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# Differential Interaction of the Ras Family GTP-binding Proteins H-Ras, Rap1A, and R-Ras with the Putative Effector Molecules Raf Kinase and Ral-Guanine Nucleotide Exchange Factor*

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**The interactions of H-Ras, R-Ras, and Rap1A with the Ras-binding domains (RBD) of the c-Raf kinase and of the Ral guanine nucleotide exchange factor (RGF) was studied biochemically in solution. From deletion cloning the RGF-RBD was defined as a 97-amino acid-long fragment from the C-terminal end of the human RGF, which is an independent folding domain with high stability. Interestingly, whereas H-Ras binds with high affinity ($K_D$ = 20 nM) to Raf-RBD and with low affinity ($K_D$ = 1 μM) to RGF-RBD, Rap1A shows the opposite behavior. The binding of both RBDs to R-Ras is weak and shows no specificity. The interaction between Rap1A and RGF-RBD shows similar characteristics to the Ras-Raf interaction because it is blocked by mutations in the effector region (D38A) and it inhibits the dissociation of guanine nucleotide, which is the basis for the quantitative measurements in this work. Furthermore, the binding of RGF-RBD inhibits the interaction between Rap1A and Rap-GAP. As long as the cellular localizations of the different proteins and their biological functions are not clarified, these biochemical data seem to indicate that Ral-guanine nucleotide exchange factors is an effector molecule of Rap1A rather than of H-Ras.**

Ras proteins play a crucial role in the transduction into the nucleus of extracellular signals that regulate different cellular responses such as growth, differentiation, and metabolism. They are activated by exchange of the protein-bound GDP for GTP catalyzed by guanine nucleotide exchange factors like Cdc25 and Sos. By its intrinsic GTPase activity Ras returns to the inactive form, and this process can be accelerated by interaction with GTPase-activating proteins like p120GAP and NF-1 (1–3).

Activated Ras interacts with downstream targets or effectors that are operationally defined as proteins interacting more tightly with the GTP-bound than with the inactive GDP-bound state. The best characterized but probably not the only effector molecule is the Raf kinase (4–9), which binds to Ras-GTP with 20 nM and to Ras-GDP with 20 μM affinity, respectively (10), and which is translocated by Ras to the plasma membrane for further activation (11–13) molecular details of which are presently not understood. The activation of Raf initiates a phosphorylation cascade involving Mek and Erk protein kinases and leads to the activation of transcription factors like Elk (14).

Recently it has become increasingly clear that the signal transduction cascade via Ras branches out to produce changes in cellular morphology in addition to induction of DNA synthesis (15) and that entirely different pathways involving Ras might exist (16, 17). A number of other bona fide or putative effector molecules such as phosphatidylinositol 3 kinase (18, 19), MEK kinase (20), RGF[1] (also termed Ral-GDS in other publications) (21–23), and a few other Ras-binding proteins (Rsb) (24) and Rin (25) have been identified by demonstrating their direct interaction with Ras-GTP. So far only for phosphatidylinositol 3 kinase has an enhancement in activity *in vivo* been shown. Among these effectors we have focused our interest in this work to RGF, which has nucleotide exchange activity specifically to Ral but not to other Ras-related proteins (26).

In addition to the variety of possible Ras effectors, the situation has become even more complicated due to the finding that other members of the Ras subfamily of GTP-binding proteins such as Rap1 and R-Ras also bind to these possible effectors (8, 21, 23, 24, 27), most likely because the amino acid sequence in their effector region is very similar. It has been shown recently by x-ray crystallography that Rap1A and Ras use a common core effector region in their interaction with Raf-RBD (28, 29). Because most of the binding reactions mentioned above have only been investigated qualitatively, mainly by using the yeast two-hybrid system, we have measured these interactions more accurately in solution to get more insight into the affinity and specificity of these interactions.

## EXPERIMENTAL PROCEDURES

*Cloning of COOH-terminal Fragments of RGF and the D38A Mutant of Rap1A*—The plasmids encoding four different protein fragments from the COOH-terminal region of Ral-guanine nucleotide exchange factor (named RGF), which contains the Ras binding domain, were made by subcloning the proper fragments from pGAD-RGF127, which encodes the 127 carboxyl-terminal amino acids of human RGF (23), into pGEX-4T3 (Pharmacia Biotech Inc.). pGEX-RGF127 was made by insertion of a 0.7-kilobase fragment of *Eco*RI- and *Sma*I-digested pGAD-RGF127 into *Eco*RI- and *Sma*I-digested pGEX. To create pGEX-RGF97, encoding a protein of 97 residues starting with the same $NH_2$-terminal residue as the 127-residue fragment, the 0.3-kilobase *Eco*RI–*Ava*II fragment (blunted with T4 DNA polymerase) from pGAD-RGF127 was subcloned into *Eco*RI- and *Sma*I-digested pGEX. This subcloning also generated pGEX-RGF83 consisting of 83 amino acids with the same $NH_2$ terminus as the proteins described above. pGEX-RGF61, which codes for a protein of 61 residues having the same $NH_2$ terminus was made by insertion of a 0.2-kilobase *Eco*RI–*Pst* (blunted with T4 DNA

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[1] The abbreviations used are: RGF, Ral guanine nucleotide exchange factor; RBD, Ras-binding domain; GST, glutathione *S*-transferase; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; mGpp(NH)p, fluorescent analogue of Gpp(NH)p carrying at position 2′ or 3′ the fluorescent mant (m) group, *N*-methylanthraniloyl; GAP, GTPase-activating protein; $GAP^{334}$, catalytic domain of Ras GAP; GDI, guanine nucleotide dissociation inhibitor; HPLC, high pressure liquid chromatography.



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polymerase) fragment from pGAD-RGF127 into *Eco*RI- and *Sma*I-digested pGEX. This subcloning lead to eight additional amino acids (GSPNSARA) between the thrombin cleavage site and the $NH_2$ terminus of RGF. Site-directed mutagenesis of the Rap-DNA was performed in the pBks vector by the "transformer" site-directed mutagenesis kit from Clontech. The mutagenic oligonucleotide was 5′CCAACGATAGAAGCTTCCTACAGAAAG3′ (substitution of $Asp^{38}$ for Ala, mutated codon underlined). The proper sequence of all plasmids was verified by DNA sequencing.

*Protein Preparations*—An overnight culture of BL21(DE3) cells containing one of the GST-RGF plasmids described was diluted 1:50 in standard I medium (Merck) including 50 mg/liter ampicilline. After it had grown to an OD = 0.8, synthesis of the GST-RGF fusion protein was induced by the addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside and by further incubation for 4 h at 37 °C. The frozen bacterial cell pellet was suspended in phosphate-buffered saline containing 1 mM dithiotreitol and 100 μM phenylmethylsulfonyl fluoride and sonicated (Branson 450). The 30,000 × *g* supernatant was run over a glutathione-Sepharose (Pharmacia) column washed with 20 mM Tris·HCl, pH 7.2, and either incubated overnight at ambient temperature with 10 units/ml thrombin (Serva) or eluted with 10 mM glutathione in the same buffer. The cleaved RGF97 protein was eluted and chromatographed over 25 ml of Q-Sepharose FF (Pharmacia) using a 0–1 M NaCl gradient. The RGF127 was chromatographed over 25 ml of SP-Sepharose FF (Pharmacia) and eluted with a 0–1 M NaCl gradient. The proteins were concentrated to 20 mg/ml (centricon 10, Amicon) and stored at −80 °C. For digestion studies of the GST fusion proteins with thrombin, phosphate-buffered saline or 20 mM Tris, pH 7.2, in the absence or the presence of 1 mM $CaCl_2$ was used. The samples were incubated for various times at 4 or 37 °C and quenched with 1 mM phenylmethylsulfonyl fluoride.

H-Ras and the D38A mutant were prepared from *Escherichia coli* as described before (30, 31). The truncated form of Rap1A (residues 1–167) was expressed in ptac in the *E. coli* strain CK600K. The cells were grown at 37 °C in standard I medium (Merck) to O.D. = 0.7, and then 0.1 mM isopropyl-1-thio-β-D-galactopyranoside was added, and the culture was incubated overnight at 32 °C. The cells were lysed using lysozyme as described for Ras. The cleared cell lysate was chromatographed over Q-Sepharose (Pharmacia), and the protein was eluted by a NaCl-gradient (0–300 mM) in a buffer containing 60 mM Tris·HCl pH 7.6, 10 mM $MgCl_2$, 0.1 mM $NaN_3$, and 10 mM dithiotreitol. The Rap-containing fractions were concentrated to 20 mg/ml using ultrafiltration membranes (10 kDa, Amicon). The protein was then run over a gel filtration column (Superdex 75, Pharmacia) where the buffer additionally contained 400 mM NaCl and 0.1 mM GDP, again concentrated to 20 mg/ml, shock frozen in liquid nitrogen, and stored at −80 °C. The D38A mutant of Rap1A was prepared as the wild type protein. The plasmid encoding R-Ras was kindly provided by Alan Hall, and the protein was purified using a Q-Sepharose column and a G75 Sephadex column. Proteins were analyzed for purity and proper molecular weight by SDS-polyacrylamide gelelectrophoresis, and protein concentrations were determined by the method of Bradford (32).

For fluorescence measurements the Ras proteins were loaded by incubation with a 1.5-fold molar excess of the fluorescent GTP analogue mGpp(NH)p prepared as described by John *et al.* (33) in the presence of 200 mM ammonium sulfate and 5 units/mg·p21 of alkaline phosphatase (Boehringer Mannheim). Loading of the proteins with GTP was achieved by incubation with a 100-fold excess of GTP in presence of 10 mM EDTA. After 2 h for H-Ras and R-Ras and 24 h for Rap1A at 4 °C, the buffer was changed to 50 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$, and 1 mM dithiotreitol (buffer A) using a gel filtration column (PD-10, Pharmacia).

*Measurement of the Interaction between Ras Proteins and Effector Molecules*—For measurement of the GTPase rates of H-Ras and Rap1A in the absence or the presence of saturating amounts of RGF or in presence of catalytic amounts of $GAP^{334}$, the catalytic domain of Ras-GAP (34), and Rap-Gap (kindly provided by T. Schweins and R. Ahmadian), respectively, the protein samples were incubated at 37 °C in buffer A. Aliquots were taken at various time intervals and analyzed for GDP and GTP content using a reversed phase $C_{18}$ HPLC column as described (30). The experiments were carried out in triplicate, and the rate of hydrolysis was obtained by fitting a single exponential curve to the decay of the GTP concentration.

The affinities between Raf-RBD or the RGF fragments and the Ras-proteins, as defined in Equation 1, were determined using the inhibition of the nucleotide dissociation from Ras by the binding of the effector to Ras. The rate constants ($k_{obs}$) of the mGpp(NH)p dissociation from the Ras protein were measured in the presence of varying effector concentrations. The reaction mixtures in buffer A were incubated at 37 °C in a fluorescence cuvette, and the mGpp(NH)p dissociation was initiated by the addition of 250 μM nonlabeled Gpp(NH)p. The fluorescence decay was monitored in a Perkin-Elmer LS50B spectrometer with excitation and emission wavelengths of 365 and 450 nm, respectively. The fluorescence traces obtained were fitted by a single exponential function yielding the rate constant for the mGpp(NH)p dissociation, $k_{obs}$. According to Scheme 1 and Equation 2, this rate constant is composed of two contributions, the dissociation of the nucleotide (N) from the Ras protein (R) alone, defined as $k_{-1}$, and from the complex with the effector (E), defined as $k_{-2}$. Due to the large excess of nonlabeled nucleotide, the rate constants for the reassociation of mGpp(NH)p, $k_{+1}$ and $k_{+2}$, can be ignored. Equations 1 and 2 are combined to give the expression for $k_{obs}$ in Equation 3. According to this equation the data are fitted using the program Grafit (Erithakus Software) to give $K_d$ within an error of 10%.

The affinity between RGF and the Ras mutant Y32W was also measured by direct titration using the tryptophan fluorescence as readout. In buffer A, the Gpp(NH)p form of Y32W was kept at a constant concentration of 0.8 μM, and the fluorescence emission (excitation wavelength, 295 nm) at 350 nm was monitored as a function of the RGF fragment concentration. A fit to the data yields a $K_d$ within an error of 10%.

## RESULTS

By biological and biochemical investigations, Raf kinase has been identified as one of the Ras effector molecules. It binds tightly to the active GTP form of Ras with a $K_d$ of 20 nM, whereas the binding to an effector region mutant such as D38A is approximately 100-fold and to the GDP-bound form 1000-fold weaker (10). The effector Raf competes with GAP and NF-1 for binding to Ras (8, 9, 35). The interaction of the Ras homologous proteins Rap and R-Ras with Raf kinase, or more specifically Raf-RBD, has also been demonstrated (8, 23, 27, 36).

In recent studies using the yeast two-hybrid system RGF has been found to be another putative Ras effector because the COOH-terminal end of the molecule binds to Ras family proteins in a GTP-dependent manner, and the interaction is interrupted by mutations that show weaker nucleotide affinity (21–23). To further define the minimal RBD of RGF and to measure its interaction with Ras and Ras-like proteins and for the comparison with the RBD of Raf kinase, protein fragments of different length from the COOH-terminal part of RGF have been expressed and characterized.

*Purification of RGF Fragments*—The DNA encoding the 127 carboxyl-terminal amino acids of human RGF, which has been identified from a yeast two-hybrid screen involving R-Ras as a bait (23), has been used for the construction of plasmids for the bacterial expression of fragments of 61, 83, 97, and 127 residues in length, named RGFxx where "xx" denotes the number of amino acids of the protein fragment. They all start with the same $NH_2$-terminal residue, which is the homologue of amino acid 726 of mouse RGF. These proteins were expressed from pGEX-4T3 as GST fusions, and all four fragments were strongly overexpressed as shown in *lanes 2–5* in Fig. 1. Only GST-RGF127 and GST-RGF97 were found in large amounts in the soluble extract whereas GST-RGF83 and GST-RGF61 appeared to be mostly insoluble (Fig. 1, *lanes 6–9*). Different *E. coli* strains and growing conditions were tried to improve the solubility, *i.e.* 20, 28, and 37 °C and concentrations of isopropyl-1-thio-β-D-galactopyranoside ranging from 10 μM to 1 mM. The cleared cell lysate was chromatographed over glutathione-Sepharose, and the fusion protein was cleaved on the column by incubation with thrombin or was eluted from the column with 10 mM glutathione. From a 10-liter bacterial culture about 100 mg of GST fusion protein of the two larger fragments were obtained, whereas only 2–3 mg of fusion protein of the two smaller fragments could be isolated. The cleaved protein fragments RGF97 and RGF127 were then fractionated by gradient elution (0–1 M NaCl) at pH 7.2 from a Q-Sepharose column and

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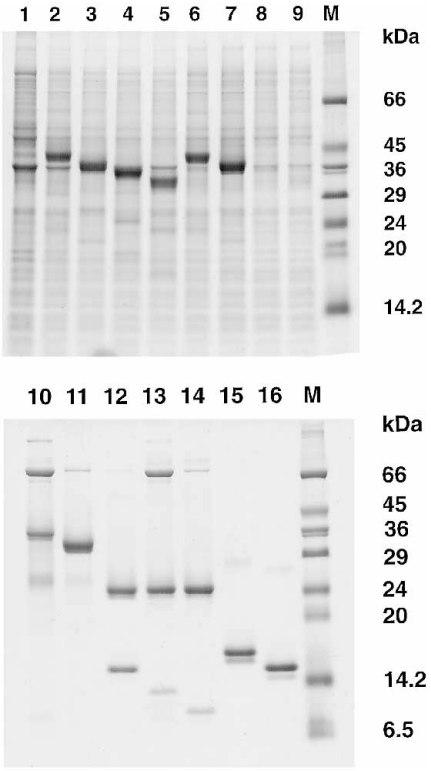



FIG. 1. **Expression and purification of the COOH-terminal RGF fragments.** SDS-polyacrylamide gel electrophoresis at 15 (*upper panel*) and 18% (*lower panel*) polyacrylamide. *Lane 1*, whole cell lysate without induction; *lanes 2–5*, whole cell lysates after induction; *lanes 6–9*, 30000 × *g* supernatant of sonicated cells; *lanes 10–14*, GST-RGF fusion proteins eluted from glutathione-Sepharose; *lanes 12–14*, thrombin-digested GST-RGF fusion proteins; *lanes 15* and *16*, RGF127 and RGF97 eluted from SP- and Q-Sepharose; respectively. *Lanes 1*, *2*, and *6*, GST-RGF127; *lanes 3*, *7*, and *12*, GST-RGF97; *lanes 4*, *8*, *10*, and *13*, GST-RGF83; *lanes 5*, *9*, *11*, and *14*, GST-RGF61.

a SP-Sepharose column, respectively. The proteins were obtained in more than 90% homogeneity as shown in Fig. 1 (*lanes 15* and *16*). The smaller RGF fragments, GST-RGF61 and GST-RGF83, could only be obtained in small amounts as GST fusions (Fig. 1, *lanes 10* and *11*). On incubation with thrombin under various experimental conditions, the two small RGF fragments were degraded for the most part as measured by densitometric scanning of the SDS gel whereas the two larger fragments were not sensitive to thrombin digestion (Fig. 1, *lanes 12–14*). It was found that particularly GST-RGF83 was tightly associated with a 70-kDa protein (Fig. 1, *lane 10*), because neither by use of a gel filtration column nor by chromatography over Q-Sepharose could this complex be separated. On the addition of 1 mM Mg-ATP to the solution, the protein precipitated. The 70-kDa protein was identified as bacterial DnaK protein by sequencing 9 $NH_2$-terminal amino acids.

*Measurement of the Affinity between H-Ras and RGF*—Raf-RBD acts as a GDI on Ras-nucleotide complexes such that the inhibition of nucleotide release can be used to measure complex formation between Ras and Raf-RBD (10). The binding of Raf-RBD to Rap and R-Ras produces a similar effect on nucleotide release, and the three-dimensional structure of the Rap1A.Raf-RBD complex has shown that this is not due to a steric blockage of the nucleotide binding site but rather due to a number of additional hydrogen bonds between nucleotide and Rap (28).

Fig. 2 shows that the interaction between RGF and Ras proteins also inhibits the dissociation of the nucleotide. This is not in contradiction with the observations of Albright *et al.* (26) where the guanine nucleotide exchange factors activity of RGF toward several Ras proteins had been addressed. Because only

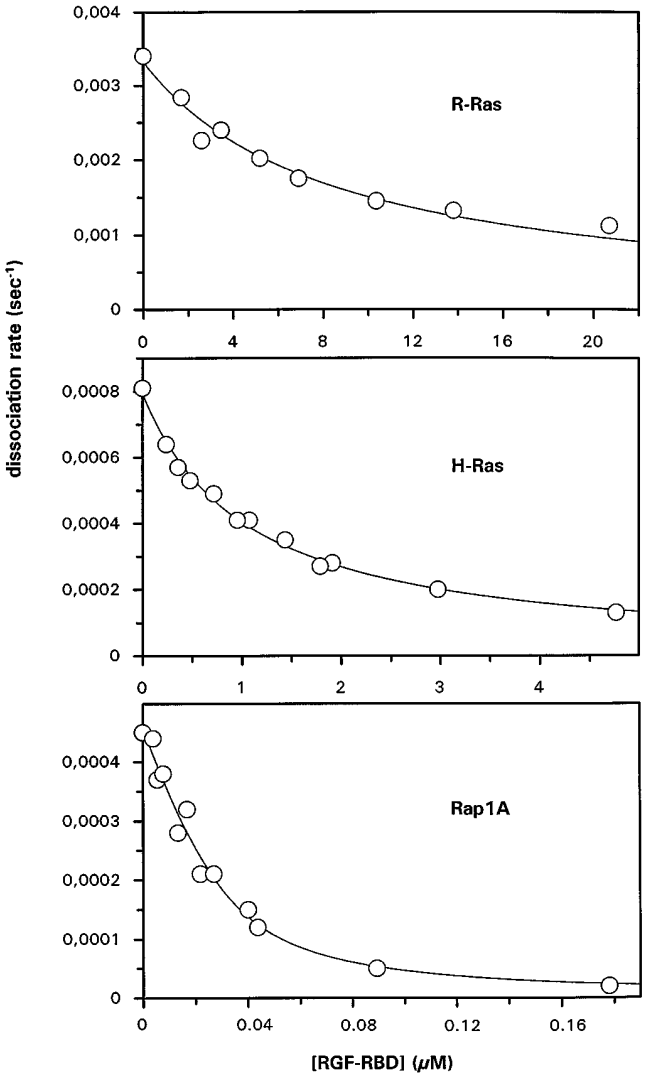
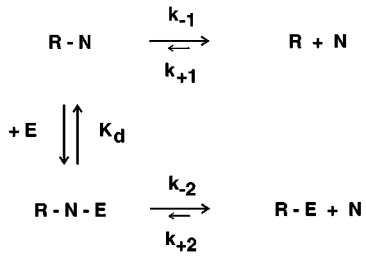



FIG. 2. **Inhibition of the nucleotide dissociation from Ras proteins by the interaction with RGF-RBD.** The dissociation rate of mGpp(NH)p from the Ras proteins (as indicated) was measured in dependence of RGF. The concentrations of H-Ras and R-Ras were 100 nM ,and Rap was at 30 nM. The curves were fitted to the data according to Equation 3 as described in the text.




SCHEME 1.

4% of the protein-bound nucleotide was dissociated, an inhibition would not be detectable. In this work the GDI effect was used as a tool to characterize the different fragments of RGF and to quantify the affinities between Ras and its putative effector molecule RGF. According to Scheme 1 and Equations 2 and 3, the dissociation rate of the nucleotide from the Ras protein depends on the effector concentration. Nucleotide dissociation in the absence of effector molecules is fast compared with the dissociation from the effector complex. Therefore $k_{-2}$ can not be determined in high accuracy, and it was estimated to

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be at least 1 order of magnitude smaller than $k_{-1}$. Whereas in Herrmann *et al.* (10) $k_{-2}$ had been neglected for this reason and the free effector concentration had been calculated iteratively, here the rate constants and $K_d$ are obtained by fitting Equation 3 to the data. With mGpp(NH)p, $k_{-1}$ was found to be 0.0008, 0.00045, and 0.0033 $s^{-1}$ for H-Ras, Rap1A, and R-Ras, respectively. (E = effector molecule, N = nucleotide, R = Ras protein, $[R_0]$ and $[E_0]$ = total concentrations.)

$$K_d = \frac{[R - N]*[E]}{[R - N - E]} \quad \text{(Eq. 1)}$$

$$k_{obs} = k_{-1}*\frac{[R - N]}{[R_0]} + k_{-2}*\frac{[R - N - E]}{[R_0]} \quad \text{(Eq. 2)}$$

$$k_{obs} = k_{-1} - (k_{-1} - k_{-2})$$

$$*\frac{([R_0] + [E_0] + K_d) - \sqrt{([R_0] + [E_0] + K_d)^2 - 4*[R_0]*[E_0]}}{2*[R_0]} \quad \text{(Eq. 3)}$$

The results of these measurements are shown in Table I. The affinities of H-Ras for RGF127 and RGF97 are the same. The fragments containing 83 and 61 amino acids could only be prepared as GST fusion proteins, and they did not inhibit nucleotide dissociation from Ras. As a control GST-RGF97 showed the same inhibition and hence the same affinity to Ras as the cleaved protein RGF97. Because the two smaller fragments are rather unstable and mostly insoluble, it appears that the smallest domain, which is stable enough for expression in *E. coli* and which at the same time shows tight binding to Ras proteins (see also below), contains between 83 and 97 amino acids. In line with this observation, it has been shown by Hofer *et al.* (22) that a fragment of 98 amino acids isolated as a GST fusion protein is sufficient for binding to Ras. All the following experiments have been done with RGF97, which in analogy to Raf-RBD is named the Ras-Binding domain of RGF, RGF-RBD.

The quantitative data on protein affinities reported here and earlier (10) are based on the inhibition of the dissociation of a fluorescently labeled nucleotide from the Ras protein by the effector molecules Raf-RBD and RGF-RBD (Fig. 2). We were able to check the validity of this method by use of an independent assay in the case of the interaction between Ras and RGF-RBD. We used the Ras mutant Y32W in order to measure directly the binding between these two proteins because the RGF fragments do not carry any tryptophans and therefore do not show too much background fluorescence. It has been shown that this Ras mutant has a normal intrinsic and GAP-catalyzed GTPase activity (37) and that it can interact with exchange factors like Cdc25 (38) in the same way as wild type Ras. On the addition of RGF-RBD to a solution of Ras-Y32W·Gpp(NH)p, the fluorescence excited at 295 nm and monitored at 350 nm is significantly decreased as shown in Fig. 3 (data set A). After the binding is saturated, the fluorescence increases linearly with further addition of RGF-RBD due to the fluorescence of tyrosines in this molecule. This increase was also measured in a second experiment where RGF-RBD was added to a buffer solution. The resulting straight line was substracted from the data obtained in the titration experiment (data set A) yielding data set B in Fig. 3. Data set B was fitted according to Equation 3 where the parameters $k_{-1}$ and $k_{-2}$ were replaced by the maximal and minimal fluorescence values, respectively, and data set A was fitted using the same equation plus a linear slope. For RGF127 and RGF97 $K_d$ = 0,7 $\mu$M and $K_d$ = 0,8 $\mu$M were obtained, respectively. In addition the mGpp(NH)p dissociation assay was performed with Ras-Y32W and RGF97. Here $K_d$ = 0.7 $\mu$M was found, which is in good keeping with the result obtained with the method described above.

The observation that the RBDs of Raf and RGF inhibit the dissociation of nucleotides from Ras and Rap can also be used to determine the concentration of active RBD in the protein preparation. The fraction of active RBD protein has been measured at concentrations of Ras-mGpp(NH)p (10 $\mu$M), which is much higher than the $K_d$ value. Under these conditions $k_{obs}$ decreases nearly linearly with increasing RBD concentration, and the data (not shown) can be fitted according to Equation 3 to obtain the active RBD concentration. For these measurements the Ras concentration has been carefully determined by Bradford (32) and HPLC analysis, and for both Raf-RBD and RGF-RBD, the concentrations of active protein were calculated to be 50–70%.

*Affinity of Ras-like GTPases for RGF-RBD and Raf-RBD*—It has been shown before using the yeast two-hybrid system or plate-binding assay with enzyme-linked immunosorbent assay that RGF also interacts with other members of the Ras subfamily such as R-Ras and Rap (21, 23). To get more quantitative data we used the GDI assay to measure these interactions. It turned out that the affinities between RGF-RBD and the different Ras proteins vary over a range of approximately 3

TABLE I
*Dissociation constants of the complexes between the Ras proteins and the Ras binding domains of Raf and RGF*

The values were obtained by measuring the inhibition of the dissociation of mGpp(NH)p from the Ras protein at 37 °C. ND, not determined.

| | | Raf-RBD | RGF127 | RGF97 |
|---|---|---|---|---|
| | | | $\mu$M | |
| R-Ras | Wild type | 1.1 | 7.0 | 6.0 |
| Rap-1A | Wild type | 1.2 | 0.013 | 0.010 |
| | D38A | >100[a] | ND | 40 |
| H-Ras | Wild type | 0.018[b] | 1.0 | 1.0 |
| | D38A | 1.3[b] | ND | >100[a] |
| | Y32W | ND | ND | 0.7 |
| | | ND | 0.7[c] | 0.8[c] |

[a] At concentrations of 100 $\mu$M no inhibition of the nucleotide dissociation was observed.
[b] From Ref. 10.
[c] These values were obtained by titration of Ras(Y32W) with RGF-RBD monitoring the change of the tryptophane fluorescence.

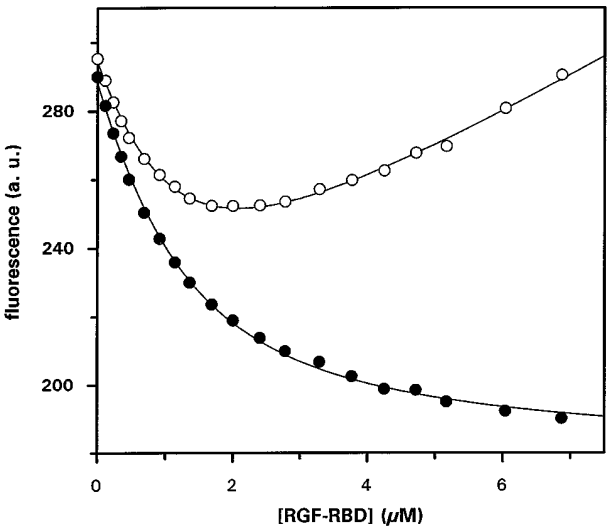



FIG. 3. **Fluorescence titration of the Y32W mutant of Ras *versus* RGF-RBD.** The concentration of Ras(Y32W) in complex with Gpp(NH)p was at 0.8 $\mu$M, and the fluorescence excited at 295 nm was monitored at 350 nm in dependence of RGF-RBD concentration, data set A (○). The fluorescence of the same RGF-RBD concentrations was measured in the absence of Ras(Y32W), and these values were subtracted from data set A yielding data set B (●). Curves were fitted to the data as described in the text.

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orders of magnitude. Whereas R-Ras binds with only micromolar affinity to RGF-RBD, Rap binds very tightly with a $K_d$ of 10 nM, 100-fold tighter than Ras (Fig. 2, Table I). It has been shown before that mutations in the effector region of Ras inhibit binding to Raf (39–41) and to RGF (22). Therefore, we have mutated the effector residue $Asp^{38}$ and show that the D38A mutant of Rap reduces the affinity 4000-fold, an even more drastic reduction than in the Ras-Raf interaction (10). Furthermore, complex formation between RGF-RBD and Rap is GTP-dependent because the affinity drops again 4000-fold when Rap is in the GDP-bound form (data not shown), which we take as further arguments in favor of RGF being an effector molecule of Rap.

Because we were surprised to find such drastic differences in affinities between RGF-RBD and the different Ras family proteins, we wanted to obtain accurate binding constants also for the interaction between Raf-RBD and the Ras family proteins, which have been shown earlier to bind to Raf (8, 23, 27, 36). Using our GDI assay we find that Rap and R-Ras bind less tightly to Raf-RBD than Ras (Table I). It is intriguing to find that the dissociation constants for the interaction of Ras and Rap with Raf-RBD and RGF-RBD are just reversed. Ras binds tightly to Raf-RBD and weakly to RGF-RBD, whereas Rap binds weakly to Raf-RBD and tightly to RGF-RBD, the differences in affinities being approximately 100-fold (Table I). We take this as an indication of high specificities of the different Ras proteins toward their possible effector molecules.

*Competitive Binding of RGF and GAP*—For Raf-RBD or larger $NH_2$-terminal fragments of Raf it was shown that it competes with GAP and NF-1 for the binding to Ras and that it has no effect on the GTPase activity of Ras (7, 8, 35, 41). Here we investigated the competition of RGF with GAP for binding to Ras and with Rap-GAP for binding to Rap. As shown in Fig. 4 the measurements of the GTPase activities of Ras and Rap were carried out in the absence or the presence of catalytic amounts of $GAP^{334}$ and Rap-GAP, respectively, in the presence of saturating concentrations of RGF fragment and in the presence of both RGF and $GAP^{334}$ or Rap-GAP. In all experiments the concentrations of Ras-GTP or Rap-GTP were 50 μM, and the intrinsic GTPase activities were 0.031 $min^{-1}$ and 0.0032 $min^{-1}$, respectively (Fig. 4). This confirms the observations made earlier that the intrinsic GTPase of Rap is approximately 10-fold lower than that of Ras (42). In the presence of 80 μM RGF-RBD, the GTPase rates of Ras and Rap were decreased to 80 and 25% of the intrinsic rates, respectively. Apparently, RGF has a considerable effect on Rap as a GTPase-inhibiting protein, which is negligible on Ras. Subsequently, the effect of RGF-RBD on the GTPase activities catalyzed by $GAP^{334}$ and Rap-GAP was measured (Fig. 4). The concentrations of $GAP^{334}$ and Rap-GAP were chosen to accelerate the GTPase activity of Ras and Rap, respectively, to a level that is still measurable on a minute time scale. The GTPase stimulating activities of $GAP^{334}$ on Ras and of Rap-GAP on Rap were strongly inhibited by the presence of 80 μM RGF-RBD. The GTPase rates found are 0.026 $min^{-1}$ and 0.0010 $min^{-1}$, respectively, and they are very close to the rates measured in the presence of RGF-RBD alone. The GTPase activity of Rap was also measured in the presence of Raf-RBD (data not shown), and there was also a little GTPase-inhibiting effect (60%) and an inhibition of the Rap-GAP activity.

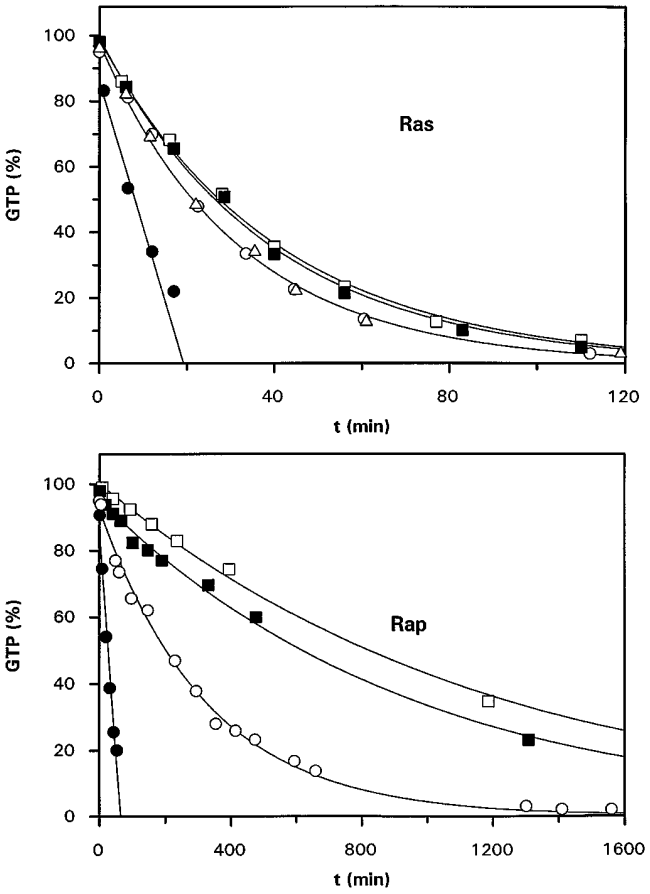



FIG. 4. **The GTPase activity of Ras and Rap in presence of RGF-RBD and GAP.** The GTPase activity of 50 μM Ras (*upper panel*) or Rap (*lower panel*) was measured in the absence (○, □) or the presence (●, ■) of 0.14 μg/ml $GAP^{334}$ or 10 μg/ml Rap-GAP, respectively, and in the absence (○, ●) or the presence (□, ■) of 80 μM RGF-RBD.

## DISCUSSION

Raf kinase has been widely characterized as a target molecule of Ras (4–9) and it was shown that it is the translocation of Raf to the plasma membrane that is essential for its activation (11–13). Other possible Ras-effector molecules such as phosphatidylinositol 3 kinase (18, 19), MEK kinase (20), RGF (21–23), protein kinase Cζ (43), Rsb's (24), and Rin (25) have since been described. Here we have focused our attention toward the Ral guanine nucleotide exchange factor (RGF). Because the effector regions among the members of the Ras protein family are very similar, it is not surprising that Rap and R-Ras were also found to interact with Raf and RGF. However, the proteins of the Ras subfamily have different if not opposing biological functions in the cell. Thus neither wild type nor a GTPase-negative Rap1A can transform cells or lead to the activation of the MAP kinase pathway, and it has in fact been shown that it can inhibit these Ras-mediated functions (44, 45). R-Ras and its homologue TC21 seem to have functions partially overlapping with those of Ras because TC21 has been shown to be transforming (46, 47), whereas R-Ras induces malignant but not morphological transformation (36, 48, 49). Here we report on a quantitative characterization of the interaction between the Ras family proteins and the effector molecules Raf and RGF, respectively, in order to address the question of specificity of the interaction between GTP-binding proteins and their effectors.

We used the inhibitory effect of the effector binding to Ras proteins upon nucleotide dissociation in order to measure the affinities in solution. The results obtained by this method were compared with a direct fluorescence titration experiment that was possible for the interaction between RGF-RBD and the Ras mutant Y32W. The good agreement of the results demonstrate the suitability of the GDI assay used in this work.

The minimal fragment of RGF that is responsible for the interaction with the Ras proteins must be 83–97 amino acids in

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length. Based on the solubility and stability of the RGF97 fragment and its CD and NMR spectroscopic properties,[2] it appears that it constitutes an independent folding domain. In contrast, the GST-RGF83 fusion protein was only poorly soluble, and it was isolated in complex with bacterial DnaK protein, which indicates that this fragment of RGF is most likely at least partially unfolded. On incubation of the protein with thrombin, the RGF83 part turned out to be mostly degraded. All these observations along with the finding that this protein did not inhibit nucleotide dissociation from Ras indicate that these 83 amino acids are not sufficient to constitute the full RBD of RGF. The question of how many amino acids can be truncated from the $NH_2$ terminus without affecting the RBD was not addressed in this work.

We find that the members of the Ras-family, H-Ras, Rap1A, and R-Ras, show greatly varying affinities to the effector molecules Raf kinase and RGF. In the case of H-Ras and Rap1A we find reversal of affinities toward these effectors. Based on these findings we would suggest that in analogy to the high affinity Ras-Raf interaction, RGF is the effector molecule of Rap1A. The negative effect of the D38A mutation on the binding to RGF-RBD, the fact that complex formation is strongly dependent on GTP, and the finding that RGF-RBD competes with Rap-GAP for binding to Rap1A are further arguments in favor of RGF being the effector of Rap1A. It is possible that the binding affinities of the full-length proteins are different from those measured with the isolated domains, but the relative affinities are likely to be the same.

Nothing is known about the function of Rap proteins except that they have been shown to be possible antagonists of Ras-dependent signal transduction (44, 45). In light of the low affinity between Rap and Raf-RBD, which is the direct downstream target of Ras, it seems unlikely that this inhibition of Ras function is due to inhibition of the Ras-Raf interaction by Rap. It might be possible that the affinity between Ras and Raf or between Rap and Raf becomes modulated by processes dependent on extracellular signals such as phosphorylation or translocation. Evidence for such a mechanism has appeared because it has been shown that Rap1A, which is geranylgeranylated at the COOH terminus and is not located in the plasma membrane but in other particulate fractions of the cell (50- 52), becomes translocated to the plasma membrane upon cell stimulation in neutrophils (51) or it is incorporated into the cytoskeleton after thrombin activation (53). Furthermore, because the intrinsic GTPase of Rap1A is 10-fold lower than that of Ras and RGF-RBD slows the GTPase rate of Rap even further, the low affinity between Raf and Rap might still be sufficient to inhibit Ras-dependent function upon overexpression of Rap. It is also possible that Rap inhibits other Ras-mediated pathways such as those leading to cellular transformation by mechanisms that are distinct from the kinase cascade activation (15, 16). A most likely candidate for such a mediator is $GAP^{334}$, which has been shown to bind tightly to Rap1A *in vitro* (42, 54) and to be associated with Rap1B, which has the same effector region as Rap1A, during activation of human platelets (55). In the future it might be useful to use transfection experiments in order to clarify the relation between these Ras proteins and their possible effectors.

Apart from being a possible antagonist of Ras, Rap1A also might have a direct target in cell signaling. Due to the high affinity of Rap1A for RGF, which is the nucleotide exchange factor for Ral, it appears that this function of Rap1A should be mediated by the GTP-binding protein Ral, whose function in the cell is, however, completely unknown at the moment, except that recently the effector of Ral has been found to be a Rho-type GAP specific for Cdc42 and Rac1 (56, 57). To further address this point the intracellular localization of the proteins involved must be investigated.

The structure of the complex between Rap1A and Raf-RBD (28) has shown that Ras should bind to the effector Raf kinase in the same way as Rap1A because the amino acid residues located in the interface are more or less conserved. Because R-Ras and TC21 also have the same residues in the effector region, which is usually defined as residues 32–40, we have to postulate that a core effector region mediates the binding interface between GTP-binding protein and effector and that residues outside this core effector region are responsible for the determination of specificity of these interactions. In contrast, there is no homology in the primary sequence among the different effector molecules, and it will be very interesting to see how these proteins, possibly by having a similar topology in their RBDs, achieve the interaction with the Ras proteins and how they fine tune their specificity.

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