Identification and Characterization of Potential Effector Molecules of the Ras-related GTPase Rap2*

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Ras proteins are monomeric GTPases that play a pivotal role in the control of cell proliferation; they function as binary switches by cycling between an inactive form bound to GDP and the active GTP-bound state (1). Activation, through the dissociation of bound GDP and subsequent binding of GTP, is catalyzed by GEFs, such as CDC25/Ras-GRF and Sos (2–4). Return to the inactive state is ensured via stimulation of the Rap exchange factor (RalGEFs) Rap GDP dissociation stimulator (RalGDS), RalGDS-like (RGL), and RalGDS-like factor (Rlf); these proteins, which also interact with activated Ras and Rap1, are effectors of Ras and mediate the activation of Rap in response to the activation of Ras. Here we show that the full-length RalGEFs interact with the GTP-bound form of Rap2 in the two-hybrid system as well as in vitro. When co-transfected in HeLa cells, an activated Rap2 mutant (Rap2Val-12) but not an inactive protein (Rap2Ala-35) co-immunoprecipitates with RalGDS and Rif; moreover, Rap2-RalGEF complexes can be isolated from the particulate fraction of transfected cells and were localized by confocal microscopy to the resident compartment of Rap2, i.e. the endoplasmic reticulum. However, the overexpression of activated Rap2 neither leads to the activation of the Rap GTPase via RalGEFs nor inhibits Ras-dependent Rap activation in vitro. Several hypotheses that could explain these results, including compartmentalization of proteins involved in signal transduction, are discussed. Our results suggest that in cells, the interaction of Rap2 with RalGEFs might trigger other cellular responses than activation of the Ras GTPase.

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The abbreviations used are: GEF, guanine nucleotide exchange factor; Gpp(NH)p, guanosine imido 5’-triphosphate; GST, glutathione S-transferase; GST-RalBD, GST fusion protein containing the Ral binding domain of BLIP76; a Rap effector; HA, hemagglutinin; RalGDS, Rap GDP dissociation stimulator; RBD, Ras binding domain; RGL, RalGDS-like; RID, Ras and Rap interaction domain of RalGEFs; Rif, RalGDS-like factor; PI3K, phosphatidylinositol 3-OH kinase.

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8737
inactive complex in quiescent Chinese hamster ovary cells that is reversed upon insulin stimulation (71). It is also possible that the function of Rap1 is independent of regulating Ras signaling, because activation of endogenous Rap1 by extracellular signals fails to interfere with Ras effector signaling in fibroblasts (72).

In contrast with Rap1, no function has yet been attributed to Rap2. Although it also contains the same effector domain as Ras, except for a single substitution of a serine to phenylalanine at position 39 (a similar substitution in Ras only moderately affects its transforming potential), its overexpression does not antagonize Ras signaling (36). In an effort to uncover the function of Rap2, we searched for potential effectors by using the yeast two-hybrid system. This enabled us to identify a novel protein, RPI8P, that specifically interacts with Rap2 and is principally expressed in brain (37). As described in this paper, we also isolated partial cDNAs encoding the C-terminal region of the RasGGEFs RasGDS, RGL, and Rf. These three related proteins, which constitute effectors of Ras, are capable of inducing activation of the Ras-related Ral GTPase, i.e. nucleotide exchange leading to the formation of active Ral-GTP complexes (12, 38, 39). Although Ras and Rap1 can both interact with RasGDS and Rf in cells, only Ras is capable of inducing activation of the GTPase Ral in vivo (12, 14). By themselves, RasGGEFs exhibit little biological activity, only slightly stimulating transcription from the c-fos promoter; however, upon co-expression with activated Raf, RasGGEFs greatly synergizes to activate c-fos promoter activity, as well as cell proliferation and morphological transformation (13, 40). Moreover, targeting Rf to the plasma membrane constitutively activates the protein, which is then able to stimulate gene induction and cell growth (38). RasGGEFs exhibit considerable homology among each other in their 130 most C-terminal residues, which constitute the Ras and Rap1 interaction domain (RID) (18). They contain a conserved central homologous to the RasGEF CDC25 that is responsible for their exchange factor activity toward Ral as well as their stimulating effects on cell growth and gene induction (16, 38).

In this study, we show that Rap2 binds to full-length RasGGEFs in vitro as well as in vivo. This interaction only occurs with active Rap2; Rap2-RasGGEF complexes are found in the particulate fraction of transfected cells, and active Rap2 is capable of recruiting RasGDS and Rf to its resident compartment, the endoplasmic reticulum, suggesting that RasGGEFs may indeed constitute effectors of Rap2 function. However, ectopic expression of activated Rap2 does not lead to the activation of the GTPase Ral, nor does it interfere with the ability of Ras to activate Rap1. These results suggest that RasGGEFs could also serve a function other than activating Rap in cells and that this novel function could be regulated by their interaction with the GTPase Rap2.

EXPERIMENTAL PROCEDURES

Two-hybrid Screens—Screening of a mouse brain cDNA library with in vitro fos promoter activity, as well as cell prolifera-
tion and gene induction (16, 38).

activity toward Ral as well as their stimulating effects on cell

as indicated above were inserted into the pcDNA3 (Invitrogen). Full-length RasGDS and RGL sequences excised from M. White (13) with BamHI and inserted into the BamHI site of pGEMMyc4; full-length Rf was excised from pCS86-Rf with SalI and NotI, blunted with Klenow at its 3′ extremity, and inserted into the 5′ SalI and 3′ PstI (blunted with T4 DNA polymerase) sites of pGEMMyc4.

For binding experiments, 10 µl of glutathione-Sepharose 4B beads bound 2 µg of GST, GST-Rap2A, GST-Ha-Ras, GST-Raf1A, and GST-RalA proteins were washed three times in ice-cold exchange buffer (25 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 5 mM diithiothreitol) and incubated for 30 min at 37 °C in 20 µl of exchange buffer containing 150 mM of Gpp(NH)p or GDP. The beads were then diluted in 180 µl of interaction buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM MgCl2, and 5 mM diithiothreitol) containing 200 µM of the appropriate nucleotide. Complexes were incubated for 3 h at 4 °C with 1 µl of exchange buffer containing [35S]methionine-labeled potential effector. Beads were washed four times with 1 ml of interaction buffer and then boiled in SDS gel sample buffer to recover bound proteins. Samples were analyzed by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels; after staining with Coomassie Blue to detect the GST and GST fusion proteins, gels were treated with Amplify (Amersham Pharmacia Biotech), dried, and exposed to film.

In Vivo Interactions—The cDNAs encoding Ras and Rap2 GTPases as well as RasGGEFs were subcloned into mammalian expression vectors under the control of the cytosmevirus promoter as follows. The coding sequences for Rap2 proteins carrying a Gly to Val substitution at position 12 (Rap2Val-12) and a Thr to Ala substitution at position 35 (Rap2Ala-35) (45) were amplified by polymerase chain reaction with Pfu DNA polymerase and subcloned at the 5′ end of the appropriate nucleotide-primed template and incubated for 3 h at 4 °C with 1 µl of exchange buffer containing [35S]methionine-labeled potential effector. Beads were washed four times with 1 ml of interaction buffer and then boiled in SDS gel sample buffer to recover bound proteins. Samples were analyzed by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels; after staining with Coomassie Blue to detect the GST and GST fusion proteins, gels were treated with Amplify (Amersham Pharmacia Biotech), dried, and exposed to film.
Results

Rap2-GTP Interacts with the C-terminal Ras Interaction Domains of RalGEFs—In order to search for potential effectors of Rap2, through which it may exert its biological effects, we performed two independent screens using the two-hybrid method in the yeast S. cerevisiae. We screened a mouse brain cDNA library with residues 1–168 of Rap2A carrying a Gly to Val substitution at position 12 (Rap2AVal-12) fused to the C terminus of the DNA binding domain of the bacterial transcription activation domain. After serum starvation for 16 h, the cells were resuspended in hypotonic buffer containing 0.1 M NaCl and recentrifuged as described above. They were solubilized in the same buffer containing 1% Nonidet P-40 (30 min on ice), and insoluble material was eliminated by a final centrifugation as described above.

Solubilized extracts were precleared with protein A-Sepharose and immunoprecipitated with 5 μg of anti-Myc 9E10 antibody (Boehringer Mannheim) followed by protein A-Sepharose as described previously (46); the presence of Rap2 in immune complexes was revealed by Western blotting with affinity-purified polyclonal anti-Rap2 antibodies (47) and visualized by ECL (Amersham Pharmacia Biotech).

TABLE I

Interaction of RIDs and full-length effectors with Ras and Rap proteins in the yeast two-hybrid system

| AD fusion | DBD fusion |
|-----------|------------|
| None      | Ha-Ras     |
| c-Raf     | Rap1A      |
| RP/IP8    | Rap2A      |
| RalGDS RID| Rap2B      |
| RAL       | R-Ras      |
| Rlf       | Rlf        |
| RalGDS    | Rlf        |
| Rlf RID   | Rlf RID    |
| Rlf       | Rlf        |

a = no interaction; +, weak interaction; ++, strong interaction; ++++, very strong interaction.

TABLE II

RalGDS and Rlf Are Potential Effectors for Rap2

Because the RIDs of RalGDS and Rlf were expressed in the yeast in the presence of 35S-methionine, to bind to the various Ras-related proteins fused to glutathione S-transferase, immobilized on glutathione-Sepharose beads, and loaded either with GDP or with a nonhydrolyzable analogue of GTP, Gpp(NH)p. Fig. 1 shows that this assay displayed results similar to those obtained by the yeast two-hybrid method: RP/IP8 and the BD of Raf-1 interacted specifically with Rap2 and Ras, respectively, whereas the C-terminal domains of all three RalGEFs bound to Ras, Rap1, Rap2, and Rlf indiscriminately. In all cases, the interaction only occurred with the GTP-bound form of the GTPases.

Hence, RalGEFs, through the direct binding of their C-terminal regions to Ras, Rap1, and Rap2 indiscriminately. In all cases, the interaction only occurred with the GTP-bound form of the GTPases.
system, full-length RalGDS and Rlf (we were unable to express a functional RGL protein in yeast) interacted with all four GTPases, albeit slightly less efficiently with Rap1A than with Ras and Rap2 (A and B). As with their RIDs, the interaction of full-length RalGEFs with Rap1 and Rap2 was unaffected by the presence or absence of the 18 C-terminal residues of these GTPases (not shown). It is noticeable that, in contrast with the results obtained with their RIDs, full-length RalGDS and Rlf could no longer interact with R-Ras, suggesting that RalGEFs do not constitute physiological effectors of the R-Ras GTPase.

Using the same in vitro binding assay as above, we extended the results obtained with the yeast two-hybrid system: Fig. 2 shows that full-length RalGDS, RGL, and Rlf interacted with Ras, Rap1, and Rap2 in their active GTP-bound form. In this assay, Rlf interacted better with Ras than with Rap1 and Rap2, in agreement with the reported high affinity of the Ras-GTP-Rlf interaction (18). Although some interaction of RalGDS and RGL occurred in certain experiments with the GDP-forms of Rap GTPases, it was always much weaker than that observed with their GTP-bound form. In summary, the data obtained with the yeast two-hybrid system as well as with an in vitro binding assay suggest that RalGEFs could indeed constitute effectors of Rap2A and Rap2B GTPases.

**FIG. 1. Interaction of the RIDs of RalGEFs with Ras, Rap1, and Rap2 GTPases.** The RIDs of RalGDS, RGL, and Rlf, along with the RBD of c-Raf and the Rap2 effector RPIP8 were transcribed and translated in vitro in the presence of [35S]methionine. They were incubated with control GST or with Ras, Rap1, Rap2A, and Rap2B proteins fused to GST and bound to glutathione-Sepharose beads that had previously been loaded with GDP (lanes a) or Gpp(NH)p (lanes b). After extensive washing, the beads were boiled in SDS-sample buffer, and the extracts submitted to SDS-polyacrylamide gel electrophoresis and fluorography. Lane T, one-half of the input labeled proteins.

**FIG. 2. Interaction of full-length RalGEFs with Ras, Rap1, and Rap2 GTPases.** Full-length RalGEFs were transcribed and translated in vitro, and their binding to the GDP or Gpp(NH)p form of Ras, Rap1, and Rap2 was assessed as described in Fig. 1.

**FIG. 3. Co-immunoprecipitation of the active form of Rap2 with RalGDS and Rlf from whole cell lysates.** HeLa cells were co-transfected with expression vectors encoding inactive (Ala-35) or constitutively active (Val-12) mutant Rap2 proteins along with vectors encoding RalGDS or Rlf carrying an exogenous Myc epitope at their N terminus. 40 h after transfection, cells were lysed with 1% Nonidet P-40. Aliquots were removed and tested for the expression of transfected constructs by Western blotting with anti-Myc or anti-Rap2 antibodies. The bulk of the extract was submitted to immunoprecipitation with anti-Myc antibodies, and the presence of Rap2 in these complexes was revealed by Western blotting. Similar results were obtained by transfecting HEK 293 cells.

**FIG. 4. A complex between Rap2 and RalGEFs is formed in the particulate fraction of cells.** HeLa cells were co-transfected with expression vectors for Rap2 and Myc-tagged RalGEFs as in Fig. 3. 40 h after transfection, cells were mechanically lysed in hypotonic buffer, and the particulate fraction of cells was isolated by ultracentrifugation, solubilized with 1% Nonidet P-40, and submitted to immunoprecipitation and Western blotting as in Fig. 3. In the middle panels, the particulate fraction was prepared from cells transfected with Rap2 or Myc-Rlf vectors only; these fractions were then mixed, solubilized, and immunoprecipitated as above. Similar results were observed after transfection of HEK 293 cells.
 Rap2 does not activate Ral in vivo. A, COS 7 cells were transfected with pMT2-HA-Ral together with expression constructs for RasVal-12, Rap2Val-12, and Myc-RalGDS as indicated. Prior to lysis, cells were labeled with [32P]orthophosphate for 5 h. HA-Ral protein 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 left 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 at least two different experiments. The right panel shows the expression levels of Myc-RalGDS, Ras, and Rap2 in the lysates as assessed by Western blotting. B, the same experiments were performed as in A except that Rlf was transfected instead of Myc-RalGDS together with Rap2Val-12 as indicated.

Under these conditions, Rap2Val-12 was present in complexes immunoprecipitated from whole cell lysates with anti-Myc antibodies (Fig. 3); such was not the case when the inactive Rap2Ala-35 mutant was expressed, showing that RalGDS and Rlf only associated in mammalian cells with the active form of Rap2. These results are similar to those obtained in control experiments with Ras (not shown).

Although they are synthesized as soluble precursors, mature Ras family GTPases are bound to cellular membranes, Ras to the plasma membrane and Rap2 to the endoplasmic reticulum, following a series of posttranslational modifications that involve prenylation and palmitoylation in the case of Ras and Rap2 (47, 50). In order to establish that the complexes between RalGEFs and Rap2 did not involve unprocessed cytosolic precursors of the GTPase but bona fide processed and membrane-associated protein, the particulate fraction of transfected cells was isolated by ultracentrifugation prior to the immunoprecipitation of RalGEFs with anti-Myc antibodies as above (Fig. 4). As previously reported (38), a significant proportion of transfected RalGDS and Rlf (10–20%) was associated with the particulate fraction of cells; this proportion did not vary with the co-expression of Ras (not shown) or Rap2 (Fig. 4) proteins and represented nonspecific association with cellular membranes (see below and Fig. 6). As with whole cell extracts, the active Rap2 protein (but not the inactive Ala-35 mutant, not shown) was co-immunoprecipitated with RalGDS and Rlf from solubilized membranes of co-transfected cells. In contrast, when membranes prepared from cells only transfected with Rap2 or RalGEFs were mixed, solubilized, and submitted to immunoprecipitation, only a very minor amount of Rap2 was recovered in the immunoprecipitates (Fig. 4); similar results were obtained after transfection in HEK 293 cells, as well as in control experiments performed with Ras (not shown). Hence, these complexes between Rap2 and RalGEFs were formed on membrane structures prior to cell lysis, suggesting that the interaction between active Rap2 and RalGEFs may indeed occur in mammalian cells.

Rap2 Does Not Lead to Activation of the Ral GTPase—In order to assess whether the observed interaction between Rap2 and members of the RalGEF family resulted in activation of the Ral GTPase, we measured the levels of Ral-GTP in transfected COS-7 cells after labeling the nucleotide pools with [32P]orthophosphate. Ectopic expression of either RalGDS or Rlf enhanced the level of Ral-GTP from 4–6% (relative to the total amount of Ral-GDP + Ral-GTP) to 15–20% (Fig. 5, A and B); co-expression of activated Ras further stimulated Ral-GTP formation up to 30–50% (Fig. 5A and Refs. 38 and 39). However, in contrast to activated Ras, overexpression of activated Rap2 did not further enhance the level of Ral-GTP induced by RalGDS (Fig. 5A) or Rlf (Fig. 5B). These results demonstrate that despite the ability of active Rap2 to form complexes with RalGEFs in vivo, active Rap2 does not stimulate the ability of RalGDS or Rlf to activate Ral under these circumstances. Therefore, it is unlikely that the Ral GTPase is involved in signal transduction downstream of Rap2.

Rap2 recruits RalGEFs to its resident compartment, which is different from that of Ras—As a possible hint to the actual inability of Rap2 to activate Ral despite its interaction in vivo with RalGEFs, we examined by confocal microscopy whether the subcellular localization of RalGDS and Rlf was similar in cells expressing activated forms of Ras and Rap2. Immunofluorescence experiments were performed on HEK cells co-transfected with expression constructs for Myc-tagged RalGEFs and constitutively inactive (Fig. 6A) or activated (Fig. 6B) GTPases, which were fixed with cold methanol, conditions that eliminate cytosolic proteins and enable to visualize only molecules present on structures such as the cytoskeleton and cellular membranes. In the absence of co-expressed activated Ras or Rap2, a faint and diffuse localization of ectopically expressed RalGDS and Rlf to intracellular membranes was observed in transfected cells (Fig. 6A, a and b). Upon co-expression with activated Ras, the membrane-associated fraction of RalGDS (Fig. 6B, a and b), as well as Rlf (Fig. 6B, c and d), was localized with Ras at the plasma membrane. In contrast, when they were co-expressed with the active form of Rap2, the membrane-associated fraction RalGDS, as well as Rlf, co-localized with this GTPase at the endoplasmic reticulum (Fig. 6B, e–h). In control experiments, co-expression of Ral GEFs with the inac-
FIG. 6. Active Ras and Rap2 recruit RalGDS and Rlf to their respective resident compartments. A, HeLa cells were transfected by electroporation with expression constructs for Myc-tagged RalGDS (a) or Myc-tagged Rlf (b) alone, or co-transfected with the following constructs encoding Myc-tagged RalGEFs and inactive GTPase mutants: RalGDS and RasN17 (c and d), Rlf and RasN17 (e and f), RalGDS and Rap2Ala-35 (g and h), and Rlf and Rap2Ala-35 (i and j). 24 h later, cells were processed for double immunofluorescence as described under “Experimental Procedures” in order to simultaneously visualize RalGEFs, stained in green, with monoclonal anti-Myc followed by fluorescein isothiocyanate-coupled anti-mouse antibodies (c, e, g, and i), and GTPases, stained in red, with rabbit polyclonal anti-Ras or anti-Rap2 antibodies followed by tetramethylrhodamine isothiocyanate-coupled anti-rabbit antibodies (d, f, h, and j) in co-transfected cells. B, HeLa cells were co-electroporated as in A with the following expression constructs for Myc-tagged RalGEFs and constitutively activated GTPase mutants: RalGDS and RasVal-12 (a and b), Rlf and RasVal-12 (c and d), RalGDS and Rap2Val-12 (e and f), Rlf and Rap2Val-12 (g and h). They were processed for double
tive RasN17 (Fig. 6A, c–f) and Rap2Ala-35 (Fig. 6A, g–j) mutants did not cause them to co-localize with the GTPases but rather to maintain the same faint and nonspecific membrane labeling as when they were expressed alone. It is noteworthy that RasN17 was localized at the plasma membrane similarly to RasVal-12, whereas inactive Rap2Ala-35 (as well as Rap2Aasn-17, not shown) did not specifically label the endoplasmic reticulum as transected Rap2Val-12 (Fig. 6B, f and h) or endogenous protein (47), but it did label various cellular membranes, including the plasma membrane. Hence, the co-localization of ectopically expressed RalGEFs with the active GTPase co-expressed in transfected cells represents recruitment of RalGDS and Rlf by active Ras and Rap2 at their resident compartment, i.e. the plasma membrane for Ras and the endoplasmic reticulum for Rap2. Such a recruitment further hints that RalGEFs may indeed act as effectors of Rap2 function and also provides an explanation for why active Rap2 does not lead to the activation of Ral.

Rap2 Does Not Interfere with Ras-mediated Ral Activation—

Given that the overexpression of activated Rap2 can recruit RalGEFs yet does not lead to Ral activation, we wished to test the hypothesis that in such a manner, activation of Rap2 could regulate Ras signaling to Ral, possibly by sequestering Rap2. To this end, we used a cell line derived from NIH 3T3 overexpressing the insulin receptor (A14), in which insulin stimulation leads to Ras-dependent Ral activation (15), and investigated whether overexpression of activated Rap2 was able to interfere with this response (Fig. 7). In these experiments, Ral activation was qualitatively measured by trapping the active Rap2-Ral complex on a GST-RalBD fusion protein as in (15). When A14 cells were transfected with HA-tagged Ral alone, insulin stimulated both the activation of endogenous Ral, representing the whole cell population, and HA-Ral ectopically expressed in transfected cells, confirming that this method may indeed be used to assess Ras to Ral signaling in transfected cells. Transfection of Rap2 expression vector caused a vast overexpression of the protein but did not affect the expression level of co-transfected HA-Ral as compared with control cells transfected with empty vector. Under basal conditions, overexpression of Rap2 was associated with a slight activation of HA-Ral. Quite remarkably, the level of HA-Ral activation in response to insulin stimulation was similar in cells overexpressing or not overexpressing activated Rap2; further enhancement of Rap2 expression level had no noticeable effect on insulin-stimulated Ral activation as well (not shown).

It should be noted that after insulin stimulation, Ras becomes strongly activated, so GTP-bound Ras and Rap2Val-12 would be expected to compete for RalGEFs binding. Our results indicate that, under these conditions, and despite its vast overexpression, Rap2 is unable to sequester the entire endogenous pool of RalGEFs, and Ras-RalGEF complexes are formed as attested by the activation of Ral in response to insulin. In conclusion, we have shown that although activated Rap2 is able to interact with RalGEFs in cells, neither does it lead to Ral activation, nor can it interfere with signaling from Ras to Ral.

**DISCUSSION**

In this study, we show that the Ras-related GTPase Rap2 is capable of binding to proteins, the normal function of which is to activate another Ras-related GTPase, Ras, yet this interaction, observed in cells overexpressing Rap2 and RalGEFs, does not lead to formation of the active Ral-GTP complex. This situation is reminiscent of what is observed with another Ras-related GTPase, Rap1, which binds RalGDS under similar experimental set-ups but does not either lead to Ral activation (12, 14). This latter observation was somewhat surprising in view of the fact that biochemical studies had revealed that the C-terminal RID of RalGDS binds Rap1 with a 10-fold higher affinity than Ras, which had led to speculate that RalGDS might well act as an effector of Rap1 function (32). The two qualitative yeast two-hybrid and *in vitro* assays described here that we performed with the C-terminal RIDs and with the full-length RalGEFs suggest that there is little differential specificity of the Ras, Rap1, and Rap2 GTPases for the three RalGEFs' RalGDS, RGL and Rlf, respectively. This observed promiscuity is not an artifact of the methods used, because Ras and Rap2 interacted specifically with Rap1 and RalGDS under similar experimental set-ups but does not either lead to Ral activation. We and others consistently find that 10–20% transfected RalGDS and Rlf are associated with cellular membranes; see Fig. 4 and 6 (38, 52) and should therefore be able to access Rap proteins that, similarly to Ras, are bound to membranes via their C-terminal extremities and remain on the cytoplasmic membrane.
face of their respective compartments (47, 53, 54). In fact, in the experiment that attempts to uncover Rap-stimulated Ral activation, Rap2 and Rlf are overexpressed and can be co-immunoprecipitated attesting of their effective interaction (not shown).

The logical second hypothesis is that Rap-RalGEF interactions are unproductive, i.e., that contrarily to Ras, Rap proteins cannot induce activation of the GEF activity of RalGDS and Rlf on Ral. Yet when posttranslationally modified forms of Rap1 and Rap2 were incorporated into liposomes, Rap1 was able to stimulate through RalGDS the dissociation of GDP from Ral (14). Moreover, ectopic overexpression of Rap1 and Rlf in COS cells resulted in a 1.5–2-fold activation of coexpressed Ral (72). However, in a similar situation, Rap2 did not lead to Ral activation through RalGDS or Rlf (Fig. 5), although a vast overexpression of activated Rap2 alone was associated with a very modest increase in Ral-GTP (Fig. 7). Therefore, at the biochemical level, Rap1 is able to activate the exchange factor activity of RalGDS and Rlf toward Ral. Our data hint that Rap2 may not be able to do the same, and biochemical experiments performed by reconstituting posttranslationally modified Rap2 and Ral in micelles together with recombinant Rap1GEFs would be required to formally address this question. As in the case of the interaction of Ras with Raf, it is not yet understood whether targeting effectors to the membrane is sufficient to cause their activation as suggested by grafting membrane targeting sequences to the C terminus of Raf-1 (55, 56), PI-3K (57), and Rlf (38) and the inability of non-prenylated Ras mutants to activate Ral in cells (38), or whether conformational changes in the effector induced by interaction with the GTP-bound GTPase also play a role in the activation mechanism as suggested in transfection experiments with Raf-1 (58, 59) and NMR spectroscopy experiments with the RID of RalGDS (60) and Rlf (61).

There are structural differences in the effector and so-called “extended effector” regions that distinguish Rap2 from Ras and Rap1 that may impair the ability of Rap2 to activate Rap1GEFs. Rap2 contains a phenylalanine at position 39, in the effector region, instead of the serine found in Ras and Rap1. Although this position does not appear critical from the three-dimensional structure of Rap2 (30), it is involved in the interaction of Rap1A with the Rap-1 RBD (29), and a replacement of serine 39 by phenylalanine reduces the transforming ability of Ras by 3–10-fold (36); one should examine whether this effect is due to a reduced ability of Ras to activate one or several of its effectors, such as Raf-1, Rap1GEFs, or PI-3K. There are also nonconservative substitutions at positions 25 and 43 that exhibit glutamine to threonine and glutamine to glutamic acid substitutions, respectively, in Rap2 as compared with Ras and Rap1, as well as the conservative replacement of valine 44 in Ras and Rap1 by isoleucine in Rap2. It is noteworthy that residues 25 and 43 do not appear to make direct contact with the Rap RBD in its crystal structure complexed to Rap1 (29); however, they could be involved in the interaction of Ras and Rap1 with the cysteine rich domain of Raf-1 (62, 63), which is necessary for optimal Ras binding and Raf-1 activation in cells (64, 65). The possibility that such residues are also necessary for Ras and Rap1 to activate Rap1GEFs in cells could be investigated by making the appropriate substitutions in Ras/Rap1 and Rap2 and assessing by transfection their ability to activate Ral via Rap1GEFs.

Finally, and perhaps most importantly, Rap2 is able to recruit Rap1GEFs to its resident compartment, the endoplasmic reticulum, as Ras recruits them to the plasma membrane. Yet under these circumstances, Rap-RalGEF complexes might not act in the same transduction pathways, whereas activation of Ral leads to the activation of Rap1.

Because overexpressed activated Rap2 is able to recruit overexpressed RalGDS and Rlf to the endoplasmic reticulum, one could have expected overexpression of activated Rap2 to sequester Rap1GEFs away from the plasma membrane and therefore inhibit Ras-dependent Rap activation. This could have represented a mechanism for Rap2, as already suggested for Rap1, to control signaling downstream from Ras. However, we have shown that in a cell line overexpressing the insulin receptor, where Rap1 is activated in the response to insulin stimulation via endogenous Ras and Rap1GEFs, the overexpression of Rap2 is unable to interfere with Rap1-dependent Rap activation. This is in line with previous observations that overexpression of Rap2 has no effect on the growth-promoting effects of Ras (36). Several plausible explanations include the possibility that a pool of membrane-associated Rap1GEFs remains in the vicinity of Ras and Rap, due to their association with a membrane microdomain or molecular scaffold, mechanisms that have been suggested to increase the efficiency of signal transduction in mammalian cells (68–70). Our results, showing that the biochemical interaction promiscuity of Ras, Rap1, and Rap2 GTPases with Rap1GEFs does not lead to functional promiscuity in cells, suggest that compartmentalization of signaling proteins is of the greatest importance to ensure the functional specificity of signaling pathways.

The possibility of a physiological Rap2-RalGEF interaction raises the question of whether Rap1GEFs might have another physiological role in addition to stimulating the activation of Ral. In fact, because activated Ral mutants cannot substitute for RalGDS or Rlf to transform cells or activate transcription from the c-fos promoter (13, 38, 40), Rap1GEFs probably also exert a Ral-independent function in the Ras signaling pathway. In the case of Rap2 signaling, a yet to be identified partner of Rap1GEFs, which might be specifically present at the surface of the endoplasmic reticulum, could serve as a target of active Rap2-RalGEF complexes. Whether Rap1GEFs play a role downstream of Rap2 by activating cellular pathways other than those involving Rap is currently under investigation.

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