Discriminatory Residues in Ras and Rap for Guanine Nucleotide Exchange Factor Recognition*

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The inability of the S17N mutant of Rap1A to sequester the catalytic domain of the Rap guanine nucleotide exchange factor C3G (van den Berghe, N., Cool, R. H., Horn, G., and Wittinghofer, A. (1997) Oncogene 15, 845–850) prompted us to study possible fundamental differences in the way Rap1 interacts with C3G compared with the interaction of Ras with the catalytic domain of the mouse Ras guanine nucleotide exchange factor Cdc25Mm. A variety of mutants in both Ras and Rap1A were designed, and both the C3G and Cdc25Mm catalyzed release of guanine nucleotide from these mutants was studied. In addition, we could identify regions in Rap2A that are responsible for the lack of recognition by C3G and induce high C3G activity by replacement of these residues with the corresponding Rap1A residues. The different Ras and Rap mutants showed that many residues were equally important for both C3G and Cdc25Mm, suggesting that they interact similarly with their substrates. However, several residues were also identified to be important for the exchange reaction with only C3G (Leu29) or only Cdc25Mm (Gln81 and Tyr46). These results are discussed in the light of the structure of the Ras-Sos complex and suggest that some important differences in the interaction of Rap1 with C3G and Ras with Cdc25Mm indeed exist and that marker residues have been identified for the different structural requirements.

Ras proteins are GTP-binding proteins that cycle between an active GTP bound form and an inactive GDP bound form. Their nucleotide binding state is tightly regulated by guanine nucleotide exchange factors (GEFs), leading to GTP binding, and by GTPase-activating proteins, inducing the transition to the GDP bound state (5, 6). Ras functions as a growth stimulus, but can also, depending on the cell type, induce cell differentiation (7). In contrast to the extensive knowledge on Ras function, relatively little is known of the function of Rap, another member of this GTPase superfamily. Rap and Ras bind to the same set of effector proteins due to their highly homologous effector region (8–10), and Rap has been described to be a suppressor of Ras function, possibly by titrating out Ras effector molecules or localizing them to a different compartment (11–14). It is, however, more likely that Rap has a function independently of Ras, since it has its own specific GEF, is activated by growth factors receptors independent of Ras activation (15–17), activates isoforms of Raf kinase (18–20), and recently Rap1B has been proposed to be a conditional oncogene of its own (21).

The cascade in which Rap1 is functioning still has to be elucidated. These studies are hampered by conflicting results concerning the putative dominant negative mutant Rap1-S17N. One group has described dominant negative effects of Rap1(S17N) on B-Raf activation (19, 20). However, B-Raf activation by Rap1A in other cell types could not be shown in a reporter gene assay, and epidermal growth factor-induced Rap activation could not be inhibited by Rap1(S17N). Furthermore, we showed that the Rap1A(S17N) mutant is unlikely to act as a dominant negative mutant on the C3G-mediated activation of Rap1 in vivo (4), since it does not titrate away the Rap1-specific nucleotide exchange factor C3G in vitro, in clear contrast to the sequestering ability of Ras(S17N) with RasGEF isoforms (22–24). These results suggested that something might be fundamentally different between the interaction of Ras and the RasGEF's Cdc25/Sos and of Rap1A and its exchange factor C3G.

Many studies made use of mutant proteins to show that three regions in Ras are important for the recognition by its GEF: the switch I region (amino acids 25–40), the switch II region (amino acids 57–75), and the stretch of amino acids at position 100–110 of Ras. Since nothing is known so far of the interaction between Rap1 and C3G, we decided to make a comparative mutational analysis of Ras-Cdc25Mm and Rap-C3G interactions. The aim of this study was 1) to identify residues in Rap1A that are important for its recognition by C3G and 2) to compare the Rap1A-C3G with the Ras-Cdc25Mm interaction in order to find similarities and more importantly possible differences. In line with our earlier findings with Rap1A(S17N), our results show that clear differences exist in the way Rap interacts with C3G compared with the Ras-Cdc25Mm interaction. In addition, our study reveals some previously unidentified novel residues as important for the GEF interaction. These results are discussed in light of the recent three-dimensional structure of the Ras-Sos complex (25).

* This work was supported by a European Community BioTech Grant Bio4CT96110. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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¶ Received a Training and Mobility of Researchers-Stipend of the European Community.
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1 The abbreviations used are: GEF, guanine nucleotide exchange factor; Cdc25Mm is the catalytic domain of the mouse Cdc25 Ras guanine nucleotide exchange factor (1) consisting of 285 residues as described earlier (2). C3G is the catalytic domain of the human Rap GEF C3G (3) consisting of 249 residues as described earlier (4). Mutations are indicated with the original and modified amino acid using the single letter code, such that Rap(Y40C) designates Rap where tyrosine 40 has been replaced by cysteine. mGDP is the fluorescent analogue of GDP analogue carrying the N-methylanthraniloyl (m) group on the 2'- or 3'-hydroxy group of the ribose.

2 C. Block, unpublished results.
3 J. L. Bos, unpublished results.
Expression and Purification of Proteins—Purification of Rap1A, Rap2A, and Ras proteins was done as described before (4, 26). The catalytic domain of Cdc25Mm consisting of 285 amino acids was purified as described before (4).

Protein-nucleotide complex was incubated at 20 °C (Ras, 100 μM) or 25 °C (Rap1A and Rap2A, 200 μM) in standard buffer (50 mM Tris-HCl, pH 7.5, 5 mM dithioerythritol, and 5 mM MgCl₂) with the indicated amount of GEF protein and an approximately 200-fold excess of unlabeled GDP. The decrease of fluorescence was monitored in a Perkin-Elmer LS50 fluorescence spectrophotometer or Fluoromax-2 from Jobin Yvon-Spex, with excitation and emission wavelengths of 366 and 450 nm, respectively. The kₗₒ were obtained after fitting the data to a single exponential function using the program Grafit (Erithacus Software). We observed that in the prolonged incubations with C3G the stimulation of the exchange rate tended to decrease. We interpret this as a slow inactivation of C3G. Analysis of these reactions by different fitting procedures resulted in similar dissociation rates with approximately 20% deviations.

RESULTS

The use of Ras(S17N) as a dominant negative mutant of normal Ras is well documented and is due to the low affinity of the mutant for guanine nucleotide resulting in the relative (to nucleotide binding) tight association with Cdc25-type of Ras guanine nucleotide exchange factors. The Rap1A(S17N) mutation also has a low affinity for guanine nucleotide but fails to inhibit the C3G-mediated guanine nucleotide exchange reaction on wild-type Rap1A in vitro. This lack of titration of C3G by Rap1A(S17N) in vitro (4) prompted us to investigate whether the activation of GEF on Rap by C3G might be different from the Ras-Cdc25Mm (or the Ras-Sos) interaction. For that purpose we designed different mutations in Ha-Ras, Rap1A, and Rap2A in those regions that have been described to interact with Ras-specific GEFs containing the Cdc25 homology region (30–40): the switch I, the switch II, and the region encoding residues 100–110 (Fig. 1). The recent structure determination of the Ras-Sos binary complex has confirmed that these regions are indeed important for the interaction, with residues 37–62 encompassing switch II being the most important (25). The mutations were chosen such that 1) highly conserved amino acids were mutated, or 2) residues in Ras were changed to those found in Rap1A and vice versa, or 3) residues in Rap2A were converted into those of Rap1A (see Fig. 1). After loading the mutant proteins with the fluorescently labeled nucleotide mGDP, the dissociation of nucleotide in the presence of a large amount of unlabeled GDP was measured using the catalytic domains of Cdc25Mm (Cdc25Mm from now on) and of C3G (C3G from now on). These domains were shown before to be large enough to comprise the actual catalytic domain of the Cdc25-like GEFs, in line with the structure determination (25). The decrease of fluorescence was followed to calculate the reaction rates which were compared with the noncatalyzed reaction rates and scored as x-fold stimulation (Figs. 2, 3, and 4 and Tables I–III).

Switch I—The core effector region, defined as residues 32–40, overlaps with switch I and is identical between Ras and Rap1A, but residues outside this region such as 31, lysine in Rap1A and glutamic acid in Ras, have been shown to be responsible for the specificity with effectors (9, 26, 41–44). We made similar mutations in the effector region of both Ras and Rap1A and studied their effect on the interaction with the GEFs. K31E in Rap1A has a weak effect and appears to stimulate the reaction, whereas T38S in both Ras and Rap1A affected the GEF activity of C3G or Cdc25Mm 2–5-fold. Unlike the interaction with effectors (9, 26, 41–44) and with GTPase-activating protein (41–43), residue 31 is not responsible for the specificity of different GEFs. Other amino acids seem more important for both GEFs: D38A strongly reduces both C3G- and Cdc25Mm-stimulated mGDP release, whereas the E37G mutation shows a weaker effect (Fig. 2A). The Y40C mutation has dramatically different effects on the C3G-Rap versus Cdc25Mm-Ras interaction. Rap1A(Y40C) does not affect the GEF activity of C3G (Fig. 2A), but the same mutation in Ras induces a 9-fold higher activity of Cdc25Mm (Table II). The Y40C mutation also has a strong effect on the specificity of Cdc25Mm as shown by the 6-fold stimulation of Cdc25Mm on Rap1A(Y40C) (Fig. 3A), whereas nucleotide exchange on WT-Rap1A is not stimulated.

Switch II—It has been shown before that residues in the switch II region of Ras between Gln41 and Asn41 are crucial for the interaction with Ras-specific GEFs, since mutations such as E62H, E62K, E63H, and E63K are completely insensitive toward Sdc25 and Cdc25 (31, 33, 34). The structure of the Ras-Sos binary complex has also indicated that residues in the switch II region are responsible for the interactions disturbing the phosphate moiety of the nucleotide binding site. Here we designed most mutations within the switch II region of Rap1A or Ras such that the amino acid of Ras was introduced in Rap1A and vice versa. In general we find that residues in the switch II region of Rap1A such as Phe64 and Leu70 are similarly important for the catalytic interaction with C3G, whereas Thr61 is not.

Two amino acids were strikingly different in their interaction with C3G versus Cdc25Mm. Mutation of T61 of Rap1A to Q

| switch I region | 25 | 30 | 35 | 40 |
|-----------------|----|----|----|----|
| K-Ras           | 2  | 2  | 2  | 2  |
| hsRap1A         | 2  | 2  | 2  | 2  |
| hsRap2A         | 2  | 2  | 2  | 2  |

| switch II region | 60 | 65 | 70 | 75 |
|------------------|----|----|----|----|
| K-Ras            | 2  | 2  | 2  | 2  |
| hsRap1A          | 2  | 2  | 2  | 2  |
| hsRap2A          | 2  | 2  | 2  | 2  |

| region 100-110  | 100 | 105 | 110 |
|-----------------|-----|-----|-----|
| K-Ras           | 2   | 2   | 2   |
| hsRap1A         | 2   | 2   | 2   |
| hsRap2A         | 2   | 2   | 2   |

FIG. 1. Sequence alignment of Ha-Ras, Rap1A and Rap2A with respect to their switch I, switch II, and the α3 regions. The asterisk indicates the different mutations used in this study.
Cdc25Mm, whereas C3G does not need Thr 61. This idea was type or mutant 200 nM Rap1A, mutated in switch I (as in Ras) did not influence the activity of C3G (Fig. 2B) but was found to introduce a recognition site for Cdc25Mm in Rap1A (Fig. 3A). This suggests that Gln51 is required for activation by Cdc25Mm, whereas C3G does not need Thr61. This idea was strengthened by the finding that Ras(Q61T) and Ras(Q61L) are both poor substrates for the Cdc25Mm-catalyzed mGDP release (Fig. 3B and Table II) in line with earlier observation on Sdc25 (31). Introduction of the corresponding Rap1A residue at position 61 to produce Ras(Q61T) did not induce C3G activity, again showing that this residue is unlikely to interact with C3G. When trying to shift the specificity of C3G and induce a reactivity toward Ras, we find that the introduction of Rap-to-Ras mutations in residues 63, 64, and 65 in Rap(T61Q) does not increase its sensitivity toward Cdc25Mm (Table I).

Mutations of residue 70 point to another difference in the recognition sites for C3G and Cdc25Mm. Rap1A(L70Q) completely blocks the GEF activity of C3G (Fig. 2B, Table I), indicating that Leu70 is important for the interaction of Rap1A with C3G, and Gln is unable to substitute the function. This was supported by the finding that Ras(Q70L) is a much better substrate for C3G than wild-type Ras (Fig. 3B, Table II). In contrast, mutation of residue 70 does not affect the interaction of Cdc25Mm with Rap1A and even increases the activity toward Ras(Q70L) almost 2-fold, indicating that residue 70 has an important role in the interaction with C3G but not as much with Cdc25Mm. Remarkably, a leucine at position 70 appears to be favorable for both reactions. Mutations of other switch II residues in Ras showed that, unlike the mutations E63H and E63K, E63A shows only a 2-fold decrease of the stimulatory action of Cdc25Mm on Ras. Furthermore the E63D mutation in Ras induces a 2-fold higher activity in the Cdc25Mm reaction than wild-type, similar to Q70L.

**Mutations in the 100–110 Region**—Mutations of residues in the 100–110 region have similar effects on the Rap1A or Ras exchange reaction: mutation of the variable residue at position 101 had no obvious effect, whereas mutation of the conserved Arg102 to leucine blocked the GEF activity with either C3G or Cdc25Mm. Sequence alignment shows the most obvious sequence difference lies in the Rap-DTED motif versus DSDD in Ras (residues 105–108, Fig. 1). Introduction of DTED into Ras had no effect on catalysis by either Cdc25Mm or C3G. However, this same stretch of amino acids introduced into Rap2A did have a major impact on recognition by C3G (see below and Fig. 4).

**Rap2A Mutants**—Previously we had shown that despite the high level of homology between Rap1A and Rap2A, C3G specifically recognizes Rap1 as a substrate and not Rap2, even with high concentrations of the GEF (4). As expected Rap2A is also not recognized by Cdc25Mm. Therefore we made mutations in Rap2A, in which we replaced residues in Rap2A in the switch II and 100–110 regions, by the corresponding residues of Rap1A. Due to the small amount of variable residues, we could design Rap2A to almost completely look like Rap1A and study whether C3G was now able to recognize the chimeric Rap2A. Interestingly, a double mutation of residues 65 and 66 (switch II region) already resulted in the 11-fold stimulation of the nucleotide release by 1 μM C3G (Table III). A similar effect was observed when the differing residues in region 100–110 were mutated (105 + 106 + 108 = DTED). Even more dramatic was the combination of these two regions: the guanine nucleotide exchange of this Rap2A mutant could now be stimulated 83-fold by 1 μM C3G. These results clearly show that both the Switch II and the 100–110 regions are necessary for efficient activation by C3G and that they synergistically contribute to the catalytic reaction. Surprisingly, this Rap2A mutant is now also a weak substrate for Cdc25Mm, in contrast to wild-type Rap1A or Rap2A. Considering the alignment of residues in the three regions studied no easy explanation for this observation is available. The most drastic difference between Rap1A and Ras versus Rap2A appears to be residue 39, which is Ser in Ras and Rap1A and Phe in Rap2A (Fig. 1), and is located adjacent to the Tyr40 shown to be crucial for the Ras-Cdc25Mm reaction.

**DISCUSSION**

In the past years, the interaction of Ras and its nucleotide exchange factors Cdc25/Sos/Sdc25 has been extensively studied by mutagenesis (30–40). From these studies three regions in Ras were found to contribute to the GEF-mediated nucleotide release. The three-dimensional structure of the Ras-Sos complex, which we assume to be similar to the Ras-Cdc25 complex in its basic features, has principally confirmed the involvement of these three regions in complex formation, with residues 60–73 supplying the most important contacts (25). In this study we make a mutational analysis of these three regions and compared Ha-Ras and Rap1A residues that are needed for the interaction with their GEF. On a global scale we find that C3G, for which a fold similar to Sos/Cdc25 can be assumed due to the albeit limited but significant sequence conservation, is likely to interact with Rap in a similar way as Ras with Sos or Cdc25Mm, since most mutations in these three regions affect the catalytic reaction. Although only limited sequence specific contacts between the switch I area of Ras and Sos have been identified in the structure, residues of switch I such as Thr36 and Asp38 are found biochemically to be important both for the Ras-Cdc25Mm and the Rap1-C3G catalytic reaction. Ras residues Tyr32, Pro34, Glu37, and Tyr40 were found to directly interact with Sos,
Wild-type and mutant Rap1A proteins were loaded with the fluorescent GDP analogue mGDP and the dissociation of the analogue in the presence of a large excess unlabelled GDP in the absence (intrinsic rate) or presence of the indicated concentrations of GEF was followed spectrophotometrically as described under "Materials and Methods." Rates are given in units of s$^{-1} \times 10^{-5}$.

| 200 nM Rap1A | Intrinsic | +50 nM C3G | Fold stimulation | +1 μM Cdc25Mm | Fold stimulation |
|---------------|-----------|------------|------------------|--------------|----------------|
| s$^{-1} \times 10^{-5}$ | s$^{-1} \times 10^{-5}$ |
| Wild-type     | 3.6 ± 1.8 | 109.0 ± 23.0 | 30× | 2.3 ± 0.8 | 1× |
| K31E          | 3.3 ± 2.5 | 130.2 ± 21.6 | 15× | 6.3 ± 0.4 | 1× |
| T35S          | 2.2 ± 0.2 | 33.8 ± 6.9 | 8× | 2.4 ± 0.1 | 1× |
| E37G          | 4.5 ± 0.3 | 34.2 ± 11.1 | 1× | 4.6 ± 0.2 | 1× |
| D38A          | 3.7 ± 1.9 | 4.6 ± 1.8 | 1× | 1.8 ± 0.1 | 1× |
| Y40C          | 5.0 ± 0.1 | 104.6 ± 2.6 | 21× | 30.6 ± 1.9 | 6× |
| T61Q          | 4.7 ± 2.6 | 117.3 ± 0.6 | 25× | 16.0 ± 2.6 | 3× |
| F64Y          | 1.3 ± 0.2 | 4.8 ± 0.8 | 4× | 1.8 ± 0.1 | 1× |
| Q63E/Q64Y     | 2.2 ± 0.8 | 2.4 ± 0.1 | 1× | 1.6 ± 0.6 | 2× |
| T61Q/Q63E/F64Y| 3.2 ± 1.3 | 3.9 ± 1.1 | 1× | 10.4 ± 2.4 | 3× |
| L70Q          | 1.8 ± 0.1 | 3.5 ± 0.4 | 2× | 2.1 ± 0.2 | 1× |
| T61Q/L70Q     | 1.7 ± 0.1 | 19.9 ± 2.1 | 12× | 4.1 ± 0.2 | 2× |
| N74T          | 2.8 ± 0.6 | 66.3 ± 13.8 | 24× | 3.0 ± 0.1 | 1× |
| L101K         | 2.8 ± 1.7 | 67.1 ± 29.7 | 22× | 2.3 ± 0.1 | 1× |
| R102L         | 2.6 ± 1.0 | 3.9 ± 1.8 | 2× | 2.6 ± 0.5 | 1× |

Wild-type and mutant Ha-Ras proteins were treated as described in Table I.

| 100 nM Ha-Ras | Intrinsic | +50 nM Cdc25Mm | Fold stimulation | +1 μM C3G | Fold stimulation |
|---------------|-----------|----------------|------------------|------------|----------------|
| s$^{-1} \times 10^{-5}$ | s$^{-1} \times 10^{-5}$ |
| Wild-type     | 1.3 ± 0.3 | 102 ± 6 | 78× | 2.4 ± 0.8 | 2× |
| T35S          | 0.6$^a$  | 23$^a$ | 38× | Not done |  |
| E37G          | 2.9$^a$  | 64$^a$ | 29× | Not done |  |
| D38A          | 0.4$^a$  | 4$^a$  | 10$^a$ | Not done |  |
| Y40C          | 4.4$^{5/0}$ | 2558$^a$ | 581$^a$ | 9.5 ± 0.7 | 2× |
| Q61L          | 2.3 ± 0.2 | 11.8 ± 0.3 | 5× | 4.5 ± 0.3 | 2× |
| Q61T          | 1.3 ± 0.2 | 12.1 ± 0.2 | 9× | 2.1 ± 0.1 | 2× |
| E63A          | 2.0 ± 0.1 | 45.0 ± 0.3 | 22× | 7.3 ± 3.0 | 4× |
| E63D          | 1.4 ± 0.2 | 184 ± 10 | 131× | 2.7 ± 0.3 | 2× |
| Q70L          | 1.2 ± 0.6 | 174 ± 13 | 145× | 16.5 ± 4.3 | 14× |
| T74N          | 1.5 ± 0.3 | 46.1 ± 1.2 | 31× | 2.2 ± 0.5 | 2× |
| K101L         | 1.0 ± 0.1 | 65.3 ± 15.3 | 65× | Not done |  |
| R102L         | 0.4 ± 0.1 | 2.1 ± 0.2 | 5× | 0.7 ± 0.1 | 2× |
| S106T/D107E   | 1.2 ± 0.3 | 97.4 | 81× | 2.8 ± 0.6 | 2× |
| D154A         | 0.5 ± 0.1 | 43.4 ± 0.4 | 87× | Not done |  |

$^a$ These data (see Footnote 4 in text) were obtained under somewhat different conditions, using 3mGDP, and 40 mM Hepes · NaOH, pH 7.5, 10 mM MgCl$_2$, 5 mM dithioerythritol. The intrinsic dissociation rate of WT-Ras under those conditions was 0.7 × 10$^{-5}$ s$^{-1}$, and the stimulated rate 67-fold faster.

Wild-type and mutant Rap2A proteins were treated as described in Table I.

| 200 nM Rap2A | Intrinsic | +1 μM C3G | Fold stimulation | +1 μM Cdc25Mm | Fold stimulation |
|---------------|-----------|------------|------------------|--------------|----------------|
| s$^{-1} \times 10^{-5}$ | s$^{-1} \times 10^{-5}$ |
| Wild-type     | 3.3 ± 0.8 | 6.1 ± 0.8 | 2× | 2.9 ± 0.3 | 1× |
| A657/S66A     | 2.2 ± 0.2 | 23.5 ± 1.5 | 11× | 2.4 ± 0.4 | 1× |
| R105D/Y106T/K108D | 3.5 ± 1.6 | 44.7 ± 10.6 | 13× | 4.0 ± 0.5 | 1× |
| A657/S66A/R105D/Y106T/K108D | 2.7 ± 0.1 | 225.2 ± 2.2 | 83× | 13.4 ± 1.2 | 5× |

Table I: GEF-catalyzed guanine nucleotide exchange on Rap1A

Table II: GEF-catalyzed guanine nucleotide exchange on Ha-Ras

Table III: GEF-catalyzed guanine nucleotide exchange on Rap2A

Gln$^{25}$, Val$^{29}$, Glu$^{31}$, Asp$^{33}$, Thr$^{35}$, and Ser$^{39}$ were positioned at the interface between Ras and Sos (25). These data fit with our observations. Studying the effect of switch I mutations is additionally important since T35S, E37G, or Y40C are extensively used as partial loss-of-function mutations in the study of Ras pathways (45–50), and their interactions with GEF have only partially been analyzed.

In this paper we show that the T35S and E37G mutations similarly reduce the interaction of Ras and Rap1A with their respective GEFs by a factor of 2–4. As noticed before, Y40C proved to be a particularly interesting mutation in that it strongly increases the Cdc25Mm-mediated effect on Ras, decreased the specificity of Cdc25Mm toward Rap1A by inducing partial reactivity, but did not influence the Rap1A-C3G interaction itself. Tyr$^{40}$, which is conserved between Ras, Rap1A and Rap2A, contacts His$^{31}$ in the Ras-Sos structure and forms an interaction mediated between the amino and the aromatic group (Fig. 5) (52). Sequence alignment between Sos, Cdc25Mm, and C3G (Fig. 6) shows that His$^{31}$ is homologous to Arg$^{135}$ in Cdc25Mm and Ser$^{966}$ in C3G. Whereas Arg could still form an amino-aromatic interaction with Tyr$^{40}$ in a similar way, the nature of a Tyr-Ser interaction would appear to be different, indicating a structural difference in the interface of the Ras-Sos/Cdc25Mm versus the Rap-C3G interaction. How the replacement of Tyr$^{40}$ by Cys leads to an increase in the reaction rate needs to be analyzed in more detail, since the overall reaction involves a number of partial reactions. A possible explanation

$^a$ R. H. Cool, G. Schmidt, C. Lenzen, H. Prinz, D. Vogt, and A. Wittinghofer, submitted for publication.
could be a decrease in the affinity of the binary Ras-Sos/Cdc25 complex. The use of Ras(Y40C), which is believed to interact mostly with PI(3)kinase but not with Raf and RalGEF, thus introduces an additional complication into unraveling signal transduction pathways via Ras, as it has a drastically increased reactivity toward Cdc25Mm, and possibly also Sos, and would thus be expected to be activated preferentially over wild-type Ras or other Ras mutants. Our study clearly show that the exchange factor interaction is, depending on the mutation, strongly or weakly disrupted by the partial loss-of-function mutations. Consequently, the GTP loading of Ras effector mutants in vivo is likely to be variable between the partial loss-of-function mutations, which should be taken into account considering the observed biological effects, which may need a different threshold concentration of activated Ras-GTP.

The structure of the Ras-Sos complex and the number of mutations and their effect on catalysis have shown that the switch II region of Ras is the most crucial site of the Ras-GEF interaction (25, 31, 33, 34; this paper). Sos interacts directly with Asp57, Ala59, Gly60, Gln61, Glu63, Tyr64, Ala66, Met67, Gln70, Tyr71 of the switch II region and Thr58, Glu62, Ser65, Arg68, and Asp69 are close to the interface (25). The data presented here confirm and extend the importance of some of these residues for both the Ras and the Rap system and also show significant differences. The nature of residue 61 in Rap1A was found to be important Cdc25Mm but only marginally so for C3G (Fig. 5). The Ras-Sos structure shows that Gln61 is involved in a donor hydrogen bond to Thr935 and the main chain CO of Gly931 of Sos and accepts a H-bond from Tyr912 from Ras and that Tyr71 is embedded in a hydrophobic pocket formed by residues in Ras and Sos and also forms a direct hydrogen bond to Tyr931. Thr935 is highly conserved in RasGEFs (Fig. 6) such as human RasGRF1 and 2, Sos, and in the Rap-specific GEFs RalGDS and Rgl (but not Rlf) and is found as Ser in CDC25 and SDC25 from yeast. The residue analogous to Thr935 in the Rap-specific GEF C3G is Gln690, and in Rap Gln61 is replaced by Thr, producing an analogous exchange in the interacting pair of Gln and Thr. Since Tyr71 is conserved between Ras and Rap proteins, the hydrogen bond network seen in the Ras-Sos structure would be similar in the Rap-C3G binary complex. The mutational data suggest, however, that either this network does not exist in the Rap-C3G complex or that its contribution to catalysis is distinctly different from the Ras-GEF interaction, maybe due to the different nature of the hydrophobic pocket surrounding Tyr71.

Mutation of residue 63 in Ras from Glu to His or Lys has been reported to abolish the interaction with yeast exchange factors SDC25 and CDC25 and with SOS (31, 33, 34, 40). Our results here show that E63A only slightly affects the interaction of Cdc25Mm, whereas E63D even weakly increases the activity. The Ras-Sos structure showed that Arg826 from Sos directly interacts with Glu63, and the single and double mutants in Rap1A suggest that residues 63 and 64 are both important for the Rap1A-C3G interaction. No mutational analysis of residue 64 in Ras has been performed, but the Ras-Sos structure shows a hydrophobic interaction of Tyr64 with residues Ile825 and Phe929 from Sos. From our Rap2A mutant proteins we can conclude that at least one of the residues Thr935 or Ala66 is important for recognition by C3G. In the Ras-Sos structure these residues are located in the interface without making any direct contact pointing at an indirect effect of the mutations or at a difference with the Rap-C3G interaction.
FIG. 5. Structural summary of the mutational analysis. A–C, surface representation of Ras\textbullet}GppNHp (53) (A) and Rap1A\textbullet}GppNHp (B), taken from the structure of the Rap1A\textbullet}RafRBD complex (51), and of nucleotide free Ras from the Sos\textbullet}Ras complex (25). Residues colored red indicate residues which effect catalysis (bright red, \( \approx 2 \)-fold; normal red, 2–5-fold; and dark red, \( \approx 5 \)-fold effect on catalysis by the corresponding GEF. Residues in blue are important for both Cdc25\textsuperscript{Mm} and C3G and in green only for Cdc25\textsuperscript{Mm} (A) or C3G (B). The yellow area in C indicates the Ras-Sos interface as determined from the structure (25). D is a stereo plot of the Ras-Sos interface around residues Tyr\textsuperscript{40} in switch I and Gln\textsuperscript{61} and Gln\textsuperscript{70} in switch II of Ras and the interacting residues from Sos.
Finally, we also studied mutants of amino acid 70 in both Ras and Rap1A. This proved to be very interesting, since the nature of residue 70 was found to be very important for C3G, but not for Cdc25Mm. In the Ras-Sos structure, Gln70 interacts with Ser76 in Sos, a conserved residue in Sos, Cdc25Mm, and C3G, and Rap1A. This proved to be very interesting, since the nature of residue 70 was found to be very important for C3G, but not for Cdc25Mm or vice versa. For the region encoding amino acids 100–110, we show that the single mutations of residue 101 in both Ras and Rap1A does not interfere with the GEF activity. This mutation had only been tested in combination with other mutations in Ras before (37, 38). Residue 102 is, however, important for recognition both by the Ras- and Rap1A-specific GEFs, supporting the structural observation where Arg102 directly interacts with Sos (25). The structure also revealed that Asp105 was located in the interface and that other amino acids from this region did not contribute to the interaction. Our mutant analysis showed residues 105, 106, and 108 to be important for the C3G-Rap1A, but not equally important for the Ras-Cdc25Mm interaction, although point mutations have not been tested.

We thus obtained evidence that many mutations have similar effects on the Ras and Rap exchange reaction, whereas others are important for C3G, but not for Cdc25Mm or vice versa. Ras and Rap have the same three-dimensional structure (9, 53), and C3G and Cdc25 most likely have a fold very similar to Sos (25). When the Ras and Rap mutations affecting catalysis are plotted onto the surface of Ras and Rap, they are all localized in what would be the interface of the Ras-Sos structure, as shown in Fig. 5. The color-coding pattern, which is related to the importance of residues for the exchange reaction, indicates that the different GTP-bonding proteins and their respective GEFs interact with each other using the same overall interface but different specific interactions which supports the notion that residues on Sos involved in the interaction with Ras are not conserved in all proteins with this topology, not even between Sos and Cdc25Mm, much less even between Sos and C3G. We thus would expect that differences exist in the kinetic and molecular mechanism of the GEF-catalyzed nucleotide exchange reaction of Ras with either Sos and Cdc25Mm and between Cdc25Mm and C3G. Our analysis has only measured the overall multipleturnover reaction which consists of several kinetic steps (2, 31, 54). A definite model for the mechanism of guanine nucleotide exchange can thus only be derived by solving the structures of the respective binary (or possibly ternary) complexes and from a more detailed kinetic analysis.

It was interesting to observe that certain mutations such as Y40C or Q70L increase the overall rate of the exchange reaction, indicating that the GEF-catalyzed exchange reaction is not optimized for fast exchange and that slight modifications of the interface can considerably increase the overall catalytic rate. We have observed before that the affinity of Cdc25Mm for Ras-GDP is in the 400 μM range (2), which is probably low enough to prevent Cdc25Mm from accidentally activating Ras and inducing futile GTPase cycles. The GEF reaction on Ras is initiated by the recruitment of GEF to the plasma membrane where Ras is located, which would mean that the catalytic efficiency in solution is kept low and explains the likelihood of finding mutants in either the GEF (55) or in the GTP-binding proteins (this paper) that increase the overall rate of the reaction.

Finally our results show that the mechanistic details of nucleotide exchange might be sufficiently different between the Ras and the Rap system such that extrapolating from one system to the other is not suitable. This was demonstrated by the finding that contrary to Ras(S17N) on Cdc25Mm and Sos, Rap(S17N) is not inhibitory to the C3G-mediated guanine nucleotide exchange reaction on Rap1A (4). Since Rap(S17N) has been used to delineate the effect of Rap1 on B-Raf and MAP kinase activation in PC12 cells, this biological activity of Rap1 is unlikely be mediated via the C3G pathway (20, 21). Two new Rap1-specific GEFs have very recently been cloned (56, 57), but the effect of Rap(S17N) on these still needs to be tested. For the C3G-dependent pathway it will be necessary to find dominant negative mutants of Rap by an approach, unbiased by the findings for Ras and other GTP-binding proteins, with either site-directed mutagenesis in the regions outlined here or by a random approach. Such a mutant would be extremely important to outline the signal transduction pathway of Rap1 via C3G, in a similar way as Ras(S17N) has been used to map Ras signal transduction pathways.

Acknowledgment—We thank Doro Vogt, Jochen Linz, Astrid Kramer, and Christiane Thesis for purification of proteins, Michael Hess for help with graphical presentation, and Rita Schebaum for secretarial assistance. We are grateful to Jean de Gunzburg for the Rap2A expression vectors and John Kuriyan for kindly supplying the coordinates of the Ras-Sos complex.

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FIG. 6. Alignment of residues from the guanine nucleotide exchange factors hSos1, Cdc25Mm, and human C3G in those areas that are important for the interactions with GTP-binding proteins as mentioned in the text.
