

# PDK1 mediates growth factor-induced Ral-GEF activation by a kinase-independent mechanism

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**Ras proteins transduce extracellular signals to intracellular signaling pathways by binding to and promoting the activation of at least three classes of downstream signaling molecules: Raf kinases, phosphoinositide-3-kinase (PI3-K) and Ral guanine nucleotide exchange factors (Ral-GEFs). Previous work has demonstrated that epidermal growth factor (EGF) activates Ral-GEFs, at least in part, by a Ras-mediated redistribution of the GEFs to their target, Ral-GTPases, in the plasma membrane. Here we show that Ral-GEF stimulation by EGF involves an additional mechanism, PI3-K-dependent kinase 1 (PDK1)-induced enhancement of Ral-GEF catalytic activity. Remarkably, this PDK1 function is not dependent upon its kinase activity. Instead, the non-catalytic N-terminus of PDK1 mediates the formation of an EGF-induced complex with the N-terminus of the Ral-GEF, Ral-GDS, thereby relieving its auto-inhibitory effect on the catalytic domain of Ral-GDS. These results elucidate a novel function for PDK1 and demonstrate that two Ras effector pathways cooperate to promote Ral-GTPase activation.**

**Keywords:** PDK1/Ral/Ral-GDS/Ras/signal transduction

## Introduction

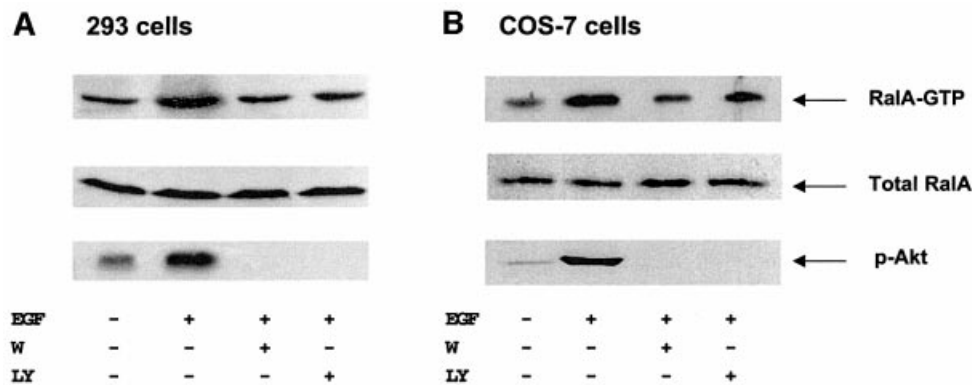
Ral-GTPases are members of the Ras superfamily of GTPases, and as such they function as molecular switches that cycle between the active GTP-bound and inactive GDP-bound states (Feig *et al.*, 1996). They become activated upon interaction with one of a family of Ral-specific guanine nucleotide exchange factors (Ral-GEFs), which promote GDP release from Ral allowing GTP to bind in its place. Ral proteins have attracted significant attention in recent years because Ral-GEFs, along with Raf and phosphoinositide-3-kinase (PI3-K) constitute the three known classes of proteins whose activities are regulated by binding to Ras proteins in cells (Takai *et al.*, 2001). Thus, Ral proteins become activated in response to ligands like epidermal growth factor (EGF) that activate Ras, and as a consequence, they mediate at least some of the cellular actions of Ras (Wolthuis *et al.*, 1998).

Active Ral proteins can influence a variety of cellular processes by interacting with a distinct set of downstream target proteins. These include RalBP1 (or RLIP) (Cantor *et al.*, 1995; Jullien-Flores *et al.*, 1995; Park and

Weinberg, 1995), which binds to a family of related EH domain proteins, Repl1 (Yamaguchi *et al.*, 1997) and POB1 (Ikeda *et al.*, 1998), and to the AP2 complex (Jullien-Flores *et al.*, 2000). These associations have implicated Ral function in the regulation of endocytosis (Nakashima *et al.*, 1999). RalBP1 is also a GTPase activating protein (GAP) for Rac and CDC42. This, and the fact that Ral-GTP also binds to the actin cross-linking protein, filamin (Ohta *et al.*, 1999), connects Ral to the regulation of the actin cytoskeleton. Recently, the exocyst (Sec6/8) complex, which is known to participate in targeting exocytic vesicles to specific sites on the plasma membrane, has been shown to be a putative downstream effector for Ral (Ikeda *et al.*, 1998; Polzin *et al.*, 2002). This may explain Ral's influence on neurosecretion through its ability to regulate the readily-releasable pool of synaptic vesicles (Polzin *et al.*, 2002). Finally, Ral proteins associate constitutively with and participate in the regulation of phospholipase D1 (Jiang *et al.*, 1995). This property may contribute to Ral's effects on vesicle function, and also to its ability to promote cell proliferation (Lu *et al.*, 2000). Ral proteins also mediate c-Src activation by EGF receptors (Goi *et al.*, 2000), promote Ras-induced Jnk kinase activation (de Ruiter *et al.*, 2000), activate cyclin D expression (Gille and Downward, 1999; Henry *et al.*, 2000) and inhibit the forkhead transcription factor (Kops *et al.*, 1999). The Ral effector proteins involved in these functions have not yet been elucidated.

Ras proteins can activate Ral proteins by binding to a set of Ral-GEFs that contain Ras-binding domains at their C-termini (Urano *et al.*, 1996; Wolthuis *et al.*, 1997). Activation of Ral is thought to occur, at least in part, by Ras-induced targeting of the Ral-GEFs to the plasma membrane where a fraction of its targets RalA and RalB exist (Kishida *et al.*, 1997; Matsubara *et al.*, 1999). However, indirect evidence has suggested that Ras-binding may not be sufficient to activate Ral-GEFs under all conditions (Rusanescu *et al.*, 2001). In addition, Ral proteins can be activated by GEFs that do not contain Ras-binding domains (Gotoh *et al.*, 2000; Rebhun *et al.*, 2000). Which extracellular signals use these Ral-GEFs is not yet clear, although calcium may be one (Hofer *et al.*, 1998).

Ras proteins can also bind to and activate the catalytic domain of PI3-K (Rodriguez-Viciana *et al.*, 1996). This function of Ras complements that of tyrosine kinase receptors, which bind to the SH2 domain of the non-catalytic subunit of PI3-K and target it to its lipid substrates in the plasma membrane. PI3-K generates the second messenger molecules, phosphoinositide-3,4-diphosphate (PtdIns-3,4-P<sub>2</sub>) and phosphoinositide-3,4,5-triphosphate (PtdIns-3,4,5-P<sub>3</sub>) (PIP<sub>3</sub>). They bind to and alter the activity of multiple cellular components, many of which contain pleckstrin homology (PH) domains (Rameh and



**Fig. 1.** PI3-K inhibitors block EGF activation of Ral. 293 (A) and COS7 (B) cells were stimulated for 10 min with EGF in the presence or absence of the PI3-K inhibitors wortmannin (W) or LY94002 (LY). Active RalA-GTP was then affinity purified from lysates of the cells with the Ral-binding domain of RalBP1 and detected by immunoblotting with anti-Ral antibodies. Total RalA in the cell lysates is also shown, as is active Akt as detected by a phospho-specific Akt antibody. Results are representative of experiments performed at least three times.

Cantley, 1999). One key PIP3-binding protein is PI3-K-dependent kinase 1 (PDK1), which plays a pivotal role in cellular signaling because it can phosphorylate a wide variety of cellular protein kinases of the AGC family, including Akt/PKB, S6 kinase, Rsk, PRKs, SGK, atypical and conventional PKCs, and p90RSK (Toker and Newton, 2000; Alessi, 2001). Through its effects on these kinases, PDK1 has the potential to regulate a wide variety of processes, including cell proliferation, differentiation and apoptosis.

In this paper, we identify PDK1 as a new participant in the mechanism of Ral activation by ligands such as EGF. PDK1 enhances the specific activity of the Ral-GEF, Ral-GDS, in response to EGF. This function complements EGF-induced Ras binding to Ral-GDS, which promotes its localization to Ral in the plasma membrane. Most strikingly, we have found that PDK1 does not use its kinase activity for this function. Rather the N-terminus of PDK1 forms a ligand-induced complex with Ral-GDS, which elevates its GEF activity.

## Results

### **EGF-induced activation of Ral-GTPases is dependent upon PI3-K activity**

To investigate the potential involvement of PI3-K in the mechanism of Ral-GTPase activation, the effect of PI3-K inhibitors on the ability of EGF to promote the GTP bound state of RalA in cells (Figure 1) was assessed. HEK 293 cells were pre-treated with either of two PI3-K inhibitors, wortmannin and LY94002, or empty buffer. The cells were then stimulated with EGF for 10 min, lysed and the amount of GTP-bound RalA was assessed by affinity purification with the Ral-binding domain of its effector protein, RalBP1 and immunoblotting with anti-RalA antibodies. As shown previously, EGF increased the amount of active GTP-bound RalA in HEK 293 cells by ~3-fold, whereas the total RalA in the sample did not change. In contrast, EGF activation of RalA in cells pre-treated with either of the two PI3-K inhibitors was suppressed (Figure 1A). Measurement of active Akt in the same cell lysates by immunoblotting with phospho-specific Akt antibodies (pT308), as an assessment of PI3-K

activation, showed that as expected EGF activation of Akt was blocked by both wortmannin and LY94002. Similar results were obtained with COS7 cells (Figure 1B) and when GTP-RalB was measured instead (data not shown).

### **Ral-GTPases can be activated by PI3-K and PDK1, but not Akt**

The experiments described above suggested that in addition to the well-characterized role of EGF induced Ras-binding to Ral-GEFs, EGF activation of Ral also requires the activation of a second Ras effector, PI3-K. To add support to this hypothesis, PI3-K was tested for its ability to activate RalA in cells. When a cDNA encoding a constitutively activated PI3-K was transfected into HEK 293 cells, the amount of endogenous GTP-RalA present in the cells increased ~2-fold (Figure 2A).

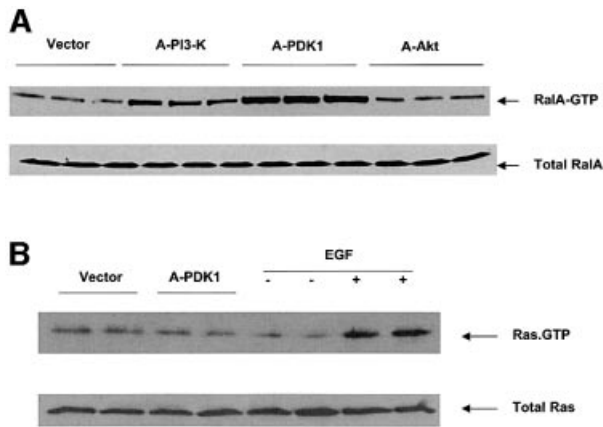
PI3-K produces the lipid mediators PtdIns-3,4-P<sub>2</sub> and PtdIns-3,4,5-P<sub>3</sub>, which have a variety of effects on cells. One of the key targets of PtdIns-3,4,5-P<sub>3</sub> is the protein kinase PDK1. To test whether PI3-K could influence RalA activation through PDK1, the cDNA encoding the kinase itself was transfected into HEK 293 cells, and its effect on GTP-Ral levels was assessed. PDK1 strongly increased RalA-GTP levels (~4-fold) (Figure 2A). In contrast, transfection of a cDNA encoding a constitutively activated mutant of Akt, a downstream target of PDK1, had no effect on GTP-RalA levels. All three components of the PI3-K pathway were active in this system since they all led to the suppression of the forkhead transcription factor (data not shown). These findings indicate that among the components of the PI3-K signaling cascade tested, PDK1 is the most effective activator of Ral.

The specificity of PDK1 action was then assessed by measuring its ability to activate Ras. Endogenous levels of GTP-Ras in transfected cells were measured by affinity purification with the Ras-binding domain of its downstream target, Raf. In contrast to its ability to activate RalA, PDK1 did not activate Ras (Figure 2B). The Ras protein in these cells was responsive to signals since it was activated by the addition of EGF (Figure 2B). This result demonstrates that PDK1 does not activate RalA through Ras activation, but rather by a novel mechanism.

### PK1 activates the intrinsic GEF activity of Ral-GDS

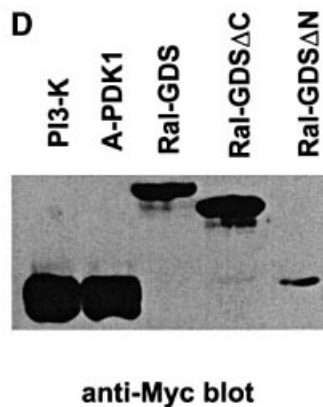
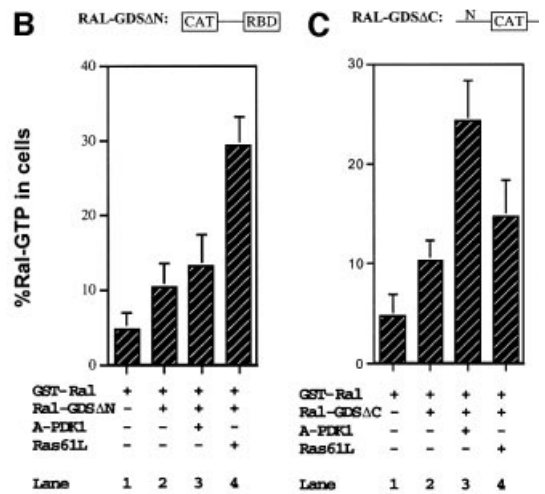
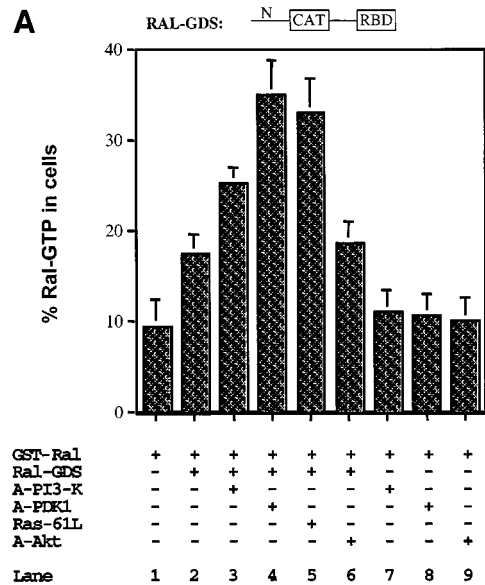
Since the primary means of regulating the activity of Ras family GTPases appears to be through changes in the activity of their GEFs, we investigated the effects of PI3-K and PDK1 on the activity of the Ral-GEF, Ral-GDS. This was accomplished with an *in vivo* assay for Ral-GEF activity that we have used previously, where the ability of the Ral-GEF to promote the GTP-bound state of co-transfected glutathione *S*-transferase (GST)-RalA was assessed (Figure 3). Transfected Ral was isolated from  $^{32}\text{PO}_4$ -labeled cells and the ratio of labeled nucleotides (GTP/GTP + GDP) bound to RalA was measured by thin-layer chromatography. When Ral was transfected alone into 293 cells, ~10% of the nucleotide bound to RalA was GTP (Figure 3A, lane 1). As we have shown previously (Urano *et al.*, 1996), the activity of co-transfected Ral-GDS was detectable by its ability to increase the proportion of GTP bound to RalA to ~18% (lane 2). When activated PI3-K was transfected along with Ral-GDS,

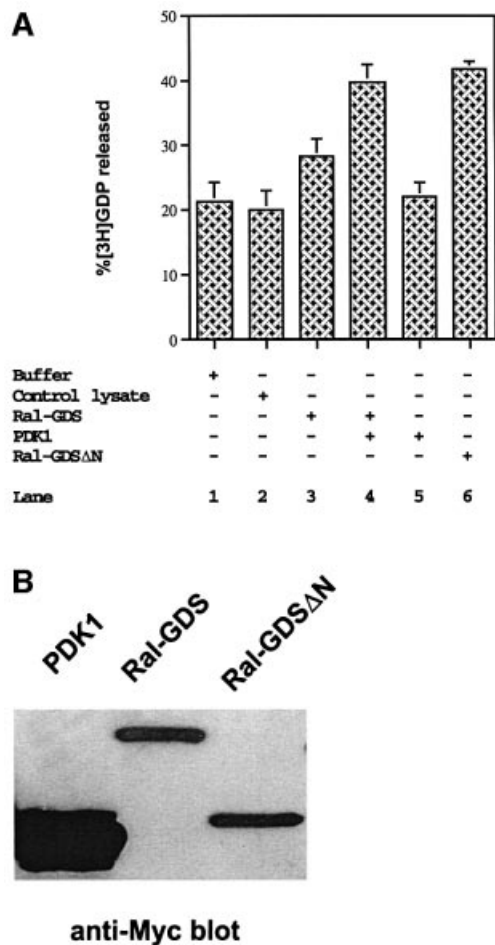
Ral-GTP levels rose to ~25% GTP (lane 3). When PDK1 was included in the transfection the proportion of Ral bound to GTP rose to even a greater degree (~35%) (lane 4), a level comparable to that observed when constitutively activated Ras was co-transfected with Ral-GDS (lane 5). In contrast, transfection of activated Akt had



**Fig. 2.** PI3-K and PDK1 but not Akt activate Ral but not Ras in cell. (A) 293 cells were transfected in triplicate with empty vector, vector expressing constitutively activated forms of PI3-K PDK1 and Akt (A-PI3-K, A-PDK1, A-Akt-CAAX box added to their C-termini). Twenty-four hours later, the cells were serum-starved and then active RalA-GTP was measured as described in the legend to Figure 1. Total RalA is also shown. (B) Ras-GTP was affinity purified from cell lysates transfected in duplicate with vector only and PDK1-expressing vector with the Ras-binding domain of Raf, and then blotted with anti-Ras antibodies. As a control, cells were incubated in duplicate with buffer only or EGF, and 10 min later the amount of GTP-Ras in cell lysates was detected as described above. Total Ras in the cell lysates is also shown. The data are representative of experiments performed three times.

**Fig. 3.** PI3-K and PDK1 activate Ral-GDS through the N-terminus of the GEF. (A) GST-Ral was transfected either alone or together with Ral-GDS and various potential activators of Ral-GDS. The cells were then labeled with  $^{32}\text{PO}_4$  for 4 h and GST-Ral was purified from cell lysates using glutathione beads. The isolated Ral was then run on thin-layer chromatography to separate labeled bound nucleotides, and the proportion of GTP bound to Ral was quantified on a PhosphorImager. In (A) wild-type Ral-GDS was used; in (B) Ral-GDS $\Delta\text{N}$  was used; and in (C) Ral-GDS $\Delta\text{C}$  was used. (D) The level of expression of the transfected proteins is shown. The functional domains of the Ral-GDS proteins used in these experiments is also shown at the top (CAT, GEF domain; RBD, Ras-binding domain). The data represent the average  $\pm$  SD of three independent experiments, each performed in duplicate.





**Fig. 4.** PDK1 enhances the intrinsic GEF activity of Ral-GDS as measured *in vitro*. (A) GST-RalA was expressed in *E. coli* and purified on glutathione beads. The bound RalA was then loaded with [ $^3$ H]GDP and incubated for 20 min with either buffer, control 293 cell lysates, or 293 cell lysates transfected with Ral-GDS alone or together with PDK1 in the presence of cold GTP. Ral was also incubated under the same conditions with PDK1 alone or Ral-GDS $\Delta$ N alone. The percentage of [ $^3$ H]GDP released was then assayed by measuring both the  $^3$ H present in the buffer and the amount remaining bound to RalA on the beads. The data represent the average  $\pm$  SD of two experiments, each performed in triplicate. (B) The levels of the Myc epitope-tagged transfected proteins in the cell lysates were determined by immunoblotting with Myc antibodies.

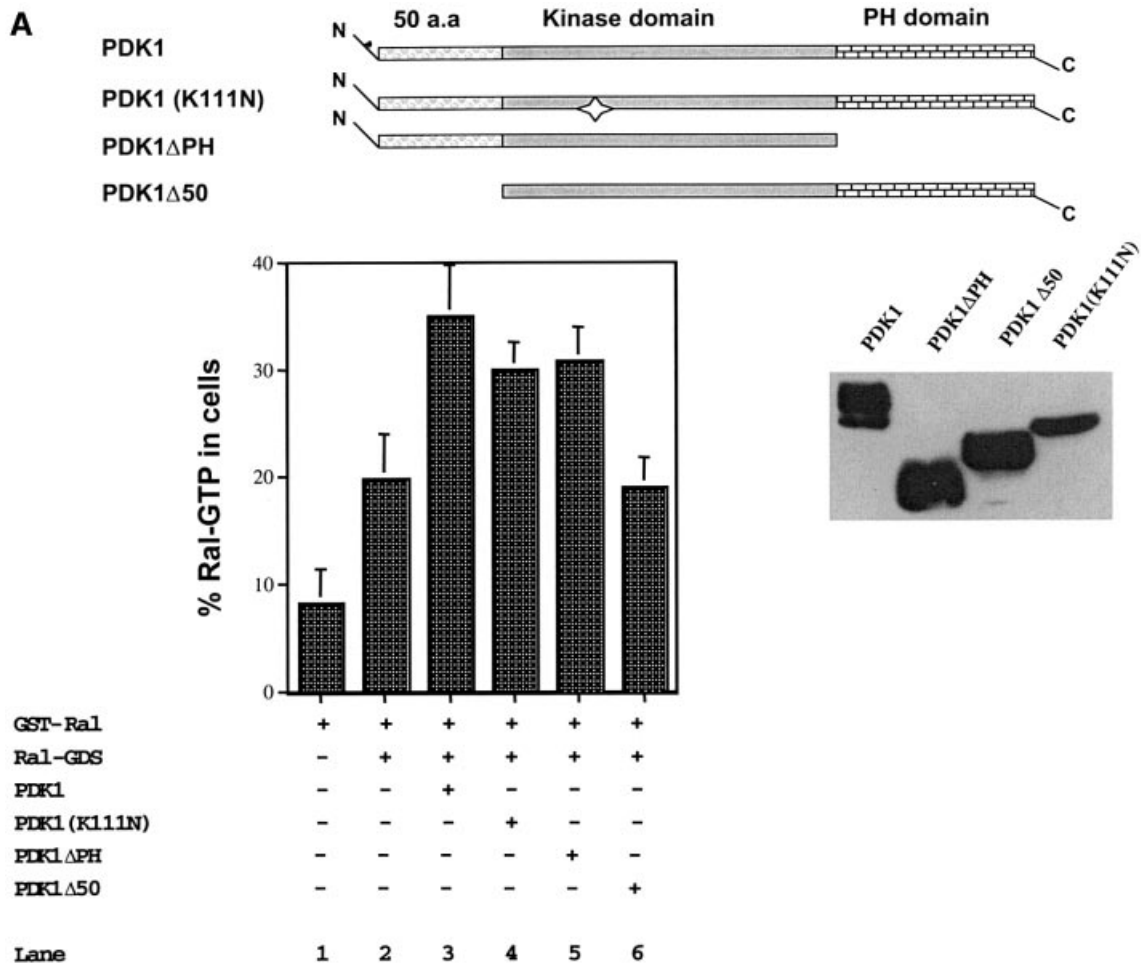
no effect (lane 6). As we have shown previously for transfected Ras, transfected PI3-K, PDK1 or Akt were not capable of increasing the proportion of GTP-Ral if Ral-GDS was not included (lanes 7–9). Presumably, endogenous Ral-GEFs are not present at high enough levels to stimulate a significant fraction of the large amounts of transfected GST-RalA. Thus, the expression of PI3-K and its downstream target, PDK1, promoted an increase in GTP-RalA through activation of Ral-GDS. This conclusion is consistent with the results reported in Figure 2A, where PI3-K and PDK1 but not Akt activated endogenous Ral in cells.

To begin to understand the mechanism of PDK1 action, the region of Ral-GDS that participates in its modulation by PDK1 was identified. Ral-GDS contains a C-terminal Ras-binding domain, a centrally located GEF catalytic

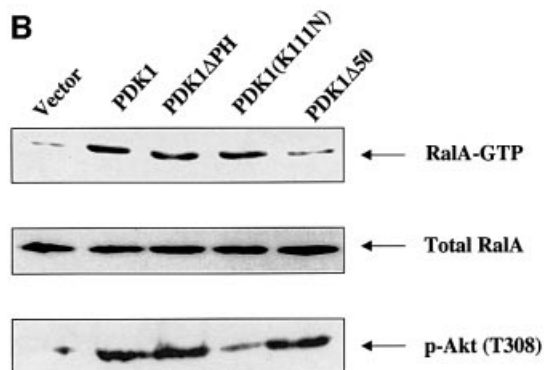
domain and an N-terminus that contains a ‘Ras exchange motif’ (REM), which in the Ras exchange factor SOS stabilizes the catalytic domain (see Figure 3A). The N-terminus of Ral-GDS was of particular interest, since we have shown previously that it negatively regulates Ral-GDS. Its deletion increased Ral-GDS activity in cells (Rusanescu *et al.*, 2001). Therefore, this deletion mutant, Ral-GDS $\Delta$ N, was transfected into 293 cells to test its responsiveness to PDK1. Note that a smaller amount of this hyperactive Ral-GDS mutant was transfected into cells compared with wild-type Ral-GDS (Figure 3D) so that they both increased Ral-GTP levels  $\sim$ 2-fold under basal conditions (Figure 3B, lane 2). Unlike wild-type Ral-GDS, however, Ral-GDS $\Delta$ N activity did not rise significantly upon PDK1 transfection (Figure 3B, lane 3). Importantly, the activity of Ral-GDS $\Delta$ N did rise upon transfection with activated Ras, since the Ras binding domain was still intact (Figure 3B, lane 4). In contrast, the activity of a mutant containing a deletion of the Ras-binding domain of Ral-GDS (Ral-GDS $\Delta$ C) increased in response to PDK1 transfection (Figure 3C, lane 3) but failed to respond significantly to activated Ras (Figure 3C, lane 4). Thus, in contrast to Ras, which activates Ral-GDS through the C-terminus, PDK1 activates Ral-GDS through the N-terminus of the GEF.

It has been shown previously that Ras binding to the C-terminus of Ral-GDS promotes Ral-GDS activity by localizing the protein to its target Ral in the plasma membrane activity (Kishida *et al.*, 1997; Matsubara *et al.*, 1999), rather than by increasing the protein’s intrinsic GEF (Spaargaren and Bischoff, 1994). Since PDK1 functioned through a distinct domain of Ral-GDS, the possibility that PDK1 complements Ras by increasing the intrinsic catalytic activity of Ral-GDS was investigated. To this end, Ral-GEF activity from control cells, cells transfected with Ral-GDS and cells transfected with Ral-GEF plus PDK1 were measured *in vitro*. To perform this assay, total cell lysates were incubated with glutathione beads containing GST-RalA isolated from *Escherichia coli* and preloaded with [ $^3$ H]GDP. The amount of [ $^3$ H]GDP released from the beads in the presence of excess unlabeled GTP was then measured as an indication of Ral-GEF activity (Figure 4A). Under basal conditions using just buffer,  $\sim$ 20% of the pre-loaded [ $^3$ H]GDP was released in the 20 min of this assay (Figure 4A, lane 1). When control cell lysates were included, no significant increase in GDP release was observed, indicating that endogenous Ral-GEF activity was too low to detect in this assay (lane 2). However, when Ral-GDS was transfected, GDP release rose to  $\sim$ 26% (lane 3) and strikingly, when PDK1 was transfected along with Ral-GDS, GDP release from RalA increased to  $\sim$ 41% (lane 4). PDK1 transfection without Ral-GDS did not significantly raise basal Ral-GEF activity in cell lysates again, presumably because the endogenous Ral-GEF activity was too low to measure (lane 5). This indicated that PDK1 increased the GDP release on RalA by increasing the intrinsic GEF activity of the transfected Ral-GDS.

Previously, we showed that deletion of the N-terminus of Ral-GDS increased the protein’s activity in cells (Rusanescu *et al.*, 2001); however, we did not investigate the mechanism involved. Because PDK1 increased the intrinsic catalytic activity of Ral-GDS through this same



**Fig. 5.** PDK1 activates the Ral signaling cascade through its N-terminus, not its catalytic activity. **(A)** PDK1 activation of Ral-GDS. GST-RalA was transfected either alone or together with Ral-GDS plus PDK1 or one of a set of PDK1 mutants. The ability of various PDK1 mutants to activate Ral-GDS's ability to promote the GTP-bound state of GST-Ral was measured as described in the legend of Figure 3. The data represent the average  $\pm$  SD of two experiments, each performed in triplicate. The levels of transfected proteins in cell lysates were determined by immunoblotting the Myc tagged proteins with anti-Myc antibodies and are shown on the right. The functional domains of PDK1 and a series of PDK1 mutants are shown at the top. **(B)** PDK1 activation of endogenous RalA. 293 cells were transfected with PDK1 or one of the mutants described above. After 24 h, the cells were serum starved and then the amount of RalA-GTP in transfected cell lysates was determined by affinity purification with the Ral-binding domain of RalBP1 and immunoblotting with anti-RalA antibodies. Total RalA in the cell lysates is shown, as is the ability of each of the PDK1 mutants to activate Akt, as assessed by immunoblotting cell lysates with T308 phospho-specific Akt antibodies. The data are representative of two independent experiments.



N-terminal region, we tested whether deletion of the N-terminus increased activity of the protein by enhancing its intrinsic catalytic activity. Although Ral-GDS and Ral-GDS $\Delta$ N were expressed at similar levels in the transfected cell lysates (Figure 4B), Ral-GDS $\Delta$ N displayed higher GEF activity (compare lanes 6 and 3). In fact, the activity of Ral-GDS $\Delta$ N was similar to wild-type Ral-GDS stimulated by PDK1 expression (compare lanes 6 and 4). These results imply that the N-terminus

of Ral-GDS acts as a negative regulator of the catalytic domain and that PDK1 activates Ral-GDS by relieving this inhibitory effect.

#### **PDK1 activates Ral-GDS by a kinase-independent mechanism**

PDK1 contains a C-terminal PH domain, a centrally located catalytic domain and a poorly defined 50 amino acid N-terminal region (Figure 5A). To determine which

region was required for Ral-GDS activation, mutant *PDK1* genes with alterations in each of these regions of the protein were tested for their ability to activate Ral-GDS and Ral in cells. To date, only the kinase domain has been implicated in PDK1 function. Thus, a kinase-dead mutant (K111N) (Chou *et al.*, 1998) was assayed first. Remarkably, this mutant retained the ability to activate Ral-GDS in experiments where PDK1, Ral-GDS and GST-Ral were all transfected into 293 cells (Figure 5A, compare lanes 3 and 4). The kinase-dead mutant also retained the ability to activate endogenous Ral when only PDK1 or the mutant PDK1 were transfected (Figure 5B, compare lanes 2 and 4). As expected for this kinase-dead mutant, it was unable to enhance the phosphorylation of T308 of AKT in the transfected cells (Figure 5B).

Deletion mutants of the PH domain (PDK1 $\Delta$ PH) and the 50 amino acid N-terminus (PDK1 $\Delta$ 50) were then assayed in a similar manner. PDK1 $\Delta$ PH still activated co-transfected Ral-GDS (Figure 5A, lane 5) and enhanced the amount of endogenous GTP-RalA in cells (Figure 5B, lane 3). In contrast, PDK1 $\Delta$ 50 did not function in either assay (Figure 5A, lane 6, and B, lane 5) despite the fact that it still activated Akt (Figure 5B, lane 5) and was expressed at levels comparable to wild-type PDK1 (Figure 5A, right panel). Thus, PDK1 activates the catalytic activity of Ral-GDS, not by its kinase activity but by a novel mechanism that involves its N-terminal 50 amino acids.

#### ***EGF induces a PI3-K-dependent association of PDK1 with Ral-GDS***

Because the kinase activity of PDK1 was eliminated as a mediator of its effects on Ral-GDS, a search was made for a physical interaction between the two proteins in cells as an alternate mechanism. Endogenous PDK1 was immunoprecipitated from serum-starved cells and then immunoblotted with antibodies against Ral-GDS. A faint Ral-GDS band of the same size as immunoprecipitated Ral-GDS (Figure 6A, lane 2) was observed in the Ral-GDS immunoblot (Figure 6A, lane 3) but not in an immunoblot performed with a control antibody (Figure 6A, lane 1). Importantly, an increase in the amount of endogenous Ral-GDS associated with endogenous PDK1 was observed after cells were stimulated for 10 min with EGF (Figure 6A, lane 4). Moreover, pre-treatment of cells with the PI3-K inhibitors, wortmanin and LY94002, which blocked EGF activation of RalA (see Figure 1), also suppressed complex formation between Ral-GDS and PDK1 (Figure 6A, lanes 5 and 6).

To identify the domains on Ral-GDS and PDK1 that are involved in binding, endogenous PDK1 was immunoprecipitated from cells co-transfected with Myc-tagged Ral-GDS and various Ral-GDS mutants. The presence of Myc-Ral-GDS in the immune complexes was then detected by immunoblotting with anti-Myc antibodies (Figure 6B). As expected, Myc-Ral-GDS behaved like endogenous Ral-GDS and was detected in PDK1 immunoprecipitates in an EGF-dependent manner that was suppressed by inhibitors of PI3 kinase (Figure 6B, lanes 1–4). Unlike control Myc-Ral-GDS (Figure 6C, lane 1), Myc-Ral-GDS $\Delta$ N, which did not respond to co-transfected PDK1, did not specifically co-precipitate with endogenous PDK1 (compare lanes 3 and 4). Moreover,

Myc-Ral-GDS $\Delta$ C, which did respond to PDK1, did appear specifically in PDK1 immunoprecipitates (compare lanes 5 and 6). Wild-type and mutant Ral-GDSs were all present at comparable levels in cell lysates (compare lanes 7–12).

Next, wild-type Myc-PDK1 or various mutants of the kinase were immunoprecipitated from cells co-transfected with Glu-tagged Ral-GDS (Figure 6D). Wild-type (Figure 6D, lane 1), PDK1 $\Delta$ PH (lane 2) and PDK1 K111N (lane 4) proteins, all of which activated Ral-GDS, all formed a complex with Ral-GDS in cells. In contrast, PDK1 $\Delta$ 50 (lane 3), which did not activate Ral-GDS in cells, failed to bind to Ral-GDS in cells. Thus, a perfect correlation was found between PDK1/Ral-GDS association and functional interaction between these proteins.

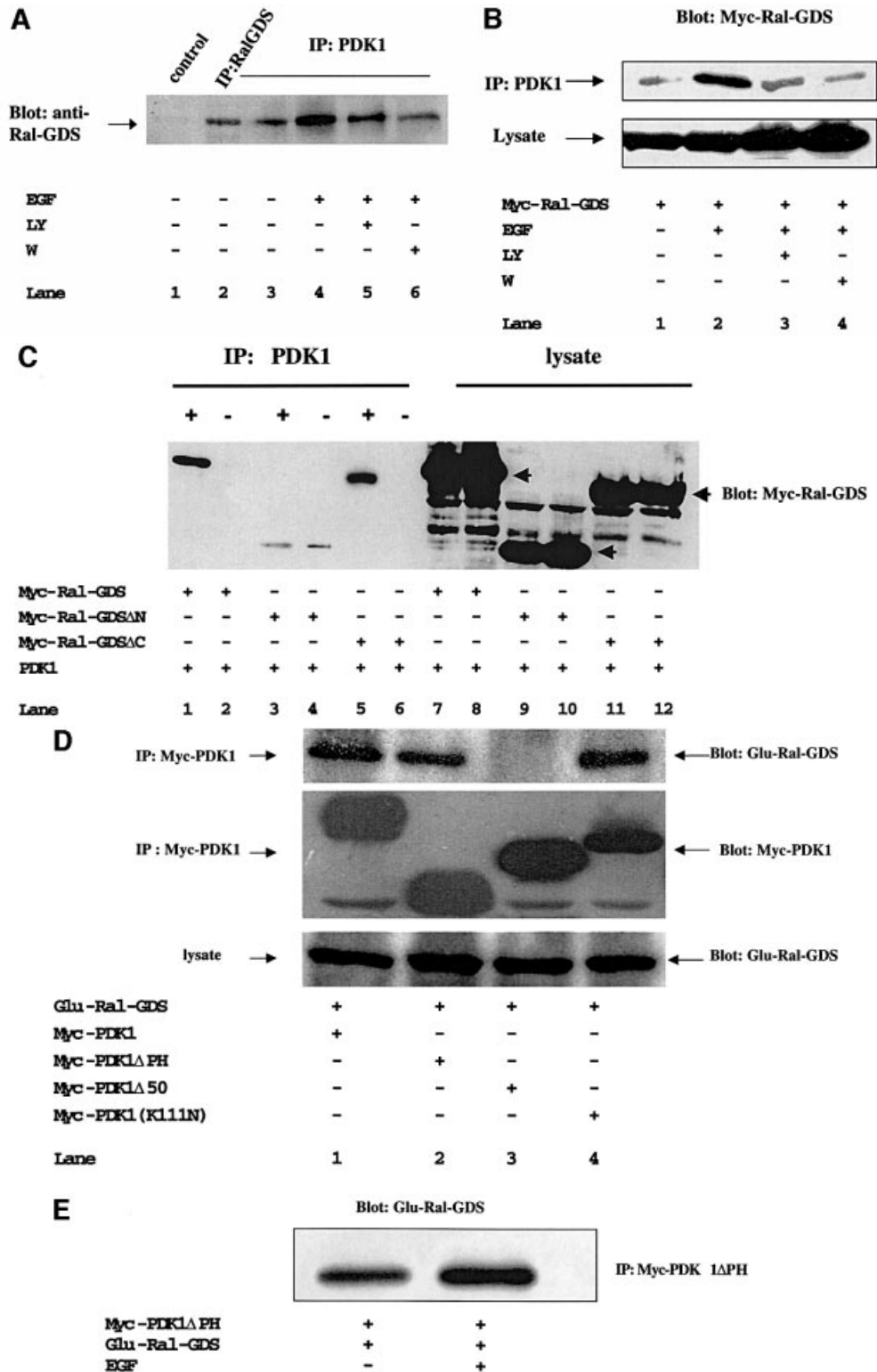
To begin to understand how EGF promotes the association of PDK1 with Ral-GDS, the role of the PH domain of PDK1 was studied, since it is known to be involved in associating PDK1 with the cell membrane by binding to the lipid products of PI3-K. To this end, Myc-PDK1 $\Delta$ PH was substituted for wild-type PDK1 in the binding experiment described above. Surprisingly, although the EGF-induced complex between PDK1 and Ral-GDS was dependent upon PI3-K activity, the PH domain of PDK1 was not necessary (Figure 6E). Thus, PI3-K must promote the association of PDK1 with Ral-GDS through some other PIP3 target in cells. Nevertheless, these binding experiments and the GEFs assays described above argue that EGF promotes Ral-GDS activation, at least in part, by promoting the association of PDK1 and Ral-GDS through their N-termini.

#### ***An N-terminal peptide of Ral-GDS prevents EGF-induced complex formation between PDK1 and Ral-GDS and suppresses EGF-induced activation of Ral***

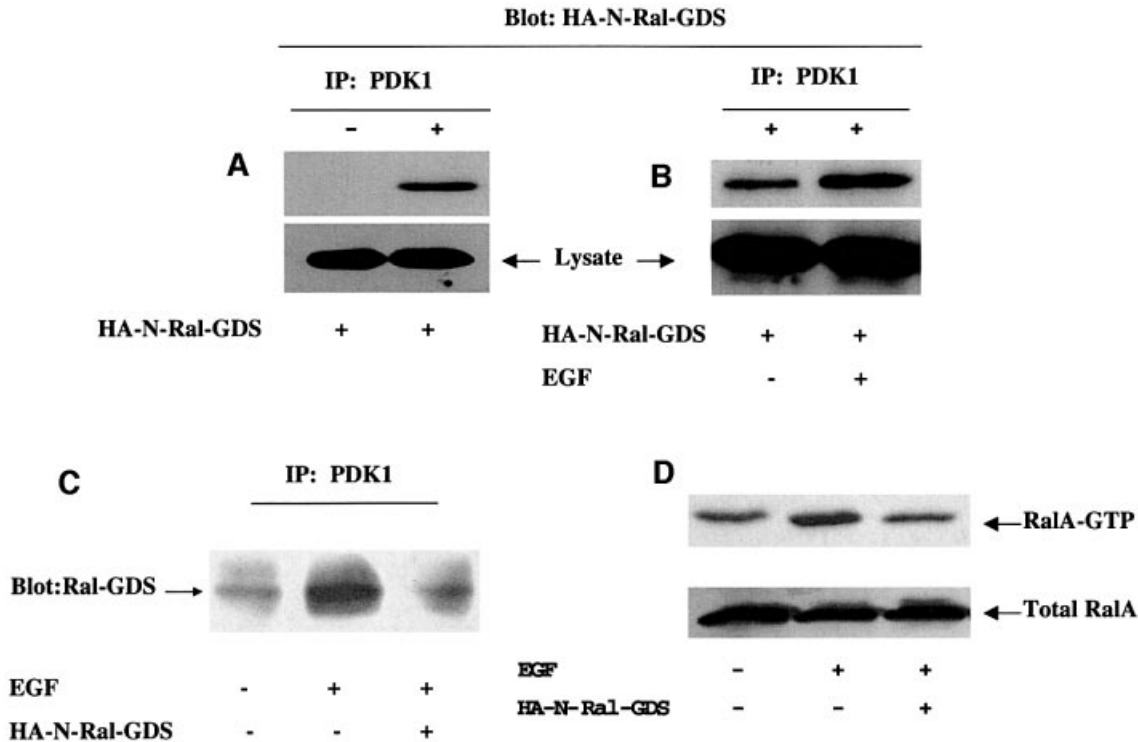
If this hypothesis is correct, then expression of just the N-terminus of Ral-GDS (N-Ral-GDS) should bind to PDK1 in cells, block binding of PDK1 to Ral-GDS and interfere with Ral activation by EGF. First, the ability of the isolated N-terminus of Ral-GDS (N-Ral-GDS) to form a complex with endogenous PDK1 in cells was demonstrated by transfecting HA-N-Ral-GDS (aa 1–297) into 293 cells and then immunoprecipitating endogenous PDK1. The presence of N-Ral-GDS in these immune complexes was then assayed by immunoblotting with HA antibodies (Figure 7). A specific complex between these two proteins was clearly observed only when PDK1 antibodies were included (Figure 7A). In fact, this small region of Ral-GDS was sufficient for regulated complex formation since the addition of EGF to serum-starved cells increased the amount of it bound to endogenous PDK1 (Figure 7B). Furthermore, the binding of N-Ral-GDS to PDK1 suppressed binding of endogenous PDK1 to endogenous Ral-GDS in cells (Figure 7C). Finally, consistent with the proposed model of Ral activation, the expression of N-Ral-GDS also suppressed EGF induction of endogenous GTP-Ral levels in these cells (Figure 7D).

## **Discussion**

PDK1 has been intensively investigated ever since it was discovered that it mediates Akt activation by PI3-K (Alessi *et al.*, 1997; Stokoe *et al.*, 1997). PDK1 attracted



**Fig. 6.** PDK1 forms an EGF-regulated complex with Ral-GDS in cells. (A) 293 cells were serum starved and then treated for 10 min with either buffer (lanes 1–3) or EGF (lanes 4–6). In some experiments, the cells were pre-treated with either LY94002 or wortmanin for 1 h. Lysates of cells were immunoprecipitated with control antibody (lane 1), anti-Ral-GDS antibody (lane 2) or anti-PDK1 antibody (lanes 3–6), and then immunoblotted with anti-Ral-GDS antibody. (B) Myc-Ral-GDS was transfected into 293 cells and the cells were treated as in (A). Endogenous PDK1 was then immunoprecipitated and the presence of Ral-GDS on the immunoprecipitate was assessed by immunoblotting with anti-Myc antibodies (C) PDK1 was transfected along with Myc-tagged Ral-GDS, Myc-Ral-GDS $\Delta$ N or Myc-Ral-GDS $\Delta$ C. PDK1 was immunoprecipitated with anti-PDK1 antibodies (+) or control antibodies (-) and the sample was immunoblotted with anti-Myc antibodies (left side, lanes 1–6). Anti-Myc-immunoblots of cell lysates detecting Ral-GDS expression are shown on the right (lanes 7–12). (D) Glu-Ral-GDS was transfected into 293 cells along with Myc-PDK1, Myc-PDK1 $\Delta$ PH, Myc-PDK1 $\Delta$ 50 or Myc-PDK1(K111N). PDK1 or its mutants were immunoprecipitated with anti-Myc antibodies and then blotted with anti-Glu antibodies to detect Ral-GDS. The PDK1 immunoprecipitates and Ral-GDS in the cell lysates were immunoblotted with either anti-Myc or anti-Glu antibodies. (E) Glu-Ral-GDS was transfected with Myc-PDK1 $\Delta$ PH and the cells were treated as in (C). The data are representative of two independent experiments.



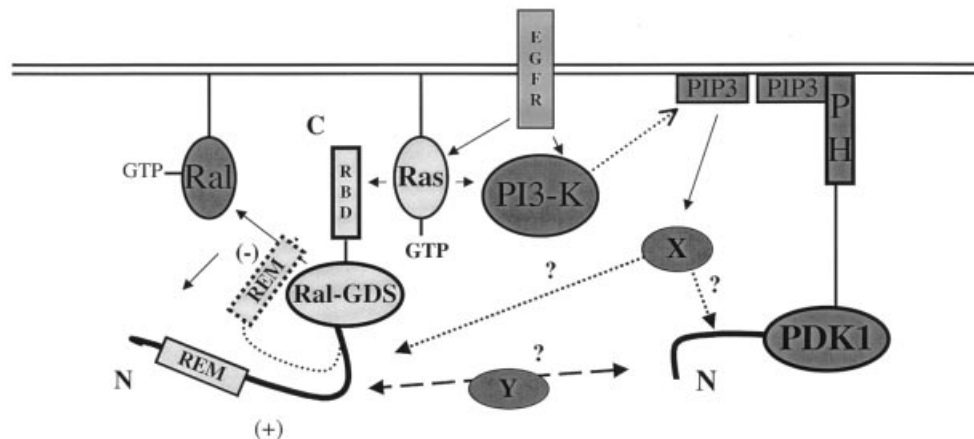
**Fig. 7.** An N-terminal peptide of Ral-GDS prevents EGF-induced complex formation between endogenous PDK1 and endogenous Ral-GDS in cells and suppresses EGF activation of Ral. (A) The N-terminal 297 amino acids of Ral-GDS were epitope tagged with HA (HA-N-Ral-GDS) and transfected into cells. Forty-eight hours later, cells were immunoprecipitated with PDK1 antibodies (+) or control antibodies (-) and the samples were immunoblotted with anti-HA antibodies. (B) Cells were prepared as in (A) except that after transfection the cells were serum starved for 12 h and then some were stimulated with EGF for 10 min. (C) 293 cells were transfected with either empty vector or vector expressing HA-N-Ral-GDS. Twenty-four hours later, cells were serum starved for 12 h and then treated with buffer or EGF for 10 min. The cells were lysed, endogenous PDK1 was immunoprecipitated and then immunoblotted for endogenous Ral-GDS. (D) Cells were treated as in (C) except that active RalA-GTP was affinity purified from cell lysates and detected by immunoblotting with anti-RalA antibodies. Total RalA and HA-N-Ral-GDS (data not shown) were also detected by immunoblotting lysates with appropriate antibodies. The experiments are representative of at least two independent experiments, each performed in duplicate.

even more attention when it became apparent that it also regulates other well-studied kinases, such as PKC, p70S6 kinase and RSK (Alessi, 2001). In all of these examples, PDK1 affects its target proteins by phosphorylation via its centrally located kinase domain. In this paper we have identified a new function of PDK1 that is mediated by its non-catalytic N-terminal 50 amino acids. This segment of the protein participates in the formation of a complex with Ral-GDS, thereby activating its Ral-GEF activity. This conclusion is supported by the finding that a catalytically inactive PDK1 mutant formed a complex with and activated Ral-GDS in cells as efficiently as wild-type PDK1 did. In contrast, a mutant PDK1 with its first 50 amino acids deleted failed to form a complex with or activate Ral-GDS, even though it retained the ability to phosphorylate Akt in cells.

In addition, the experiments presented in this study show that this newly discovered function of PDK1 plays a critical role in the mechanism by which EGF promotes the activation of Ral in cells. First, EGF promoted the association of endogenous PDK1 with endogenous Ral-GDS, concomitant with Ral activation in cells. Secondly, the PI3-K inhibitors wortmanin and LY94002 blocked both EGF activation of Ral and EGF-induced association of PDK1 with Ral-GDS. Finally, an isolated N-terminal

peptide of Ral-GDS formed an EGF-dependent complex with endogenous PDK1 in cells and blocked its association with endogenous Ral-GDS. Importantly, this peptide also blocked EGF activation of endogenous Ral.

Until now the only mechanism known that explained how EGF leads to the activation of Ral-GTPases was EGF receptor-induced activation of Ras, which then binds to the C-terminus of Ral-GDS. This association does not alter the specific activity of Ral-GDS but instead localizes it to the membrane fraction of cells where its targets, the Ral-GTPases, reside. However, we recently described experiments suggesting that Ras binding to Ral-GDS may not be sufficient for activation upon ligand stimulation (Rusanescu *et al.*, 2001). In those experiments we found conditions where Ras bound to Ral-GDS but did not activate it. Here we identify a new EGF-induced, PDK1-mediated mechanism that is distinct from and complementary to that which is used by Ras. In particular, PDK1 increases the intrinsic GEF activity of Ral-GDS. While Ras binds to the C-terminus of Ral-GDS, PDK1 forms a complex with the N-terminus. Interestingly, deletion of the N-terminus of Ral-GDS activates the intrinsic catalytic activity of the protein to levels comparable to that found when the wild-type protein associates with PDK1. Thus, PDK1 likely activates Ral-GDS by relieving the



**Fig. 8.** Model of how PDK1 and Ras may cooperate to activate Ral-GDS. EGF activates Ras, which then binds to the Ras-binding domain (RBD) of Ral-GDS to localize it to the membrane where its target Ral is located. Concomitantly, Ras, together with the EGF receptor, activates PI3-K. PI3-K enhances the catalytic activity of Ral-GDS by promoting the association of the GEF with PDK1. This may be a direct interaction or through an as yet unidentified protein (Y). Although PI3-K is known to function through the binding of its product, PIP3, to the PH domain of PDK1 to enhance the kinase's ability to phosphorylate Akt, some other mechanism must participate in promoting the binding of PDK1 to Ral-GDS, thereby activating it. This may occur through some other region of PDK1 or through the N-terminus of Ral-GDS, and involve another PIP3 target (X). Usually the N-terminus of Ral-GDS (possibly through its REM domain) blocks the catalytic domain of the GEF. The association of the N-terminus of PDK1, either directly or indirectly with the N-terminus of Ral-GDS, relieves its inhibitory action on Ral-GDS and thus activates the catalytic activity of the GEF.

inhibitory effect of its N-terminus on its catalytic domain. A comparison between the way Ras and PDK1 activate Ral-GDS is shown schematically in Figure 8.

The idea that Ras binding to Ral-GDS is not sufficient for Ral activation by ligands may seem at odds with previous experiments using Ras effector domain mutants (White *et al.*, 1995). These point mutations in Ras allow it to preferentially bind to one of the three known classes of Ras effectors, Raf, PI3-K or Ral-GEFs. Characterization of those mutants showed that Ras37G, which is capable of binding to only Ral-GEFs like Ral-GDS, is able to activate Ral-GEFs in cells (White *et al.*, 1996; Wolthuis *et al.*, 1997). A reasonable explanation for this apparent paradox is that the high levels of activated Ras used in these types of experiments exaggerates the importance of redistributing Ral-GEFs to Ral. As such, it may mask the importance of other regulatory pathways. An analogous explanation may explain why overexpression of PDK1 reported here was able to activate Ral without a contribution from Ras. Under physiological stimulation such as exposure of cells to EGF, the experiments described here imply that both Ras and PDK1 mechanisms are required. The apparent complementary functions of Ras and PDK1 for Ral-GDS activation are analogous to the mechanisms used to activate other Ras effectors like Raf. In that case, Ras also targets the protein to the membrane fraction where a complementary mechanism, phosphorylation, increases the intrinsic catalytic activity of the kinase (Hagemann and Rapp, 1999).

The finding that PDK1 associates with Ral-GDS upon EGF stimulation of cells has intriguing implications. For example, although the kinase domain of PDK1 is not used to activate Ral-GDS, it is nonetheless present as part of the Ral signaling complex induced by EGF. This suggests that PDK1 phosphorylates one or more components of the Ral signaling cascade. This could alter some property of Ral-GDS other than its intrinsic catalytic activity or affect the activity of individual downstream targets of Ral.

Furthermore, although we have shown that PDK1 alters the activity of Ral-GDS, Ral-GDS may also influence the activity of PDK1. This possibility may be relevant to a common observation in studies on the Ral signaling pathway. Expression of a Ral-GEF induces phenotypes that are not always mimicked by expression of the activated GTPase (Wolthuis *et al.*, 1996; de Ruiter *et al.*, 2000). Such a phenomenon might be explained in part by Ral-GEF-induced alterations in PDK1, which is known to regulate a wide variety of protein kinases. Finally, the 50 amino acid region of PDK1 that associates with Ral-GDS may have the capacity to associate with and regulate, or be regulated by, other cellular proteins. These possibilities are all presently under investigation.

It also remains to be determined how EGF promotes the association of PDK1 with Ral-GDS. The process clearly requires PI3-K activity, since it is blocked by PI3-K inhibitors. The most obvious mechanism would involve PIP3 binding to the PH domain of PDK1, a process that is known to be involved in targeting PDK1 to the plasma membrane and activation of Akt. Yet, we have shown that a mutant PDK1 that lacks its PH domain still forms a complex with Ral-GDS in response to EGF. This result does not preclude a role for the PH domain of PDK1 in Ral activation, since it might be masked by overexpression of the mutant in cells. However, it does indicate that a different mechanism, independent of the PH domain of PDK1, must also be involved. PIP3 is known to influence a wide variety of signaling molecules, such as exchange factors for Rho family GTPases, tyrosine kinases and scaffolding proteins (Alessi, 2001). Presumably, PIP3 uses one of these other proteins to influence the N-termini of PDK1 and Ral-GDS and promote complex formation (see Figure 8). This latter mechanism would be analogous to how PIP3 induces PDK1 phosphorylation of Akt, where the lipid functions through both PDK1 and its substrate (Alessi, 2001). In fact, we have already demonstrated that the N-terminus of Ral-GDS is responsive to other intra-

cellular signals. PKC activity leads to the phosphorylation of this region and blocks Ral-GDS activation by Ras (Rusanescu *et al.*, 2001).

We also do not yet know whether PDK1 binds directly to Ral-GDS or through an intermediary protein. We do know that the isolated N-terminus of Ral-GDS produced in *E.coli* fails to bind to *E.coli*-produced PDK1 (our unpublished observation), even though it can bind to PDK1 *in vivo*. Thus, either binding is through an intermediary protein or post-translational modification of one of the proteins is necessary. This interpretation is consistent with the fact that PDK1–Ral-GDS binding is regulated by growth factor stimulation. Moreover, the locus within the N-terminus of Ral-GDS that negatively regulates the catalytic domain needs to be identified. One reasonable possibility is the so-called REM, which is the only recognizable motif in this region of Ral-GDS (see Figure 8). A REM is found in many GEFs for Ras family GTPases, and in the Ras-GEF SOS, it has been shown to interact with the catalytic domain, but its only function appears to be to stabilize it (Boriack-Sjodin *et al.*, 1998). This raises the possibility that in Ral-GDS the REM takes on the additional role of suppressing the catalytic domain possibly by interfering with its interaction with the target GTPase. Binding of PDK1 to the N-terminus might alter the REM, thereby freeing the catalytic domain to interact with GTPases (see Figure 8). Interestingly, some Ral-GEFs that do not contain Ras-binding domains also do not contain REM motifs (Rebhun *et al.*, 2000). These proteins are probably regulated by different mechanisms.

In conclusion, this study has determined that Ral activation by EGF is more complex than was previously appreciated, in that it involves the cooperation of two downstream effectors of Ras, Ral-GDS and PI3-K. The interplay between PI3-K and another Ras effector has been observed before; however, in that case, the PI3-K-dependent enzyme involved was Akt and it suppressed the activity of the Raf kinase (Rommel *et al.*, 1999; Zimmermann and Moelling, 1999). Even more striking, this study reveals that PDK1 signaling is more complex than was previously appreciated. The protein uses at least two regulatory mechanisms, protein phosphorylation and protein binding, through distinct domains to alter the activities of a diverse set of cellular signaling molecules.

## Materials and methods

### Cell culture and transfection

293T and COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) iron-enriched bovine calf serum (Hyclone). 293T and COS-7 cells were plated at a density of  $5 \times 10^5$  cells per 60-mm diameter dish, 1 day before transfection. Each plasmid DNA was introduced into 293 or COS-7 cells by the calcium phosphate precipitation procedure or by the use of LipofectAMINE (Gibco-BRL) (where ~75% transfection efficiency was obtained). Twenty-four hours later, cells were serum deprived overnight or not, as necessary. Forty-eight hours after transfection, cells were treated as described.

### Plasmid construction

The expression constructs for pMT3-Myc-Ral-GDS, pMT3-Myc-Ral-GDS $\Delta$ N, pMT3-Myc-Ral-GDS $\Delta$ C, pMT3-RasH(61L), pMT3-Glu-Ral-GDS, pEBG-GST-Ral and pGEX2T-Ral have been described previously (Urano *et al.*, 1996; Gotoh *et al.*, 2001). The N-terminal domain of Ral-GDS (1–297 codons of Ral-GDS) was generated by PCR and cloned into pJ3 $\Omega$ -HA-vector using *Bam*HI–*Eco*RI sites (HA-N-Ral-GDS). Bacterial expression vectors containing the Ral-GTP binding

domain of RalBP1(GST–RalBD) in pGEX2TK or the Ras-GTP binding domain of c-Raf (GST–RafBD) in pGEX2T were as described previously (Goi *et al.*, 2000). The expression constructs for pCMV-Myc-PI3-K-CAAX, pCMV-PDK1, pCMV-HA-Akt-CAAX (A-Akt), pCMV-Myc-PDK1 $\Delta$ PH (deletion of the C-terminal from 451–556 amino residues of PDK1), pCMV-Myc-PDK1 $\Delta$ 50 (deletion of the N-terminal 50 amino residues of PDK1), pCMV-Myc-PDK1(K111N) and the kinase negative mutant of PDK1 were all as described previously (Chou *et al.*, 1998).

### Measurement of GTP-bound state of endogenous Ral and Ras

293 and COS-7 cells were pretreated with wortmannin (W) or LY94002 (LY) or dimethyl sulfoxide (DMSO) for 1 h, 10 min before harvesting the cells, EGF was added as indicated. For the effects of Ral-GTP or Ras-GTP on A-PI3-K, A-PDK1, A-Akt, HA-RalGDS-N and different PDK1 mutants, plasmids were transfected for 48 h as indicated. All cells were serum starved overnight. The cells were lysated in buffer A [50 mM Tris–HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 200 mM NaCl, 2% NP-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 1 mM NaVO<sub>3</sub>, 1 mM dithiothreitol (DTT)]. Nucleus-free supernatants were affinity purified using a GST fusion with either Ral-GTP binding domain of RalBP1(GST–RalBD) or Ras-GTP binding domain of c-Raf (GST–RafBD) immobilized on S-hexylglutathione–agarose beads (Sigma) by incubating for 1 h at 4°C, after washing three times with buffer B (25 mM Tris–HCl pH 7.5, 30 mM MgCl<sub>2</sub>, 40 mM NaCl, 1% NP-40, 1 mM DTT). Immunoblots were visualized with either anti-Ral antibodies or anti-Ras antibodies (Transduction Laboratories) by enhanced chemiluminescence (ECL; NEN). To quantify differences in Ral-GTP or Ras-GTP levels, serial dilutions of protein purified with GST–RalBP1 or GST–Raf were used in western blotting and were compared before and after experimental treatment.

### In vivo assay of Ral-GDS activity

Measurement of Ral-GDS GEF activity *in vivo* was as described previously (Gotoh *et al.*, 2001). Briefly, 293 cells were transfected with expression vectors containing GST–Ral and different plasmids as indicated. Forty-eight hours later, cells were metabolically labeled for 4 h in phosphate and serum-free media with 0.05 mCi/ml of <sup>32</sup>P<sub>4</sub>. GST-fused Ral protein were collected by incubation of cell lysates with S-hexylglutathione–agarose beads for 1 h and washed with lysis buffer. Ral-bound <sup>32</sup>P-labeled guanine nucleotides were separated by thin-layer chromatography (Aldrich) and quantified by PhosphorImager analysis (Molecular Dynamics). The percentage of GTP was calculated as counts of GTP/GTP + GDP.

### In vitro nucleotide exchange assay

GST–RalA was purified from *E.coli* with glutathione beads. The nucleotide exchange reaction was carried out as described previously (Shou *et al.*, 1992) with minor modification. Briefly, 20 pmol of GST–RalA together with [<sup>3</sup>H]GDP (100 nM) was loaded into a 20  $\mu$ l reaction buffer containing 20 mM Tris–HCl pH 7.5, 1 mM MgCl<sub>2</sub>, 100 mM NaCl, 20 mM EDTA, 5% glycerol, 1 mg/ml bovine serum albumin (BSA), 1 mM DTT for 5 min at 30°C. After the incubation, 30 mM MgCl<sub>2</sub> was added to stabilize the binary complex and washing three times with buffer containing 20 mM Tris–HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 100 mM NaCl, 5% glycerol, 1 mg/ml BSA, 1 mM DTT. The nucleotide exchange reaction was carried out in 100  $\mu$ l reaction volume with 20 pmol of GTP-binding protein–[<sup>3</sup>H]GDP complex in reaction buffer (20 mM Tris–HCl pH 7.5, 3 mM MgCl<sub>2</sub>, 100 mM NaCl, 1% NP-40, 1 mM PMSF, 1.5 mM GTP, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 1 mM NaVO<sub>3</sub>, 1 mM DTT) alone, with buffer containing control cell lysates, or buffer with different transfected cell lysates. The samples were incubated for 20 min at 30°C and then centrifuged at 4°C and the supernatants were collected. The pellets were washed with buffer and the radioactivity of the supernatants (S) and the pellets (P) were counted using liquid scintillation counter (model 2200CA; Packard), respectively. The relative percentage of [<sup>3</sup>H]GDP release was calculated as a ratio of S/S + P.

### Formation of complex of PDK1 with Ral-GDS in cells

To detect complex formation between endogenous PDK1 and endogenous Ral-GDS, cells were lysed in buffer containing 10 mM Tris–HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM PMSF, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin and 1 mM NaVO<sub>3</sub>. PDK1 was immunoprecipitated with anti-PDK1 antibodies (generated against GST–PDK1 made in *E.coli*) for 1 h and then protein G–Sepharose

was added for another 1 h. The immune complexes were washed four times with lysis buffer and once with 0.5 M LiCl (pH 8.0) and then subjected to SDS-PAGE and immunoblotting with antibodies against Ral-GDS (Transduction Laboratories). To detect complex formation between different domains of Ral-GDS with PDK1, a 60 mm dish of semi-confluent 293 cells was transfected with pMT3-Myc-Ral-GDS, pMT3-Myc-Ral-GDSΔN or pMT3-Myc-Ral-GDSΔC, and all the cells were transfected with pMT3-PDK1. Forty-eight hours later, cells were lysed in buffer described above and samples were incubated with or without anti-PDK1 polyclonal antibody as described above and then immunoblotted with anti-Myc antibodies. For complex formation of Ral-GDS and PDK1 mutants, pMT3-Glu-RalGDS together with pCMV-Myc-PDK1, pCMV-Myc-PDK1ΔPH, pCMV-Myc-PDK1Δ50 or pCMV-Myc-PDK1ΔN (K111N) was transfected into 293 cells, the cleared lysates were incubated with anti-Myc antibodies and immunoblotted with anti-Glu antibodies.

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## References

- Alessi,D.R. (2001) Discovery of PDK1, one of the missing links in insulin signal transduction. Colworth Medal Lecture. *Biochem. Soc. Trans.*, **29**, 1–14.
- Alessi,D.R., James,S.R., Downes,C.P., Holmes,A.B., Gaffney,P.R., Reese,C.B. and Cohen,P. (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balph. *Curr. Biol.*, **7**, 261–269.
- Boriack-Sjodin,P.A., Margarit,S.M., Bar-Sagi,D. and Kuriyan,J. (1998) The structural basis of the activation of Ras by Sos. *Nature*, **394**, 337–343.
- 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.
- Chou,M.M., Hou,W., Johnson,J., Graham,L.K., Lee,M.H., Chen,C.S., Newton,A.C., Schaffhausen,B.S. and Toker,A. (1998) Regulation of protein kinase C $\zeta$  by PI 3-kinase and PDK-1. *Curr. Biol.*, **8**, 1069–1077.
- de Rooter,N.D., Wolthuis,R.M., van Dam,H., Burgering,B.M. and Bos,J.L. (2000) Ras-dependent regulation of c-Jun phosphorylation is mediated by the Ral guanine nucleotide exchange factor-Ral pathway. *Mol. Cell. Biol.*, **20**, 8480–8488.
- Feig,L.A., Urano,T. and Cantor,S. (1996) Evidence for a Ras/Ral signaling cascade. *Trends Biochem. Sci.*, **21**, 438–441.
- Gille,H. and Downward,J. (1999) Multiple Ras effector pathways contribute to G<sub>1</sub> cell cycle progression. *J. Biol. Chem.*, **274**, 22033–22040.
- Goi,T., Shipitsin,M., Lu,Z., Foster,D.A., Klinz,S. and Feig,L.A. (2000) An EGF receptor/Ral-GTPase signaling cascade regulates c-Src activity and substrate specificity. *EMBO J.*, **19**, 623–630.
- Gotoh,T., Cai,D., Tian,X., Feig,L.A. and Lerner,A. (2000) p130Cas regulates the activity of AND-34, a novel Ral, Rap1 and R-Ras guanine nucleotide exchange factor. *J. Biol. Chem.*, **275**, 30118–30123.
- Gotoh,T., Tian,X. and Feig,L.A. (2001) Prenylation of target GTPases contributes to signaling specificity of Ras-guanine nucleotide exchange factors. *J. Biol. Chem.*, **276**, 38029–38035.
- Hagemann,C. and Rapp,U.R. (1999) Isozyme-specific functions of Raf kinases. *Exp. Cell Res.*, **253**, 34–46.
- Henry,D.O., Moskalenko,S.A., Kaur,K.J., Fu,M., Pestell,R.G., Camonis,J.H. and White,M.A. (2000) Ral GTPases contribute to regulation of cyclin D1 through activation of NF- $\kappa$ B. *Mol. Cell. Biol.*, **20**, 8084–8092.
- Hofer,F., Berdeaux,R. and Martin,G.S. (1998) Ras-independent activation of Ral by a Ca<sup>2+</sup>-dependent pathway. *Curr. Biol.*, **8**, 839–842.
- Ikeda,M., Ishida,O., Hinoi,T., Kishida,S. and Kikuchi,A. (1998) Identification and characterization of a novel protein interacting with Ral-binding character 1, a putative effector protein of Ral. *J. Biol. Chem.*, **273**, 814–821.
- 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.
- Jullien-Flores,V., Dorseuil,O., Romero,F., Letourneur,F., Saragosti,S., Berger,R., Tavittian,A., Gacon,G. and Camonis,J.H. (1995) Bridging Ral GTPase to Rho pathways. *J. Biol. Chem.*, **270**, 22473–22477.
- Jullien-Flores,V., Mahe,Y., Mirey,G., Leprince,C., Meunier-Bisceuil,B., Sorkin,A. and Camonis,J.H. (2000) RLIP76, an effector of the GTPase Ral, interacts with the AP2 complex: involvement of the Ral pathway in receptor endocytosis. *J. Cell Sci.*, **113**, 2837–2844.
- Kishida,S., Koyama,S., Matsubara,K., Kishida,M., Matsuura,Y. and Kikuchi,A. (1997) Colocalization of Ras and Ral on the membrane is required for Ras-dependent Ral activation through Ral GDP dissociation stimulator. *Oncogene*, **15**, 2899–2907.
- Kops,G.J., de Rooter,N.D., De Vries-Smits,A.M., Powell,D.R., Bos,J.L. and Burgering,B.M. (1999) Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature*, **398**, 630–634.
- Lu,Z. *et al.* (2000) Phospholipase D and RalA cooperate with the epidermal growth factor receptor to transform 3Y1 rat fibroblasts. *Mol. Cell. Biol.*, **20**, 462–467.
- Matsubara,K., Kishida,S., Matsuura,Y., Kitayama,H., Noda,M. and Kikuchi,A. (1999) Plasma membrane recruitment of RalGDS is critical for Ras-dependent Ral activation. *Oncogene*, **18**, 1303–1312.
- Nakashima,S., Morinaka,K., Koyama,S., Ikeda,M., Kishida,M., Okawa,K., Iwamatsu,A., Kishida,S. and Kikuchi,A. (1999) Small G protein Ral and its downstream molecules regulate endocytosis of EGF and insulin receptors. *EMBO J.*, **18**, 3629–3642.
- Ohta,Y., Suzuki,N., Nakamura,S., Hartwig,J.H. and Stossel,T.P. (1999) The small GTPase RalA targets filamin to induce filopodia. *Proc. Natl Acad. Sci. USA*, **96**, 2122–2128.
- Park,S.H. and Weinberg,R.A. (1995) A putative effector of Ral has homology to Rho/Rac GTPase activating proteins. *Oncogene*, **11**, 2349–2355.
- Polzin,A., Shipitsin,M., Goi,T., Feig,L.A. and Turner,T.J. (2002) Ral-GTPase influence the regulation of the readily-releasable pool of synaptic vesicles. *Mol. Cell. Biol.*, in press.
- Rameh,L.E. and Cantley,L.C. (1999) The role of phosphoinositide 3-kinase lipid products in cell function. *J. Biol. Chem.*, **274**, 8347–8350.
- Rebhun,J.F., Chen,H. and Quilliam,L.A. (2000) Identification and characterization of a new family of guanine nucleotide exchange factors for the Ras-related GTPase Ral. *J. Biol. Chem.*, **275**, 13406–13410.
- Rodriguez-Viciana,P., Warne,P.H., Vanhaesebroeck,B., Waterfield,M.D. and Downward,J. (1996) Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation. *EMBO J.*, **15**, 2442–2451.
- Rommel,C., Clarke,B.A., Zimmermann,S., Nunez,L., Rossman,R., Reid, K., Moelling,K., Yancopoulos,G.D. and Glass,D.J. (1999) Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science*, **286**, 1738–1741.
- Rusanescu,G., Gotoh,T., Tian,X. and Feig,L.A. (2001) Regulation of ras signaling specificity by protein kinase c. *Mol. Cell. Biol.*, **21**, 2650–2658.
- Shou,C., Farnsworth,C.L., Neel,B.G. and Feig,L.A. (1992) Molecular cloning of cDNAs encoding a guanine-nucleotide releasing factor for Ras p21. *Nature*, **358**, 351–354.
- Spaargaren,M. and Bischoff,J.R. (1994) Identification of the guanine nucleotide dissociation stimulator for Ral as a putative effector molecule of R-ras, H-ras, K-ras and Rap. *Proc. Natl Acad. Sci. USA*, **91**, 12609–12613.
- Stokoe,D., Stephens,L.R., Copeland,T., Gaffney,P.R., Reese,C.B., Painter,G.F., Holmes,A.B., McCormick,F. and Hawkins,P.T. (1997) Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science*, **277**, 567–570.
- Takai,Y., Sasaki,T. and Matozaki,T. (2001) Small GTP-binding proteins. *Physiol. Rev.*, **81**, 153–208.
- Toker,A. and Newton,A.C. (2000) Cellular signaling: pivoting around PDK-1. *Cell*, **103**, 185–188.
- Urano,T., Emkey,R. and Feig,L.A. (1996) Ral-GTPases mediate a distinct downstream signaling pathway from Ras that facilitates cellular transformation. *EMBO J.*, **15**, 810–816.
- White,M.A., Nicolette,C., Minden,A., Polverino,A., Van Aelst,L., Karin, M. and Wigler,M.H. (1995) Multiple Ras functions can contribute to mammalian cell transformation. *Cell*, **80**, 533–541.
- White,M.A., Vale,T., Camonis,J.H., Schaefer,E. and Wigler,M.H. (1996) A role for the Ral guanine nucleotide dissociation stimulator in mediating Ras-induced transformation. *J. Biol. Chem.*, **271**, 16439–16442.
- Wolthuis,R.M., Baur,B., van't Veer,L.J., De Vries-Smits,A.M.M.,

- Cool,R.H., Spaargaren,M., Wittinghofer,A., Burgering,B.M.T. and Bos,J.L. (1996) RalGDS-like factor (Rlf) is a novel Ras and Rap1A associating protein. *Oncogene*, **13**, 353–362.
- Wolthuis,R.M., de Ruiten,N.D., Cool,R.H. and Bos,J.L. (1997) Stimulation of gene induction and cell growth by the Ras effector Rlf. *EMBO J.*, **16**, 6748–6761.
- Wolthuis,R.M., Zwartkruis,F., Moen,T.C. and Bos,J.L. (1998) Ras-dependent activation of the small GTPase Ral. *Curr. Biol.*, **8**, 471–474.
- Yamaguchi,A., Urano,T., Goi,T. and Feig,L.A. (1997) An Eps homology (EH) domain protein that binds to the Ral-GTPase target, RalBP1. *J. Biol. Chem.*, **272**, 31230–31234.
- Zimmermann,S. and Moelling,K. (1999) Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science*, **286**, 1741–1744.

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