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# EMBO MEMBER'S REVIEW

# All in the family? New insights and questions regarding interconnectivity of Ras, Rap1 and Ral

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**Ras, Rap1 and Ral are related small GTPases. While the function of Ras in signal transduction is well established, it has been recognized only recently that Rap1 and Ral also are activated rapidly in response to a large variety of extracellular signals. Between the three GTPase an intriguing interconnectivity exists, in that guanine nucleotide exchange factors for Ral associate with the GTP-bound form of both Ras and Rap1. Furthermore, Rap1 is considered to function as an antagonist of Ras signalling by trapping Ras effectors in an inactive complex. Here, I summarize the recent developments in understanding the functional relationship between these three GTPase and argue that Rap1 functions in a signalling pathway distinct from Ras, while using similar or identical effectors.**
*Keywords*: GTPase/Ral/Rap1/Ras/signalling

## Introduction

At the surface of cells, many different kinds of receptors are expressed which allow the cell to respond to signals provided by its environment. Activation of these receptors leads to a large variety of biochemical events in which small GTPases play a crucial role. These proteins cycle between two conformations induced by the binding of either GDP or GTP. Guanine nucleotide exchange factors (GEFs) induce the dissociation of GDP to allow association of the more abundant GTP, which in its turn is hydrolysed to GDP by the intrinsic GTPase activity in combination with GTPase-activating proteins (GAP). These processes have been visualized in the co-crystallization studies of Ras with the catalytic domain of the RasGEF SOS (Boriack-Sjodin *et al*., 1998) and of Ras with the catalytic domain of p120RasGAP (Scheffzek *et al*., 1997).

The Ras-like small GTPases (Figure 1) are a family of proteins which includes Ras, Rap1, Rap2, R-ras, TC21, Ral, Rheb (Bos, 1997) and the recently identified M-ras (R-ras3) (Kimmelman *et al*., 1997; Matsumoto *et al*., 1997). These proteins are classified in this group due to similarities in the effector domain, a region that in Ras interacts with downstream targets. While Ras is clearly the best known and best-studied member of the family, both Rap1 and Ral recently have attracted much attention: Ral because Ral-specific GEFs are regulated by direct binding to Ras, and Rap1 because of its proposed role as an antagonist of Ras signalling. In this review I will summarize our recently gained knowledge on Ras signalling, highlighting the RalGEF–Ral effector pathway. In addition, I will discuss the Rap1 signalling pathway and will argue that Rap1 may not function as an antagonist of Ras signalling, but serves in a signalling pathway independently of Ras.

## The Ras signalling pathway

### *Ras, the paradigm of Ras-like GTPases in signal transduction*

The Ras proteins (H-ras, K-ras and N-ras) achieved notoriety as products of genes which were found mutated in ~15% of all human tumours (Bos, 1989). In particular, the presence of mutated K-ras in ~50% of adenocarcinoma of the colon and 90% of adenocarcinoma of the pancreas contributes to this relatively frequent occurrence. Ras proteins are ubiquitously expressed and located at the inner side of the plasma membrane. Clearly, there is redundancy between the different Ras proteins, since H- and N-ras knock-out mice are completely viable. However, K-ras knock-out mice die between day 12 and 14 of gestation, showing that this gene performs some unique function (Johnson *et al*., 1997).

### *Activation of Ras*

A large variety of signals induce the activation of Ras, most notably signals that induce receptors with intrinsic or associated tyrosine kinase activity (Pronk and Bos, 1994). Phosphotyrosines serve as docking sites for the assembly of a complex containing the Ras-specific GEF SOS and the adaptor protein Grb2 (Figure 2). Receptors not directly associated with tyrosine kinases, such as serpentine receptors, may activate Ras indirectly through src-like kinases or ligand-independent activation of receptor tyrosine kinases (van Biesen *et al*., 1995; Daub *et al*., 1997; Lopez-Ilasaca *et al*., 1997). Calcium and diacylglycerol can activate Ras directly by activation of specialized Ras-specific GEFs. RasGRF, an IQ-motif-containing GEF predominantly expressed in brain, is regulated by calcium-bound calmodulin (Farnsworth *et al*., 1995). RasGRP, a GEF also expressed predominantly in brain, binds calcium directly through a structure resembling the calcium-binding 'EF hands' and, in addition, RasGRP is regulated by direct binding of diacylglycerol (Ebinu *et al*., 1998).

### *Ras effectors*

In the GTP-bound conformation, Ras binds to and activates effector proteins (Figure 2). Currently, a large number of proteins have been identified which bind specifically to the active, GTP-bound conformation of Ras (Katz and McCormick, 1997). For only a few of these proteins, additional biochemical and genetic evidence has been provided indicating that these proteins are genuine effector

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          G37 C40
        S35|E38|
FVDEYDPTIEDSYRKQVV    Ras
--EK-------------E    Rap1
--ED-E--KA-----K--    Ral

-IEK-------F---EIE    Rap2
--SD---------T-ICS    R-ras
--TD---------T--C-    TC21
--PD---------L-HTE    M-ras
---S------NTFT-LIT    Rheb
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**Fig. 1.** The switch 1 region of the family of Ras-like GTPases. This family consists of Ras (N-, K- and H-ras), Rap1 (A and B), Ral (A and B), Rap2 (A and B), R-ras, TC21, M-ras/R-ras3 and Rheb. Sequence comparison of the region in Ras between residue 28 and 45. This region is responsible for binding of Ras to effectors (effector region) and shows a dramatic conformational difference between the GTP-bound and the GDP-bound form (Wittinghofer and Nassar, 1996). Indicated are the mutations in Ras that affect specific binding to effectors: S35 and E38 do not bind to RalGEFs and PI-3 kinase, nor G37 to Raf1 and PI-3 kinase or C40 to Raf1 and RalGEFs.

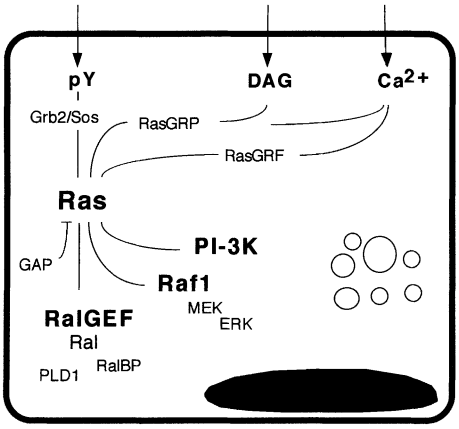


**Fig. 2.** The Ras signalling pathway. Ras is activated by association of Grb2/Sos with phosphotyrosine-containing proteins. In addition, predominantly in brain, specific RasGEFs are present that respond directly to the second messengers calcium and diacylglycerol. Downstream from Ras, three groups of distinct effectors have been established, RalGEFs, members of the Raf family and PI-3 kinase (PI-3K). The open circles represent vesicular structures and the black oval the nucleus.

proteins of Ras. These are the members of the Raf family (Raf1, B-raf and A-raf), the p85–p110 phosphatidylinositol 3 kinase (PI-3 kinase) and members of the RalGEF family (RalGDS, Rlf and Rgl).

The involvement of multiple functional effectors pathways of Ras was demonstrated by the observation that a functionally inactive effector domain mutant of Ras can be complemented by another functionally inactive effector domain mutant (White *et al*., 1995). Interestingly, there are three complementation groups each binding specifically to one of the three currently established effectors (Figure 1). Thus, RasV12S35 and, particularly, RasV12E38 only bind Raf and not RalGEF and PI-3 kinase, whereas RasV12G37 only binds RalGEF, and RasV12C40 only PI-3 kinase (White *et al*., 1996; Rodriguez-Viciana *et al*., 1997).

The best-characterized downstream effector of Ras is the serine-threonine kinase Raf1 and its close relatives B-raf and A-raf. The mechanism(s) of activation for Raf may be rather complex, including translocation to the plasma membrane, induction of a conformational change

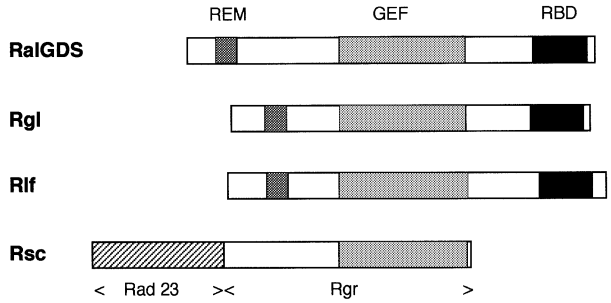


**Fig. 3.** The family of Ral guanine nucleotide exchange factors (RalGEFs). Indicated are the four currently known RalGEFs: RalGDS, the RalGDS-like factors Rgl and Rlf, and the *rsc* oncogene product Rsc. Rsc is a fusion protein of a protein homologous to the yeast Rad23 protein and a RalGDS-related protein (Rgr). This protein was found in an NIH-3T3 focus formation assay (D'Adamo *et al*., 1997).

by Ras and modification by phosphorylation. Activation of these kinases culminates in activation of the well-described MEK-ERK kinase (for reviews see Burgering and Bos, 1995; Marshall, 1996).

Another established effector of Ras is PI-3 kinase. This enzyme is a heterodimer consisting of a p85 regulatory subunit and a p110 catalytic subunit. Ras activates the p85–p110 complex by direct binding to the p110 catalytic subunit (Rodriguez-Viciana *et al*., 1996).

The most recently established Ras effectors are the members of the RalGEF family, which connect Ras to the small GTPase Ral.

## The Ral signalling pathway

### *RalGEF–Ral, a genuine effector pathway of Ras*

Until recently, little was known about the regulation and function of Ral (A and B), but this changed when RalGDS, a RalGEF, was found to interact with the GTP-bound form of Ras (and several other Ras-like GTPases) in a yeast two-hybrid screen (Hofer *et al*., 1994; Spaargaren and Bischoff, 1994). In addition, two distant relatives, Rgl and Rlf, with similar structural and Ras-binding properties were identified (Figure 3; Kikuchi *et al*., 1994; Wolthuis *et al*., 1996). These three ubiquitously expressed RalGEFs exhibit exchange activity towards Ral *in vivo* which is augmented by co-expression of active Ras (Urano *et al*., 1996; Murai *et al*., 1997; Wolthuis *et al*., 1997). Importantly, insulin- and epidermal growth factor (EGF)-induced activation of Ral is inhibited by dominant-negative Ras, establishing that the RalGEF–Ral pathway is a downstream signalling pathway of Ras (Wolthuis *et al*., 1998b). However, Ras-independent activation of Ral does occur as well, for instance in response to elevated levels of calcium (Hofer *et al*., 1998; Wolthuis *et al*., 1998a). Perhaps this activation is mediated by other Ras-like GTPases, as suggested for calcium-induced Ral activation in platelets, where Rap1 activation correlated with the activation of Ral (Wolthuis *et al*., 1998a). Alternatively, calcium may activate Ral through the binding of calmodulin to a region in the C-terminus of RalA (Wang *et al*., 1997). Activation of Ras does not always lead to Ral activation, since in neutrophils granulocye–macrophage colony-stimulating factor (GM-CSF) induces Ras activation, but not Ral (L.M'Rabet, P.Coffer, F.Zwartkruis, R.Wolthuis, L.Koenderman and J.L.Bos, submitted). Thus, although

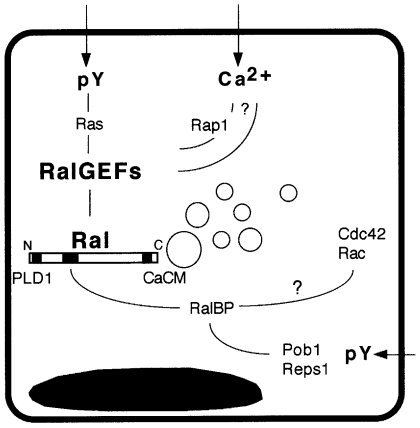


**Fig. 4.** The Ral signalling pathway. RalGEFs are activated by binding to both Ras and Rap1 and by a calcium-dependent pathway, and subsequently to the small GTPase Ral, which is localized both in the plasma membrane and in endocytic/exocytic/lysosomal vesicles (yellow). The N-terminus of both RalGTP and RalGDP forms a complex with phospholipase D1 (PLD1), whereas the C-terminus binds to calcium-bound calmodulin (CaCM). The effector domain of RalGTP binds to Ral-binding protein (RalBP), which is a GTPase-activating protein for the small GTPases Cdc42 and Rac. RalBP interacts with Reps1 or Pob1, related proteins that are tyrosine phosphorylated after EGF stimulation.

Ral is a clear downstream element in Ras-mediated signalling, the activation of Ral is controlled by both Ras-dependent and Ras-independent events (Figure 4).

### *The function of RalGEF-mediated signalling*

To activate the Ras-RalGEF pathway selectively, the RasV12G37 mutant (binds RalGEFs, but not Raf1 or PI-3 kinase) and RalGEFs were used. In NIH-3T3 cells, both RasV12G37 and RalGEF synergize with Raf1 in the induction of cell transformation, and constitutively active Rlf allows the cells to grow in low serum (Khosravi-Far *et al*., 1996; Urano *et al*., 1996; White *et al*., 1996; Wolthuis *et al*., 1997). Introduction of the dominant-negative mutant RalN28 inhibited Ras- and Raf-induced cell transformation (Urano *et al*., 1996). From these results, it was concluded that the RalGEF–Ral pathway contributes to cell transformation. This conclusion is supported further by the identification of the Rsc oncogene. The oncogenic potential of this rearranged gene is provided by the catalytic region of another Ral-specific GEF, Rgr (D'Adamo *et al*., 1997). The effects of the RalGEF–Ral pathway on cell transformation may be mediated by the induced expression of transformation-related genes such as Fos (Murai *et al*., 1997; Wolthuis *et al*., 1997). Surprisingly, the GTP-bound RalV23 is rather ineffective in inducing these processes as compared with RasV12G37 and active RalGEFs (Urano *et al*., 1996; Wolthuis *et al*., 1997). This may imply that RasV12G37 and RalGEFs activate, in addition to Ral, other downstream signalling pathways. Alternatively, RalV23 may not generate an output signal, for instance because Ral needs to cycle between the GTP- and GDP-bound form for proper functioning.

### *Ral effectors and function*

In a yeast two-hybrid screen, the GTP-bound form of Ral was found to interact with Ral-binding protein (RalBP), a GAP for the Rho-like GTPases Cdc42 and Rac (Feig *et al*., 1996). These GTPases are involved in the regulation of the cytoskeleton and are essential for Ras-induced oncogenic transformation (Symons, 1996). Whether the association of RalBP with active Ral results in an activation or an inactivation of these GTPases awaits further investigation. RalBP was also found to form a complex with two related proteins, Pob1 (Ikeda *et al*., 1998) and Reps1 (Yamaguchi *et al*., 1997). These proteins have a RalBP-binding domain, proline-rich sequences that can interact with the SH3 domains of Grb2 and Crk, and an Eps15 homology (EH) domain. Upon EGF stimulation, Pob1 and Reps1 are tyrosine phosphorylated and Pob1 was found to complex with the EGF receptor, presumably through the Grb2 adaptor protein. Although the biological relevance of these interactions still remains to be established, it may indicate that Ral is involved in endocytosis and/or receptor downregulation (Yamaguchi *et al*., 1997; Ikeda *et al*., 1998). In support of this notion, Ral is localized in the plasma membrane and in the membranes of endocytic and exocytic vesicles (Feig *et al*., 1996). Another Ral-associated protein, phospholipase D1 (PLD1), is also implicated in vesicular trafficking. This enzyme produces phosphatidic acid by hydrolysis of phosphatidylcholine. PLD1 associates *in vitro* with the N-terminus of Ral independently of the nucleotide bound to Ral. This PLD1 activity could be induced by Src and Ras and was inhibited by dominant-negative Ral (Jiang *et al*., 1995).

## The Rap1 signalling pathway

Rap1 (A and B) is a Ras-like GTPase localized mainly at endocytic and lysosomal vesicles (Pizon *et al*., 1994). Rap1 was identified in a screen for cDNAs that revert the morphology of K-Ras-transformed cells (Kitayama *et al*., 1989). Interestingly, Rap1 has an effector domain virtually identical to that of Ras (Figure 1), indicating that both GTPases may interact with similar effectors. However, Ras and Rap1 differ considerably in several aspects. For instance, a characteristic feature of Rap1 is its threonine residue at position 61. In Ras and most other GTPases, this residue is a glutamine. Substitution of threonine for glutamine in Ras resulted in a strongly reduced intrinsic and GAP-induced GTPase activity. Indeed, Rap1 has a 10-fold lower intrinsic GTPase activity than Ras (Noda, 1993). Also the guanine nucleotide exchange reaction may be different for Ras and Rap1. This is suggested by the observation that, while the RasN17 mutant tightly associates with RasGEFs, RapN17 does not associate with the Rap1GEF C3G (van den Berghe *et al*., 1997). As a consequence, Rap1N17 may not function as a dominant-negative mutant for Rap1 signalling in a similar way to RasN17 for Ras signalling.

Rap1 is ubiquitously expressed but particularly abundant in platelets, neutrophils and brain. Interestingly, in platelets, Rap1 is one of the major substrates of protein kinase A (PKA) (Bos *et al*., 1997). Also, in other cells, activation of PKA results in the phosphorylation of a serine adjacent or close to the C-terminal $C^{181}$AAX-motif (Ser180 of Rap1A and Ser179 of Rap1B) (Altschuler and Lapetina, 1993). The function of this phosphorylation is still largely elusive. The site of phosphorylation suggests that it may affect the processing and/or the membrane localization of Rap1, but other functions cannot be excluded.

### Activation of Rap1

The first evidence that Rap1 is involved in signal transduction came from the observation that cAMP induces an increase in the GTP-bound form of epitope-tagged Rap1 (Altschuler *et al*., 1995). Subsequently, in various cell types, a number of stimuli were found to induce the activation of Rap1; e.g. in platelets by thrombin and thromboxane A2 (Franke *et al*., 1997), in lymphocytes by T cell receptor and B cell antigen receptor activation (Boussiotis *et al*., 1997; McLeod *et al*., 1998; Reedquist and Bos, 1998), in neutrophils by fMetLeuPhe (fMLP) and platelet-activating factor (M'Rabet *et al*., 1998) and in fibroblast by, among others, EGF, platelet-derived growth factor (PDGF), endothelin and LPA, but not by, for instance, insulin (Zwartkruis *et al*., 1998). In most cases, activation is very rapid, i.e. in seconds in platelets and within minutes in fibroblasts, suggesting that Rap1 activation is a receptor-proximal event. In platelets and fibroblasts, inhibition of phospholipase C (PLC) by pharmacological inhibitors largely abolishes the activation of Rap1 by all signals tested, except cAMP. Also Rap1 activation by the B cell antigen receptor is greatly reduced in B cells deficient for PLCγ (McLeod *et al*., 1998), indicating that PLC mediates Rap1 activation. Interestingly, both second messengers generated by PLC activation, calcium and diacylglycerol, are able to induce Rap1 activation, but in a cell type-specific way. In platelets, thrombin-induced Rap1 activation is mediated almost exclusively by calcium (Franke *et al*., 1997), whereas in B cells diacylglycerol mediates antigen receptor-induced Rap1 activation (McLeod *et al*., 1998). In fibroblasts, both calcium and diacylglycerol are able to induce Rap1 activation (Zwartkruis *et al*., 1998). Thus, at least three distinct second messenger pathways, calcium, diacylglycerol and cAMP, and perhaps others (M'Rabet *et al*., 1998), are able to induce Rap1 activation (Figure 4). Clearly, Rap1 activation is a surprisingly common event, which suggests a function that is central in signal transduction processes.

### Regulation of Rap1 activation

How these three pathways induce Rap1 activation is still unclear, but the picture is emerging that these second messengers may activate Rap1-specific GEFs directly, in analogy with RasGEFs directly activated by calcium and diacylglycerol (Farnsworth *et al*., 1995; Fam *et al*., 1997; Ebinu *et al*., 1998).

Indeed, 12-*O*-tetradecanoyl-13-acetate (TPA)-induced Rap1 activation is insensitive to inhibitors of protein kinase C (PKC) (M'Rabet *et al*., 1998; L.M'Rabet, P.Coffer, F.Zwartkruis, R.Wolthuis, L.Koenderman and J.L.Bos, submitted), and cAMP-induced Rap1 activation is insensitive to the PKA inhibitor H89. In addition, forskolin and 8-Br-cAMP induce Rap1 normally in CHO cells lacking PKA (J.de Rooij, F.Zwartkruis, M.Verheijen, S.Nijman and J.L.Bos, submitted). This implies that diacylglycerol and cAMP do not activate Rap1 via their predominant targets, PKC and PKA, respectively, but perhaps through second messenger-responsive GEFs. Interestingly, we recently identified a Rap1-specific GEF regulated by cAMP (J.de Rooij, F.Zwartkruis, M.Verheijen, S.Nijman and J.L.Bos, submitted) and one regulated by calcium and diacylglycerol (J.de Rooij and J.L.Bos, in preparation).

C3G is a Rap1-specific GEF containing a proline-rich domain which interacts with the SH3 domain of members of the Crk adaptor proteins, Crk I, Crk II and Crk L (Gotoh *et al*., 1995; Kiyokawa *et al*., 1997). In general, this association is constitutive, but tyrosine phosphorylation of Crk may disrupt the interaction (Okada *et al*., 1998). The SH2 domain of Crk binds directly to various activated receptor tyrosine kinases and phosphotyrosine-containing adaptor proteins (Kiyokawa *et al*., 1997). This association of Crk–C3G with these complexes may enhance GEF activity of C3G (Ichiba *et al*., 1997). Thus, in analogy with the RasGEF SOS, it is plausible to assume that complex formation and dissociation of C3G regulates Rap1 activation by tyrosine kinases. However, it is puzzling as to how to reconcile involvement of C3G with the above-described observation that tyrosine kinase-induced Rap1 activation is mediated by PLCγ. Moreover, in a human Jurkat T cell leukaemia line, T cell receptor-dependent induction of a Cbl–CrkL–C3G signalling complex does not activate Rap1 (Reedquist and Bos, 1998). Therefore, resolution of how C3G complex formation is coupled to Rap1 regulation awaits further study.

Although several Rap1-specific GAPs have been identified (Bos *et al*., 1997), currently little is known about their role in the regulation of Rap1 signalling. However, considering the very low intrinsic GTPase activity of Rap1, these GAPs should serve an important role.

### Rap1, an inhibitor of Ras signalling?

The most acknowledged model suggests that Rap1 functions as an antagonist of Ras signalling by trapping Ras effectors, in particular Raf1, in an inactive complex. This hypothesis originated from the observed similarities between the effector domains of Ras and Rap1, and the finding that introduction of Rap1 into K-ras-transformed cells reverts the transformed phenotype (Kitayama *et al.*, 1989). This model was supported by the observation that introduction of RapV12 inhibits LPA-induced, Ras-dependent ERK activation (Cook *et al*., 1993).

Two recent papers further developed the model that Rap1 is an antagonist of Ras signalling. First, in T lymphocytes, induction of anergy (functional unresponsiveness) correlated both with the phosphorylation of Cbl, which is in a complex with Crk-L and C3G, and with a constitutively active Rap1 complexed to Raf1 (Boussiotis *et al*., 1997). One of the characteristics of anergic cells is that T cell receptor ligation does not result in the activation of the Raf1–MEK–ERK pathway and, therefore, inhibition of this pathway by sequestration of Raf1 by active Rap1 is a plausible model. However, in anergic cells, T cell receptor-dependent conversion of Ras in the GTP-bound state is already silenced (Fields *et al*., 1996; Li *et al*., 1996), suggesting that the block is upstream from Ras, rather than downstream. So, although Rap1 activation may provide a safeguard mechanism to block fortuitous Ras activation, it is equally possible that Rap1 plays some role in anergic T lymphocytes independently of Ras signalling.

The second paper describes that insulin treatment of CHO cells overexpressing the human insulin receptor resulted in a decrease in Rap1-GTP and a decrease in Raf1 co-immunoprecipitated with Rap1 (Okada *et al*., 1998). Concomitantly, Ras is activated and found in a complex with Raf1. These results were interpreted as indicating that insulin treatment results in the derepression of Rap1 inhibitory function on the Raf1 kinase.

In contrast to the model of Rap1 functioning as a repressor of Ras signalling are results obtained by comparing the activation of Rap1 with those of the Raf1 signalling pathway (Zwartkruis *et al*., 1998). In this study, it was found that most signals that activate Raf1, such as PDGF and EGF, activate rather than inhibit Rap1. Furthermore, increasing the amount of GTP-bound Rap1 by TPA in Rat1 cells did not inhibit Ras-dependent activation of ERK by PDGF and EGF detectably. From these and other results, it was concluded that activation of endogenous Rap1 does not interfere with growth factor-induced Raf1 activation. Thus, while overexpression of Rap1 may interfere in Ras signalling, presumably by trapping Raf1 in an inactive complex, it is unlikely that growth factors activate Rap1 to repress Ras-effector signalling.

### *Rap1 effectors*

An alternative model for the function of Rap1 is that Rap1 is involved in signalling pathways distinct from Ras, while using similar or identical effector pathways. Examples of a clear positive effect of Rap1 are the induction of DNA synthesis and oncogenic transformation in Swiss 3T3 cells, effects normally attributed to Ras (Yoshida *et al*., 1992; Altschuler and Ribeiro-Neto, 1998). A candidate effector for mediating the Rap1 effect is B-raf, a close relative of Raf1, which can bind to and is activated by Rap1 *in vitro* (Ohtsuka *et al*., 1996). Support for the effector function of B-raf comes from studies in PC12 cells which demonstrate that cAMP and nerve growth factor (NGF)-induced B-raf activation is mediated by Rap1 and functions in the establishment of a sustained ERK activation essential for differentiation (Vossler *et al*., 1997; York *et al*., 1998). It is unclear whether this interaction is regulated by an increase in Rap1GTP or an increased phosphorylation of Rap1 by PKA, since cAMP-induced B-raf, but not Rap1 activation is dependent on PKA. However, whatever the mechanism, these results support the hypothesis that Rap1 functions independently of Ras signalling, utilizing effectors similar or identical to those of Ras, like Raf family members.

Also RalGEFs may belong to a class of effectors that are shared between Ras and Rap1. RalGDS, Rgl and Rlf associate with Rap1GTP *in vitro* and *in vivo* (Spaargaren and Bischoff, 1994; Wolthuis *et al*., 1996; Kishida *et al*., 1997). In addition, in co-transfection experiments, Rlf (Zwartkruis *et al*., 1998), but apparently not RalGDS (Urano *et al*., 1996), can mediate Rap1- and C3G-induced Ral activation. However, in fibroblasts, activation of Rap1 by the phorbol ester TPA and by cAMP did not result in Ral activation (Zwartkruis *et al*., 1998). These results show that Rap1, in principle, is able to activate the RalGEF–Ral pathway, but it may not necessarily do so.

Another potential effector of Rap1 is Krit1, an ankyrin repeat-containing protein that specifically interacts with the GTP-bound form of Rap1, but not Ras (Serebriiskii *et al*., 1997). The function of this protein is, however, unknown.

### *Rap1 in lower eukaryotes*

Rap1 is a highly conserved protein also present in *Drosophila* and yeast. In *Drosophila*, the *Roughened* mutation was found to be a gain-of-function mutation in Rap1. The dominant F157L mutation disrupts eye development. In particular, the ommatidia of the adult eye frequently lack one of the photoreceptor cells, most commonly the R7 cell (Hariharan *et al*., 1991). However, the *Roughened* mutation does not interfere directly in the Ras pathway (Li *et al*., 1997). Loss-of-function mutations in Rap1 are lethal at the larval stage (Hariharan *et al*., 1991) and embryos that lack maternally supplied Rap1 develop abnormally and display a variety of morphological abnormalities including defects in closure of the ventral furrow and head involution. The migration of the cells of the germline (the pole cells) and the mesodermal cells is perturbed (H.Asha and I.K.Hariharan, personal communication). These results suggests a function for Rap1 in regulating morphogenesis.

In the budding yeast *Saccharomyces cerevisiae*, the Rap1 orthologue Bud1 (Rsr1) is involved in the selection of the bud site (Chant *et al*., 1991). This bud normally is formed either in a region directly adjacent (in haploid cells) or opposite (in diploid cells) to the previous site of cell division. Bud1 null cells, however, display a random bud site selection. Two other factors involved in this process are Bud5, a GEF for Bud1, and Bud2, a GAP for Bud1. Interestingly, Bud5 and Bud2 null cells have the same phenotype as Bud1 null cells, strongly indicating that Bud1 needs to cycle between the GTP- and GDP-bound state to exhibit its function (Bender, 1993; Park *et al*., 1993). The function of Bud1 is to recruit polarity establishment factors to target bud formation (Chenevert *et al*., 1992; Michelitch and Chant, 1996; Park *et al*., 1997). These polarity establishment factors are Cdc24, which is a GEF for the small GTPase Cdc42, Cdc42 itself, and Bem1, an SH3-domain-containing protein. Cdc24 associates with the GTP-bound form of Bud1, whereas Bem1 appears to associate specifically with the GDP-bound form of Bud1 (Park *et al*., 1997). Thus both the GDP- and the GTP-bound conformations of Bud1 are involved in the formation of the complex. Multiple cycles of Bud1GDP–GTP exchange are thought to be essential to obtain sufficient amounts of Bem1 and Cdc24 at the target site. Cdc24 activates Cdc42, which, together with Bem1, directs actin polymerization to form the future bud.

### *Function of Rap1*

From the genetic analysis in lower eukaryotes, we can conclude that Rap1 has a distinct function in morphogenesis and/or in the recruitment of protein complexes to target sites. Furthermore, Rap1 has to cycle between the GTP- and GDP-bound conformation to exhibit its effect. Although these conclusions may not account for Rap1 in mammalian cells, it does indicate that Ras may not be a correct paradigm to understand the function of Rap1. Unfortunately, despite many studies addressing the function of Rap1 in mammalian cells, no clear picture has yet crystallized. However, the localization of Rap1 in endocytic/lysosomal vesicles in fibroblasts, its abundant expression and rapid activation in specialized cell types such as platelets and neutrophils, and its regulation by three very common second messengers indicate that Rap1 functions in a general process in signal transduction, which may be linked to endocytosis/exocytosis. Receptor recycling and downregulation, regulated secretion and formation of localized protein complexes are a few of the possibilities.

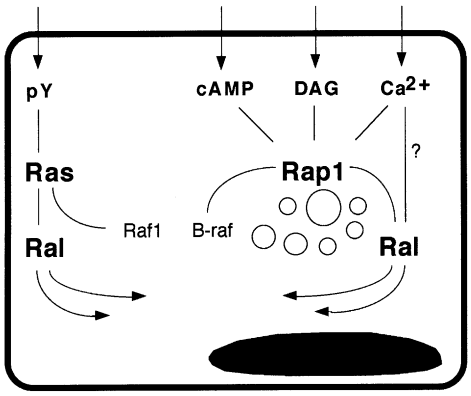


**Fig. 5.** Model for the interconnectivity between Ras, Rap1 and Ral. The model suggests that Ras and Rap1 serve in different compartments of the cell, i.e. Ras at the plasma membrane and Rap1 in endocytic/exocytic/lysosomal vesicles (open circles), to transduce signals to effector pathways. For further explanation, see text.

## Concluding remarks

The Ras signalling pathway is in general considered to be a well-understood signalling pathway, but with the continuing discovery of novel effectors we have to acknowledge that the picture is still not complete. In the meantime, two other family members have stepped into the spotlight: Ral and Rap1. Both GTPases are expressed rather abundantly in all cell types tested and are activated rapidly by a large variety of extracellularly regulated signals. This indicates that they mediate common events in signal transduction. In contrast to common belief, the function of Rap1 is unlikely to be the repression of Ras-effector signalling, but the transduction of common signals to distinct targets. An interesting difference in the activation of Ras and Rap1 is that, while the predominant mechanism of Ras activation is the direct association of GEFs with membrane-bound receptors, Rap1 is activated by highly motile second messengers. This may indicate that Ras mainly serves a function in the proximity of a receptor, whereas Rap1 functions more intracellularly, compatible with the subcellular localization of the two proteins, i.e. Ras at the plasma membrane and Rap1 at endocytic/lysosomal vesicles (Figure 5). Each at their location may be able to activate identical or similar effectors. One of these effector pathways may be the RalGEF–Ral pathway which can be activated by both Ras and Rap1. This may result in the activation of Ral in different subcellular compartments, and, as a consequence, different biological effects. Clearly, a lot of benchwork needs to be done before these three GTPases disclose all of their secrets.

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