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Ral-GTPases mediate a distinct downstream signaling pathway from Ras that facilitates cellular transformation

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Ral proteins (RalA and RalB) comprise a distinct family of Ras-related GTPases (Feig and Emkey, 1993). Recently, Ral-GDS, the exchange factor that activates Ral proteins, has been shown to bind specifically to the activated forms of RasH, R-Ras and Rap1A, in the yeast two-hybrid system. Here we demonstrate that although all three GTPases have the capacity to bind Ral-GDS in mammalian cells, only RasH activates Ral-GDS. Furthermore, although constitutively activated RalA does not induce oncogenic transformation on its own, its expression enhances the transforming activities of both RasH and Raf. Finally, a dominant inhibitory form of RalA suppresses the transforming activities of both RasH and Raf. These results demonstrate that activation of Ral-GDS and thus its target, Ral, constitutes a distinct downstream signaling pathway from RasH that potentiates oncogenic transformation.

Keywords: GTPase/Ral/Ras/signal transduction/transformation

Introduction

RalA and RalB constitute a family of GTP binding proteins within the superfamily of Ras-like GTPases (Chardin and Tavitian, 1986). Ral proteins are one of the closest relatives of Ras, displaying 55% amino acid sequence identity. This similarity is reflected in the similar way the two proteins interact with guanine nucleotides. Thus like Ras, Ral proteins have very high affinities for GTP and GDP and low intrinsic GTPase activity. Moreover, analogous mutations generate GTPase-deficient versions of both proteins (Frech *et al.*, 1990). These mutants also fail to respond to their respective GTPase activating proteins (GAPs) (Emkey *et al.*, 1991). Unlike some other members of the Ras superfamily, both Ras and Ral proteins are found exclusively in membrane fractions (Bhullar *et al.*, 1990).

As expected, Ras and Ral are distinct in many important respects. First, they interact with unique GAPs such as Ras-GAP and Ral-GAP (Boguski and McCormick, 1993), which are responsible for inactivating Ras and Ral respectively. They also become activated by distinct nucleotide exchange factors, such as SOS and Ral-GDS (guanine nucleotide dissociation stimulator) (Feig, 1994). Second, Ras and Ral have distinct effects on cells. Constitutively activated Ras promotes oncogenic transformation, whereas a similar Ral mutant does not (Feig and Emkey, 1993). This is likely to be the consequence of differences in the effector domains of the two GTPases. Whereas Ras has been shown to bind to and participate in the activation of both the Raf kinase and phosphatidyl inositol-3-phosphate kinase (PI_3 kinase) (for review see Feig and Schaffhausen, 1994), Ral has recently been shown to bind to its own downstream target, Ral binding protein 1 (Cantor *et al.*, 1995). This protein contains a GAP domain with preference for the Rho family member CDC42.

Ras and Ral also differ in their subcellular distribution. Ras is confined to the inner surface of the plasma membrane, whereas Ral has been found mostly in vesicles associated with endocytic and exocytic vesicles (Bielinski *et al.*, 1993; Feig and Emkey, 1993; Volknandt *et al.*, 1993) although Ral can also be detected in plasma membrane fractions of cells (R.Emkey and L.A.Feig, submitted). This may be a consequence of distinct C-termini and differential post-translational processing. Whereas Ras is farnesylated (Casey *et al.*, 1989), Ral is geranylgeranylated (Kinsella and Maltese, 1991).

Recently, a variety of groups, using the two-hybrid system in yeast, have demonstrated that Ral-GDS and a related Ral exchange factor, RGL, bind specifically to the activated forms of RasH, R-Ras and Rap1A (Hofer *et al.*, 1994; Kikuchi *et al.*, 1994; Spaargaren and Bischoff, 1994). These closely related GTPases have the same effector domains, and bind to other potential downstream targets of Ras such as Raf, at least *in vitro*. However, for reasons that are not yet clear, only Ras promotes full transformation in cells (Cox *et al.*, 1994). These findings raised the possibility that Ral-GDS, and thus Ral, are downstream targets of at least one of these GTPases.

We show here that although all three of these GTPases can bind to Ral-GDS in mammalian cells, only Ras can activate Ral-GDS *in vivo*. That Ral-GDS and Ral represent a distinct downstream pathway from Ras is also supported by our finding that activated RalA enhances the transforming activities of Ras and Raf, and dominant interfering RalA suppresses the transforming activities of these two oncogenes.

Results

R-Ras, Rap 1A and RasH can all bind to Ral-GDS when overexpressed in mammalian cells

RasH, R-Ras and Rap1A have similar effector domains (Chardin, 1993) and have been shown to bind to the same set of potential downstream signaling molecules *in vitro*, including Ral-GDS (Hofer *et al.*, 1994; Kikuchi *et al.*, 1994; Spaargaren and Bischoff, 1994). In order to determine which, if any, of these GTPases influences Ral signaling *in vivo*, we first determined whether they can bind to Ral-GDS in mammalian cells. COS-7 cells were

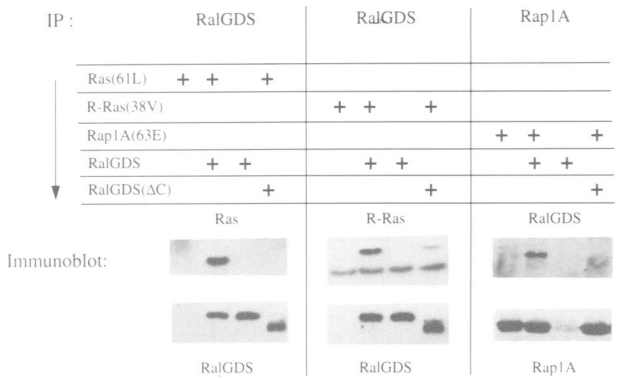

Fig. 1. Co-precipitation of RasH, R-Ras and Rap1A with Ral-GDS but not mutant Ral-GDS from COS-7 cells. COS-7 cells were transiently transfected with Ral-GDS or Ral-GDS(ΔC), along with either of the following constitutively activated GTPase mutants: RasH(61L), Myc-epitope tagged R-Ras(38V) or Glu-epitope tagged Rap1A(63E). For co-transfections with Ras and R-Ras, Glu-epitope tagged GDS was used and it was immunoprecipitated with anti-Glu antibodies. The immunoprecipitates were then immunoblotted with antibodies to either Ras or Myc (to detect Myc-R-Ras). Because the size of Rap1A was the same as immunoglobulin light chains, the Glu-Rap1A was immunoprecipitated with anti-Glu antibodies and Ral-GDS was detected in immune-complexes by immunoblotting with anti Myc antibodies. Expression levels of proteins were detected in the bottom panel by immunoblotting immunoprecipitates with either anti-Glu antibodies [for Ral-GDS and Ral-GDS(ΔC)], or monoclonal antibodies against Rap1A. A similar proportion of each GTPase (~1%) was found to be complexed with Ral-GDS.

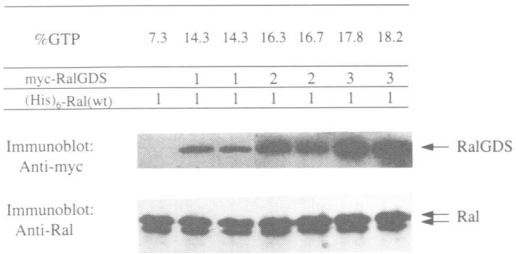

Fig. 2. An assay for Ral-GDS activity *in vivo*. COS-7 cells were transfected with 1.0 μg of (His_6)-RalA DNA plus various amounts of DNA (1–3 μg) encoding Myc-tagged Ral-GDS. 48 h later, the cells were metabolically labeled with $^{32}PO_4$ (0.25 mCi/ml) in phosphate-free D-MEM for 4 h and RalA was precipitated using nickel resin beads. The labeled nucleotides were separated by TLC and GTP and GDP spots were quantitated using a PhosphorImager. The data are expressed as the percentage of Ral-GTP/Ral-GTP + Ral-GDP. Results from duplicate transfections are shown. Extracts from similarly transfected cells were immunoblotted with either anti-Myc antiserum to detect Ral-GDS or anti-RalA antisera to detect exogenous RalA (upper band).

transiently transfected with constitutively activated mutants of RasH(12V), Rap1A(63E) or R-Ras(38V), along with Ral-GDS (Figure 1). For experiments with RasH and R-Ras, Ral-GDS was immunoprecipitated and the precipitates were then immunoblotted with antibodies that recognized the transfected GTPase. Because Rap1A migrated in gels at the same position as the light chain of antibodies used in the immunoprecipitation, Rap1A was immunoprecipitated and precipitates were then immunoblotted with antibodies that detected transfected Ral-GDS. Figure 1 shows that complexes were detected between Ral-GDS and all three GTPases, R-Ras, Rap1A and RasH.

It has previously been shown that binding between these GTPases and Ral-GDS in the yeast two hybrid system and *in vitro* was mediated by the non-catalytic C-terminal 130 amino acids of the exchange factor (Hofer *et al.*, 1994; Kikuchi *et al.*, 1994; Spaargaren and Bischoff, 1994). Thus, we tested the specificity of the binding we observed *in vivo* by substituting a mutant Ral-GDS with its final 130 amino acids deleted [Ral-GDS(ΔC)] for wild-type Ral-GDS. As expected, this mutant Ral-GDS failed to co-immunoprecipitate efficiently with RasH, R-Ras or Rap1A (Figure 1).

RasH, but not R-Ras or Rap1A, activates Ral-GDS in vivo

We then determined whether any of these GTPases could activate Ral-GDS. To this end we developed an assay system for Ral-GDS activity *in vivo* by detecting its ability to promote the GTP-bound form of RalA in cells. Ral-GDS was transfected transiently into COS-7 cells along with (His_6)-tagged RalA. 48 h later, the cells were metabolically labeled with $^{32}PO_4$ and (His_6)-RalA was precipitated on nickel-resin beads. The proportion of RalA bound to GTP was then quantitated after separating labeled nucleotides bound to RalA by thin layer chromatography (TLC; Figure 2). We have used a similar assay recently to study the regulation Ras exchange factors (Farnsworth *et al.*, 1995). When (His_6)-RalA was expressed alone, ~7% of the protein was bound to GTP (Figure 2). When increasing amounts of Ral-GDS DNA were co-transfected (from 1 to 3 μg) with a constant amount of (His_6)-RalA, we observed a dose-dependent increase in both the expression of Ral-GDS and the proportion of Ral bound to GTP.

We then tested the ability of activated RasH to influence Ral-GDS activity in this system (Figure 3). As previously shown in Figure 2, 1.0 μg of Ral-GDS DNA increased the proportion of Ral-GTP levels from ~7 to ~14% (Figure 3A). When activated RasH was included in the transfection, the proportion of Ral-GTP rose even further to ~20%. Importantly, little if any changes in the levels of Ral-GDS or Ral were detected. Figure 2 shows that such small changes in Ral-GDS could not have accounted for the Ras-enhanced Ral-GDS activity observed. Thus, activated Ras can increase the exchange activity of Ral-GDS *in vivo*. As expected, wild-type Ras had a smaller effect on Ral-GDS activity (Figure 3A).

To show that the observed increase in Ral-GDS activity was dependent upon Ras binding to Ral-GDS, the mutant Ral-GDS that failed to bind Ras *in vivo* [Ral-GDS(ΔC)] was studied (Figure 3B). Ral-GDS(ΔC) enhanced Ral-GTP levels only slightly *in vivo*, and its activity did not rise when constitutively activated Ras was co-expressed. This defect was not due to faulty catalytic activity, because the mutant Ral-GDS purified from COS-7 cells displayed intrinsic nucleotide exchange activity *in vitro* that was similar to that of wild-type Ral-GDS (data not shown). Apparently, the C-terminus of Ral-GDS is needed for both basal and Ras-induced Ral-GDS activity *in vivo*.

In contrast to the results with Ras, constitutively activated forms of R-Ras and Rap1A had no significant effect on the activity of Ral-GDS (Figure 3B) despite the fact that both GTPases bound the Ral exchange factor in cells under these conditions (see Figure 1). Thus, of the Ras

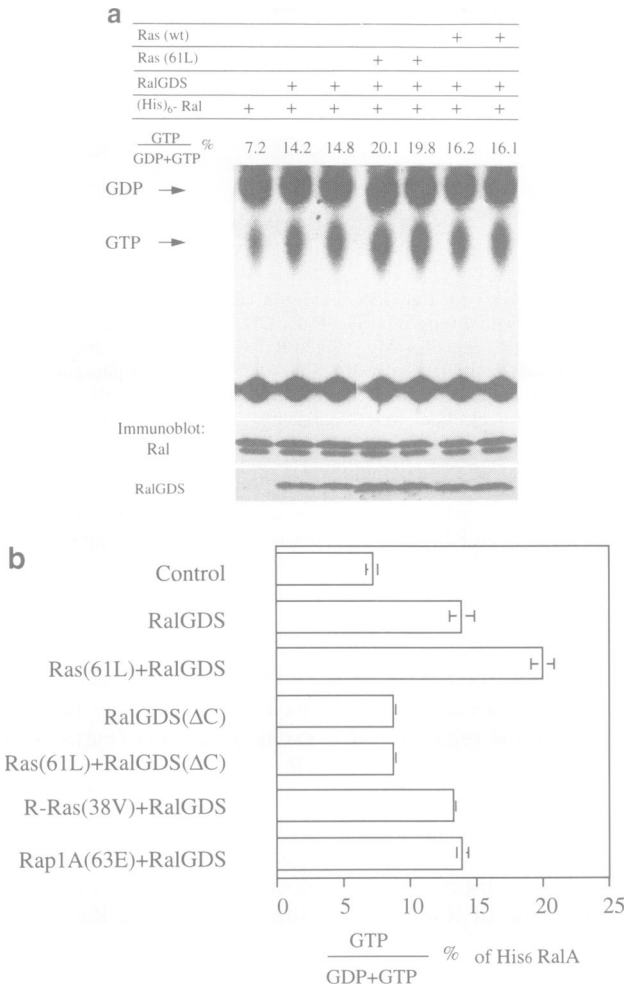

Fig. 3. RasH but not R-Ras or Rap1A activates Ral-GDS activity *in vivo*. **(A)** COS-7 cells were transfected with (His_6)-RalA plus Ral-GDS, Ral-GDS plus RasH(61L), or wild-type RasH. 48 h later, the cells were metabolically labeled with $^{32}PO_4$ (0.25 mCi/ml) in phosphate-free D-MEM for 4 h and RalA was precipitated using nickel resin beads. The labeled nucleotides were separated by TLC and GTP and GDP spots were quantitated using a PhosphorImager. Results from duplicate transfections are shown. Similar results were obtained in all of five independent experiments. Cells in some dishes were used to make lysates for immunoblotting to detect levels of Myc-Ral-GDS and RalA in transfected cells. The lower band represents endogenous Ral and the upper band (His_6)-RalA. **(B)** COS-7 cells were transfected with (His_6)-RalA together with either Ral-GDS or Ral-GDS(ΔC) along with RasH(61L), R-Ras(38V) or Rap1A(63E). 48 h later the proportion of (His_6)RalA bound to GTP was determined as in (A). The data represent the average from duplicate transfections from a representative experiment. The range of results are indicated. Similar results were obtained in each of at least three independent experiments.

superfamily members with the potential to bind Ral-GDS, only Ras can increase Ral-GDS activity *in vivo*.

Expression of activated RalA enhances transforming activity of Ras and Raf

The data presented above suggest that activation of Ral-GDS, and thus Ral, constitutes a distinct downstream signaling pathway from Ras. If this were the case, one might expect this pathway to contribute to some of the phenotypes induced by Ras in cells, such as cellular proliferation and oncogenic transformation. We therefore tested the ability of activated RalA to produce transformed

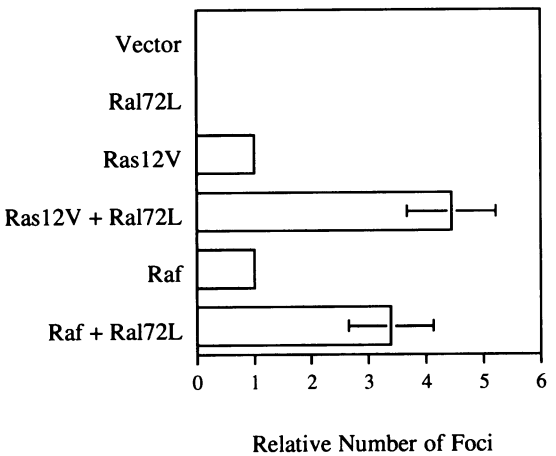

Fig. 4. Activated Ral enhances the transforming activities of RasH and Raf. Non-saturating amounts of oncogenic RasH(12V) or of a partially activated c-Raf deletion mutant were transfected together with vector alone or with the same amount of vector containing constitutively activated RalA(72L) into NIH 3T3 cells. The number of foci of transformed cells were counted 14 days later. Similar results were obtained when fully activated v-Raf was used. The data are expressed as the number of foci relative to that obtained with either oncogenic Ras (~12 foci/dish) or Raf (~13 foci/dish) and represent the average (± SEM) of four independent transfection experiments each performed in duplicate.

foci upon transfection of NIH 3T3 cells. We also tested its ability to influence focus formation induced by oncogenic Ras, and oncogenic Raf. The latter stimulates an additional downstream pathway from Ras.

RalA(72L) has a mutation that is analogous to the Ras oncogenic mutation (61L) such that the protein has a defective intrinsic GTPase activity (Frech *et al.*, 1990) and fails to respond to Ral-GAP (Emkey *et al.*, 1991). Unlike its Ras counterpart however, RalA(72L) failed to produce transformed foci upon transfection into NIH 3T3 cells (Figure 4). Cells stably expressing RalA(72L) also failed to proliferate in reduced serum conditions (unpublished observation). However, transfection of constitutively activated RalA stimulated the focus-forming activity of a submaximal amount of Ras(61L) ~4-fold (Figure 4). Transfection of Ral(72L) also enhanced the focus forming activity of a partially transforming allele of Raf ~3-fold (Figure 4). Thus, RalA activity can facilitate transformation induced by the Ras/Raf signaling pathway.

To begin to understand how activated Ral enhances cellular transformation by Ras, we investigated whether Ral can influence the activity of Erk proteins. This family of Ser/Thr kinases acts downstream of Ras in a signaling cascade that contributes to Ras-induced cell transformation. 293T cells were transfected with a Myc-tagged Erk-2 clone alone or together with Ral(72L). Two days later cells were serum starved for 12 h and then harvested. Erk2 was immunoprecipitated and its activated state was detected by its slower mobility in SDS gels (Figure 5). Expression of activated Ral failed to activate Erk2, consistent with its inability to promote transformation on its own. Furthermore, expression of Ral(72L) did not enhance Erk activation induced by EGF. This negative result was not obtained because Erk2 was already maximally stimulated by EGF, since even greater Erk2 activation was observed when activated Ras was transfected (Figure

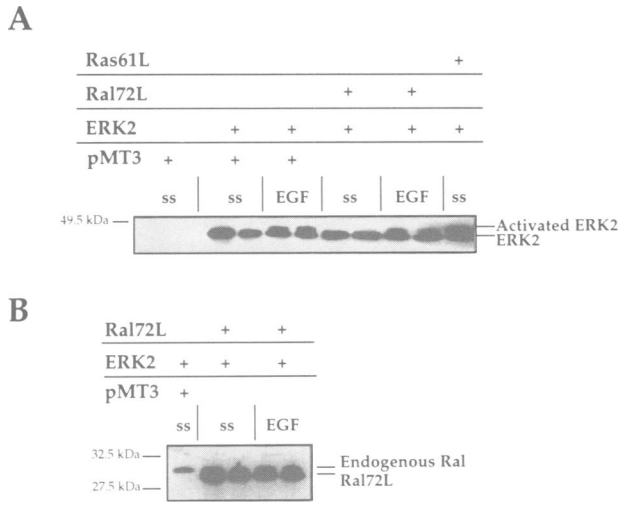

Fig. 5. Ral does not activate Erk2. 293T cells were transiently transfected with (His_6)Erk2 (kindly provided by M.Cobb) and either empty vector or Ral(72L) or Ras(61L). After 24 h, cells were serum starved for 12 additional hours and then stimulated with either 25 ng/ml EGF for 10 min or buffer. Cells were then extracted and ERK2 was purified on nickel resin and detected after SDS–PAGE by immunoblotting (**A**) with anti-Erk2 antiserum (kindly provided by M.Cobb). Alternatively, cell extracts were fractionated by SDS–PAGE and immunoblotted to document the expression of transfected Ral(72L) (**B**). ss, serum starved.

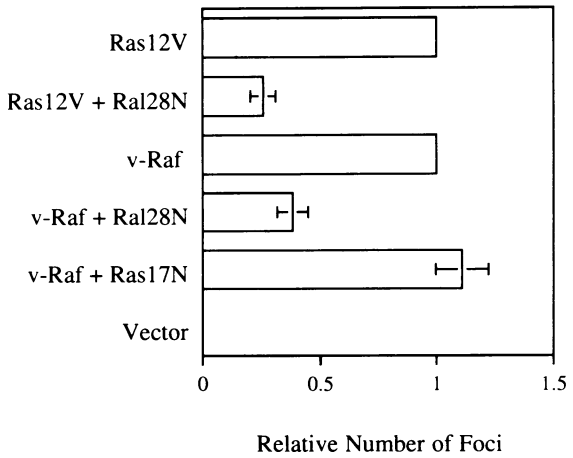

Fig. 6. Dominant inhibitory Ral(28N) inhibits the transforming activities of Ras and Raf. Non-saturating amounts of RasH(12V) or v-Raf were transfected together with empty vector or with the same amount of plasmid containing dominant inhibitory Ral(28N) or Ras(17N) into NIH 3T3 cells. Fourteen days later the number of transformed foci of cells were quantitated. The data are expressed as the number of foci relative to that obtained with either oncogenic Ras (~12 foci/dish) or Raf (~13 foci/dish) and represent the average (± SEM) of four independent transfection experiments each performed in duplicate.

5). These results argue that Ral does not potentiate cellular transformation by enhancing the Ras/Raf/Mek/Erk cascade.

Inhibition of Ral function in vivo suppresses cellular transformation

The significance of Ras-induced Ral activation in cellular proliferation and oncogenic transformation was investigated by blocking Ral activity in cells by expression of a dominant inhibitory RalA(28N) mutant (Figure 6). This mutant is analogous to Ras(17N) that has been shown to interfere with Ras exchange-factor activation of endogenous Ras (Farnsworth and Feig, 1991; Schweighoffer *et al.*, 1993). Transfection of the analogous RalA(28N) in a neo^r-containing vector into NIH 3T3 cells generated much fewer G418-resistant colonies than control vector, and those cells that did grow failed to express measurable Ral(28N) protein above endogenous Ral levels (data not shown). These results are consistent with Ral function being required for cell proliferation. Transfection of Ral(28N) also inhibited focus formation induced by Ras(61L), indicating that active Ral is also necessary for full Ras transforming activity (Figure 7). Interestingly, transfection of RalA(28N) also suppressed focus formation induced by Raf (Figure 6). This is contrary to results obtained with dominant negative Ras(17N), which failed to block Raf transformation.

Discussion

The evidence presented in this study argues that Ral-GDS is a distinct downstream target of Ras proteins (Figure 7). It also supports the idea that Ras-induced activation of Ral-GDS and its substrate Ral, contributes to the growth regulatory effects of Ras. These conclusions are based on the fact that Ras was shown to bind to and activate Ral-GDS in cells. Moreover, expression of constitutively activated RalA enhanced the transforming activities of both Ras and Raf; and expression of dominant interfering RalA suppressed the transforming activities of these two oncogenes.

The first hint of this new signaling pathway came from previous two-hybrid screens in yeast, where Ral-GDS was shown to bind to the activated form of Ras. However, Ral-GDS also bound to activated R-Ras and Rap1A proteins, that are known to possess the same effector domain as Ras. Thus, it was not clear from these studies which, if any of these GTPases regulate Ral-GDS *in vivo*. Here we showed that all three GTPases bound to Ral-GDS when the proteins were overexpressed in COS-7 cells, but that only Ras activated Ral-GDS.

Ras induced Ral-GDS activation was dependent upon the two proteins binding *in vivo*, since a mutant Ral-GDS with its Ras-binding C-terminus deleted, failed to respond to Ras expression. Interestingly, this mutant also showed reduced basal activity *in vivo* despite normal exchange activity *in vitro*. These results suggest that the C-terminus of Ral-GDS plays a role in targeting Ral-GDS to Ral even in unstimulated cells.

It is not yet clear how Ras binding activates Ral-GDS. One possible mechanism that is analogous to receptor activation of the SOS Ras exchange factor, is Ras-mediated redistribution of Ral-GDS to its target protein Ral. Like Ras, RalA is present on the inner face of the plasma membrane (R.Emkey *et al.*, submitted). However, the majority of Ral has been detected in endocytic (Feig and Emkey, 1993) and exocytic vesicles (Bielinski *et al.*, 1993; Volknandt *et al.*, 1993), suggesting it spends only a short time associated with the plasma membrane. Presumably R-Ras, whose location in the cell has not been clearly established, and Rap1A that is localized to late endosomes

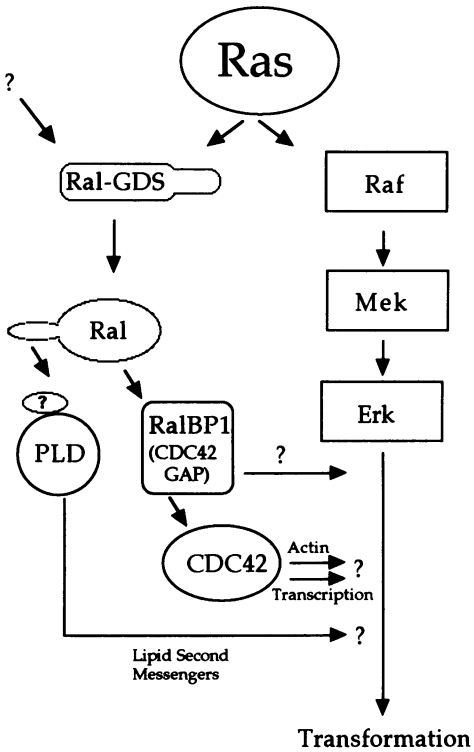

Fig. 7. Signal transduction pathways emanating from Ras through Raf and Ral-GDS. The data presented in this paper demonstrate that activation of Ral-GDS and subsequently Ral, constitutes a distinct downstream signaling pathway from Ras. One potential downstream target of Ral is the recently cloned Ral binding protein 1 (RalBP1) that is a GAP for the Rho family GTPase CDC42 (Cantor *et al.*, 1995). This protein binds to a region of Ral that is similar to the effector domain of Ras. We have recently found that Ral also interacts with phospholipase D (PLD) (either directly or indirectly) at a second region of Ral involved in effector function located at its N-terminus (manuscript submitted). Ral does not induce transformation on it own, but does facilitate transformation induced by activated Ras and activated Raf. This effect could be mediated by RalBP1 through its effect on CDC42, by an additional undefined function of RalBP1, or by second messengers produced by PLD. The question mark pointing to Ral-GDS refers to the possibility that other signals besides Ras activate Ral-GDS since dominant negative Ras did not give the same phenotype as dominant negative Ral (see Figure 6).

in fibroblasts (Pizon *et al.*, 1994) and plasma membrane and granules in platelets (Berger *et al.*, 1994), cannot deliver bound Ral-GDS to Ral in COS-7 cells. It remains to be determined whether Ras binding increases the intrinsic exchange activity of Ral-GDS.

Experiments with inhibitory RalA(28N) suggest that Ral function plays a vital role that is required for cells to proliferate. Like the analogous inhibitory Ras(17N) mutant, high level expression of Ral(28N) did not seem to be compatible with cell proliferation of NIH 3T3 cells. The loss of Ral function *in vivo* also reduced the efficiency with which both Ras and Raf induced oncogenic transformation. These effects distinguish Ral from Ras function in that inhibitory Ras(17N) did not block transformation by oncogenic Raf (Figure 6) (Feig and Cooper, 1988; Stacey *et al.*, 1991).

This set of experiments also suggests that Ral activity is not completely dependent upon Ras function (see Figure 7). If it was, inhibition of Ras by Ras(17N) expression would be expected to completely block Ral activity and thus suppress Raf transformation. This Ras independent Ral activity [that is blocked by inhibitory RalA(28N)] is apparently required for full transforming activity by Raf.

A striking finding of this study is that although activated RalA cannot promote oncogenic transformation on its own, its expression in NIH3T3 cells can enhance the transforming potential of activated Ras and Raf. How Ral activity alters cellular proliferation is not yet known. However, it is clear that on its own activated RalA does not dramatically influence ERK activation by upstream signals. One clue to Ral function has emerged from our recent cloning of a putative downstream target of RalA. This Ral-binding protein, RalBP1, interacts with the region of GTP-bound Ral that is analogous to the effector domain of Ras. RalBP1 contains a GAP domain that preferentially effects the GTPase activity of the Rho family member, CDC42 (Cantor *et al.*, 1995). Since CDC42 has been shown to promote filopodia formation (Kozma *et al.*, 1995; Nobes and Hall, 1995), Ral may influence oncogenic transformation through changes in cell shape (see Figure 7). CDC42 has also been shown to activate the JNK/SAPK kinases (Coso *et al.*, 1995; Minden *et al.*, 1995), suggesting Ral may influence cell growth by altering gene transcription. Interestingly, the Rho family member Rac, which promotes membrane ruffling and the JNK/SAPK pathway, has also been shown to facilitate cell transformation by Ras and Raf (Qiu *et al.*, 1995).

We have recently documented that Ral is also involved in the activation of phospholipase D (PLD) by Src (Jiang *et al.*, 1995). This pathway is dependent upon a distinct domain of Ral at its unique N-terminus. PLD is activated by a wide variety of ligands that activate tyrosine kinase receptors (Foster, 1993). The enzyme hydrolyzes phosphatidylcholine to produce phosphatidic acid (PA), a known mitogen. PA can be further metabolized to diacylglycerol, which activates protein kinase C. Ral may therefore facilitate oncogenic transformation by promoting the generation of these second messenger molecules (see Figure 7).

Thus, Ral-GDS and RalA join Raf, PI_3 kinase and Ras-GAP as likely effectors of Ras function (Feig and Schauffhausen, 1994). RalB also responds to Ral-GDS (Albright *et al.*, 1993), so that it also likely functions as a Ras effector. In addition to activating the well-known protein kinase cascade involving Raf, MEK and MAP kinase, the data presented here show that Ras initiates a cascade of GTPase cycles (see Figure 7). By activating Ral GTPases, Ras also participates in the modulation of phospholipid metabolism through PLD. This complex signaling array emanating from Ras accentuates its critical role in signal transduction.

Materials and methods

Plasmid construction

Ral-GDS was inserted into an altered version of pMT3 that contained a modified Glu (MEFMPME) (Farnsworth *et al.*, 1995) or Myc epitope (MEQKLISEEDL) 5′ to the cloning site by PCR. The 3′ deletion mutation of Ral-GDS [Ral-GDS(ΔC)], lacking the final 130 codons, was generated by isolating a *Pst*I fragment of Ral-GDS containing the first 722 codons and recloning it back into pMT3. pMT3-RasH(61L) was constructed by placing *Eco*RI linkers on Ras removed from pXCR Ras(61L) (Feig *et al.*, 1986). The *Eco*RI fragment was then cloned into

the *Eco*RI cloning site of pMT3. pMT3-Myc R-Ras(38V) was a gift from Dr Alan Hall. pMT3-Glu Rap1A was constructed as follows. The Glu-tagged Rap1A(63E) fragment was excised from plasmid pZIP Rap1A(63E) (a gift of Dr Channing Der). *Eco*RI linkers were added, and the resulting fragment was subcloned into the *Eco*RI site of pMT3. An epitope containing six histidines was added to the N-terminus of RalA by PCR. The PCR product was then cloned into the expression vector pMT3. The 778 bp *Eco*RI–*Hin*dIII fragment encoding the *ral* mutants were isolated from ptacRalA(28N) and ptacRalA(72L) (gift of P.Chardin). *Bam*HI linkers were added and the genes were cloned into the *Bam*HI site of pZipneoSV(X).

Transient transfections

COS-7 cells (5×10^5 cells in a 60 mm culture dish) were transfected with various forms of Ral-GDS DNA by the DEAE–Dextran method (Ausubel *et al.*, 1987). 293T cells (Pear *et al.*, 1993) (5×10^5 cells in a 60 mm culture dish) were transfected by the HEPES-buffered calcium phosphate precipitation procedure (Ausubel *et al.*, 1987).

Detection of protein complexes

Transfected COS-7 cells were lysed in 0.5 ml buffer A [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM PMSF and 10 μg/ml aprotinin] and immunoprecipitated with 20 μl of anti-Glu epitope monoclonal antibody bound to protein G–Sepharose. Immunoblots were visualized with anti-Myc, anti-Glu epitope (gift from S.Powers), anti-Rap1A monoclonal (gift from Koichi Furukawa) or anti-Ras polyclonal antibody (UBI) by ECL (Amersham).

In vivo Ral-GDS assay

COS-7 cells were transfected with 1.0 μg of pMT3-(His_6)-Ral, plus mixtures of the following DNAs: 1.0 μg of pMT3 Ral-GDS, 1.0 μg pMT3 Ral-GDS(ΔC), 0.5 μg pMT3 RasH(61L) 0.5 μg pMT3 RasH, 0.5 μg pMT3 R-Ras(38V), 0.5 μg pMT3 Rap1A(63E) or 0.5 μg pMT3 only. Cells were metabolically labeled 48 h later with $^{32}PO_4$ (0.25 mCi/ml) in phosphate-free D-MEM for 4 h. The cells were lysed in 0.5 ml buffer B [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1% Triton X-100, 1 mM PMSF and 10 μg/ml aprotinin]. After removing insoluble material by centrifugation at 10 000 *g* for 5 min, the extract was incubated for 60 min with 30 μl of a 50% slurry of Ni^{2+}-NTA-agarose (Qiagen) which had been prewashed in buffer H [50 mM NaH_2PO_4 (pH 8.3), 0.3 M NaCl]. The beads were then washed four times in buffer H plus 10 mM imidazole. After a final wash in phosphate-buffered saline, nucleotides precipitated with Ral were eluted from the beads and separated by PEI-cellulose TLC. The % GTP was calculated as counts in GTP/(counts in GTP + GDP) with a PhosphorImager (Molecular Dynamics), after normalizing for moles of phosphate.

Focus-forming assays

NIH 3T3 cells were transfected by the calcium phosphate precipitation method as described (Feig and Cooper, 1988). Sixty ng of pM1 [a 6.0 kb genomic clone of RasH12V in pBR322 (Feig and Cooper, 1988)] or 20 ng of Raf20A [a partially activated allele of c-Raf with 313 amino acids deleted from the N-terminus (Stanton *et al.*, 1989)], were transfected along with 2 μg of pZipneoSV(X) or 2 μg of pZipneoSV(X) containing RalA(72L), RalA(28N) or RasH(17N) (Feig and Cooper, 1988).

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References

Albright,C.F., Giddings,B.W., Liu,J., Vito,M. and Weinberg,R.A. (1993) Characterization of a guanine nucleotide dissociation stimulator for a ras-related GTPase. *EMBO J.*, **12**, 339–347.

Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (Eds) (1987) *Current Protocols in Molecular Biology*. Wiley and Sons, New York.

Berger,G., Quarck,R., Tenza,D., Levy-Toledano,S., de Gunzburg,J. and Cramer,E.M. (1994) Ultrastructural localization of the small GTP-binding protein Rap 1 in human platelets and megakaryocytes. *Br. J. Haematol.*, **88**, 3777–3782.

Bhullar,R.P., Chardin,P. and Haslam,R.J. (1990) Identification of multiple ral gene products in human platelets that account for some but not all of the platelet G-proteins. *FEBS Lett.*, **260**, 48–52.

Bielinski,D.F., Pyun,N.Y., Linko-Stentz,K., Macara,I. and Fine,R.E. (1993) Ral and Rab3a are major GTP binding proteins of axonal rapid transport vesicles of synaptic vesicles and do not redistribute following depolarization stimulated synaptosomal exocytosis. *Biochim. Biophys. Acta*, **1151**, 246–256.

Boguski,M.S. and McCormick,F. (1993) Proteins regulating Ras and its relatives. *Nature*, **366**, 643–654.

Cantor,S., Urano,T. and Feig,L.A. (1995) Identification and characterization of RalBP1, a potential downstream target of Ral GTPases. *Mol. Cell. Biol.*, **15**, 4578–4584.

Casey,P.J., Solski,P.A., Der,C.J. and Buss,J.E. (1989) p21Ras is modified by a farnesyl isoprenoid. *Proc. Natl Acad. Sci. USA*, **86**, 8323–8327.

Chardin,P. (1993) Structural conservation of Ras-related proteins and its functional implications. In Lacal,J.C. and McCormick,F. (eds), *GTPases in Biology I*. Springer-Verlag, Berlin, pp. 159–176.

Chardin,P. and Tavitian,A. (1986) The *ral* gene: a new ras-related gene isolated by the use of a synthetic probe. *EMBO J.*, **5**, 2203–2208.

Coso,O.A., Chiariello,M., Jin-Chen,Y., Teramoto,H., Crespo,P., Xu,N., Miki,T. and Gutkind,J.S. (1995) The small GTP-binding proteins Rac and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell*, **81**, 1137–1146.

Cox,A.D., Brtva,T.R., Lowe,D.G. and Der,C.D. (1994) R-Ras induces malignant, but not morphologic, transformation of NIH3T3 cells. *Oncogene*, **9**, 3281–3288.

Emkey,R., Freedman,S. and Feig,L.A. (1991) Characterization of a GTPase activating protein for the Ras-related Ral protein. *J. Biol. Chem.*, **266**, 9703–9706.

Farnsworth,C.L. and Feig,L.A. (1991) Dominant inhibitory mutations in the Mg^{2+} binding site of Ras blocks its activation by GTP. *Mol. Cell. Biol.*, **11**, 4822–4829.

Farnsworth,C.L., Freshney,N.W., Rubin,L.B., Ghosh,A., Greenberg,M.E. and Feig,L.A. (1995) Calcium activation of Ras mediated by the neuronal exchange factor Ras-GRF. *Nature*, **376**, 524–527.

Feig,L.A. (1994) Guanine-nucleotide exchange factors: a family of positive regulators of Ras and related GTPases. *Curr. Opin. Cell Biol.*, **6**, 204–211.

Feig,L.A. and Cooper,G.M. (1988) Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP. *Mol. Cell. Biol.*, **8**, 3235–3243.

Feig,L.A. and Emkey,L.A. (1993) Ral Gene products and their regulation. In Lacal,J.C. and McCormick,F. (eds), *The ras Superfamily of GTPases*. CRC Press, London, pp. 247–258.

Feig,L.A. and Schauffhausen,B. (1994) The hunt for Ras targets. *Nature*, **370**, 508–509.

Feig,L.A., Pan,B.T., Roberts,T.M. and Cooper,G.M. (1986) Isolation of ras GTP-binding mutants using an *in situ* colony-binding assay. *Proc. Natl Acad. Sci. USA*, **83**, 4607–4611.

Foster,D.A. (1993) Intracellular signalling mediated by protein-tyrosine kinases: networking through phospholipid metabolism. *Cell. Signalling*, **5**, 389–399.

Frech,M., Schlichting,I., Wittinghofer,A. and Chardin,P. (1990) Guanine nucleotide binding properties of the mammalian RalA protein produced in *Escherichia coli*. *J. Biol. Chem.*, **265**, 6353–6359.

Hofer,F., Fields,S., Schneider,C. and Martin,G.S. (1994) Activated Ras interacts with the Ral guanine nucleotide dissociation stimulator. *Proc. Natl Acad. Sci. USA*, **91**, 11089–11093.

Jiang,H., Luo,J.-Q., Urano,T., Frankel,P., Lu,Z., Foster,D.A. and Feig,L.A. (1995) Involvement of Ral GTPase in v-Src-induced phospholipase D activation. *Nature*, **378**, 409–412.

Kikuchi,A., Demo,S.D., Ye,Z.-H., Chen,Y.W. and Williams,L.T. (1994) ralGDS family members interact with the effector domain of ras p21. *Mol. Cell. Biol.*, **14**, 7483–7491.

Kinsella,B.T. and Maltese,W.A. (1991) Carboxyl-terminal isoprenylation of ras-related GTP binding proteins rac1, rac2 and ralA. *J. Biol. Chem.*, **266**, 9786–9794.

Kozma,R., Ahmed,S., Best,A. and Lim,L. (1995) The Ras-related protein CDC42 and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. *Mol. Cell. Biol.*, **15**, 1942–1952.

Minden,A., Lin,A., Claret,F.-X., Abo,A. and Karin,M. (1995) Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and CDC42Hs. *Cell*, **81**, 1147–1157.

Nobes,C.D. and Hall,A. (1995) Rho, Rac and CDC42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell*, **81**, 53–62.

Pear,W.S., Nolan,G.P., Scott,M.L. and Baltimore,D. (1993) Production of high-titer helper free retroviruses by transient transfection. *Proc. Natl Acad. Sci. USA*, **90**, 8392–8396.

Pizon,V., Desjardins,M., Bucci,C., Parton,R.G. and Zerial,M. (1994) Association of Rap1A and Rap1B proteins with the late endocytic/phagocytic compartments and Rap2A with the Golgi complex. *J. Cell Sci.*, **107**, 1661–1670.

Qiu,R.-G., Chen,J., Kirn,D., McCormick,F. and Symons,M. (1995) An essential role for Rac in Ras transformation. *Nature*, **374**, 457–459.

Schweighoffer,F., Cai,H., Chevallier,M.C., Fath,I., Cooper,G.M. and Tocque,B. (1993) The *Saccharomyces cerevisiae* SDC25 C-domain gene product overcomes the dominant inhibitory activity of Ha-Ras Asn-17. *Mol. Cell. Biol.*, **13**, 39–43.

Spaargaren,M. and Bischoff,J.R. (1994) Identification of the guanine nucleotide dissociation stimulator for Ral as a putative effector molecule of R-Ras, N-Ras, K-Ras and Rap. *Proc. Natl Acad. Sci. USA*, **91**, 12609–12613.

Stacey,D.W., Roudebush,M., Day,R., Mosser,S.D., Gibbs,J.B. and Feig,L.A. (1991) Dominant inhibitory Ras mutants demonstrate the requirement for Ras activity in the action of tyrosine kinase oncogenes. *Oncogene*, **6**, 2297–2304.

Stanton,V.P., Nichols,D.W., Laudano,A.P. and Cooper,G.M. (1989) Definition of the human raf amino-terminal regulatory region by deletion mutagenesis. *Mol. Cell. Biol.*, **9**, 639–647.

Volknandt,W., Pevsner,J., Elferink,L.A. and Scheller,R.H. (1993) Association of three small GTP-binding proteins with cholinergic synaptic vesicles. *FEBS Lett.*, **317**, 53–56.

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