

GTP-Ras has been shown to interact physically with numerous downstream targets and to activate several different signaling pathways (1, 2). One of the best characterized Ras-activated pathways is the Raf-MEK1-ERK pathway, also known as the mitogen-activated protein (MAP) kinase cascade (6). Ras directly binds Raf in a GTP-dependent manner, and this interaction appears to be critical for the activation of Raf. Activated Raf phosphorylates and activates MEK, which in turn phosphorylates and activates the MAP kinase, ERK. Activation of ERK is essential for numerous Ras-induced cellular responses including transcription activation of immediate early genes, such as c-fos (6–9).

The promoter of the proto-oncogene c-fos has been characterized extensively and is now considered a paradigm of transcription regulation in response to extracellular signals, including serum (7, 9). The serum response element (SRE) within the c-fos promoter confers serum responsiveness to a basal promoter and functions via a transcription factor complex consisting of a dimeric serum response factor and, in some cases, an associated ternary complex factor (TCF) family member (7, 9). One well characterized member of the TCF family is a ubiquitously expressed 62-kDa protein, Elk-1. MAP kinases phosphorylate numerous serine and threonine residues in the COOH-terminal transactivation domain of Elk-1 and in doing so, increase its transactivation potential (10–15). TCFs are thought to play significant roles in the induction of c-fos in response to oncogenic Ras and a variety of growth factors and cytokines (10, 11, 16–18). Thus, phosphorylation of TCFs by activated MAP kinases reveals a linear pathway from Ras activation to transcriptional regulation.

N17Ras is a dominant negative Ras mutant that binds GDP with preferential affinity over GTP. This property allows N17Ras to inhibit endogenous Ras activation by sequestering Ras-GEFs (5, 19–26). Expression of N17Ras can effectively inhibit serum-dependent cell proliferation, and this effect can be reversed by coexpression of oncopgenic Ras or Ras-GEFs (5, 20, 21). Therefore, N17Ras has been proposed to inhibit selec-
tively wild-type, but not oncogenic, Ras (23). In contrast to N17Ras, L61S186 is a cytoplasmic, GTP-bound interfering Ras mutant (23, 27). L61S186Ras interferes with signaling via a mechanism that is likely to involve titration of effectors away from the endogenous, membrane-associated Ras. Consistent with this, L61S186Ras appears to block signaling from both wild-type and oncogenic Ras (23). These observations suggest that N17Ras should always be recessive to V12Ras.

However, the dogma that the active GTP-bound form of Ras is dominant with respect to GDP-Ras is somewhat perplexing. For instance, a single copy of an active Ha-ras gene, in the presence of a single copy of a wild-type Ha-ras gene, is not sufficient to transform Rat-1 cells (28). Furthermore, loss of a normal copy of ras has been observed in numerous tumors containing active mutant ras alleles (29–32). These observations suggest that the absence of normal Ras gene product may facilitate transformation by the remaining activated mutant ras allele. Moreover, a GDP-bound Ras mutant, N17Ras, can effectively block transformation or neuronal survival induced by oncopgenic Ras mutants (5, 25).

We examined the effects of expressing the dominant interfering Ras mutant, N17Ras, on growth factor and phorbol ester-induced signaling. Phosphorylation and activation of Elk-1, a well known MAP kinase substrate, in response to PMA was specifically inhibited by N17Ras expression. However, MAP kinase activity stimulated by phorbol esters was not affected by N17Ras. Expression of either N17Ras or A15Ras, another GDP-bound interfering mutant, inhibited Elk-1 activation induced by V12Ras. The ability of N17Ras to inhibit Elk-1 requires Ras membrane association. In contrast, the ability to inhibit Ras activation is not required for N17Ras to inhibit Elk-1 because N17N69Ras, a noninterfering GDP-bound Ras mutant, retains the ability to block Elk-1. Furthermore, we observed that focus formation in NIH3T3 cells induced by GTP-Ras (V12Ras) is inhibited by N17Ras although it does not affect the nucleotide loading of V12Ras. These observations suggest that N17Ras may have functions in addition to interfering with endogenous Ras activation.

MATERIALS AND METHODS

Cell Culture and Transfection—COS-1 and CV-1 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Life Technologies, Inc.). NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Life Technologies, Inc.). Transfections were performed using either DEAE-dextran as described previously (33) or LipofectAMINE (Life Technologies, Inc.) as recommended by the manufacturer.

Plasmid Construction—Expression vectors encoding Ras mutants were constructed by amplifying the appropriate mutant cDNA (templates encoding mutant Ha-Ras cDNAs were generously provided by Drs. L. Quilliam, Indiana University) by polymerase chain reaction followed by subcloning them into the mammalian expression vector pcDNA3.1 (Invitrogen) or pcDNA3-HA (33). The identities of all constructs were confirmed by DNA sequencing. Expression vectors encoding Elk-1, HA-ERK1, active MEK1 (MEK1*), active MEK3 (MEK3-DE), p38 MAP kinase, and V12Ras have been described (33). Expression vectors for I-OS (34) (pEF-FLAG-SC16), HA-ERK1 (33) (pMT2-HA-ERK1), and N17Rap1B (pcDNA-N17Rap1B) were kindly provided by Drs. J. Pessin (University of Iowa), Y. Zhao (University of Michigan), and P. Stork (Oregon Health Sciences University), respectively.

Luciferase Assays—In general, CV-1 or NIH3T3 cells in 3.5-cm wells were transfected with 50 ng of Ga4 TCF chimeras (10, 18) or Ga4-ATF2 (36), 100 ng of a 5x Ga4-luciferase reporter, and 100–250 ng of each expression vector, c-fos-luciferase (18), 4x AP-1 luciferase (Stratagene), and 5x NF-κB luciferase (Stratagene). Reporter genes were typically used at a concentration of 0.25 μg/3.5-cm well. Total DNA was kept constant by the addition of the appropriate amount of pcDNA3.1 for all transfections. Luciferase assays were performed as described previously (33) and normalized for transfection efficiency and using a cotransfected β-galactosidase expression vector.

Immunoblots—Whole cell extracts were separated by SDS-poly-
strong inhibitory effect of N17Ras on TCF-dependent reporter activity is that, in contrast to previous reports, N17Ras may block the activation of MAP kinase in our cell lines in response to PMA. To test this possibility, we expressed HA-tagged ERK1 (HA-ERK1) in COS-1 cells and measured PMA- or EGF-induced HA-ERK1 activity in an immunocomplex kinase assay using myelin basic protein as a substrate. Expression of N17Ras, which resulted in >80% inhibition of Gal4-ElkC reporter activity, had no effect on PMA-stimulated HA-ERK1 activity (Fig. 2A, compare columns 3 and 6). EGF-stimulated HA-ERK1 activity, however, was inhibited by N17Ras (Fig. 2A, compare columns 2 and 5). In contrast to N17Ras, dnMEK expression blocked both PMA- and EGF-stimulated HA-ERK1 activity (Fig. 2A, columns 2 and 8).

The activation state of ERK was also determined using a phosphorylation state-specific antibody (α-pERK). This antibody specifically recognizes the dual threonine/tyrosine-phosphorylated ERK1 and ERK2. The ERK polyclonal antibodies (α-ERK) used were raised against recombinant ERK1 and recognize endogenous ERK1 (Fig. 2B, middle bands), transfected HA-ERK1 (Fig. 2B, upper bands), and weakly recognize ERK2 (Fig. 2B, lower bands). Expression of N17Ras had no effect on HA-ERK1 phosphorylation induced by PMA (Fig. 2B, compare lanes 3 and 6). In contrast, N17Ras strongly inhibited EGF-stimulated HA-ERK1 phosphorylation (Fig. 2B, compare lanes 4 and 7). Similar results were observed in CV-1 cells (data not shown). In addition, we performed a time course from 0 to 8 h monitoring HA-ERK1 phosphorylation in response to PMA in the presence or absence of N17Ras. Expression of N17Ras had little effect on HA-ERK1 phosphorylation from 0 to 8 h of PMA treatment (Fig. 2C).

Together, these results support the idea that the two stimuli tested, EGF and PMA, are likely to activate ERK by distinct mechanisms, only one of which is sensitive to inhibition by N17Ras, as is the case with EGF (40). This is consistent with previously published reports that N17Ras expression does not interfere with the activation of ERK in response to PMA treatment in COS-1 cells (40, 41). However, these results do not explain our observation that PMA-stimulated Elk-1 activity is inhibited by N17Ras, because Elk-1 is a direct target of active MAP kinases.

We also examined the effects N17Ras on the activity of another ERK substrate, RSK1. RSK1 is a serine/threonine kinase whose activity is enhanced upon phosphorylation by ERK in vivo (35, 45). N17Ras had little effect on HA-RSK activity as determined by an immunocomplex kinase assay, both under basal and PMA-stimulated conditions. In contrast, expression of dominant negative MEK1 significantly abrogated PMA-stimulated HA-RSK1 activity (Fig. 2D). These observations indicate that HA-RSK activity is not inhibited by N17Ras, although ERK activation appears to be required for RSK activity.

Previous reports have established that the transactivation activity of Elk-1, as well as other TCF members, is enhanced by MAP kinase phosphorylation at specific Ser/Thr residues in its activation domain (13, 14, 18). In the case of Elk-1, phosphorylation at serine 383 leads to a significant enhancement of its trans-activation activity (10, 11, 15). Using an Elk-1 phosphoserine 383-specific antibody, we observed that N17Ras reduced PMA-stimulated serine 383 phosphorylation of Elk-1 (Fig. 2E). N17S186Ras, which is exclusively cytosolic (data not shown), was unable to inhibit Elk-1 phosphorylation, suggesting that membrane localization of N17Ras may be important for its ability to inhibit Elk-1 (Fig. 2E). These results support our above observation that N17Ras expression specifically reduces TCF transcription activity and further demonstrate that N17Ras inhibits Elk-1.

Effects of N17Ras or L61S186Ras on MEK-induced Elk-1 Phosphorylation—We tested whether the inhibition of Elk-1 is a common feature of dominant interfering Ras mutants or
FIG. 2. Elk-1 phosphorylation, but not ERK1 or RSK1 activity, is inhibited by N17Ras expression. Panel A, EGF- but not PMA-stimulated HA-ERK1 activity is inhibited by N17Ras expression. COS-1 cells were cotransfected with HA-tagged ERK1 together with either N17Ras, dnMEK1, or vector. Cells were either left untreated (solid bars) or stimulated for 5 min with 50 ng/ml EGF (light hatched bars) or 100 ng/ml PMA (dark hatched bars). HA-ERK1 activity was determined by an immunocomplex kinase assay using myelin basic protein as a substrate (upper panel). Lane 0 denotes transfection control without HA-ERK1. A portion of each kinase reaction was blotted and probed with α-ERK antibody (lower panel). Panel B, EGF-stimulated HA-ERK1 phosphorylation is blocked by N17Ras expression. COS-1 cells were cotransfected with HA-ERK1 and N17Ras or vector as in panel A. Quiescent cells were stimulated with either PMA or EGF for 5 min and harvested. Whole cell extracts were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting with α-ERK (upper panel) and α-pERK (lower panel). The two lower bands detected by ERK antibodies are caused by endogenous ERK1 and ERK2. Panel C, extended time course of ERK activation. COS-1 cells were transfected as in panel A. After serum starvation, cells were stimulated with PMA for the indicated times followed by lysis and immunoblotting. Panel D, PMA-stimulated HA-RSK1 activity is not altered by N17Ras. COS-1 cells were transfected with HA-tagged RSK1 in the presence or absence of N17Ras, dnMEK1, or vector. Serum-deprived cells were stimulated with PMA for 20 min. HA-RSK1 kinase activity was determined by an immunocomplex kinase assay (upper panel). A portion of each kinase reaction was blotted and probed with α-HA (lower panel). Shown for each are representative examples of at least three independent experiments. Panel E, N17Ras expression blocks PMA-stimulated Elk-1 phosphorylation at serine 383. COS-1 cells were cotransfected with expression vectors for Elk-1 and either vector, N17, or N17S186HRas. Cells were stimulated with PMA for 5 min, and extracts were blotted and probed with α-Elk-1 (middle panel), α-phospho-Elk-1 (upper panel), and α-Ha-Ras (lower panel α-HRas) as indicated.
unique to N17Ras. L61S186Ras is a cytosolic GTP-bound Ras mutant that dominantly interferes with Ras signaling (23, 27). Ectopic expression of L61S186Ras is likely to prevent membrane recruitment of Ras targets, such as Raf, by sequestering them from membrane-targeted GTP-Ras (23, 27). In contrast, N17Ras inhibits GTP-Ras formation. Therefore, L61S186Ras expression is thought to interfere with both wild-type and oncogenic Ras signaling (23). We therefore tested whether this mutant would inhibit Elk-1 phosphorylation like N17Ras. Expression of active MEK1* resulted in a large increase in both HA-ERK1 and Elk-1 phosphorylation as detected by phospho-specific antibodies (Fig. 3A, compare lanes 1–3). N17Ras expression resulted in an inhibition of MEK1*–induced Elk-1, but not HA-ERK1 phosphorylation (Fig. 3A, upper panels, compare lanes 3 and 4). In contrast, L61S186Ras had no detectable effect on either Elk-1 or HA-ERK1 phosphorylation induced by MEK1* (Fig. 3A, upper panels, compare lanes 3–5). However, the L61S186Ras used in these experiments inhibited endogenous GTP-Ras signaling because it effectively blocked EGF-stimulated HA-ERK1 and Elk-1 phosphorylation (Fig. 3A, compare lanes 7 and 9). These results suggest that the inhibition of Elk-1 by N17Ras is not likely to be the result of inhibition of endogenous Ras functions, and they demonstrate that the inhibition of Elk-1 phosphorylation is unique to N17Ras.

Several recent reports have demonstrated that other MAP kinase family members in addition to ERKs are able to activate TCFs by phosphorylation (13, 14, 18, 46). We therefore asked whether N17Ras expression would inhibit Ga4-ElkC activity induced by MAP kinases other than ERK. Expression of active MEK3-DE and p38 MAP kinase resulted in a synergistic activation of the Ga4-ElkC reporter (Fig. 3B, solid bars) and cotransfection of N17Ras significantly reduced this activity (Fig. 3B, solid bars). This result is surprising because Ras function has not been directly linked to p38 activation, nor does N17Ras expression inhibit MEK3-DE-induced p38 kinase activity (data not shown). As with Ga4-ElkC, MEK3-DE/p38 expression significantly elevated Ga4-ATF2 (36) activity (Fig. 3B, hatched bars), and cotransfection of N17Ras had no significant effect (Fig. 3B, hatched bars). These results suggest that the inhibitory effect of N17Ras is specific to TCFs like Elk-1 and does not depend on activation of the ERK MAP kinase.

Negative Regulation of Elk-1 by a Noninterfering Version of N17Ras, N17N69Ras—We examined whether we could experimentally distinguish the two functions of N17Ras observed here, namely inhibition of Elk-1 phosphorylation versus inhibition of endogenous Ras activation. As mentioned previously, N17Ras inhibits GTP-Ras formation by targeting Ras-GEFs, like SOS. N17N69Ras is a GDP-bound form of Ras (see Fig. 5C) that no longer functions as a dominant interfering mutant because of the substitution of asparagine for aspartic acid at position 69 of human Ha-Ras (20, 21). Therefore, we tested whether N17N69Ras would inhibit Elk-1. N17N69Ras expression significantly elevated Gal4-ATF2 reporter activity (Fig. 4A, hatched bars). As with N17Ras, cotransfection of N17N69Ras reduced Gal4-ATF2 expression by approximately 10-fold (Fig. 4A), and coexpression of either N17 or N17N69 reduced SRE and c-fos reporter activity to near basal levels (Fig. 4A). A15Ras, another
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interfering Ras mutant that blocks Ras activation (19), also inhibited V12Ras-induced Gal4-ElkC (Fig. 4B). The fact that expression of the MAP kinase phosphatase HVH-1 (47) also inhibited reporter activity suggests that V12Ras-induced c-fos promoter requires MAP kinase activation (Fig. 4A and B). Activity of Gal4-TCF chimeras was also tested. V12Ras-induced activation of Gal4-ElkC, Gal4-Sap1C, or Gal4-Sap2C (7, 9) was inhibited by expressing N17, N17N69, or A15Ras (Fig. 4B). Furthermore, expression of N17, N17N69, or A15Ras inhibited V12Ras-induced focus formation in NIH3T3 cells (Fig. 4C). These results suggest that certain events leading to transcription activation and cellular transformation induced by V12Ras remain sensitive to inhibition by coexpression of N17 and other GDP-bound Ras mutants.

N17Ras Selectively Blocks Nucleotide Loading of Wild-type, but Not Oncogenic, Ras—These observations are not readily explained by current models of Ras function in which GTP-Ras is active and GDP-Ras is inactive and in which N17Ras displays a dominant negative effect by simply interfering with endogenous Ras activation. This model predicts that N17Ras should always be recessive to phenotypes elicited by V12Ras. Two simple scenarios, however, would explain our observations. First, N17Ras may interfere with the ability of V12Ras to bind GTP in vivo. Second, N17Ras may have a discreet function in signaling Elk-1 via an unknown mechanism.

We performed experiments to examine directly the effects of N17Ras expression on the nucleotide binding status of either V12 or wild-type Ras in COS-1 cells. To this end, COS-1 cells were transfected with HA-V12Ras or HA-Ras in the presence or absence of a non-epitope-tagged version of N17Ras. Serum-starved cells were labeled with 32PO4 for 4 h prior to stimulation and immunoprecipitation with anti-HA. Nucleotides bound to the immunoprecipitated Ras were eluted and resolved by TLC. Our results indicate that HA-V12Ras was mostly complexed with GTP (Fig. 5A, left panel, lane 2) and this was not affected by coexpression of N17Ras (Fig. 5A, left panel, compare lanes 2 and 3). In contrast, wild-type HA-Ras was mainly GDP-bound, and treatment of cells with EGF for 2 min resulted in a significant increase in GTP-bound HA-Ras which was inhibited completely by N17Ras (Fig. 5A, left panel, compare lanes 4–7), suggesting that N17Ras was capable of inhibiting an EGF-stimulated Ras-GEF. Similarly, expression of FLAG-SOS significantly increased the amount of GTP-bound HA-Ras (Fig. 5A, left panel, lane 8), and expression of N17Ras reduced this significantly (Fig. 5A, left panel, compare lanes 8 and 9). Immunoblot analysis of lysates from identically transfected cells with α-Hu-Ras and α-FLAG revealed that all cDNAs were expressed evenly (Fig. 5A, right panel). Consistent with our in vivo labeling experiments, expression of N17Ras did not alter the amount of HA-V12Ras that could bind to the Ras binding domain of c-Raf (RBD) as determined by GST pull-down and immunoblotting (Fig. 5B, left panel), although N17Ras was expressed efficiently (Fig. 5B, right panel). These data demonstrate that V12Ras is not subject to regulation by N17Ras in vivo and confirm that in COS-1 cells N17Ras can effectively interfere with wild-type Ras activation in response to EGF via Ras exchange factors, such as SOS.

**Fig. 4. Oncogenic Ras-induced transformation and transcriptional activation are inhibited by N17Ras. Panel A, V12Ras-induced SRE and c-fos promoter activity is blocked by GDP-Ras mutants. NIH3T3 cells were cotransfected with either SRE or c-fos luciferase constructs in the presence V12Ras. Where indicated, Ras mutants, HVH-1, or vector was included. Panel B, inhibition of V12Ras-induced Gal4-ElkC, Gal4-Sap1C, and Gal4-Sap2C activity by GDP-Ras mutants. NIH3T3 cells were transfected with Gal4-luciferase and the indicated Gal4 chimeras together with the indicated Ras expression vector. All luciferase activity was normalized to a cotransfected β-galactosidase activity. Shown are representative examples from at least four independent experiments performed in duplicate. Panel C, N17Ras blocks V12Ras-induced focus formation in NIH3T3 cells. Low passage NIH3T3 cells were transfected with the indicated Ras expression vectors in duplicate. 14 days post-transfection, foci were stained with crystal violet and scored. Shown is one of three independent experiments that yielded very similar results.**
N17Ras Is GDP-bound in Vivo—In vitro, under limiting Mg
and nucleotide concentrations, N17Ras binds GDP with
preferential affinity, although 60-fold less effectively than wild-
type Ras (5, 25). To our knowledge, however, it has not been
demonstrated that N17Ras is GDP-bound in vivo, although it
has been predicted based on the known binding constants and
intracellular Mg and GDP concentrations (5, 25). We there-
therefore determined the in vivo nucleotide binding specificity of
various Ras mutants by in vivo labeling and immunoprecipita-
tion experiments. Our results indicate that greater than 90% of
the nucleotides complexed with both N17 and N17N69 are GDP
in vivo (Fig. 5C). Surprisingly, A15Ras, previously shown to be
nucleotide-free using bacterial expressed protein (19), was
GDP-bound (Fig. 5C) in vivo. In contrast, the majority of nu-
cleotides complexed with V12Ras were GTP, whereas wild-type
Ras is also largely GDP-bound (Fig. 5A). It is important to note
that all Ras proteins tested in this assay were associated with
comparable amounts of radioactivity. Therefore, assuming that
all of the wild-type Ras is bound to nucleotide, N17, N17N69,
and A15Ras all appear to be loaded with GDP, whereas V12Ras
is largely GTP-bound.

DISCUSSION

Experiments using Ras mutants have been instrumental in
eliciting biological and biochemical functions of Ras. N17Ras is
used extensively as a dominant negative mutant to probe Ras function because it interferes with Ras activation in vivo by formation of nonproductive complexes with exchange
factors (5, 19–26). In fact, overexpression of N17Ras is usually
the sole indicator for determining whether a particular signal-
ing event involves Ras activation. The analogous mutant ver-
sions of Ras-related GTPases, such as Rac, Rho, and CDC42,
also act as dominant interfering mutants and are utilized fre-
quently to determine roles for GTPases in signaling. However,
to ascertain the involvement of a small GTPase in a given
signaling pathway, it is essential to understand the mechanism
of function of such dominant interfering mutants. This report
describes a novel effect induced by dominant negative N17Ras,
in addition to its ability to block Ras activation. Our results
demonstrate that expression of N17Ras can inhibit Elk-1 acti-
vation independently of blocking endogenous Ras activation.
These observations suggest that caution should be taken in
interpreting data that rely upon dominant negative mutants to implicate a small GTPase in signaling.

Expression of N17Ras alone can effectively inhibit serum-dependent cell proliferation, and this effect can be reversed by coexpression of oncogenic Ras or Ras-GEFs (5, 20, 21). It has therefore been assumed that the only function of N17Ras is to inhibit Ras activation. The data presented here, however, suggest that N17Ras may have functions besides inhibiting Ras activation. We base this argument on several observations. First, N17Ras expression can negatively regulate Elk-1 (Figs. 1, 2E, 3, and 4), a substrate of MAP kinases, yet have no effect on the activity of MAP kinase itself (40–42, 48). Second, N17Ras expression inhibits active MEK3-p38 induced Elk-1 activity as well as active MEK1-induced Elk-1 phosphorylation. This result is surprising because no GTP-dependent Ras function has been identified which regulates the direct activation of a MAP kinase by a MAP kinase kinase. Third, we compared the abilities of two different classes of dominant negative Ras mutants to inhibit MEK-induced Elk-1 phosphorylation. We found that only the GDP-bound N17, but not the GTP-bound L61S186 mutant, inhibited Elk-1 in response to constitutively active MEK expression. Fourth, N17N69Ras also inhibits Elk-1 phosphorylation and oncogenic transformation, yet it neither inhibits cell growth (20, 21) nor ERK activation. Furthermore, membrane association appears important for N17Ras function because a cytosolic mutant, N17S186Ras, can no longer inhibit either Elk-1 or transformation. Lastly, N17Ras expression inhibits TCF activity induced by V12Ras, which is not subject to negative regulation by N17Ras.

The observations presented here can be explained by at least two hypothetical models. In the first model, an unidentified GTP-dependent Ras function, which is required for Elk-1 activation, is inhibited by N17Ras. This would explain why we observe inhibition of Elk-1, but not ERK1. However, at the moment it is difficult to hypothesize a role for this unknown Ras effector in Elk-1 regulation. Furthermore, we cannot explain clearly how a particular GTP-dependent Ras function such as Elk-1 activation could be inhibited by N17Ras whereas another such as ERK activation would not be affected in the same cells. One possibility is that distinct intracellular pools of Ras may be inhibited by N17Ras expression. For example, different pools of Ras, each regulated by distinct Ras-GEFs, may participate in ERK activation and Elk-1 activation, respectively. In the second model, N17Ras may regulate, directly or indirectly, the activity of an unidentified component(s) involved in Elk-1 regulation. Because GEFS are the only known targets for N17Ras, it is possible that N17Ras may regulate another GEF for a small GTPase.

In vitro, N17Ras displays reduced nucleotide binding to both GDP and GTP, although the latter is much more severe (5, 25, 26). In vivo, the nucleotide binding status of N17Ras has not been examined. The results from our in vivo labeling and immunoprecipitation experiments confirm directly that this N17Ras is mainly GDP-bound in vivo (Fig. 5C). Although there are no known GDP-dependent targets of Ras, inhibition of Elk-1 and transformation may be physiological function of GDP-Ras because N17Ras is constitutively GDP-bound under physiological conditions. Furthermore, A15Ras that is also GDP-bound in vivo displays functions similar to N17Ras. Therefore, GDP-Ras itself may signal to an unknown effector molecule that leads to Elk-1 inhibition and suppression of transformation. Interestingly, recent evidence indicates that a Ras-related GTPase, Bud1, which functions in bud site selection in yeast, interacts directly with one of its targets in a GDP-dependent manner (49). In addition, similar phenomena have been observed for another small GTPase, Ran1, which regulates nuclear protein transport (50). These examples provide direct evidence for a GDP-bound form of small GTPase in signaling.

Our observations have relied upon transfected GDP-bound Ras mutants, and it is intrinsically difficult to demonstrate that endogenous GDP-Ras functions in signaling in the types of experiments presented here. However, studies of cancer progression provide genetic evidence that endogenous wild-type Ras, which is primarily GDP-bound, participates in suppressing the oncogenic potential of active Ha-ras alleles. For example, Brenner and Balmain (31) have observed that loss of wild-type, but not active, Ha-ras alleles occurs at high frequencies during skin tumor progression in mice. Loss of wild-type Ha-ras was observed frequently in tumors that harbor an activated Ha-ras allele. Thus, amplification of active Ha-ras alleles and/or loss of the wild-type copy of Ha-Ras appears to be consistent features of skin tumor development in mice. Similar results have been observed with Nrass in mouse thymic lymphomas (29) as well as with K and Nrass in clonal murine lymphoma (30) and with Ha-ras in human cervical cancers (32). It is also interesting to note that a single copy of active Ha-ras is not dominant with respect to a single copy of wild-type Ras in transforming the Rat-1 fibroblast cell line (28). Furthermore, the spontaneously transformed cells that arose from this V12Ras/Ras heterozygous cell line were found to contain either amplification of the active Ha-ras allele or deletion of the wild-type copy (28). These observations suggest that wild-type Ras, which is mainly in a GDP-bound form, may have an inhibitory effect on oncogenic transformation in the presence of active Ha-ras alleles.

Other investigators have raised questions concerning the mechanism of N17Ras function in vivo. For example, it has been demonstrated recently that GTP-Ras dependent functions, such as c-Raf activation, are inhibited by neutralizing Ras antibody injection, but not by N17Ras expression (40). In addition, N17Ras has also previously been reported to inhibit cellular transformation induced by V12Ras (5), v-Raf induced transcription activation of the T-cell receptor β gene (51), and V12Ras-induced neuronal survival (25), suggesting that GDP- and GTP-Ras may be co-dominant in some cases.

The data presented here suggest that caution must be taken when interpreting data that rely upon N17Ras as the sole means of implicating Ras function in signaling. This notion is certainly underscored by the fact that mutant Ras proteins, such as N17N69, which poorly interferes with EGF-stimulated Ras activation (Fig. 3C) and cell growth (20, 21), inhibit Elk-1 activation (Fig. 3C and 4, A and B) and NIH3T3 transformation (Fig. 4C). In addition, N17N69Ras attenuates c-fos promoter activity as effectively as N17 or A15Ras (Fig. 4A). Further evidence is still needed to prove unequivocally that N17Ras has targets other than Ras-GEFs, however. Identification of in vivo targets of N17Ras should clarify the mechanism of action of these mutants and add to our understanding of GTPase regulation in general.

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