

A Role for the Ral Guanine Nucleotide Dissociation Stimulator in Mediating Ras-induced Transformation*

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Oncogenic Ras transforms cells through the activation of multiple downstream pathways mediated by separate effector molecules, one of which is Raf. Here we report the identification of a second ras-binding protein that can induce cellular transformation in parallel with activation of the Raf/mitogen-activated protein kinase cascade. The Ral guanine nucleotide dissociation stimulator (RalGDS) was isolated from a screen for Ras-binding proteins that specifically interact with a Ras effector-loop mutant, ras(12V,37G), that uncouples Ras from activation of Raf1. RalGDS, like ras(12V,37G), cooperates synergistically with mutationally activated Raf to induce foci of growth and morphologically transformed NIH 3T3 cells. RalGDS does not significantly enhance MAP kinase activation by activated Raf, suggesting that the cooperativity in focus formation is due to a distinct pathway acting downstream of Ras and parallel to Raf.

The Ras GTPases function as important nodes in signal transduction networks regulating proliferation and differentiation, integrating extracellular signals to a variety of downstream cellular responses. The importance of this role is highlighted by oncogenic ras, which can induce growth and morphological transformation of many cell lines (1). It is becoming increasingly apparent that Ras activity is mediated through multiple effector pathways (2–4). The best characterized pathway is the Raf/MAP¹ kinase (mitogen-activated protein kinase) cascade where, upon Ras binding, Raf is activated and in turn activates MAP kinase through the activation of

MEK (mitogen-activated or extracellular signal-regulated kinase kinase) (5–8). MAP kinase activation is a critical step in cellular transformation induced by oncogenic ras (9, 10).

Through the use of effector-specific ras mutants, which separate the ability of Ras to interact with different downstream targets, we have previously shown that multiple Ras functions can contribute to cellular transformation. Only one of these involves Raf activation (3). ras(12V,37G) was defective in Raf1 binding and did not transform cells. However, ras(12V,37G) retained activity that complemented the transformation defect of a different ras mutant, ras(12V,35S). ras(12V,35S) could bind Raf1 and activate MAP kinase, but was presumably defective in other target interactions preserved by ras(12V,37G). The cooperativity between ras(12V,37G) and ras(12V,35S) suggested the presence of a novel pathway mediating Ras-induced cellular transformation in parallel to Raf activation (3).

Here we report the identification of a mammalian ras-binding protein, RalGDS (11, 12, 13), that interacts with ras(12V,37G). Using focus formation assays in NIH 3T3 cells, we show that both ras(12V,37G) and RalGDS cooperate synergistically with mutationally activated Raf to transform cells. This cooperativity is not due to additive effects on MAP kinase activation, suggesting that RalGDS, like ras(12V,37G), contributes to cellular transformation through an activity distinct from activation of the Raf/MAP kinase cascade.

EXPERIMENTAL PROCEDURES

Plasmids—All ras variants used were in the vector pDCR, for mammalian expression, or pBTM116, for expression in yeast, as described previously (3). pBTM116-Lamin was provided by A. Vojtek (14). pSRα-raf-BXB was provided by A. Minden and M. Karin. pCEP4-RalGDS contains the entire coding sequence of mouse *ralGDS* inserted as a *Bam*HI fragment into the *Bam*HI site of pCEP4 (Invitrogen Corp.). pCEP4-ralA(26A) and pCEP4-ralA(23V) were derived from pBTM116-ralA(26A) and pBTM116-ralA(23V), respectively (15). Ral coding sequences were removed as blunt-ended *Xba*I-*Kpn*I fragments and ligated into pCEP4 at blunt-ended *Bam*HI and *Kpn*I sites.

Yeast Two-hybrid Screens and Tests—The yeast reporter strain L40 (14) was used for all two-hybrid analysis. A random-primed, size-selected, mouse embryo cDNA library expressed as fusions to the VP16 activation domain (14) and an oligo(dT)-primed PC12 cDNA library expressed as fusions to the GAL4 activation domain in pGADGH (provided by S. Tsui and S. Halegua) were screened for fusions that interacted with ras(12V,37G) fused to the LexA DNA-binding domain. Approximately 10 million transformants were screened from each library. Positives were tested for specificity of interaction by standard techniques and sequenced (16).

Mammalian Cell Transfections—Stable transfections of NIH 3T3 cells were performed by the calcium phosphate precipitation method as described (17). 24 h post-transfection, the cells were split into Dulbecco's modified Eagle's medium (ICN Biomedicals, Inc.) plus 5% calf serum (Life Technologies, Inc.) for focus formation assays, and Dulbecco's modified Eagle's medium plus 10% calf serum supplemented with 0.5 mg/ml G418 sulfate (Life Technologies, Inc.). Foci of growth and morphologically transformed cells were scored under magnification after 14 days of incubation in 5% serum. G418-resistant colonies were counted after 10 days of growth in 10% serum plus G418. Transient transfections were performed using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's protocols.

MAP Kinase Assays—Stably transfected NIH 3T3 cells were lysed in a modified RIPA buffer supplemented with protease and phosphatase inhibitors (18). ERK1 and ERK2 were immunoprecipitated from 200 μg of total protein with the anti-ERK1 C-16 polyclonal antibody (Santa Cruz Biotechnology, Inc.). The immunocomplex was incubated with 12 μg of myelin basic protein (MBP) in a kinase assay for 30 min at room temperature (18). Reactions were separated by SDS-polyacrylamide gel electrophoresis and visualized on a PhosphorImager (Molecular

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¹ The abbreviations used are: MAP, mitogen-activated protein; MEK, mitogen-activated or extracellular signal-regulated kinase kinase; MBP, myelin basic protein; RalGDS, Ral guanine nucleotide dissociation stimulator.

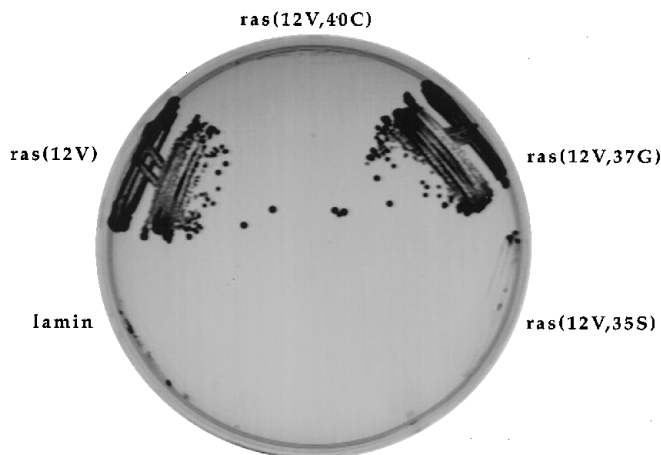


FIG. 1. Two-hybrid interactions between ras mutants and RalGDS. Ras variants and lamin, as a control, were expressed as fusions to the LexA DNA-binding domain. RalGDS was expressed as a fusion to the GAL4 activating domain. Transformants, from the yeast reporter strain L40, selected to express GAL4 activating domain-RalGDS and the indicated LexA DNA-binding domain fusions, were tested for the ability to grow on media lacking histidine. Growth on the selective plate indicates a positive two-hybrid interaction. Transformants that were His⁺ also gave a positive indication of β -galactosidase activity using filter assays (data not shown). At least four independent transformants were tested for each pair.

Dynamics).

Antibodies and Protein Expression—Protein expression from exogenously introduced cDNAs was determined by Western blot analyses of cell lysates. raf-BXB expression was detected, from 20 μ g of total protein, using the anti-Raf1 C-20 antibody (Santa Cruz Biotechnology, Inc.). Endogenous, activated forms of ERK1 and ERK2 were detected, from 20 μ g of total protein, by using the 759B (Promega Corp.) antibody that selectively recognizes activated MAP kinase. Total ERK1/ERK2 levels were detected using the anti-ERK1 C-16 antibody. RalA was detected, from 20 μ g of total protein, using a mouse anti-RalA monoclonal antibody from Transduction Laboratories.

RESULTS

ras(12V,37G) Interacts with RalGDS—To identify the effector molecule(s) that may mediate signaling from ras(12V,37G), we used the yeast two-hybrid system to screen cDNA libraries for clones that interact with ras(12V,37G) but not ras(12V,35S). In this way, the previously characterized ras-binding protein RalGDS was isolated from libraries derived from mouse embryo and PC12 cells. RalGDS was originally identified as a guanine nucleotide dissociation stimulator (GDS) for the Ral GTPase (19). Human and rodent RalGDS interact directly with Ras in a GTP-dependent manner (11–13), and RalGDS has been shown to associate with Ras in COS cells upon stimulation with epidermal growth factor (20). The two-hybrid interactions of full-length RalGDS with ras(12V,37G) versus other ras effector-domain mutants are shown in Fig. 1. Although ras(12V,35S) does not interact with full-length RalGDS, a truncated version of RalGDS, expressing amino acids 703–814, was isolated from the mouse embryo library which interacts with both ras(12V,37G) and ras(12V,35S) (data not shown). This suggests that the specificity of the RalGDS-Ras interaction is affected by residues outside of the minimal Ras interaction domain (12). A different ras effector mutant rasV12C40 did not interact with any form of RalGDS isolated (Fig. 1 and data not shown).

ras(12V,37G) and RalGDS Cooperate with Activated raf to Transform Cells—ras(12V,37G) is defective in Raf1 binding, but retains a function that cooperates with Raf1 to transform cells (3). The binding of RalGDS with ras(12V,37G) suggests the possibility that RalGDS may partially or fully mediate this function. To test this, we compared the abilities of RalGDS and

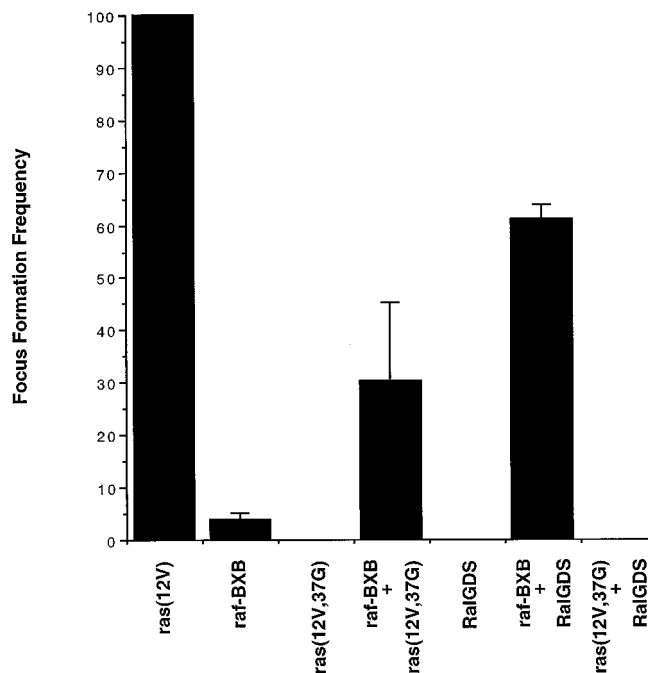


FIG. 2. ras(12V,37G) and RalGDS cooperate with raf-BXB to transform cells. NIH 3T3 cells were transfected with the constructs expressing the indicated proteins. Each transfection included the appropriate empty vector(s) as needed to achieve equivalent DNA concentrations and to allow G418 selection. Focus formation frequency for each transfection was determined as number of foci per number of G418-resistant colonies, normalized to the focus formation frequency induced by ras(12V), which was set at 100. Ras variants were introduced (100 ng/transfection) in pDCR which contains the G418 resistance gene. RalGDS was introduced (500 ng/transfection) in pCEP4. raf-BXB was introduced (100 ng/transfection) in pSR α . Transfections with only empty vectors yielded no foci. Results shown represent one experiment with each transfection performed in duplicate. Error bars indicate the variation observed between the duplicate transfections. Three additional experiments yielded similar results.

ras(12V,37G) to enhance focus formation induced by activated raf. raf-BXB is a Raf1 variant with a deletion of the amino-terminal regulatory domain containing the Ras-binding site (14, 21). This results in a mutationally activated protein that signals constitutively to downstream components (21). NIH 3T3 cells were transfected with rasV12, raf-BXB, ras(12V,37G), and wild-type RalGDS alone and in the indicated combinations (Fig. 2). raf-BXB alone induced a low level of focus formation. Both RalGDS and ras(12V,37G) had no focus forming activity when expressed alone or together. However, ras(12V,37G) acted synergistically with raf-BXB to transform cells, resulting in an approximately 8-fold induction of focus formation above the level observed with raf-BXB alone. Similarly, RalGDS acted synergistically with raf-BXB to transform cells, resulting in an approximately 12-fold induction of focus formation above the level observed with raf-BXB alone. raf-BXB expression levels, as determined by Western blotting of pooled G418-resistant colonies, were equivalent in cells transfected with raf-BXB alone and in combination with ras(12V,37G) or RalGDS (data not shown).

Ectopic Expression of RalGDS Does Not Contribute to MAP Kinase Activation by raf-BXB—The activation of MAP kinase is an important step in cellular transformation induced by Ras and Raf (9, 10). It is possible that the cooperativity in focus formation between ectopically expressed RalGDS and raf-BXB converges at the level of MAP kinase activation. To test this, the MAP kinases ERK1 and ERK2 were immunoprecipitated from pooled G418-resistant colonies derived from cells transfected with pDCR-ras(12V) or pSR α -raf-BXB alone and in com-

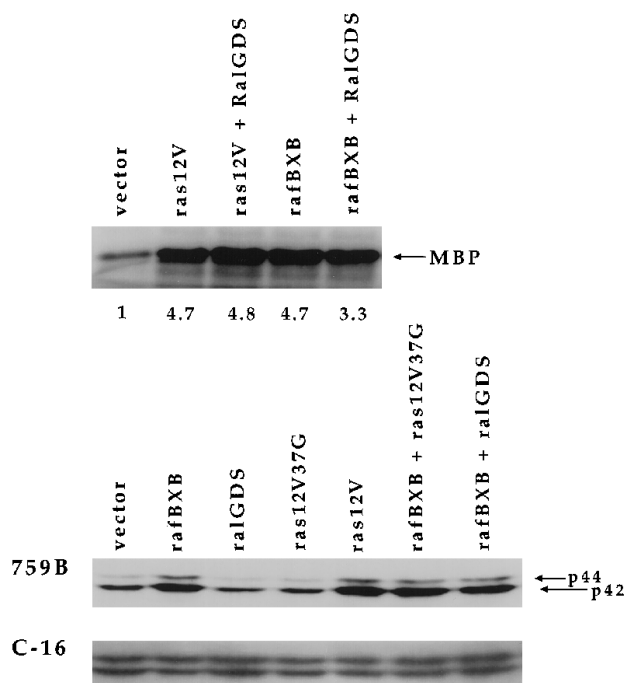


FIG. 3. RalGDS does not contribute to MAP kinase activation by activated raf. *A*, MAP kinase activity in stable transfected cell lines, expressing the indicated proteins, was measured by MBP phosphorylation using anti-ERK1/ERK2 immune complexes. Numbers indicate fold elevation relative to vector-transfected cells. raf-BXB expression levels were equivalent in cells transfected with raf-BXB alone or in combination with RalGDS (data not shown). Two additional experiments yielded similar results. *B*, NIH 3T3 cells were transiently transfected with the indicated constructs (1 μ g/transfection) and empty vector as required (2 μ g/transfection of total DNA). The level of activated cellular MAP kinase was measured by Western blotting using the polyclonal antibody 759B which selectively recognizes the activated phosphorylated forms of ERK1 and ERK2. Blots were stripped and reprobed with the C-16 antibody which recognizes both active and inactive forms of ERK1 and ERK2. A repeated experiment yielded similar results.

bination with pCEP4-RalGDS. The number of colonies derived from individual transfections were equivalent (\sim 250/plate). Activity of the immunoprecipitated ERK1 and ERK2 was assayed by *in vitro* phosphorylation of MBP. The level of MAP kinase activation in cells expressing raf-BXB was similar to that in cells expressing ras(12V). Coexpression of RalGDS with raf-BXB resulted in no detectable induction of MAP kinase above the level observed with raf-BXB alone (Fig. 3A).

Transient transfection assays yielded similar results. To detect activation of endogenous MAP kinase in transiently transfected cells, it was necessary to use the 759B antibody which selectively recognizes the activated phosphorylated forms of ERK1 and ERK2. Cells transiently expressing RalGDS or ras(12V,37G) showed no increase in the levels of activated MAP kinase as compared to cells transfected with empty vectors. Importantly, RalGDS and ras(12V,37G) did not significantly enhance the level of activated MAP kinase induced by raf-BXB. Total levels of cellular MAP kinase were equivalent among transfections (Fig. 3B).

Contribution of the Ral GTPase to transformation by Ras and Raf—RalGDS stimulates guanine nucleotide exchange on the Ras-related GTPase Ral (19). Therefore, the promotion of cellular transformation by RalGDS may be a consequence of Ral activation. To test this, we examined the contribution of Ral to focus formation by Ras and Raf.

ralA(23V) has a mutation resulting in a defective intrinsic GTPase activity homologous to the ras(12V) activating mutation (22). Expression of pCEP4-ralA(23V) resulted in a 3-fold

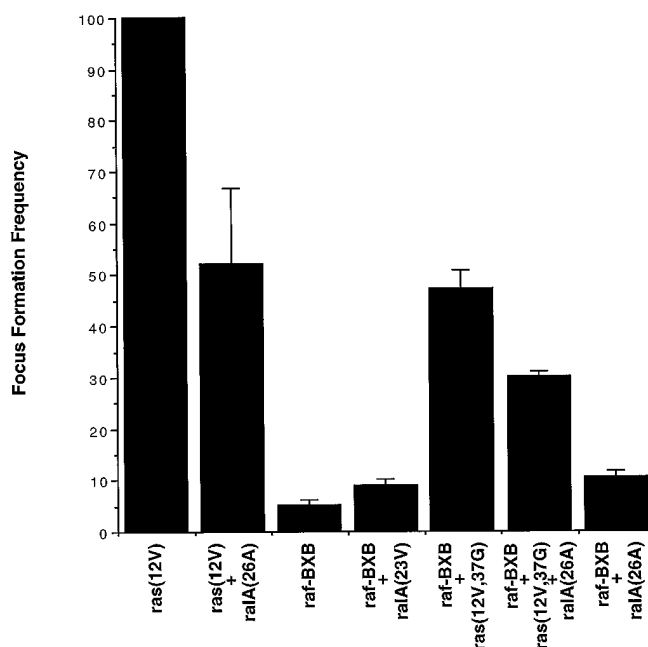


FIG. 4. Contribution of ral(26A) and ral(23V) mutants to focus formation induced by activated ras and raf. NIH 3T3 cells were transfected with plasmids expressing the indicated proteins, and focus formation frequencies were determined as described in Fig. 2. Ras variants, RalGDS, and raf-BXB were introduced as described in Fig. 2. Ral mutants were introduced (500 ng/transfection) in pCEP4, resulting in a 2- to 3-fold elevation of total Ral protein as determined by Western blot (data not shown). Results represent one experiment with each transfection performed in duplicate. Error bars indicate the variation observed between the duplicate transfections. Two additional experiments yielded similar results.

elevation of total RalA protein but did not cooperate with raf-BXB to transform cells (Fig. 4). This lack of cooperativity suggests that RalGDS may contribute to raf-BXB-induced focus formation through a RalA-independent mechanism. Alternatively, the 23V mutation may not result in the same degree of functional activation of RalA as ectopic expression of RalGDS.

ralA(26A) contains a mutation with structure and sequence homology to the dominant negative mutant ras(15A) (23). ras(15A) has been shown to form unproductive complexes with Ras guanyl nucleotide exchange factors and is predominantly in the GDP-bound state (24, 23). We hoped that expression of ralA(26A) would inhibit cellular Ral function by sequestering Ral guanyl nucleotide exchange factors in a manner analogous to the block of cellular Ras function that occurs upon expression of ras(15A). Expression of pCEP4-ral(26A) resulted in a 2.5-fold elevation in total detectable RalA protein. ralA(26A) had no significant effect on focus formation induced by raf-BXB. ralA(26A) expression did result in a small but reproducible reduction in foci induced by ras(12V) and by coexpression of raf-BXB and ras(12V,37G) (Fig. 4). This reduction may be a result of sequestration of RalGDS through unproductive binding to ralA(26A). Consistent with this, ralA(26A) interacts with RalGDS in the yeast two-hybrid system whereas wild-type RalA and ralA(23V) give no detectable interaction (data not shown).

DISCUSSION

ras(12V,37G) is defective in Raf1 interaction and cellular transformation. However, ras(12V,37G) does retain an activity that complements the transformation defect of a different Ras mutant, ras(12V,35S). ras(12V,35S) binds Raf1, but is presumably defective in other target interactions that mediate cellular

transformation. The transformation defect of ras(12V, 37G) is rescued by coexpression of a Raf1 mutant that rescues binding to ras(12V,37G). We have interpreted this as evidence that Ras transforms cells through the activation of Raf1 as well as other, as yet unidentified, molecules (3). Here, we show that the ras-binding protein RalGDS interacts with ras(12V,37G) but not ras(12V,35S), suggesting that it may mediate the complementary transformation activity of ras(12V,37G). Consistent with this, RalGDS, like ras(12V,37G), cooperates with mutationally activated raf to transform cells.

While the results described here establish RalGDS as a positive regulator of transformation, it remains to be demonstrated that this activity is mediated by Ras interaction. In addition, RalGDS is not the only candidate Ras effector other than Raf which may mediate Ras-induced cellular transformation. Additional Ras-binding proteins have been identified, including phosphatidylinositol-3-OH kinase (2), AF6 (25), Rin-1 (26), NF1 (27), ζ PKC (28), and MEK kinase 1 (29), some or all of which may have roles in mediating Ras activity, including induction of cellular transformation.

Raf1 activates MAP kinase through direct binding and activation of MEK, a MAP kinase kinase (8). MAP kinase activation is an important step in the induction of cellular transformation by oncogenic ras (9, 10). The complementary transforming activity of RalGDS does not appear to converge at the level of MAP kinase activation, as it does not activate MAP kinase and does not enhance activation of MAP kinase by activated Raf. This suggests that RalGDS contributes to cellular transformation by activating a pathway that is distinct from the Raf-MAP kinase cascade.

Obvious candidates for targets that mediate RalGDS transforming activity are the Ral GTPases (30). The use of "activated" and "dominant negative" mutants of RalA was unsuccessful in revealing a role for Ral GTPases in Ras-induced cellular transformation. Similar experiments with RalB mutants were also negative (data not shown). However, while this manuscript was in preparation, Urano *et al.* (31) published observations that ralA(72L) cooperated with ras(12V) to transform cells, and ralA(28N) interfered with cellular transformation by both ras(12V) and v-raf. Parallel studies with these same reagents have not been done by us, and we cannot rule out a role for RalA in mediating the activities we observe with RalGDS. Further studies are underway, using mutants of RalGDS that effect Ral binding and guanyl nucleotide exchange, as well as RalGDS mutants that restore interaction with ras mutants,

that should clarify the roles of RalGDS and Ral in mediating Ras-induced cellular transformation.

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