Extracellular signal-regulated activation of Rap1 fails to interfere in Ras effector signalling

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The small GTPase Rap1 has been implicated in both negative and positive control of Ras-mediated signalling events. We have investigated which extracellular signals can activate Rap1 and whether this activation leads to a modulation of Ras effector signalling, i.e. the activation of ERK and the small GTPase Ral. We found that Rap1 is rapidly activated following stimulation of a large variety of growth factor receptors. These receptors include receptor tyrosine kinases for platelet-derived growth factor (PDGF) and epithelial growth factor (EGF), and G protein-coupled receptors for lysophosphatidic acid (LPA), thrombin and endothelin. At least three distinct pathways may transduce a signal towards Rap1 activation: increase in intracellular calcium, release of diacylglycerol and cAMP synthesis. Surprisingly, activation of endogenous Rap1 fails to affect Ras-dependent ERK activation. In addition, we found that although overexpression of active Rap1 is able to activate the Ral pathway, activation of endogenous Rap1 in fibroblasts does not result in Ral activation. Rap1 also does not negatively influence Ras-mediated Ral activation. We conclude that activation of Rap1 is a common event upon growth factor treatment and that the physiological function of Rap1 is likely to be different from modulation of Ras effector signalling.

Keywords: ERK/GTPases/Ral/Rap/Ras

Introduction

Rap1 is a small GTPase that was found in a screen for Ras homologous proteins (Kawata *et al.*, 1988; Pizon *et al.*, 1988) and independently as a suppressor of K-ras transformation (Kitayama *et al.*, 1989). Interestingly, Rap1 has an effector domain which is virtually identical to the effector domain of Ras. The active GTP-bound form of Rap1 was found to bind *in vitro* to most Ras effectors, e.g. the Raf family kinases c-Raf1 and B-raf (Nassar *et al.*, 1995; Vossler *et al.*, 1997), and the Ral guanine nucleotide exchange factors (RalGEFs) RalGDS, Rgl and Rlf (Kikuchi *et al.*, 1994; Spaargaren and Bischoff, 1994; Ikeda *et al.*, 1995; Urano *et al.*, 1996; Wolthuis *et al.*, 1996). This has led to the hypothesis that Rap1 functions as an antagonist of Ras effector signalling by trapping Ras effectors in an inactive complex. This hypothesis is

supported by the effects observed after introduction of active Rap1 into cells. For instance, overexpression of active Rap1 inhibits both Ras-dependent germinal vesicle breakdown in *Xenopus* oocytes (Campa *et al.*, 1991) and activation of ERK1 and ERK2 in Rat-1 cells (Cook *et al.*, 1993). In transient transfection experiments, activation of the c-Fos promoter by K-Ras, but not by c-Raf1, is blocked by active Rap1 (Sakoda *et al.*, 1992). Also, in Jurkat cells, Ras-dependent transcription of the IL-2 gene is inhibited by overexpression of Rap1 (Boussiotis *et al.*, 1997). It is less clear whether endogenous Rap1 can antagonize Ras. cAMP has been reported to be an activator of Rap1 (Altschuler *et al.*, 1995) and is a potent inhibitor of Ras-induced ERK activation at the level of Raf1 in certain cell lines (Burgering *et al.*, 1993b; Cook and McCormick, 1993; Wu *et al.*, 1993), suggesting that Rap1 may mediate cAMP-induced inactivation of ERK (Burgering *et al.*, 1993b; Altschuler *et al.*, 1995; Vossler *et al.*, 1997). However, alternative mechanisms for cAMP-induced inhibition of c-Raf1 have also been suggested (Wu *et al.*, 1993; Hafner *et al.*, 1994; Mischak *et al.*, 1996). It has recently been reported that insulin down-regulates Rap1 activity and thereby allows Ras to bind to Raf-1 (Okada *et al.*, 1998).

In contrast, for cells expressing B-raf, such as PC12 cells, it was suggested that Rap1 activation results in the activation of ERK through the direct interaction with B-raf (Vossler *et al.*, 1997; York *et al.*, 1998). Indeed, *in vitro* Rap1 is able to activate B-raf (Ohtsuka *et al.*, 1996), and in PC12 cells cAMP and NGF stimulation were found to result in the formation of a Rap1/B-raf complex (Vossler *et al.*, 1997; York *et al.*, 1998).

In addition to the Raf-ERK pathway, the RalGEF-Ral pathway has also been implicated in both Ras (Wolthuis *et al.*, 1998a) and Rap1 signalling. *In vitro* GTP-bound Rap1 tightly associates with RalGDS (Herrmann *et al.*, 1996), and in reconstitution experiments with lipid vesicles, processed Rap1 stimulates RalGEF-mediated Ral activation (Kishida *et al.*, 1997). In platelets Ral activation was found to correlate with Rap1 activation rather than Ras activation (Wolthuis *et al.*, 1998b).

In this study, various growth factors were found to increase the level of Rap1^{GTP} in various unrelated cell types. We investigated the effect of Rap1 activation on two signal transduction pathways that were previously shown to be critically dependent on Ras in the cell lines used, namely stimulation of ERK2 (de Vries-Smits *et al.*, 1992; Burgering *et al.*, 1993a) and RalGEFs (Wolthuis *et al.*, 1998a). Our results do not support the idea that Rap1 activation by growth factors serves to modulate Ras signalling, either quantitatively or qualitatively.

Fig. 1. Growth factor-mediated activation of Rap1. Time course of activation of endogenous Rap1 in Rat-1 cells (A), and CCL39 cells and NIH3T3-A14 cells (B). Serum-starved cells were stimulated with different stimuli for the time in minutes as indicated. Rap1^{GTP} was isolated and detected by Western blot analysis as described in the Materials and methods. In CCL39 cells, Rap1^{GTP} levels remained elevated for >5 min in the case of PDGF, or 15 min in the case of α -thrombin (data not shown). (C) Quantification of the increase in endogenous Rap1^{GTP}. Rap1^{GTP} isolated from Rat-1 cells unstimulated (–) or stimulated for 5 min with PDGF (+). A sample from the stimulated cells was serially diluted prior to gel electrophoresis (1, 1/2, 1/3, 1/4). (D) Activation of myc-Rap1 by EGF. Cos-7 cells were transfected with 2 μ g pCDNA3-mycRap1, starved overnight, labelled with [³²P]orthophosphate for 5 h, and either left untreated or incubated with 50 ng/ml EGF for 5 min. Myc-Rap1 was immunoprecipitated with the 9E10 anti-myc monoclonal antibody, and bound nucleotides were separated using thin layer chromatography, followed by PhosphorImager detection. Positions of GDP and GTP were determined by addition of standards.

Results

Growth factors activate Rap1

We first investigated whether Rap1 can be activated by growth factors in fibroblasts. To measure activation of endogenous Rap1 we used an assay based on the differential affinity of the Rap binding domain (RBD) from RalGDS for Rap1^{GTP} versus Rap1^{GDP} (Franke *et al.*, 1997). Serum-starved Rat-1 cells were given platelet-derived growth factor (PDGF) and cell lysates were made at various time points after treatment. Rap1^{GTP} was collected specifically from cell lysates by a GST–RalGDS–RBD fusion protein precoupled to glutathione–agarose beads and subsequently visualized by Western blotting. PDGF clearly increased the amount of endogenous Rap1^{GTP}. Epithelial growth factor (EGF), that also activates a receptor tyrosine kinase, and ligands for G-protein-coupled receptors such as LPA and endothelin, could also activate Rap1 in Rat-1 cells. Time course analysis revealed that activation occurred within minutes of stimulation and, depending on the growth factor used, was sustained for 20 min (Figure 1A). Rap1 is also activated by the mitogens thrombin and PDGF in CCL39 cells. In NIH3T3-A14 cells (which express the human insulin receptor), Rap1 is activated by endothelin and EGF, but not by insulin (Figure 1B; data not shown).

To quantify the increase in Rap1^{GTP} following growth factor stimulation, fractions of Rap1^{GTP} isolated from treated cells were compared with Rap1^{GTP} isolated from untreated cells and quantified by densitometric scanning of autoradiograms. This revealed a 2.3-fold increase in the amount of Rap1^{GTP} after stimulation of Rat-1 cells with PDGF and a similar increase after treatment of NIH3T3-A14 cells with endothelin (Figure 1C and Table I). This observation is in agreement with the 2.4-fold increase in Rap1^{GTP} seen in transient transfection experiments with myc-tagged Rap1 in Cos-7 cells (Figure 1D and Table I). Together, these results show that Rap1 can be activated by a variety of stimuli in various cell lines.

Table I. Induction of Rap1 by various growth factors

Cell line	Rap1	Stimulus	Fold induction
Rat-1	endogenous	PDGF (25 ng/ml)	2.3 $\pm$ 0.2
NIH3T3-A14	endogenous	endothelin (100 nM)	2.5 $\pm$ 0.3
NIH3T3-A14	endogenous	insulin (1 μ g/ml)	1.0 $\pm$ 0.1
Cos-7	transfected	EGF (20 ng/ml)	2.4 $\pm$ 0.2

Relative levels ($\pm$ S.E.M.) of endogenous Rap1^{GTP} in unstimulated cells and cells stimulated for 5 min with the listed growth factors as determined by densitometric scanning of autoradiograms (see Materials and methods). The values shown are the result of at least three independent experiments.

Rap1 is activated by phospholipase C-mediated signalling

Previously, we reported for platelets that thrombin-induced activation of Rap1 was mediated by phospholipase C (PLC) and the subsequent increase in intracellular calcium (Franke *et al.*, 1997). To investigate whether PLC mediates PDGF-induced Rap1 activation we used porcine aorta endothelial (PAE) cells, stably expressing either a wild-type or a Y1009F/Y1021F mutant PDGF receptor (kindly provided by Dr L. Claesson-Welsh). In this mutant receptor, two tyrosine residues essential for binding and activation of PLC are replaced by phenylalanine (Ronnstrand *et al.*, 1992). PDGF readily activated Rap1 via the wild-type receptor, but binding of PDGF to the mutant receptor had no effect on Rap1 (Figure 2A). In addition, the PLC inhibitor U73122 strongly diminished Rap1 activation by PDGF in Rat-1 cells. This inhibition was even more pronounced when cells were treated with U73122 and the intracellular calcium chelator BAPTA-AM simultaneously. By itself, BAPTA-AM only marginally inhibited Rap1 activation (Figure 2B). A very similar pattern of inhibition was found for endothelin-induced Rap1 activation in NIH3T3-A14; BAPTA-AM reduced Rap1^{GTP} levels to 88 $\pm$ 17% (n = 5) and U73122 to 46 $\pm$ 15% (n = 2) of those found in the absence of inhibitors. When applied

Fig. 2. Analysis of Rap1 activation in PAE cells (A) or Rat-1 cells (B and C) measured as in Figure 1. (A) Activation of endogenous Rap1^{GTP} in PAE cells carrying a wild-type or Y1009F/Y1021F-mutant PDGF receptor. Serum-starved PAE cells were treated with PDGF or endothelin for the time indicated in min. (B) Inhibition of PDGF induced Rap1 activity by U73122 and BAPTA-AM. Serum-starved Rat-1 cells were pretreated with U73122 for 5 min, with BAPTA-AM for 30 min, or both before addition of PDGF (25 ng/ml) for 5 min. Numbers shown above the blot indicate relative Rap1 activity compared with untreated cells. A representative experiment of two experiments is shown. (C) Elevation of intracellular calcium or addition of TPA activates Rap1. Serum-starved Rat-1 cells were treated with A23187 or TPA for the indicated time in minutes.

together, Rap1 induction was <1.2-fold ($n = 2$). These results suggest that PLC mediates activation of Rap1 after stimulation of both receptor tyrosine kinases and G protein-coupled receptors. This also explains the failure of insulin to induce Rap1 activation in NIH3T3-A14 cells, since in these cells insulin does not activate PLC γ (de Vries-Smits *et al.*, 1992). Treatment of Rat1 cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which mimics diacylglycerol, and agents that increase intracellular calcium levels, such as A23187, ionomycin and thapsigargin, independently induce Rap1 activation (Figure 2C; data not shown). This implies that both second messengers generated by PLC activation are able to activate Rap1 independently.

Rap1 activation does not affect ERK activation

Activation of Rap1 by growth factors may serve one of several functions. First, it may act in parallel with Ras to activate common signalling pathways. Secondly, it may generally interfere with the action of Ras or perhaps in a more subtle fashion prevent the action of a single class of Ras effector proteins. Finally, Rap1 may have a function unrelated to Ras. To test these different possibilities without overexpressing Rap1, we took advantage of the finding that TPA and endothelin selectively activate Rap1, but not Ras, in Rat-1 cells (Figure 3A). Stimulation with TPA or endothelin did not lead to ERK activation, whereas EGF did (Figure 3B). Rat-1 cells express a high-molecular-mass (85 kDa) isoform of B-raf, the Raf family member

implicated in Rap1-induced ERK activation (Vossler *et al.*, 1997) (Figure 3C). When this 85 kDa isoform of B-raf or c-Raf1 was immunoprecipitated from Rat-1 cells stimulated with TPA or endothelin, they did not display enhanced phosphorylation of GST-MEK (Figure 3B). Clearly, in Rat-1 cells Rap1 activation is not sufficient to activate B-Raf, c-Raf1 or ERK.

We further examined the possibility that activation of Rap1 by growth factors functions to negatively regulate the action of Ras. In Rat-1 cells PDGF induces both Ras, Rap1 and ERK activity, and activation of ERK2 by PDGF is critically dependent on Ras activity (de Vries-Smits *et al.*, 1992). We elevated Rap1^{GTP} levels, prior to addition of PDGF, by pretreatment with TPA. This also caused a further increase in Rap1 activity during PDGF treatment. However, TPA pretreatment did not detectably inhibit (or stimulate) PDGF-induced ERK activation (Figure 3D). Also, EGF-induced ERK activation was not affected by TPA (Figure 3E). These results demonstrate that activation of endogenous Rap1 does not lead to an inhibition of ERK either. Furthermore, these results exclude the possibility that the failure to induce ERK activation by TPA in Rat-1 cells is due to the induction of an additional pathway which inhibits ERK activation.

cAMP is a potent inhibitor of Ras-mediated ERK2 activation in Rat-1 cells at the level of c-Raf1 (Burgering *et al.*, 1993b) and has been reported previously to activate Rap1 in fibroblasts (Altschuler *et al.*, 1995). When Rat-1 cells were treated with 10 μ M forskolin, an activator of adenylate cyclase, or 8-bromo-cAMP, Rap1 was activated (Figure 4A; data not shown). To investigate whether cAMP mediates ERK inhibition via Rap1, we titrated the concentration of forskolin required to block PDGF-induced ERK activity (Figure 4B). PDGF-induced ERK activation was completely blocked at all concentrations tested. In contrast, Rap1 activation was dependent on the concentration of forskolin used and not seen at the lowest concentration tested (0.2 μ M: fold induction is 1.0 ± 0.1 , $n = 5$). At this concentration forskolin slightly enhanced Rap1 activation by PDGF (e.g. 1.9- to 2.6-fold in Figure 4C). However, a direct comparison of the amount Rap1^{GTP} in TPA- and forskolin-pretreated cells revealed that there is no correlation between the level of Rap1^{GTP} and inhibition of ERK (Figure 4C). A more extensive time course analysis ruled out the possibility that Rap1 peaked at earlier time points (Figure 4D). Similar results were obtained with forskolin and insulin in NIH3T3-A14 cells (data not shown). From these results we conclude that activation of endogenous Rap1 does not interfere in Ras-mediated ERK activation.

Rap1 and ERK activation in PC12 cells

Recently, it was shown for PC12 cells that NGF, but not EGF, activates ectopically expressed Rap1, suggesting a role for Rap1 in PC12 cell differentiation (Vossler *et al.*, 1997; York *et al.*, 1998). We have investigated whether endogenous Rap1 is also activated by these stimuli. In addition, we compared Rap1 activation with the activation of Ras and ERK (Figure 5). To our surprise, EGF treatment led to a clear but transient Rap1 activation, whereas NGF did not activate Rap1 ($n = 4$). Rap1 activity induced by EGF peaked around 2 min and returned to basal levels at 4–10 min post-stimulation. As previously reported (e.g.

Fig. 3. Rap1 activity does not influence ERK activity in Rat-1 cells. (A) Endothelin and TPA do not activate Ras in Rat-1 cells. Serum-starved cells were treated with endothelin, TPA or EGF for the time indicated in minutes. Ras^{GTP} was isolated and detected by Western blot analysis, as described in Materials and methods. (B) Rap1 activity does not lead to ERK, B-Raf or c-Raf1 activity. Serum-starved cells were left untreated (lane a) or treated with endothelin (lane b), EGF (lane c), or TPA (lane d) for 5 min. Activation of ERK was determined by the occurrence of a mobility shift: ERK2^{PP} is the active form that migrates more slowly. c-Raf1 and B-raf activity was measured in an immunocomplex assay using [γ -³²P]ATP and GST-MEK as substrate, followed by gel separation and autoradiography. (C) A high-molecular-mass B-raf isoform is expressed in Rat-1 cells. B-Raf was immunoprecipitated from Rat-1 cells using the Raf-B (C-19) antibody (Santa Cruz), and following immunoblotting detected with the same antibody (lane a). For comparison, total-cell lysates from Cos-7, transfected with the high molecular weight neuronal B-Raf isoform (lane b), or mock transfected Cos-7 (lane c) cells are shown. The transfected form runs slightly slower than endogenous B-raf from Rat1 cells. (D and E) Increasing Rap1 activity does not inhibit PDGF- or EGF-induced ERK activity. Serum-starved Rat-1 cells were treated with TPA for 10 min (D and E), PDGF for 5 min (D) or EGF for the indicated time (E). In the case of co-stimulation, the ligands were added for the indicated time prior to cell lysis. Rap1^{GTP} and ERK activity were determined as described. Numbers above the panels indicate fold-induction of Rap1^{GTP}.

Dikic *et al.*, 1994; Traverse *et al.*, 1994), EGF treatment led to a transient ERK activation, while ERK activity following NGF treatment was slightly lower but more sustained. Activation of Ras by EGF and NGF correlated well with ERK activity. Since it was previously reported that EGF- and NGF-induced activation of ERK was dependent on Ras (Wood *et al.*, 1992), we conclude that in the PC12 cells tested Rap1 is not involved in the activation of ERK.

Rap1 can activate RalA in transient transfection assays

We have previously obtained correlative evidence in platelets that Ral activation may be mediated by Rap1 rather than by Ras (Wolthuis, 1998b). To investigate whether Rap1 could activate the RalGEF-Ral pathway, we first performed cotransfection experiments in Cos-7 cells. Active Rap1 (HA-Rap1^{G12V}) or a membrane-targeted, activated version of the Rap1-specific exchange factor C3G (C3G-CAAX; Gotoh *et al.*, 1995) were co-transfected with the RalGEF Rlf and myc-tagged RalA. Ral activation was analysed by measuring radiolabelled GTP and GDP bound to myc-Ral. We observed that active Rap1, like active Ras, induces Ral activation. C3G-CAAX was equally effective in stimulating Ral, strongly suggesting that Rlf can be activated via endogenous Rap1 (Figure 6A). In addition, Myc-tagged RalA, Rlf and HA-Rap1^{G12V} were co-expressed in NIH3T3-A14 cells and Ral activation was measured using GST-RalBD as an activation specific probe (Wolthuis, 1998b). Again, Rap1^{G12V} activated Ral as it increased the fraction of myc-RalA isolated with GST-RalBD from lysates that contain identical levels of myc-RalA. No strong increase was seen for endogenous Ral, which can be explained by the fact that only a small percentage of the cells are transfected.

From these results we conclude that in principle, activation of Rap1 can result in the activation of the RalGEF-Ral pathway (Figure 6B).

Rap1 does not affect Ral activation in fibroblasts

To investigate whether growth factor-mediated activation of endogenous Rap1 leads to Ral activation, we again used TPA or a high dose of forskolin to activate Rap1 but not Ras. Remarkably, activation of Rap1 by TPA or forskolin did not induce Ral activation (Figure 6C and D). Thus, activation of Rap1 does not necessarily lead to Ral activation. We recently established that Ral activation by insulin and EGF in NIH3T3-A14 cells is a Ras-dependent process (Wolthuis *et al.*, 1998a). Activation of Rap1 by TPA or forskolin appeared not to interfere in ligand-induced Ral activation; in NIH3T3-A14 cells, elevation of Rap1^{GTP} levels by TPA had no effect on insulin-induced Ral activation (Figure 6C). Similarly, activation of Rap1 following pretreatment of Rat-1 cells with TPA or high doses of forskolin did not interfere in PDGF-mediated Ral activation (Figure 6D and data not shown). Together, these data show that in the cell lines tested Rap1 activation did not affect Ral activation.

Discussion

Regulation of Rap1 activity

The data presented here show that Rap1 activity is regulated by a variety of tyrosine kinase and G-protein-coupled receptors in fibroblasts as well as in several unrelated cell lines (PC12, Cos-7, PAE). Previously, we found Rap1 activation following stimulation of the thrombin receptor in platelets and the T-cell receptor in T-cells (Franke *et al.*, 1997; Reedquist and Bos, 1998). These receptors all have in common the ability to induce PLC

Fig. 4. Rap1 activation does not correlate with inhibition of ERK activity in Rat-1 cells. **(A)** Elevation of intracellular cAMP activates Rap1. Serum-starved cells were incubated with forskolin for the indicated time in minutes and Rap1^{GTP} was detected. **(B)** 0.2 μ M of forskolin effectively blocks PDGF-induced ERK activity but does not induce Rap1 activity. Serum-starved Rat-1 cells were stimulated with increasing concentration of forskolin for 10 min. Where indicated, PDGF was added during the last 5 min of forskolin treatment. Rap1^{GTP} and ERK activity was determined in the same extract. **(C)** Rap1 activity does not correlate with inhibition of ERK2 activity. Serum-starved Rat-1 cells were treated either with forskolin or TPA for 10 min or PDGF for 5 min, or in combination. Rap1^{GTP} and ERK activity were determined. **(D)** Rap1 activity is not increased during early timepoints of costimulation with forskolin and PDGF. Serum-starved Rat-1 cells were either pretreated or not with forskolin for 5 min. Subsequently PDGF was added and Rap1 activity was determined at the indicated time points. Numbers shown above the panels in (C) and (D) indicate fold-induction of Rap1^{GTP}.

activity. Indeed, inhibition of PLC abolished receptor-induced Rap1 activation. PLC hydrolyses phosphatidylinositol-4,5-diphosphate to release inositol-1,4,5-triphosphate (IP₃) and diacylglycerol. IP₃, in its turn, mobilizes calcium from internal stores. Both second messengers appear to mediate Rap1 activation independently, since both an increase in Ca²⁺ and stimulation with TPA, which mimics diacylglycerol, activate Rap1. Thus, together with the previously reported cAMP pathway (Altschuler *et al.*, 1995), there are at least three distinct pathways which regulate Rap1. These three pathways do not operate in all cell types. In platelets for instance, Ca²⁺ is the major second messenger involved in Rap1 activation (Franke *et al.*, 1997). In these cell fragments cAMP inhibits rather than activates Rap1. Also, in other cell types cAMP may not induce Rap1 activation. For instance, cAMP induces Rap1 activation in Rat-1 cells, but not in NIH3T3-A14 cells (J.de Rooij, F.J.T.Zwartkruis, M.H.G.Verheijen, S.M.B.Nijman and J.L.Bos, manuscript in preparation).

How the various second messengers activate Rap1 is currently unclear. To date, a single Rap1-specific exchange factor, C3G, has been described (Gotoh *et al.*, 1995), and

Fig. 5. Activation of Rap1, Ras and ERK in PC12 cells. PC12 cells were treated with 100 ng/ml EGF or 50 ng/ml NGF for the time indicated in minutes. Rap1^{GTP} (upper panel), Ras^{GTP} (middle panel) and ERK activity (lower panel) were determined. Densitometric scanning of several experiments did not reveal a significant increase or decrease in Rap1 activity following NGF stimulation. The bottom panel shows Rap1^{GTP} levels at shorter time points after EGF stimulation.

at least four distinct GTPase-activating proteins (GAPs); Rap1GAP, Spa-1, GapIP4BP and tuberlin (Rubinfeld *et al.*, 1991; Cullen *et al.*, 1995; Wienecke *et al.*, 1995; Kurachi *et al.*, 1997). C3G associates with the Crk adaptor proteins CrkI, CrkII and Crk-L (Tanaka *et al.*, 1994; Gotoh *et al.*, 1995; Ichiba *et al.*, 1997). These Crk proteins bind to tyrosine-phosphorylated Cbl, resulting in a heterotrimeric complex (Reedquist *et al.*, 1996). It has been suggested that formation of this heterotrimeric complex regulates Rap1 activation. It is unclear if and how PLC is involved in the formation of this complex. However, preliminary data suggest that in NIH3T3-A14 cells Cbl is phosphorylated following EGF stimulation, but not after endothelin activation, suggesting that C3G might not be the only exchange factor involved in Rap1 activation (K.Reedquist and J.L.Bos, unpublished results). It is presently also unclear how cAMP activates Rap1. Although direct phosphorylation of Rap1 by PKA may lead to activation (Hata *et al.*, 1991), mutation of the phosphorylation site did not abolish cAMP-induced Rap1 activation (Altschuler *et al.*, 1995).

Rap1 does not affect Ras-effector signalling

Given the well-documented transformation inhibitory effects of Rap1, we were initially surprised to find that many growth factors, including mitogenic ones, activate Rap1 in various cell types. Although the function of Rap1 is largely unknown, the current hypothesis is that Rap1 modulates Ras effector signalling (see Introduction). Clearly, Rap1 does not contribute to ERK2 activation in Rat-1 cells, as is evident from the finding that three stimuli selectively activating Rap1 but not Ras (forskolin, TPA and endothelin) do not stimulate ERK2 in Rat-1 cells. Our results do not support a negative role for Rap1 on Ras signalling towards ERK2 either. This is concluded

Fig. 6. Rap1 can activate transfected Ral. (A) Rap1^{G12V} activates RalA upon overexpression with Rlf in Cos-7 cells. Cos-7 cells were transfected with 1 μ g of pcDNA3-mycRalA alone, or in combination with 1 μ g of pcDNA3-Rlf together with 1 μ g pSVE-Ras^{G12V}, 1 or 2 μ g pMT2-HA-Rap1^{G12V} or 1 μ g C3G-CAAX. GDP and GTP, bound to transfected myc-RalA, was determined as described for myc-Rap1 in the Figure 1 legend. (B) Rap1^{G12V} activates RalA upon overexpression with Rlf in NIH3T3-A14 cells. pcDNA3-mycRalA (1 μ g) and pcDNA3-Rlf (1 μ g) were co-transfected in the absence or presence of pMT2-HA-Rap1^{G12V} (2 μ g) into NIH3T3-A14 cells. After 48 h cells were lysed, and Ral^{GTP} was isolated using GST-RalBD and detected by Western blotting with an anti-RalA antibody which detects both transfected (myc-RalA) and endogenous Ral (Ral). (C and D) Activation of endogenous Rap1 does not stimulate or inhibit Ral activation. Serum-starved NIH3T3-A14 cells were treated with insulin for 5 min, TPA for 10 min, or both (C) and serum starved Rat1 cells were stimulated with PDGF for 5 min, forskolin (40 μ M) for 10 min, or both (D). Rap1^{GTP} and Ral^{GTP} were detected as described.

from the fact that increasing Rap1^{GTP} by TPA in Rat-1 cells does not detectably interfere in the Ras-dependent ERK2 activation by PDGF or EGF. Although this result seems to be contradictory to many results obtained by overexpression of Rap1, it is in line with the outcome of genetic studies where Rap1 has so far not been found to be an enhancer or suppressor of Ras (Hariharan *et al.*, 1991; Li *et al.*, 1997). The effects seen in overexpression studies with Rap1 may then be explained either by the increased absolute level of GTP-bound Rap1 or by the presence of Rap1^{GTP} for prolonged periods of time. It was reported recently that in CHO cells Rap1 activity is downregulated by insulin and that this permits binding of Ras to Raf-1 (Okada *et al.*, 1998). In NIH3T3-A14 cells, insulin did not stimulate nor decrease Rap1 activity. (Figure 1B and Table I). Clearly, our results indicate that downregulation of Rap1 following growth factor treatment is the exception rather than the rule, and not a strict requirement for c-Raf1 activation by Ras.

In PC12 cells we observed a very rapid activation of Rap1 by EGF and no activation of Rap1 by NGF. These data are in contrast to those reported by Stork and co-workers (Vossler *et al.*, 1997; York *et al.*, 1998), who showed that Rap1 was activated by NGF rather than EGF. Possibly, the EGF-induced activation has been missed by these authors because of the transient nature of the Rap1 activity. For NGF the difference may be explained by clonal variation between PC12 cell lines. Nonetheless, in our PC12 cells, NGF induced sustained ERK-activation, which correlated with Ras activation and differentiation, indicating that Rap1 activation is not essential for these processes. This would be in good agreement with results from Blenis and co-workers who found that RasN17 under the control of an inducible promoter could block both

early and late ERK activation following NGF treatment in PC12 cells (Wood *et al.*, 1992).

Endogenous Rap1 activates Ral but not in fibroblasts

We also studied the effects of Rap1 on the Ras effector pathway RalGEF-Ral. Using an overexpression system, we showed that this pathway in principle can be activated by Rap1 as well as by C3G-CAAX, a constitutively active GEF acting on Rap1. In addition, in platelets, activation of Ral correlated with the activation of Rap1 rather than Ras (Wolthuis, 1998b). However, activation of Rap1 in fibroblasts by forskolin or TPA does not lead to Ral activation. Thus, also in the case of RalGEFs there is a clear difference in results obtained by overexpression studies and measurements of endogenous activity. Furthermore, Rap1 activation did not interfere in growth factor-induced Ral activation. These results were rather surprising to us since the affinity of one of the RalGEFs, RalGDS, for Rap1 is much higher than that for Ras and in the same order of magnitude as the affinity of Raf1 for Ras (Herrmann *et al.*, 1996). The fact that forskolin does not alter RalGEF activity but blocks ERK2 activity completely is intriguing as it shows that endogenous Ras-effectors are differentially regulated by cAMP.

The function of Rap1 remains elusive. Rap1 can induce neuronal differentiation of PC12 cells by activating ERK via the neuronal-specific isoform of B-Raf (Vossler *et al.*, 1997). However, the fact that a variety of growth factor receptors can induce Rap1 in unrelated cell lines suggests that Rap1 has a more general function in addition. Rap1 is required for cell viability in *Drosophila* (Hariharan *et al.*, 1991). In platelets Rap1 is activated by stimuli that induce aggregation (Franke *et al.*, 1997). This process

depends on cytoskeletal rearrangements, transport of cell adhesion molecules to the surface and secretion. The fact that we do not find any effect of activation of endogenous Rap1 on two established Ras-signalling pathways suggests that the biological significance of the transformation suppressing activity seen following overexpression of Rap1 should be interpreted with care.

Materials and methods

Materials

The following stimuli and inhibitors were used at concentrations indicated, unless stated otherwise: A23187 (0.5 μ M), TPA (100 ng/ml), LPA (2 μ M), EGF (25 ng/ml), insulin (1 μ g/ml), α -thrombin (2 U/ml), all from Sigma; U73122 (10 μ M) from Calbiochem; PDGF (25 ng/ml) from Amersham; endothelin (100 nM) from Novabiochem; BAPTA-AM (25 μ M) from Molecular Probes; forskolin (0.2 μ M) from ICN; and NGF (50 ng/ml) from Boehringer Mannheim. PVDF membranes and enhanced chemiluminescence (ECL) were from DuPont NEN.

Plasmids

pMT2-HA-Rap1A contained the coding sequence of murine Rap1A, isolated after digestion with *Rsa*I and *Nru*I, and blunting into the *Sma*I site of pMT2-HA vector. The mutagenesis kit Quick Change from Stratagene was used to create a G12V mutation. pCDNA3-mycRap1A was cloned by first inserting a myc-tag encoding oligonucleotide into the *Hind*III–*Bam*HI site of pCDNA3, followed by subcloning of Rap1A encoding insert from pMT2-HA-Rap1A as a *Sal*I–*Not*I fragment. All other constructs have been described previously (Wolthuis *et al.*, 1997).

Cell culture

Rat-1, NIH3T3-A14 (NIH3T3 cells overexpressing the human insulin receptor), CCL39 and Cos-7 cells were grown in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal calf serum (FCS) and serum-starved in DMEM without FCS for 16 h, except for Cos-7 cells, which were serum-starved at 1.5% FCS. PAE cells were grown in Ham's F12 medium, containing 10% FCS and PC12 cells in RPMI with 5% FCS and 10% donor horse serum. PAE and PC12 cells were serum-starved in media containing 1% FCS.

Rap1, Ral and Ras, and ERK2 activation assays

Transfection and RalA and Rap1 loading assays with [32 P]orthophosphate-labelled Cos-7 cells were performed exactly as described in Wolthuis *et al.* (1997). For measurements of Rap, Ral or Ras activation by means of activation-specific probes, cells were grown to 70% confluency in 9 cm dishes and serum-starved as described above. Following growth factor stimulation, cells were washed twice with ice-cold PBS and lysed in Ral-buffer (10% glycerol, 1% Nonidet P-40, 50 mM Tris pH 7.4, 200 mM NaCl, 2.5 mM MgCl₂, 250 μ M phenylmethylsulfonyl fluoride, 1 μ M leupeptin, 0.1 μ M aprotinin, 10 mM NaF and 1 mM Na₂VO₄). Lysates were clarified by centrifugation and supernatants were incubated with 15 μ g GST–RalGDS–RBD to isolate Rap1^{GTP}, GST–RalBD to isolate Ral^{GTP}, or GST–Raf1–RBD to isolate Ras^{GTP} (de Rooij and Bos, 1997; Wolthuis, 1998b). These GST-fusion proteins were precoupled to glutathione–agarose beads (7 μ l packed beads). After incubation for 45 min at 4°C, beads were washed four times in Ral buffer. The remaining fluid was aspirated and beads were resuspended in SDS–Laemmli sample buffer. Samples were analyzed by SDS–polyacrylamide gel electrophoresis (15%) followed by transfer to PVDF membranes which were blocked with Blotto for 1 h and probed with anti-Rap1 and anti-RalA, both from Transduction Laboratories, or anti-Ras (Y13–259). Isolated proteins were detected by ECL. All GTPase activation assays were performed at least twice.

For ERK2 activation part of the cell lysates used for GST–RalGDS–RBD fish experiments were mixed immediately after centrifugation with 5 $\times$ concentrated SDS sample buffer and analysed as described previously (Leevers and Marshall, 1992). c-Raf1 and B-Raf assays were described by Alessi *et al.* (1995).

Quantification of Rap1^{GTP}

The fold-increase of Rap1^{GTP} levels was quantified by densitometric scanning using a Personal Densitometer (Molecular Dynamics). Autoradiographs were scanned and compared with a serial dilution of Rap1^{GTP} isolated from stimulated cells (see Figure 1C for an example). Only autoradiographs with a linear density in the serial dilution were used.

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