Stimulation of gene induction and cell growth by the Ras effector Rlf

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Rlf is a ubiquitously expressed distinct relative of RalGDS that interacts with active Ras *in vitro*. We now demonstrate that Rlf, when co-expressed with Ras mutants, associates *in vivo* with RasV12 and the effector-domain mutant RasV12G37, but not with RasV12E38 or RasV12C40. Rlf exhibits guanine nucleotide exchange activity towards the small GTPase Ral and, importantly, Rlf-induced Ral activation is stimulated by active Ras. In addition, RasV12 and RasV12G37 synergize with Rlf in the transcriptional activation of the c-*fos* promoter. Rlf, when targeted to the plasma membrane using the Ras farnesyl attachment site (Rlf–CAAX), is constitutively active, inducing both Ral activation and c-*fos* promoter activity. Rlf–CAAX-induced gene expression is insensitive to dominant negative Ras and the MEK inhibitor PD98059, and involves activation of the serum response element. Furthermore, expression of Rlf–CAAX is sufficient to induce proliferation of NIH 3T3 cells under low-serum conditions. These data demonstrate that Rlf is an effector of Ras which functions as an exchange factor for Ral. Rlf mediates a distinct Ras-induced signalling pathway to gene induction. Finally, a constitutively active form of Rlf can stimulate transcriptional activation and cell growth.
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Introduction

Ras is a pivotal small GTPase that couples growth factor signals to a variety of cellular processes, including transcription, DNA synthesis and differentiation (Pronk and Bos, 1994). The best known effectors of Ras are the serine–threonine kinases of the Raf family, most notably Raf1, B-Raf and A-Raf, each of which can induce the activation of the MEK–ERK signalling pathway (Marais and Marshall, 1996). However, Ras may also activate several other pathways. Genetic support for this idea comes from studies that have addressed the ability of oncogenic Ras with mutations in the effector-domain to induce transformed foci in fibroblasts. Recently, it has become clear that these mutants are impaired in the association with some effector proteins, but can still interact with and activate others. For instance, RasV12S35—a Ras effector-domain mutant that still induces Raf-activation—is only moderately transforming, but transforming potential can be restored upon co-expression of RasV12G37, which does not activate Raf (White *et al.*, 1995). Indeed, several additional effector molecules have been identified. First, phosphatidylinositol 3-kinase (PI-3K), which generates 3′-phosphorylated inositol phospholipids. PI-3K is induced by many growth factors that also activate Ras, but more importantly, RasGTP can interact with the catalytic p110 subunit, and oncogenic Ras can stimulate PI-3K activity both *in vitro* and *in vivo* (Rodriguez-Viciana *et al.*, 1996, 1997). Since activation of PI-3K is also stimulated by binding of the p85 regulatory subunit to tyrosine kinase receptors, growth factor-induced PI-3K activation can be mediated directly via the activated receptor or via RasGTP formation (Kauffmann-Zeh *et al.*, 1997). Interestingly, PI-3K does not bind to RasV12G37 or RasV12S35, but can interact with the effector-domain mutant RasV12C40 (Rodriguez-Viciana *et al.*, 1997).

Secondly, the Ral guanine nucleotide exchange factors (GEFs) RalGDS and Rgl have been identified as potential Ras effectors (Feig *et al.*, 1996; Murai *et al.*, 1997). These RalGEFs bind to RasV12G37, the effector-domain mutant of Ras that synergizes with RasV12S35 in a foci formation assay, but not to the other two effector-domain mutants (White *et al.*, 1996; Okazaki *et al.*, 1997; Rodriguez-Viciana *et al.*, 1997). RalGEFs mediate the activation of the small GTPase Ral, the function of which is largely unknown. Ral has been shown to bind to phospholipase D (PLD) activity via its N-terminus (Jiang *et al.*, 1995), and to a GTPase-activating protein for CDC42, RalBP (also known as RLIP or RIP) via its effector-domain (Cantor *et al.*, 1995; Jullien-Flores *et al.*, 1995; Park and Weinberg, 1995). Other candidate Ras effectors, which have been shown to interact specifically with the GTP-bound form of Ras, have been identified and include Rin (Han and Colicelli, 1995) and AF6/Canoe (Kuriyama *et al.*, 1996). However, whether these latter proteins function as Ras effectors is as yet unknown (Marshall, 1996).

In a yeast two-hybrid screen, we recently identified a protein that associates with the GTP-bound form of Ras and its close relative Rap1. This protein, Rlf, has in its N-terminal part homology to CDC25-like GEFs, and in its C-terminus a Ras-binding domain (RBD). Rlf is ubiquitously expressed and reveals ~30% identity to RalGDS (Wolthuis *et al.*, 1996).

Here we investigated the potential role of Rlf as a Ras effector. We demonstrate that Rlf binds to Ras *in vivo* and that Rlf is a GEF for Ral. Rlf is activated by GTP-bound Ras, and membrane-targeting of Rlf is sufficient to induce constitutive activation of Rlf. Importantly, activated Rlf

induces transcriptional activation of the c-*fos* promoter, independent of the Raf–MEK–ERK pathway, and stimulates proliferation under low-serum conditions. We conclude that Rlf is a genuine Ras effector which mediates a distinct pathway of Ras-induced gene expression.

Results

Rlf interacts with RasV12 and the effector-domain mutant RasV12G37 in Cos7 cells

We have previously shown that the Ras binding domain (RBD) of Rlf associates *in vitro* with the GTP-bound, but not the GDP-bound form of Ras (Wolthuis *et al.*, 1996). To investigate whether Rlf interacts with active Ras *in vivo*, Cos7 cells were co-transfected with HA-epitope-tagged Rlf and RasV12. Anti-Ras immunoblotting demonstrated the association of RasV12 with HA-Rlf in HA-immunoprecipitates (Figure 1A, upper panel). Reciprocally, anti-HA immunoblotting revealed the association of Rlf with endogenous Ras in anti-Ras (Y13-238) immunoprecipitates. Expression of RasV12 increased Rlf-association with Ras (Figure 1A, lower panel). *In vivo* association of Rlf with active Ras was further indicated by the observation that expression of HA-tagged Rlf-RBD inhibited Ras-dependent activation of myc-tagged ERK2 in a concentration-dependent manner (Figure 1B).

Association of Rlf with several effector-domain mutants of Ras was also tested. These included RasV12S35 and RasV12E38, which associate with and activate the Raf-1 kinase (White *et al.*, 1995; Khosravi-Far *et al.*, 1996; Rodriguez-Viciana *et al.*, 1997), RasV12C40, which interacts with and activates PI-3K (Rodriguez-Viciana *et al.*, 1997), and RasV12G37, which interacts with RalGDS and Rgl (Murai *et al.*, 1997; Okazaki *et al.*, 1997; Rodriguez-Viciana *et al.*, 1997). We found that Rlf formed complexes *in vivo* with RasV12G37, but not RasV12E38 and RasV12C40. A weak interaction between Rlf and RasV12S35 was also found (Figure 1A). Equivalent expression levels of Ras mutants and Rlf were confirmed by anti-Ras (Figure 1A, upper panel) and anti-HA immunoblotting (Figure 1A, lower panel)

From these results we conclude that Rlf associates with active Ras *in vivo*, and that this interaction is mediated by the effector domain of Ras.

Rlf is a GEF for the small GTPase Ral

The N-terminal part of Rlf reveals homology with CDC25-like GEFs. In order to determine whether indeed Rlf is a GEF and to identify its target, the GEF activity of Rlf for several Ras-like proteins *in vitro* was tested. Partially purified GST-Rlf (Figure 2A, left panel) was incubated with Ras-like proteins loaded with a fluorescent homologue of GDP (mant-GDP) in the presence of an excess of unlabelled GDP. Since the fluorescence signal of the mant-nucleotide is stronger when the nucleotide is bound to the GTPase than when it is released, the exchange reaction was monitored by measuring the decrease of fluorescence in time (Lenzen *et al.*, 1995). Figure 2A (right panel) reveals that increasing amounts of GST-Rlf promoted guanine nucleotide exchange activity of mant-GDP-bound RalA and RalB, but not of mant-GDP-bound H-Ras, the closest relative of Ral based on sequence comparison. In addition, HA-tagged Rlf isolated from transfected Cos7

cells stimulated the exchange of GDP bound to Ral for labelled GTP (Figure 2B). As expected for CDC25-like exchange factors, no exchange activity towards the Rho-like GTPase Rac could be detected (data not shown). These data demonstrate that Rlf is a GEF for Ral, at least *in vitro*.

To determine whether Rlf can activate Ral *in vivo*, we transfected increasing amounts of Rlf in Cos7 cells together with epitope-tagged Ral, and analysed the ratio of GTP versus GDP bound to Ral. Cells were metabolically labelled with [^{32}P]orthophosphate, Ral was immunoprecipitated, and bound nucleotides were eluted and separated by thin-layer chromatography. Whereas the basal level of RalGTP in Cos7 cells is ~7%, transfection of 1–3 μg of Rlf increased the level of RalGTP to 17–22% (Figure 2C, upper panel). Increases in RalGTP correlated with the amount of Rlf detected by anti-Rlf-immunoblotting (Figure 2C, lower panel). These results demonstrate that Rlf acts as a GEF for Ral *in vivo*.

Ras activates Rlf

We next addressed the question whether Ras can activate the exchange activity of Rlf *in vivo*. Cos7 cells were transfected with epitope-tagged Ral and RasV12 alone, or with RasV12 and Rlf. Transfection of 1 μg of the RasV12 expression vector induced only a small activation of Ral, presumably as a result of activation of endogenous RalGEFs (Figure 3A). Increasing the amount of transfected RasV12 did not further enhance the observed RalGTP levels, indicating that the endogenous levels of RalGEFs are limiting under these conditions. However, ectopic expression of Rlf together with Ras could strongly stimulate Ral activation, resulting in RalGTP levels between 30–50%, depending on the amount of Ras and Rlf transfected (Figure 3A).

To exclude the possibility that the observed increase in GTP was due to co-immunoprecipitation of RasGTP molecules in the anti-HA-immunoprecipitates, epitope-tagged RalN28 was used, which is locked in the GDP-bound state. After immunoprecipitation of RalN28 from cells expressing 3 μg of both Rlf and RasV12, we observed only 3% GTP in the eluates of the immunoprecipitates (Figure 3A). This clearly demonstrates that in this assay the observed increases in GTP indeed reflect Ral activation. From these results we conclude that active Ras can activate Rlf *in vivo*.

Finally, we tested whether proper membrane localization of Ras is required for the activation of Rlf. Therefore, Rlf was co-transfected with a mutant of Ras that remains cytoplasmic, RasL61S186. As shown in Figure 3B, RasL61, but not RasL61S186, was able to activate Rlf, demonstrating that membrane localization of Ras is necessary for Rlf activation.

Rlf synergizes with Ras in the induction of gene expression

Activation of Ras induces transcriptional activation of the c-*fos* gene (Cahill *et al.*, 1996; Treisman, 1996). To examine the possible role of Rlf in mediating Ras-induced gene expression, we investigated the effect of Rlf on the c-*fos* promoter. Transient transfection of active Ras (RasL61) in A14 cells (NIH 3T3 cells that express high levels of the human insulin receptor) resulted in a 6- to
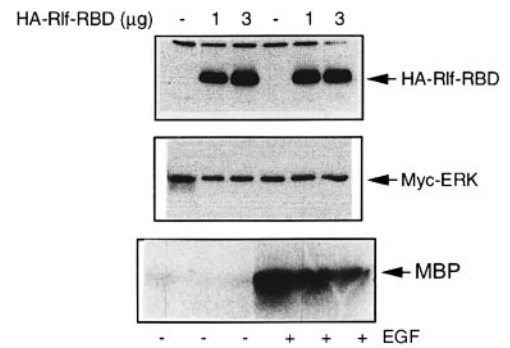
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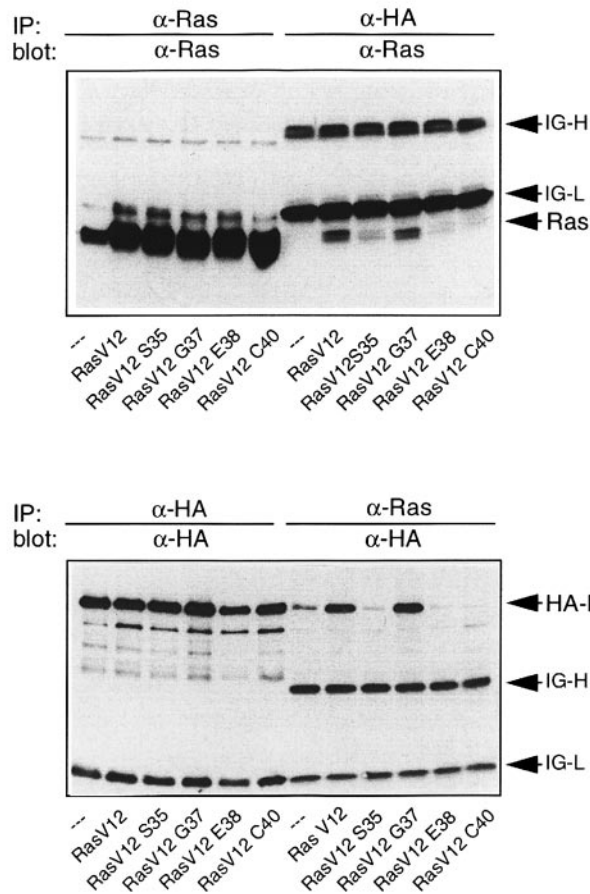
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Fig. 1. Rlf interacts with active Ras *in vivo*. (**A**) Cos7 cells were co-transfected with expression plasmids encoding HA-tagged full-length Rlf (2 μg) and pSG5-RasV12, RasV12S35, RasV12G37, RasV12E38 and RasV12C40 (5 μg). HA-Rlf was immunoprecipitated using the 12CA5 antibody, and Ras proteins were collected by Y13238. Immunocomplexes were analysed by SDS–PAGE and immunoblotting. The upper panel shows a Western blot probed with anti-Ras antibody (Transduction Laboratories), revealing the expression of the several Ras proteins on the left (20% of the total immunoprecipitate), and the Ras proteins co-immunoprecipitating with HA-Rlf on the right. The lower panel shows HA-Rlf expression in 12CA5 immunoprecipitates (lanes 1–6; 20% of the total immunoprecipitate) and HA-Rlf in Ras-immunoprecipitates (lanes 7–12). The observed association between ectopically expressed Rlf and endogenous Ras is reproducible. The absence of this association in the RasV12E38- and RasV12C40-expressing cells may be due to competition between mutant Ras (high concentration, low affinity) and endogenous Ras (low concentration, high affinity). (**B**) Inhibition of EGF-induced ERK2 activation by the expression of HA-Rlf-RBD. A14 cells were transfected with 1 or 3 μg of pMT2-HA-Rlf-RBD, serum-starved overnight and stimulated with 40 ng/ml EGF for 5 min. MYC-ERK2 was collected from the lysates and assayed *in vitro* using myelin basic protein (MBP) as a substrate. Expression of HA-Rlf-RBD and MYC-ERK2 were verified by immunoblotting (upper panels). The lower panel shows the autoradiograph of the reaction mixture separated on a 15% polyacrylamide gel.

8-fold induction of promoter activity, 40 h after transfection, as was reported previously (Medema *et al.*, 1992). Virtually no induction of c-*fos* promoter activity was found when Rlf was transfected alone. However, transfection of RasL61 together with Rlf resulted in an 11-fold induction of c-*fos* promoter activity, whereas RasL61S186 and Rlf did not induce transcriptional activation (Figure 4A). These data demonstrate that active Ras and Rlf synergistically stimulate transcriptional activation of c-*fos*, and emphasize the possible role of Rlf as a Ras effector involved in the induction of gene expression.

Next, we investigated whether endogenous Rlf or any other RalGEF is involved in Ras-mediated c-*fos* promoter induction. We therefore introduced RalN28, a putative dominant negative mutant of Ral that is constitutively GDP-bound (Figure 3A). This mutant will bind to and trap endogenous RalGEFs. As shown in Figure 4B, RalN28

partially inhibited RasL61-induced c-*fos* promoter activity. Co-expression of RalN28 also inhibited synergistic activation of the c-*fos* promoter by Rlf and RasL61, indicating that RalN28 indeed can inhibit RalGEF function (Figure 4B). This implies that RalGEFs mediate in part Ras-induced activation of the c-*fos* promoter.

Since RalGEFs have been shown to synergize with activated mutants of Raf, we investigated whether Ras-induced activation of the Raf–MEK–ERK pathway, which also leads to stimulation of c-*fos* promoter activity, was involved in the observed transcriptional activation. To test this possibility, the cells were incubated with the MEK-inhibitor PD98059. At the concentration used, this inhibitor blocks growth factor-stimulated ERK activation in A14 cells completely (data not shown). As expected, PD98059 only partially inhibited Ras-induced c-*fos* induction (Figure 4B). Importantly, in the presence of PD98059,
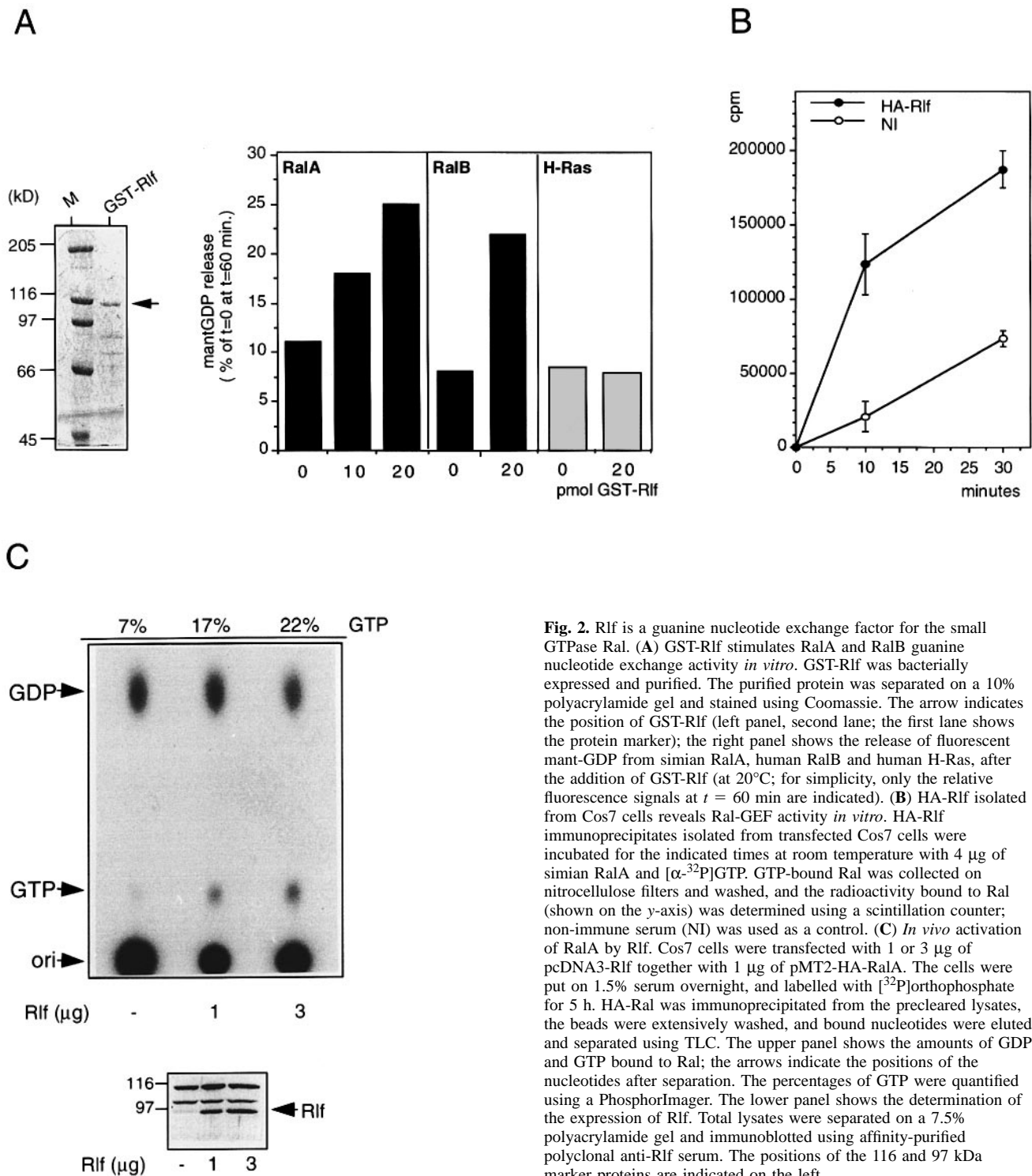
A

B

C

Fig. 2. Rlf is a guanine nucleotide exchange factor for the small GTPase Ral. (**A**) GST-Rlf stimulates RalA and RalB guanine nucleotide exchange activity *in vitro*. GST-Rlf was bacterially expressed and purified. The purified protein was separated on a 10% polyacrylamide gel and stained using Coomassie. The arrow indicates the position of GST-Rlf (left panel, second lane; the first lane shows the protein marker); the right panel shows the release of fluorescent mant-GDP from simian RalA, human RalB and human H-Ras, after the addition of GST-Rlf (at 20°C; for simplicity, only the relative fluorescence signals at $t = 60$ min are indicated). (**B**) HA-Rlf isolated from Cos7 cells reveals Ral-GEF activity *in vitro*. HA-Rlf immunoprecipitates isolated from transfected Cos7 cells were incubated for the indicated times at room temperature with 4 µg of simian RalA and [α-^{32}P]GTP. GTP-bound Ral was collected on nitrocellulose filters and washed, and the radioactivity bound to Ral (shown on the *y*-axis) was determined using a scintillation counter; non-immune serum (NI) was used as a control. (**C**) *In vivo* activation of RalA by Rlf. Cos7 cells were transfected with 1 or 3 µg of pcDNA3-Rlf together with 1 µg of pMT2-HA-RalA. The cells were put on 1.5% serum overnight, and labelled with [^{32}P]orthophosphate for 5 h. HA-Ral was immunoprecipitated from the precleared lysates, the beads were extensively washed, and bound nucleotides were eluted and separated using TLC. The upper panel shows the amounts of GDP and GTP bound to Ral; the arrows indicate the positions of the nucleotides after separation. The percentages of GTP were quantified using a PhosphorImager. The lower panel shows the determination of the expression of Rlf. Total lysates were separated on a 7.5% polyacrylamide gel and immunoblotted using affinity-purified polyclonal anti-Rlf serum. The positions of the 116 and 97 kDa marker proteins are indicated on the left.

co-transfection of Rlf could still significantly stimulate Ras-induced c-*fos* promoter activity. The stimulation by Rlf was again blocked by RalN28. Finally, the RasV12G37 effector-domain mutant, which binds to Rlf, but not to Raf1 (Figure 1A; White *et al.*, 1995), stimulated c-*fos* in a RalN28-sensitive manner. In contrast, RasV12E38, which can only activate the Raf–MEK–ERK pathway (Rodriguez-Viciana *et al.*, 1997), was insensitive to expression of dominant negative Ral (Figure 4C). RasV12C40, which can still activate PI-3K-dependent pathways, gave

only a very small induction of c-*fos* in these cells (Figure 4C). From these results we conclude that Ras can induce a Raf–MEK–ERK-independent activation of the c-*fos* promoter which is mediated by RalGEFs, like Rlf.

Membrane targeting of Rlf
Artificial targeting of Raf1 to the plasma membrane results in constitutively active Raf1, independent of Ras (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). Additionally, the requirement for Ras association with the plasma membrane for
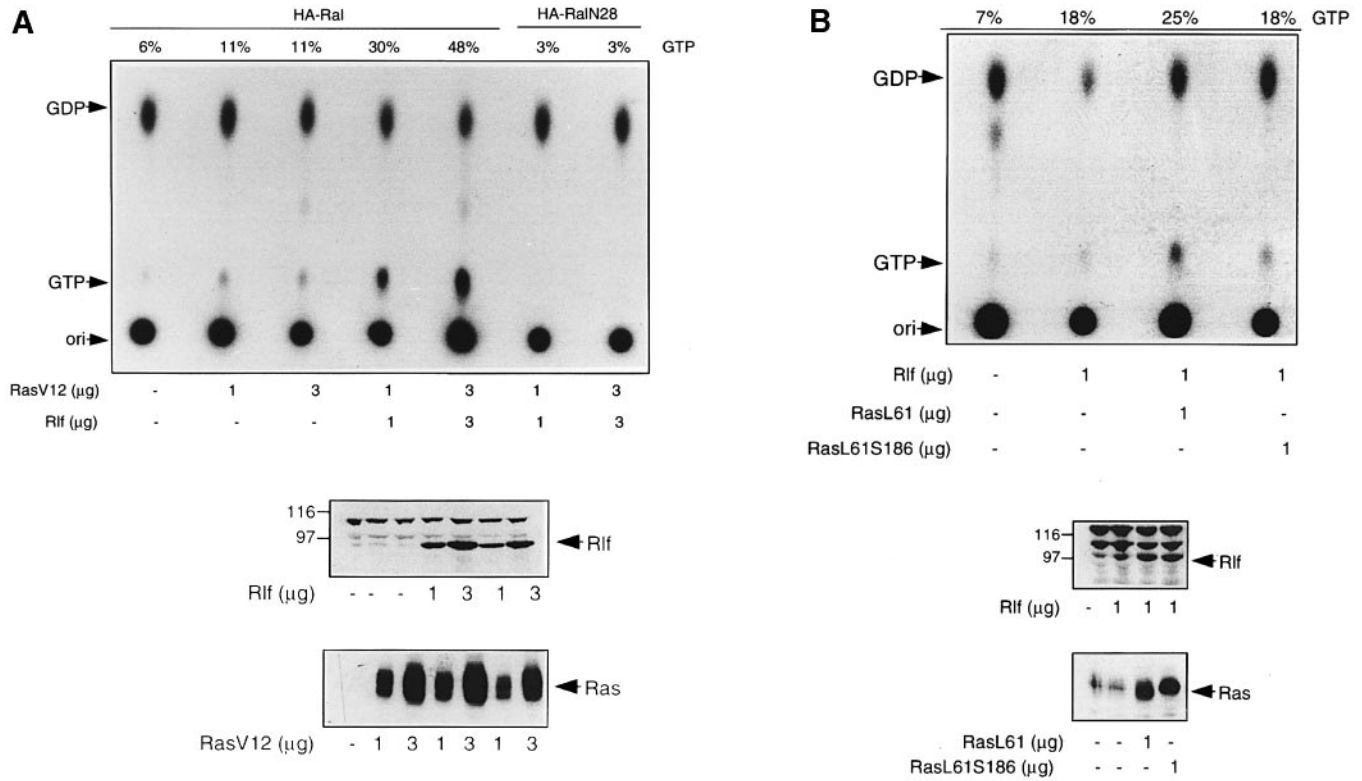
Fig. 3. Ras-induced Ral-activation is stimulated by Rlf. (**A**) Cos7 cells were transfected with 1 μg of pMT2-HA-RalA alone, together with 1 or 3 μg of pSVE-RasV12 or with RasV12 and 1 or 3 μg pcDNA3-Rlf. Prior to lysis, the cells were labelled with [^{32}P]orthophosphate for 5 h. HA-Ral was immunoprecipitated from the precleared lysates using 12CA5-protein A–Sepharose, the beads were extensively washed, and bound nucleotides were eluted and separated using TLC. The upper panel shows the amounts of GDP and GTP bound to Ral; the arrows indicate the positions of the nucleotides after separation. The percentages of GTP were quantified using a PhosphorImager. Similar results were obtained in different experiments. In the case of the last two lanes, HA-RalN28 was transfected instead of wild-type HA-Ral, together with RasV12 and Rlf. The lower panels show the expression levels of Rlf and Ras. Total lysates were separated on 7.5% or 13% polyacrylamide gels and immunoblotted using affinity-purified polyclonal anti-Rlf serum or anti-Ras antibody. (**B**) RasL61, but not RasL61S186, can stimulate Rlf-induced Ral activation. Cos7 cells were transfected with pcDNA3 or Rlf-pcDNA3, in the presence or absence of the RSV-driven expression plasmids for RasL61 or RasL61S186. The Ral-GTP levels were determined and are indicated above the upper panel. The lower panels show the expression of Rlf and Ras. Note that the Ras expression is lower, compared with the levels in the experiment shown in (A), where a RasV12 plasmid containing the SV-40 replication sequence was used, which leads to plasmid amplification in Cos7 cells. The RSV-RasL61 and RSV-RasL61S186 do not contain the amplification signal (Medema *et al.*, 1991). Similar results were obtained in three different experiments.
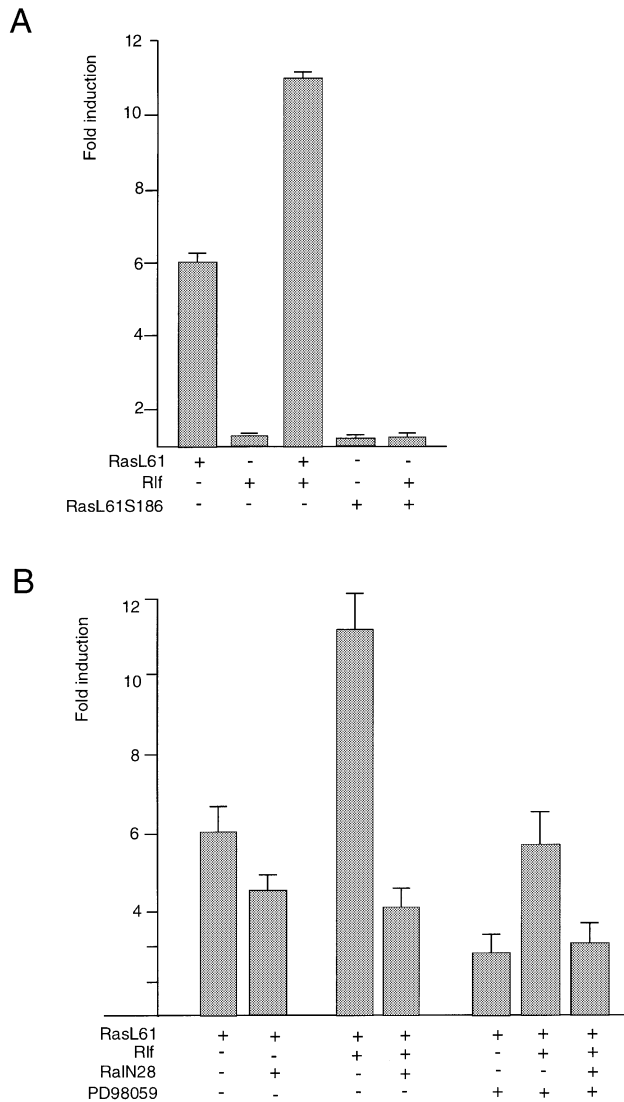
activation of Rlf suggested that membrane translocation of Rlf might play a role in its function. To examine whether recruitment of Rlf to the plasma membrane would also result in a constitutively active protein, we replaced the C-terminus of Rlf for the C-terminal region of K-Ras, containing the farnesylation and palmitoylation site. This results in a construct, Rlf–CAAX, in which the RBD of Rlf is replaced by the membrane localization signal of Ras. First we investigated whether the Rlf–CAAX protein has an intrinsic guanine nucleotide exchange activity that is comparable with that of full-length Rlf. Therefore, the epitope-tagged Rlf and Rlf–CAAX constructs were transiently expressed in Cos7 cells (Figure 5A and B). Cells were lysed and Rlf or Rlf–CAAX was collected from the lysate by immunoprecipitation, and the immunocomplexes were used in an *in vitro* Ral exchange assay. We found that Rlf and Rlf–CAAX stimulated guanine nucleotide exchange to comparable levels *in vitro*, showing that the catalytic activity was not significantly altered by the replacement of the RBD by the CAAX motif (Figure 5B). Localization of Rlf–CAAX to the plasma membrane was confirmed by subcellular fractionation and immunofluorescence analyses. Rlf–CAAX was transiently

expressed in A14 cells and the cell lysate fractionated into soluble and particulate fractions by ultracentrifugation. Immunoblotting revealed that Rlf–CAAX was predominantly present in the particulate fraction whereas Rlf was mainly cytosolic (Figure 5C, upper panel). Immunofluorescence analysis with confocal laser scanning microscopy clearly demonstrated that Rlf was most abundant in the cytoplasm, whereas Rlf–CAAX was detected at the plasma membrane (Figure 5C, lower panel).

The capacities of Rlf and Rlf–CAAX to stimulate RalGTP formation was examined in Cos7 cells. As shown in Figure 5D, normal Rlf induced RalGTP from 5 to 19%, whereas Rlf–CAAX significantly induced RalGTP from 5 to 41%. *In vivo* activation of H-Ras, Rap1 or R-Ras by Rlf–CAAX was not observed (data not shown). Thus, the Ral activation induced by Rlf–CAAX is comparable with Ral activation observed after co-transfection of active Ras and Rlf in this assay. From these results we conclude that Rlf–CAAX is a constitutively active form of Rlf.

Induction of gene expression by Rlf–CAAX

From the above results, we predicted that Rlf–CAAX can bypass the requirement for Ras for the induction of gene

A

RasL61	+	-	+	-	-
Rlf	-	+	+	-	+
RasL61S186	-	-	-	+	+

B

RasL61	+	+	+	+	+	+	+
Rlf	-	-	+	+	-	+	+
RalN28	-	+	-	+	-	-	+
PD98059	-	-	-	-	+	+	+

C

+ RalN28

Fig. 4. Rlf stimulates Ras-induced transcriptional activation of the c-*fos* promoter. (**A**) Rlf stimulates c-*fos* induction by active Ras. A14 cells were transfected with 0.5 μg of a c-*fos* luciferase reporter construct in combination with empty 1.0 μg of pMT2SM-HA, pRSV-RasL61, pMT2SM-HA-Rlf and/or pRSV-RasL61S186 expression vector per 5 cm dish. The next day, the cells were allowed to recover in the presence of 10% FCS for 6 h and put on serum-free medium for 16 h. Lysates were made 40 h after transfection and luciferase activity was determined. In each experiment, duplicate dishes were analysed. Transfection efficiency was monitored by co-transfection of an RSV–lacZ construct and measuring β-galactosidase activity. The increases in luciferase activity are shown as folds induction over the activity in cells transfected with control plasmids alone. The results represent the average of at least five independent experiments. The error bars represent the standard deviations of the values. Protein expression levels were regularly controlled by immunoblotting. (**B**) The synergism between Rlf and Ras is blocked by dominant negative Ral, but not by PD98059. A14 cells were transfected with 1.0 μg of RasL61 vector and/or pMT2SM-HA-Rlf, together with 1.0 μg of pMT2-HA-RalN28 or 1.0 μg of empty pMT2-HA vector, and incubated with or without the MEK-kinase inhibitor PD98059 (40 μM) for 16 h. The induction of c-*fos* promoter activity was determined by measuring the increase in luciferase activity as described in (A) The results represent the average of five independent experiments. (**C**) The effector-domain mutant RasV12G37, but not RasV12E38 or RasV12C40, induces Ral-dependent activation of c-*fos*. NIH 3T3 cells, stably expressing HA-Rlf, were transfected with 1.5 μg of the pSG5-RasV12 effector-domain mutants together with 1.0 μg of a control plasmid (left panel) or with RalN28 expression vector (right panel). Cells were serum-starved for 24 h. The results represent the average of three independent experiments. Although the levels of induction by RasV12G37 and RasV12E38 varied between the repetitive experiments, only RasV12G37 was significantly inhibited by RalN28.

expression. We therefore tested the effect of Rlf–CAAX on the induction of the c-*fos* promoter, as well as on two other promoter elements known to be induced by active Ras, the collagenase promoter and an artificial promoter containing AP1 binding elements (3× TRE) (de Groot and Kruijer, 1990; Angel and Karin, 1991; Medema *et al.*, 1991). Figure 6 shows that indeed, Rlf–CAAX did not require RasV12 and efficiently induced promoter activity by itself. Importantly, a Rlf–CAAX control construct in which the scr-1 region of the catalytic domain was deleted, Rlf-ΔCAT–CAAX (Figure 5A), did not induce promoter activity, stressing the involvement of the guanine nucleotide exchange activity of Rlf. Furthermore, Rlf–CAAX induced a level of gene expression similar to that stimulated by active Ras. From these results we conclude that constitutively active Rlf can strongly induce gene expression.
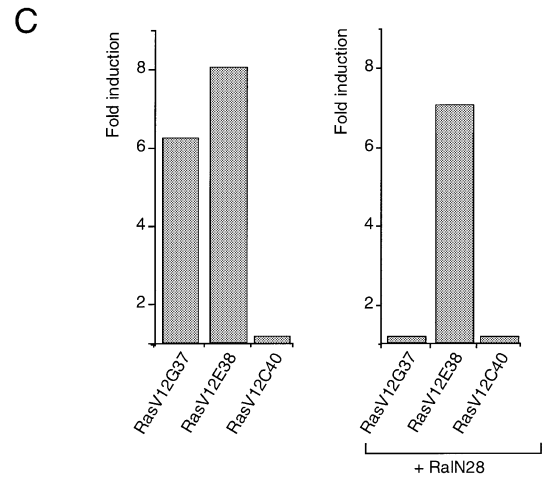
Rlf–CAAX-induced gene expression is independent of Ras and MEK.

To characterize the pathway which is used by Rlf–CAAX to induce c-*fos* promoter activity, we investigated first whether Rlf–CAAX induces a pathway independent of Ras activation and independent of the Raf1–MEK–ERK pathway. We therefore inhibited Ras activation with the dominant negative RasN17, and MEK with the inhibitor PD98059. As a positive control for c-*fos* activation, A14 cells were treated with insulin. Insulin stimulation induced c-*fos* promoter activation, and this was completely inhibited by RasN17 (Figure 7A), in agreement with previous results (Medema *et al.*, 1992). However, RasN17 did not inhibit Rlf–CAAX-induced c-*fos* promoter activation (Figure 7A). PD98059 also failed to inhibit activation of the c-*fos* promoter by Rlf–CAAX, whereas this inhibitor partially inhibited Ras-induced c-*fos* promoter activity
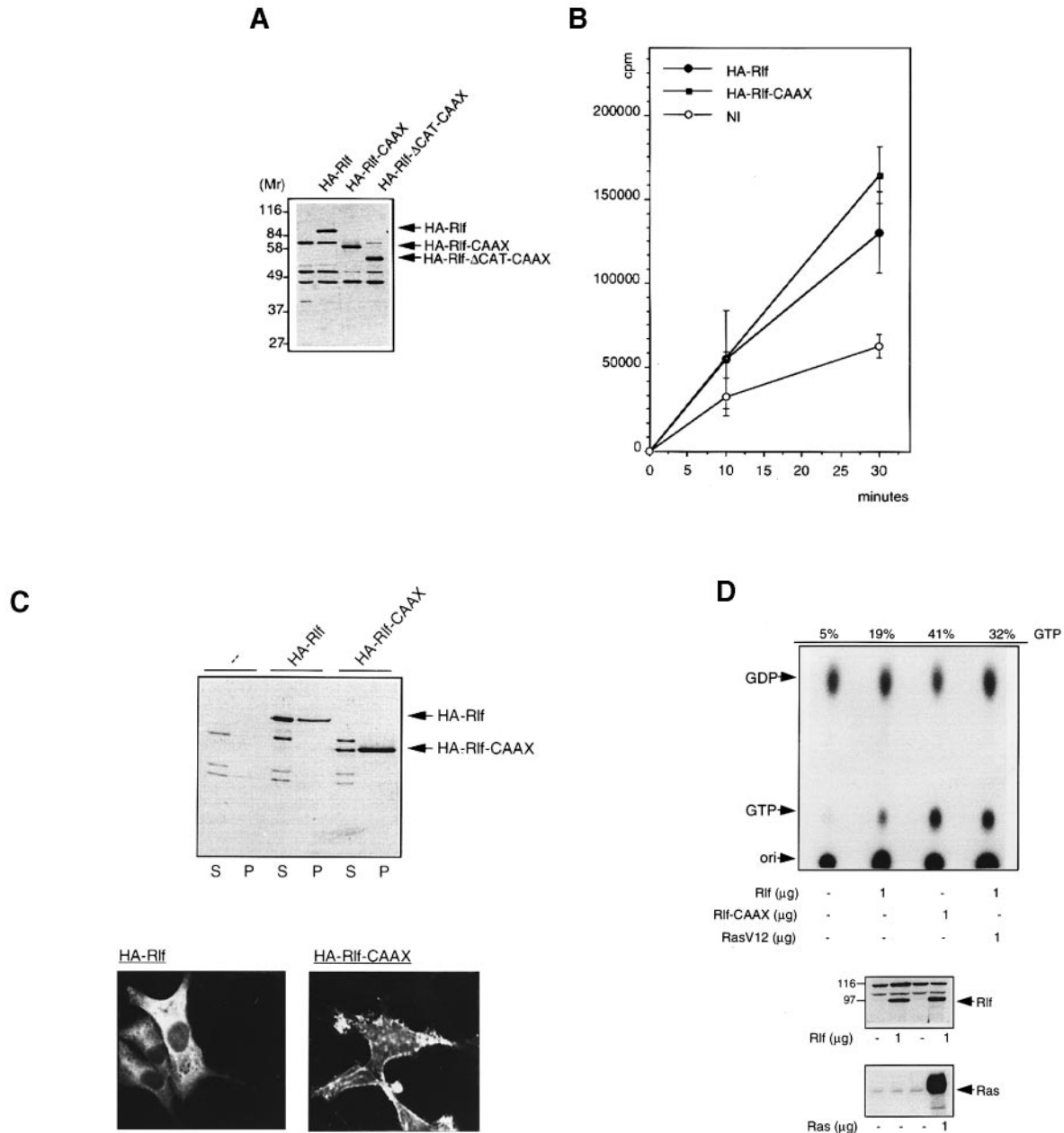
Fig. 5. A membrane targeted form of Rlf, Rlf–CAAX, activates Ral *in vivo*. (**A**) Expression of HA-Rlf–CAAX. A14 cells were transfected with 1 μg of HA-Rlf, 1 μg of HA-Rlf–CAAX or 1 μg of HA-Rlf–ΔCAT–CAAX. HA-Rlf–CAAX is a derivative of Rlf that contains the Ras-membrane localization motif, the CAAX-box, in place of the C-terminally located Ras binding domain. HA-Rlf–ΔCAT–CAAX is a mutant of HA-Rlf–CAAX, from which the scr-1 region in the catalytic domain is removed. Total lysates were analysed by immunoblotting; the arrows indicate the position of the expressed Rlf-proteins in the gel. The positions of the molecular weight markers are shown on the left. (**B**) The intrinsic GEF-activity of Rlf and Rlf–CAAX are similar. HA-Rlf and HA-Rlf–CAAX were transfected into Cos7 cells, immunoprecipitated and used in an *in vitro* Ral exchange assay, in the presence of radiolabelled [α-^{32}P]GTP. The increase in Ral-[^{32}P]GTP was determined by counting radioactivity of the Ral protein that was collected on nitrocellulose filters (designated c.p.m. on the y-axis). (**C**) Subcellular localization of Rlf and Rlf–CAAX. A14 cells were transiently transfected with pMT2-HA-Rlf or pMT2-HA-Rlf–CAAX. Following transfection, the isolated cells were Dounce homogenized, the nuclear fraction was removed, and the lysates were fractionated by ultracentrifugation. The soluble (S) and particulate (P) fractions were collected in sample buffer and analysed by immunoblotting. The positions of HA-Rlf and HA-Rlf–CAAX are indicated. The left two lanes represent untransfected cells. In the lower panel, confocal laser scan images of transfected A14 cells reveal cytoplasmic localization of HA-Rlf, and plasma membrane localization of Rlf–CAAX. A14 cells, grown on coverslips, were transiently transfected with 1.0 μg of pMT2-HA-Rlf or pMT2-HA-Rlf–CAAX, serum-starved, and 16 h later fixed, permeabilized and stained with the 12CA5 monoclonal antibody, and a secondary antibody conjugated with FITC. (**D**) Activation of Ral by Rlf–CAAX *in vivo*. Cos7 cells were transfected with 1 μg of pMT2-HARal and 1 μg of pcDNA3, pcDNA3-Rlf, or pcDNA3-Rlf–CAAX. As a control, 1 μg of Rlf was co-transfected with 1 μg of pSVE-RasV12 (right lane), and *in vivo* Ral-activation was determined as described in the legend of Figure 3. The Ral-GTP levels were determined as specified in the legend of Figure 3, and are indicated above the upper panel. Similar results were found in four independent experiments. The lower panels show the expression of Rlf and Ras. Rlf–CAAX was not recognized by the Rlf-antiserum. However, transfection of pcDNA3 HA-Rlf–CAAX and pcDNA3 HA-Rlf gave rise to the same protein expression levels as analysed by the 12CA5 antibody. Furthermore, untagged and tagged Rlf–CAAX identically induced transcriptional activation (see Figure 6; results not shown).
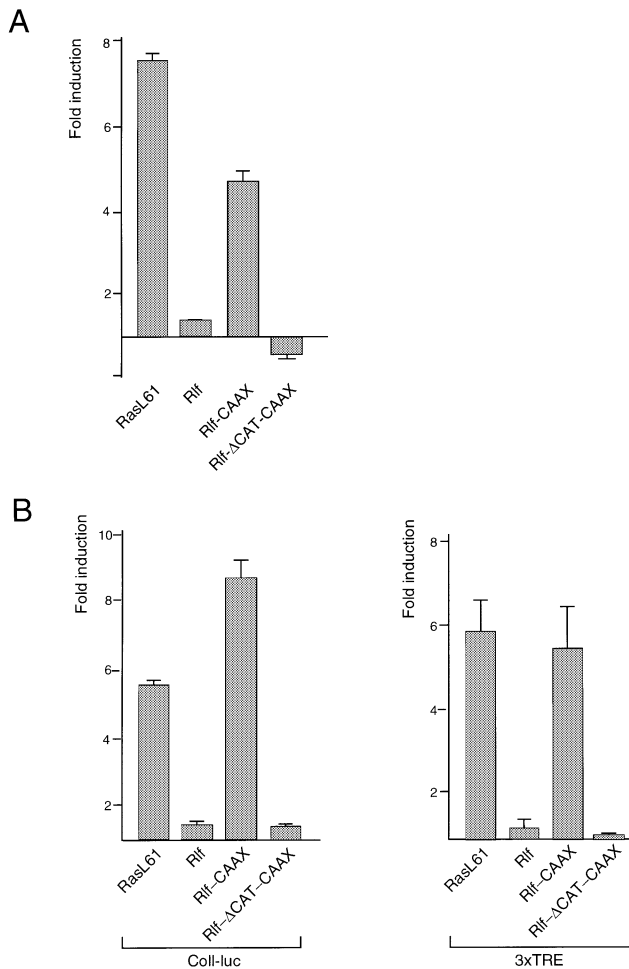
A

B

Coll-luc

3xTRE

Fig. 6. Membrane targeting of Rlf is sufficient to induce transcriptional activation of the c-*fos* promoter. (**A**) Rlf–CAAX induces c-*fos* promoter activity. A14 cells were transfected with a c-*fos* luciferase reporter together with 1.0 µg of RSV-RasL61, pMT2-HA-Rlf, pMT2-HA-Rlf–CAAX and pMT2-HA-Rlf-ΔCAT–CAAX. pMT2-HA-Rlf-ΔCAT–CAAX is a control construct that lacks the scr-1 region of the catalytic domain (see Figure 5A). Luciferase activity was assayed as indicated in the legend of Figure 4. (**B**) Rlf–CAAX stimulates the Collagenase promoter, and the TRE. A14 cells were transfected with 0.5 µg of a collagenase–luciferase reporter construct (left panel) or 0.5 µg of a chloramphenicol acetyltransferase (CAT) reporter construct under the control of three copies of the TPA responsive element (TRE) (right panel) in combination with 1.0 µg of expression vectors for the indicated proteins per 5 cm dish. Luciferase activity was assayed as described in the legend of Figure 4. Results show the average of at least five independent experiments.

(Figure 7A). From these results we conclude that Rlf–CAAX can activate a pathway which is independent of Ras activation or activation of the Raf1-MEK–ERK pathway.

Next, we investigated whether Rlf or any other RalGEF is necessary for Raf1-induced c-*fos* promoter induction. Transfection of Raf–CAAX, which is an active form of Raf1 (Leevers *et al.*, 1994), gave rise to a comparable c-*fos* gene induction as Rlf–CAAX. Whereas Rlf–CAAX-driven c-*fos* induction is strongly inhibited by RalN28, the stimulatory effect of Raf–CAAX is not affected by dominant negative Ral (Figure 7B). These data demonstrate that Raf-induced c-*fos* promoter activation is independent of RalGEFs and thus that both Rlf and Raf mediate separate pathways from Ras to induce gene

expression. In agreement with this, suboptimal amounts of Rlf–CAAX and Raf–CAAX cooperated in the activation of the c-*fos* reporter construct (Figure 7C).

To specify further the nature of the c-*fos* promoter activation, we used a promoter construct containing three copies of the c-*fos* serum response element, that still contains a Ternary Complex Factor (TCF) and a Serum Response Factor (SRF) binding site, fused to a CAT reporter (SRE-CAT; de Groot and Kruijer, 1990; Treisman, 1992). A14 cells were transfected with Rlf–CAAX, with and without RalN28. We found that Rlf–CAAX induced the SRE to a level comparable with the induction observed for the c-*fos* promoter (5- to 10-fold), and that the induction could be inhibited by co-expression of dominant negative Ral (Figure 7D). Two other elements present in the c-*fos* promoter, the cyclic AMP responsive element (CRE) and the SIS-inducible element (SIE) (Treisman, 1996), were not induced by Rlf–CAAX (data not shown).

Active Ral does not mimic active Rlf

Since Rlf is an exchange factor for Ral, we predicted that expression of active Ral would give similar effects as Rlf–CAAX. We therefore investigated whether the observed gene-induction can also be stimulated by activated Ral itself. Surprisingly, transfection of mutationally activated Ral, RalV23, of which 85% is bound to GTP *in vivo* (data not shown), gave little, if any, activation of the c-*fos* promoter by itself (Figure 7B). Also, after co-transfection of RalV23 with RasV12, we observed no stimulatory effect of RalV23 (data not shown). These observations suggest that Rlf can either stimulate other processes besides Ral activation or that ectopic expression of RalV23 does not mimic the activation of endogenous Ral.

Biological effects of Rlf–CAAX

It has been reported that although RasV12 has a profound effect on cell morphology and actin cytoskeletal organization, RasV12G37, which still binds Rlf, does not induce cytoskeletal rearrangements (Rodriguez-Viciana *et al.*, 1997). Therefore, we investigated the effects of Rlf–CAAX on cytoskeletal reorganizations in different cell types. In agreement with a role for Rlf as an effector of RasV12G37, Rlf–CAAX did not induce actin rearrangements or any other clear morphological changes (data not shown).

In order to assess the importance of the pathway activated by Rlf in proliferation, we characterized the growth properties of NIH 3T3 cells stably expressing Rlf, Rlf–CAAX or Rlf-ΔCAT–CAAX. In the presence of growth medium supplemented with 10% serum, the cells expressing Rlf–CAAX and Rlf displayed enhanced proliferation, whereas the cells expressing the catalytically inactive Rlf-ΔCAT–CAAX displayed growth rates similar to cells expressing the control vector alone (Figure 8A). Furthermore, if the cells were maintained in culture medium supplemented with low serum (0.5%), only the cells that expressed Rlf–CAAX were able to grow (Figure 8B). These observations show that Rlf can stimulate proliferation of murine fibroblasts in normal growth medium. Importantly, in medium supplemented with 0.5% serum, where normal Rlf is apparently inactive, Rlf–CAAX expression is sufficient to induce cell growth.
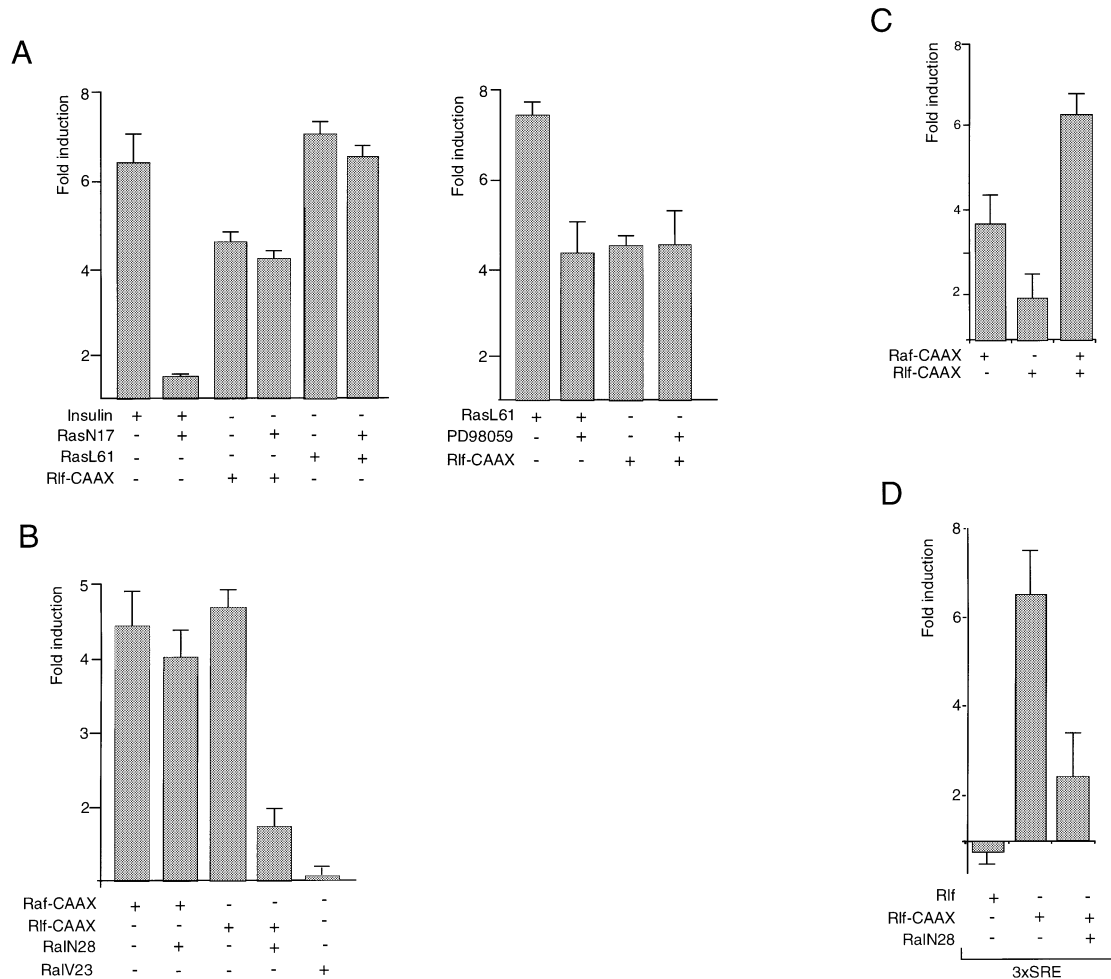
A

B

C

D

Fig. 7. Rlf–CAAX stimulates a novel signalling pathway that leads to gene induction. (**A**) Rlf–CAAX-induced transcriptional activation is not blocked by dominant negative Ras or the MEK inhibitor PD98059. A14 cells were transfected with 1.0 µg of the indicated expression vectors, and luciferase activity was determined as described in the legend of Figure 4. Insulin stimulation was for 16 h (1.0 µM). (**B**) Rlf–CAAX stimulated c-*fos* induction is blocked by RalN28, whereas Raf–CAAX stimulated c-*fos* induction is not inhibited. A14 cells were transfected with 1.0 µg of Rlf–CAAX or Raf–CAAX expression vectors, in the presence of 1.0 µg pMT2-HA-RalN28 or empty pMT2-HA vector, and luciferase activity was determined. The last bar represents c-*fos* induction by pMT2-HA-RalV23. These results are the average of five different experiments. (**C**) Cooperation of Raf–CAAX and Rlf–CAAX in c-*fos* induction. A14 cells were transfected with 0.5 µg of Raf–CAAX or Rlf–CAAX or both, and the luciferase activities were determined and controlled for transfection efficiencies. The average of three different experiments is shown. (**D**) Rlf–CAAX stimulates transcriptional activation via the serum response element. A CAT-reporter construct containing three copies of the serum response element (3× SRE; 1.0 µg) was co-transfected with 1.0 µg of pMT2-HA-Rlf, and pMT2-HA-Rlf–CAAX with 1.0 µg of pMT2-HA or pMT2-HA-RalN28. After transfection, the cells were put on serum-free medium for 24 h. At 40 h after transfection, the cells were collected and CAT-activity was determined. Protein levels were controlled by Western blotting; the results shown here are the average of three independent experiments.

These results are in agreement with the observation that the Ras effector-domain mutant RasV12G37 can enhance the growth properties of NIH 3T3 cells (Khosravi-Far, 1996).

In conclusion, our data suggest that Rlf may contribute to the biological effects of oncogenic Ras by inducing transcriptional activation and stimulating cell growth.

Discussion

Rlf is an effector of Ras and functions as a GEF for Ral

Rlf was originally identified as a protein that interacts with GTP-bound Rap1A and Ras *in vitro* via its RBD (Wolthuis *et al.*, 1996). Here we demonstrate first that Rlf functions as a GEF for Ral: (i) bacterially expressed full-length Rlf protein reveals specific guanine nucleotide exchange activity for RalA and RalB *in vitro*; (ii) immuno-

complexes of Rlf isolated from Cos7 cells exhibit Ral guanine nucleotide exchange activity *in vitro*; and (iii) overexpression of Rlf in Cos7 cells increases the levels of Ral-GTP. Although for instance the closest relative of Ral, H-Ras (55% amino acid identity; Chardin and Tavitian, 1986), is not activated by Rlf, our results do not exclude the possibility that Rlf may also activate other unknown small GTPases.

Secondly, we show that Rlf is a genuine effector of Ras: (i) Rlf binds to active Ras *in vivo*, but not to the effector-domain mutants RasV12E38 and RasV12C40; (ii) Ras activates Rlf *in vivo*, as shown both by the increased Rlf dependent activation of Ral and the synergy between Ras and Rlf in the induction of c-*fos* promoter activity; and (iii) Ras-induced gene expression is (partially) inhibited by dominant negative Ral. Since dominant negative Ral is assumed to trap and inactivate RalGEFs, this
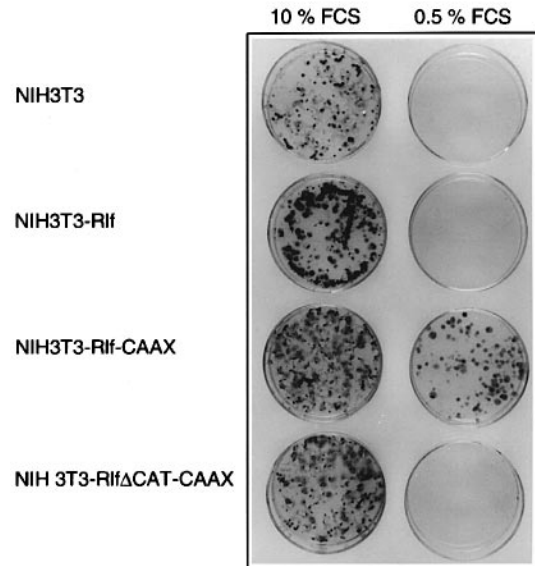
A
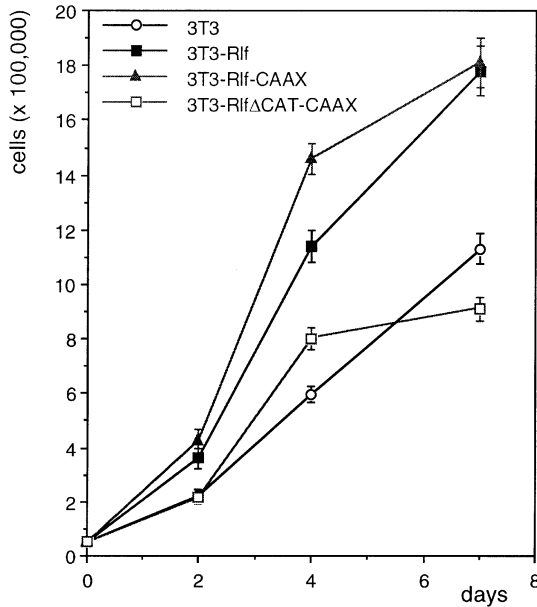
B

Fig. 8. Growth properties of Rlf, Rlf–CAAX and Rlf-ΔCAT–CAAX expressing NIH 3T3 cells. (**A**) Enhanced growth rates of Rlf and Rlf–CAAX expressing cells. NIH 3T3 cells stably expressing either the control vector or comparable protein levels of HA-Rlf, HA-Rlf–CAAX or HA-Rlf-ΔCAT–CAAX were plated at a density of 5×10^4 cells/60 mm dish. The cells were maintained until the indicated days, trypsinized and counted. The data represent the average of two experiments. (**B**) Rlf–CAAX induces low-serum growth. The cell lines as in (A) were plated in 60 mm dishes at a density of 1×10^3 cells/dish. Culture medium containing either 10% or 0.5% fetal calf serum (FCS) was refreshed every 2–3 days. Cells were stained with 0.5% crystal violet after 15 days. Similar results were obtained in independent experiments and with different cell lines.

result implies that a RalGEF mediates Ras-induced gene expression.

Rlf is constitutively activated by membrane targeting

Results obtained with two other effector molecules of Ras, Raf1 and PI-3K demonstrated that membrane targeting could result in constitutive activation (Leevers *et al.*, 1994; Klippel *et al.*, 1996). Here we show that membrane targeting of Rlf, by replacement of the C-terminal RBD by the H-Ras membrane localization signal, also leads to constitutive activation of Rlf. From these results one may conclude that the only function for Ras in the activation of Rlf is the membrane localization. Indeed, a Ras mutant which lacks the membrane attachment site does not activate Rlf. However, whether more subtle effects may be induced by replacing the RBD sequence for the CAAX motif, as for instance observed for Raf1, cannot yet be excluded (Marais *et al.*, 1995, 1997; Jelinek *et al.*, 1996). Importantly, constitutively active Rlf may be an excellent tool to study the function of RalGEF-mediated signalling pathways.

Rlf mediates a distinct signalling pathway to c-fos promoter activation

Rlf mediates a distinct signalling pathway involved in signal transduction. This conclusion is based on the following observations: (i) whereas Rlf by itself is not active in inducing transcriptional activation, Rlf stimulates the Ras-induced activation of the c-*fos* promoter in fibroblasts. This synergism is independent of activation of the MEK–ERK pathway, and sensitive to inhibition by

dominant negative Ral; (ii) Rlf–CAAX induces c-*fos* and AP-1 promoter activity by itself, which is independent of Ras activation and ERK activation; (iii) Raf–CAAX-mediated gene-induction is insensitive to dominant negative Ral. These data emphasize that the Ras effectors Raf and Rlf are both important activators of immediate early genes, but regulate transcription via distinct signalling pathways.

Previously it has been shown that RalGDS synergizes with constitutively active Raf–CAAX to induce *fos* promoter activity. This supports our conclusion that both Raf and RalGEFs mediate distinct pathways to c-*fos* promoter activity. A variance with our results is that RalGDS alone can induce c-*fos* promoter activity, whereas Rlf needs to be activated by Ras. These different observations may be explained by differences in the basal levels of RasGTP in the cell lines used. For instance, we noted that in Cos7 cells, in which expression of Rlf can induce an increase in RalGTP, the level of endogenous RasGTP is already rather high. Also in apparent contrast to our conclusions is the observation that dominant negative Ral inhibits v-Raf-induced focus formation (Urano *et al.*, 1996). However, it is clearly possible that RalGEFs are necessary for v-Raf-induced transformation, but not for Raf-induced c-*fos* promoter activation.

Besides induction of c-*fos*-driven expression, we observed that Rlf–CAAX could regulate transcriptional activation via the c-*fos* serum response element (SRE) alone. c-*fos* SRE activation is the result of ternary complex formation of serum response factor (SRF) and a ternary complex factor (TCF) such as the Ets domain proteins (Elk1, Sap-1 and Sap-2) (Treisman, 1992; Cahill *et al.*,

1996). Ras-induced activation of the SRE via the Raf–MEK–ERK pathway requires the activation of TCF. SRE promoter constructs that lack the TCF-binding site but contain the SRF-binding site can still be activated by Ras, but also by at least two other pathways, involving the Rho family of GTPases (Hill *et al.*, 1995). However, we did not observe inhibition of Rlf–CAAX-induced transcriptional activation after blocking Rho signalling by dominant negative Rho or C3-toxin, indicating that the pathway induced by Rlf does not involve Rho activation (data not shown).

The idea that Rlf mediates a distinct signalling pathway was further supported by our results with the effector-domain mutants. Rlf does not interact with the effector-domain mutants RasV12E38 or RasV12C40, which have been shown to associate with Raf1 kinase and PI-3K (White *et al.*, 1995; Khosravi-Far *et al.*, 1996; Rodriguez-Viciana *et al.*, 1997). However, Rlf binds to the effector-domain mutant RasV12G37. Besides Rlf, RalGDS and Rgl have also been shown to interact with RasV12G37 *in vivo* (Okazaki *et al.*, 1997; Rodriguez-Viciana *et al.*, 1997). These observations suggest that the effects of RasV12G37 may contribute to the activation of RalGEFs, although other effectors of these Ras-mutants may still be unknown. We observed that Rlf synergized with RasV12G37-stimulated transcriptional activation of the c-*fos* promoter, a process which could be blocked by dominant negative Ral. These results indeed implicate RalGEFs in RasV12G37-induced effects. RasV12G37 is a mutant that is defective in morphological transformation, but complements transformation when co-transfected with activated Raf or RasV12S35, RasV12E38 or RasV12C40. Cells expressing RasV12G37 were found to grow in low serum (Khosravi-Far *et al.*, 1996). In agreement with a role for Rlf in mediating the effects of RasV12G37, we observed that NIH 3T3 cell lines expressing Rlf–CAAX also gain the ability to grow under low serum conditions.

Ral does not mimic Rlf effects

In contrast to the good correlation between the Rlf-induced RalGTP formation and the Ral-dependency of the observed gene expression, ectopic expression of RalV23 (constitutively GTP-bound) is not sufficient to stimulate transcriptional activation. It should be noted that in general RalV23 has little, if any, biological effect (Lee *et al.*, 1996; Urano *et al.*, 1996; White *et al.*, 1996; Okazaki *et al.*, 1997). This may imply that both Rlf and RalGDS also activate other downstream targets besides RalGTP. Alternatively, and perhaps more likely, RalV23 does not mimic active Ral, for instance because GTP hydrolysis is necessary to activate downstream targets. This mechanism has been described, for example, for EF-Tu (Sprinzl, 1994). Alternatively, besides Ral, another protein becomes activated during the Ral-guanine nucleotide exchange reaction.

Assuming that Ral indeed is the major target for RalGEF in the induction of gene expression, the next question to answer is how Ral can activate transcription. The only putative effector of Ral known to date is RalBP1 (also termed RLIP and RIP) (Cantor *et al.*, 1995; Jullien-Flores *et al.*, 1995; Park and Weinberg, 1995). This protein interacts with GTP-bound Ral *in vitro* and, interestingly, exhibits GTPase activity for the small Rho-like GTPase Cdc42. This may provide a link to the Rho signalling

pathway known to activate the c-*fos* promoter. However, at present it is unclear whether RalBP1 is indeed a genuine Ral effector. A second protein which interacts with active as well as inactive Ral is phospholipase D (PLD) (Jiang *et al.*, 1995). Importantly, dominant negative Ral inhibits Ras-induced PLD activity, indicating that Ral mediates Ras-induced PLD activation. One of the downstream responses of the Rlf pathway may therefore be PLD activation.

RalGEFs represent a distinct class of effector proteins

We have characterized a third member of the RalGEF family, which serves as an effector of Ras. A recently discovered fourth member of this family, Rgr, does not have a Ras binding domain and thus is most likely not an effector of Ras (D'Adamo *et al.*, 1997). The question can be raised why three different members act as Ras effectors, of which at least Rlf and RalGDS are ubiquitously expressed. It is important to note that Rlf is only ~30% identical to RalGDS and Rgl at the amino acid level, which indicates that Rlf is a rather distinct RalGDS family member. For a comparison, the Ras-effector protein family of the Raf-kinases c-Raf1, B-Raf and A-Raf share 50–60% identity at the amino acid level. The significant difference in primary structure makes it likely that Rlf will have its own specificity in mediating Ras signals. For instance, the three RalGEFs may be subject to specific additional regulation, elusive in the type of experiments performed so far. Alternatively, the three RalGEFs may have different abilities to become activated by other Ras-family members. In analogy with this concept, *in vitro* association experiments between RalGDS family members and Ras-like proteins have revealed remarkable differences in their relative affinities. For instance, the RBD of Rlf has a much higher *in vitro* affinity for Ras than the RBDs of RalGDS and Rgl. In contrast, the RBD of RalGDS, but not Rlf, exhibits a very high affinity for GTP-bound Rap1 (Herrmann *et al.*, 1996; Wolthuis *et al.*, 1996; B.Bauer and R.H.Cool, unpublished observations). Thus far, *in vivo* activation of RalGEFs by other Ras-like proteins has not been observed, but this may imply that the correct conditions or cell types need still to be found (Urano *et al.*, 1996). In this respect a lesson can be learned from the recent observation that Rap1 can activate B-raf in specific cell types only (Vossler *et al.*, 1997).

Materials and methods

Plasmids

Full-length Rlf was isolated as described previously (Wolthuis *et al.*, 1996); the 5′-regions of the Rlf open reading frame obtained from cDNA expression libraries were subcloned into pBluescript, and subsequently a *Sal*I–*Spe*I Rlf-fragment (including the pBluescript multiple cloning site and the Rlf 3′-UTR and ORF, bp 1–1585) was subcloned in *Sal*I–*Spe*I-digested PC86-LEU2-Rlf-RBD (Wolthuis *et al.*, 1996). This generated PC86-LEU2-Rlf (ATG), from which a *Eco*RI–*Not*I fragment was isolated and ligated into the *Eco*RI–*Not*I-cut pcDNA3 vector (Invitrogen), producing the expression vector pcDNA3-Rlf, in which the Rlf-translation initiation site is used.

A *Sal*I–*Bgl*II–*Sac*II oligonucleotide linker (5′-GTCGACAGATCT-CCCGCGG-3′) was ligated to PC86-LEU2-Rlf(AUG) that was linearized by partial digestion at the Rlf *Sac*II site (bp +10), which generated PC86-LEU2-Rlf (*Sal*I–*Bgl*II–*Sac*II), from which a *Sal*I–*Not*I fragment was subcloned into the AdMLP-driven mammalian expression vector

pMT2-HA (*Sal*I–*Not*I-digested), creating HA-epitope-tagged Rlf (amino acids 2–778).

The HA-Rlf-RBD construct contains the last 235 amino acids of Rlf, and was generated by ligation of a *Sal*I–*Not*I fragment isolated from PC86-Rlf-RBD, isolated from the yeast two-hybrid screen (Wolthuis *et al.*, 1996) into pMT2-HA.

The CAAX-construct was generated using a *Sph*I–*Not*I K-Ras CAAX sequence, which was ligated into *Spe*I–*Not*I-digested pMT2-HA-Rlf together with a *Spe*I–*Sph*I oligonucleotide linker (5′-ACTAGTGAT-CACACAGCATG-3′) to generate pMT2-HA-Rlf–CAAX, or with a *Spe*I–*Not*I-digested Rlf cloning vector, from which Rlf–CAAX was subsequently subcloned as a *Eco*RI–*Not*I fragment into the mammalian expression vector pcDNA3 (Invitrogen), to generate pcDNA3-Rlf–CAAX.

pMT2-HA-Rlf–ΔCAT–CAAX encodes the Rlf–CAAX protein from which amino acids 212–327 (the scr-1 region of the catalytic domain) are deleted. This construct was generated by the release of two *Apa*I fragments from pMT2-HA-Rlf–CAAX.

pGEX-GST-Rlf was generated by subcloning of a *Sal*I–*Not*I fragment from PC86-Rlf (*Sal*I–*Bgl*II–*Not*I) into *Xho*I–*Not*I-digested pGEX 4T3.

The wt-RalA and RalA-N28 expression plasmids were made by subcloning of a *Sal*I–*Sma*I fragment from pBluescript-RalA (Wolthuis *et al.*, 1996) into *Sal*I–*Sma*I-digested pMT2-HA. The RalA-V23construct was generated using the Stratagene QuikChange site-directed muta-genesis kit and two oligonucleotide primers containing the desired mutation.

The pSVE-RasV12, RSV-RasL61 and RSV-RasL61S186 plasmids used were described earlier (Medema *et al.*, 1991). Raf–CAAX was obtained from Drs S.Leevers and C.Marshall (Leevers *et al.*, 1994). The RasV12 and respective effector-domain mutants, a kind gift of P.Warne and Dr J.Downward, were cloned as *Bam*HI fragments in pSG5 (Stratagene) (Rodriguez-Viciana *et al.*, 1997).

The integrities of all cloning sites and point mutations were established by sequencing.

Cell culture and cell lines
A14 cells (Burgering *et al.*, 1991), Cos7 and NIH 3T3 cells were routinely grown in DMEM, 10% FCS and 0.05% glutamine. Cell lines were generated by transfecting NIH 3T3 cells with the indicated constructs or control vector and a neomycin vector, and selecting with 300 µg/ml G418. Single colonies were isolated. Cell lines revealing comparable expression levels of the desired proteins as monitored by Western blotting were used for the assessment of growth properties.

Purification of GST-Rlf
Escherichia coli strain AD202-bacteria (Nakano *et al.*, 1994), expressing the GST-Rlf construct, were grown in LB + 0.4% glucose until the start of log-phase and induced with 0.2 mM IPTG for 8 h at 30°C. Isolated bacteria were resuspended, and freeze–thawed. The suspension was incubated with lysozyme at a concentration of 1 mg/ml for 30 min at 4°C, and then sonicated. Triton X-100 was added to a final concentration of 0.5%. Lysates were cleared by centrifugation (20 000 *g*, 60 min). Glycerol was added to a final concentration of 10% and the lysate was loaded on a Q-Sepharose column. The column was washed with PBS containing 10% glycerol, 250 mM NaCl, 1 mM EDTA and 5 mM DTT, and eluted with 5 vols PBS containing 10% glycerol, 600 mM NaCl, 1 mM EDTA and 5 mM DTT. The eluate was then loaded on a glutathione–Sepharose column; the column was washed with the same buffer and with elution buffer (50 mM Tris, pH 7.5, 10% glycerol, 100 mM NaCl, 5 mM MgCl$_2$, 5 mM DTT) and GST-Rlf was eluted with elution buffer containing 15 mM glutathione. The protein was dialysed against elution buffer and concentrated. The obtained fractions were 50–70% pure and contained ~0.1 µg/µl GST-Rlf as determined by gel-staining and Western blotting, and two Coomassie-stainable peptides of low molecular weight at varying levels. Comparison of different fractions in *in vitro* nucleotide exchange experiments demonstrated that the level of exchange activity correlated only with the levels of GST-Rlf, and not with the background bands.

In vitro guanine nucleotide exchange reactions
In vitro guanine nucleotide exchange reactions were performed using different Ras-like proteins loaded with mant-GDP. H-Ras protein was purified as described (Lenzen *et al.*, 1995), whereas simian RalA (Frech *et al.*, 1990) and human RalB (Chardin and Tavitian, 1989) were purified as C-terminally truncated GST-fusion proteins; the GST-moiety was removed by thrombin cleavage, and the proteins were further purified by gel filtration chromatography. After concentration and dialysis to

reaction buffer (50 mM Tris, pH 7.4, 5 mM MgCl$_2$, 5 mM DTE), the protein solution was snap frozen and stored at –80°C (Herrmann *et al.*, 1996). Exchange reactions were performed at 20°C by measuring the decrease in fluorescence, caused by the release of mant-GDP from the protein (excitation wavelength 336 nm, emission wavelength 450 nm; Perkin Elmer fluorometer LS 50B). The reaction was started by adding 20 µM GDP to a cuvette containing 100 nM of Ral-mant-GDP or Ras-mant-GDP and the indicated estimated amounts of GST-Rlf, in 1200 µl of reaction buffer. The fluorescence level after full release of mant-GDP was determined after addition of 15 mM of EDTA.

To investigate the exchange activity in immunocomplexes, Cos7 cells were transfected with pMT2-HA-Rlf or pMT2-HA-Rlf–CAAX using the calcium phosphate technique, as described previously (Burgering and Coffer, 1995). At 40 h after transfection, the cells were washed with PBS and lysed in Triton X-100 lysis buffer (1% Triton X-100, 25 mM Tris, pH 7.4, 0.5 mM MgCl$_2$, 150 mM NaCl, 10 mM NaF, 1 mM Na$_3$VO$_4$, 1 mM PMSF, 1 µM leupeptin, 10 µg/ml soybean trypsin inhibitor, 0.1 µM aprotinin). After removal of the nuclei by centrifugation, HA-Rlf/HA-Rlf–CAAX proteins were immunoprecipitated using protein A–Sepharose beads precoupled to monoclonal 12CA5 antibody, or to non-immune rabbit serum. Immunoprecipitates were washed with lysis buffer and subsequently with high salt buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 0.1% Triton X-100, 5 mM MgCl$_2$ and 0.01% SDS). Nucleotide exchange activity was measured as described for Ras-GRF (Downward, 1995) using 5 mM MgCl$_2$, 0.1% Triton X-100 and 4 µg of bacterially expressed C-terminally truncated simian RalA protein in the exchange buffer.

Ras–Rlf interactions
Cos7 cells were transfected with 2 µg of HA-Rlf together with 5 µg of RasV12 or the RasV12 effector-domain mutants. Cells were lysed in NP-40 buffer (1% NP-40, 25 mM Tris, pH 7.4, 10 mM MgCl$_2$, 15% glycerol, 1 µM leupeptin, 10 µg/ml soybean trypsin inhibitor, 0.1 µM aprotinin). Immunoprecipitation was performed using 12CA5 precoupled to protein A–Sepharose or Y13-238 precoupled to protein G–Sepharose. Immunocomplexes were washed four times in NP-40 buffer, proteins were collected in sample buffer, separated by SDS–PAGE and Western blotted. Blots were incubated with 12CA5 or anti-Ras (Transduction Laboratories).

For inhibition of growth factor-induced ERK activation, A14 cells were transfected with the indicated amounts of HA-Rlf-RBD plasmid together with a MYC-epitope-tagged ERK2 construct (Cowley *et al.*, 1994). *In vitro* kinase activity was determined as described earlier (Burgering and Coffer, 1995).

Fractionation experiments and immunofluorescence
A14 cells transfected with 1.0 µg of HA-Rlf or HA-Rlf–CAAX, were lysed in lysis buffer (20 mM HEPES, pH 7.4, 5 mM EGTA, 1 mM PMSF, 1 µM leupeptin, 0.1 µM aprotinin), Dounce-homogenized on ice, and the nuclear components removed by centrifugation for 10 min at 14 000 r.p.m. Next, the samples were centrifuged at 100 000 *g* at 4°C for 90 min and the upper layer was collected as the soluble fraction; the particulate fraction was dissolved in buffer containing 1% Triton X-100, 50 mM HEPES, pH 7.4, 50 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 µM leupeptin and 0.1 µM aprotinin, and centrifuged (14 000 r.p.m., 5 min) to remove insoluble material. Soluble and particulate fractions were analysed by SDS–PAGE and immunoblotting.

Immunofluorescence studies, using the 12CA5 monoclonal antibody, were carried out as described (van Weering and Bos, 1997). Subcellular localization was examined using a confocal laser scan microscope.

In vivo Ral-loading assay
Cos7 cells grown in 5 cm dishes were transfected with 1 µg of HA-Ral (WT or N28), together with the indicated amounts of expression vectors (pcDNA3-Rlf, pcDNA3-Rlf–CAAX, pSVE-RasV12, RSV-RasL61 or RSV-RasL61S186; all samples were transfected with comparable amounts of the several different expression constructs) using the calcium-phosphate precipitation technique. Cells were washed 16 h after transfec-tion and maintained at 1.5% serum overnight. The next day, the cells were washed and labelled in phosphate-free DMEM containing 0.15 mCi [^{32}P]orthophosphate for 5 h, washed with cold PBS, and lysed in 750 µl of 50 mM Tris, pH 7.4, 140 mM NaCl, 1% Triton X-100, 1% NP-40, 1 mM KCl, 2 mM MgCl$_2$, 1 mM PMSF, 1 µM leupeptin, 10 µg/ml soybean trypsin inhibitor, 0.1 µM aprotinin. Lysates were precleared for 5 min with protein A–Sepharose. HA-Ral was immunoprecipitated with 12CA5-protein A–Sepharose (45 min). Washing of the immunocom-plexes and elution of nucleotides was as described for Ras activation

studies (Burgering *et al.*, 1991). The ratios of GTP versus GDP were determined by PhosphorImager.

For the controls of Rlf-expression levels we used anti-Rlf serum that was isolated from rabbits immunized with a peptide corresponding to the C-terminus of Rlf (NH$_2$-Pro-Arg-Ile-Lys-Ala-Thr-Gly-Arg-Lys-Ile-Ala-Arg-Ala-Leu-Phe-COOH). For affinity purification of the crude serum, CNBr-activated Sepharose, coupled to this peptide, was used as a column and purified antibody was eluted with Tris/glycine and neutralized.

Gene induction studies

A14 cells were plated at ~10% confluency and transfected with 0.5 µg of c-*fos* luciferase or Collagenase luciferase reporter construct together with the indicated amounts of expression plasmids (Medema *et al.*, 1991, 1992); calf thymus DNA or control vectors were added so that the total amount of DNA used per transfection (5 cm dish) was 4.5 µg, or 9 µg in the case of the Ras effector-domain mutants. The cells were washed the day after the transfection, maintained in 10% FCS for 6 h and left without serum overnight. Lysis and determination of luciferase activity was carried out 40 h after transfection and performed as described (Medema *et al.*, 1992). For the c-*fos* luciferase experiments, the expression of co-transfected LacZ, measured by assaying β-galactosidase activity, was used as an internal control. Duplicate dishes were analysed in all experiments, with duplicate experiments repeated at least five times; standard deviations of fold induction were then determined. For measurement of the gene induction by the several Ras effector-domain mutants, two different clones of NIH 3T3 cells stably expressing HA-Rlf, were transfected with 1.5 µg of the Ras mutants, 1 µg pMT2-HA or pMT2-HA-RalN28, 0.5 µg of c-*fos* luciferase construct, and carrier DNA (sheared calf thymus DNA).

For the chloramphenicol acetyltransferase (CAT) assays, cells were transfected with 0.5 µg of 3× TRE-CAT construct or 1.0 µg of a 3× SRE-CAT construct (containing three times the sequence corresponding to the –324 to –298 region of the human c-*fos* promoter) and analysed as described (de Groot and Kruijer, 1990). For the SRE-CAT assays, cells were put on serum-free medium 12 h after transfection and lysed 40 h after transfection. Expression levels of the transfected proteins were controlled by immunoblotting.

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