Guanine Nucleotide Binding Properties of Rac2 Mutant Proteins and Analysis of the Responsiveness to Guanine Nucleotide Dissociation Stimulator[†,⊥]

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ABSTRACT: The Rac GTPases are currently being subjected to intensive study due to their involvement in a wide array of cellular phenomena. Many studies of Rac function have relied upon the use of relatively uncharacterized Rac dominant active, dominant negative, and effector domain mutants on the basis of the analogy to Ras structure. We have generated and purified such Rac2 mutants and characterized their guanine nucleotide binding properties *in vitro*. The Rac2 G12V and Q61L activating mutations were shown to hydrolyze bound GTP very slowly and were unresponsive to p190 Rac GTPase-activating protein. Distinct differences in the kinetics of nucleotide binding to individual mutant proteins were observed, accounting for the behavior of these proteins in biological assays. The structural features required for the responsiveness of Rac2 to the guanine nucleotide exchange protein smgGDS were examined. We show that guanine nucleotide exchange by smgGDS is dependent upon intact switch 1 and switch 2 regions in Rac2. Functional interactions between the switch 1 and switch 2 regions and the G12V mutation of Rac2 are described. These data form the basis for rational use of Rac mutants in biological studies.

The members of the Rho family of GTP binding proteins are important regulators of both normal and abnormal cell function. These closely related GTPases (Rho, Rac, and Cdc42) have been implicated in cellular motile events, inflammatory responses, transformation, and tumor metastasis. The Rac proteins in particular regulate the formation of filamentous actin structures associated with lammelipodia and membrane ruffles (Ridley, 1992, 1996), the formation of toxic oxygen metabolites used by phagocytic leukocytes for the purpose of bacterial killing (Bokoch, 1995; Knaus et al., 1991), and are necessary for the transforming effects of the Ras oncogene (Qui et al., 1995). A family of p21-activated kinases (Paks) have been shown to be direct targets of Rac and Cdc42 in their GTP-bound forms (Manser et al., 1994), and these Ser/Thr kinases appear to mediate some of the effects of Rac on mammalian cell function. The involvement of Pak(s) in regulating phagocyte oxidant production (Knaus et al., 1995), actin assembly (M. A. Sells, U. G. Knaus, S. Bagrodia, D. Ambrose, G. M. Bokoch, and J. Chernoff, unpublished observations), and the activity of the stress-activated MAP kinases (p38 and Jun kinase) (Zhang et al., 1995; Bagrodia et al., 1995) has been reported.

The mechanisms by which Rac proteins are activated by hormone and chemoattractant receptors have not been well defined. Proteins able to modulate the GTP/GDP state of Rac have been identified (Ridley, 1995; Boguski & McCormick, 1993). These include a number of potential negative regulatory proteins which stimulate the hydrolysis of GTP by Rac (GTPase-activating proteins or GAPs). Currently, only the Bcr Rac GAP, implicated in regulating Rac activity associated with NADPH oxidase activation and the responsiveness to inflammatory stress in a mouse transgenic model (Voncken et al., 1995), has been shown to regulate Rac activity under physiological conditions. In contrast to the relatively large number of Rac GAPs, only two proteins have been identified as being capable of activating Rac by stimulating the exchange of GTP for GDP. The Rac guanine nucleotide exchange factors (GEFs) include smgGDS, a 56 kDa protein capable of stimulating guanine nucleotide dissociation on a number of low-molecular mass GTPases (Yamamoto et al., 1990; Kaibuchi et al., 1991; Hiraoka et al., 1992; Chuang et al., 1994). More recently, the Tiam1 protein, which was originally identified on the basis of its ability to regulate the metastatic capability of T cell tumors, has been shown to be a Rac GEF (Habets et al., 1994; Michiels et al., 1995).

Many studies of Rac function in cells have relied upon the generation of Rac dominant active, dominant negative, or effector domain mutants. Very little biochemical characterization of the guanine nucleotide binding and hydrolysis properties of such Rac mutants has been reported however (Menard et al., 1992; Knaus et al., 1992). These modified Racs were prepared by mutating Rac1 or Rac2 at amino acid residues previously shown to produce such phenotypes in the mammalian Ras proteins (Barbacid, 1992; Der, 1989; Grand & Owen, 1991; Krengel et al., 1990; Marshall, 1993). In an earlier study (Xu et al., 1994), we demonstrated the inability of Rac mutations in the classical Ras effector domain (amino acids 32−40) to activate the phagocyte

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NADPH oxidase or actin assembly in fibroblasts. Analysis of these Rac mutants revealed the existence of a second "effector" domain required for GAP responsiveness. In the present study, we provide a detailed characterization of the guanine nucleotide binding and hydrolysis capabilities of a series of commonly utilized Rac2 mutants and use these proteins to characterize the structural basis for regulation of Rac by the guanine nucleotide exchange factor smgGDS.

METHODS

Preparation of Proteins. Rac mutants were prepared and isolated as described in Xu et al. (1994). smgGDS was prepared as in Chuang et al. (1994). GST−smgGDS was prepared by transformation of *Escherichia coli* strain DH10B (Gibco BRL) and induction of protein expression with 1 mM IPTG in cells growing at 37 °C when the OD_{600} reached 0.7−0.9. The cells were then grown for an additional 2 h at 37 °C, harvested by centrifugation, and washed once with cold phosphate-buffered saline. The cell pellet from a 2 L culture was suspended in 50 mL of ice-cold 25 mM Tris (pH 7.5), 1 mM EDTA, 5 mM $MgCl_2$ (TEM buffer) plus 1 mM PMSF, 1 mM DTT, and 0.2 mg/mL lysozyme and disrupted by sonication on ice. Triton X-100 was added to a final concentration of 1% and the lysate incubated at 4 °C for 30 min with stirring and then centrifuged at 10000*g* for 15 min. The supernatant (50 mL) was incubated with 4 mL of a slurry of glutathione-Sepharose 4B equilibrated with TEM buffer for 2 h at 4 °C with gentle agitation. The beads were washed with 50 mL of TEM buffer, and then the bound protein eluted with 10 mM glutathione in TEM. All proteins utilized in these studies were greater than 90% pure as assessed by silver staining and typically were at least 50% active as determined by the ratio of bound [^{35}S]GTPγS[1] to total protein (Wray et al., 1981; Xu et al., 1994).

GTP Hydrolysis. GTP hydrolysis was determined as described in Xu et al. (1994) using Rac proteins at a final concentration of 250 nM in the assay. Hydrolysis was routinely determined after 15 min at 30 °C.

[^{35}S]GTPγS Binding. Binding of [^{35}S]GTPγS (3.22 μM, 2.5×10^4 cpm/pmol) to Rac proteins was determined as described previously (Chuang et al., 1994; Knaus et al., 1992). In some experiments, Rac proteins were pre-exchanged with unlabeled GDP or GTPγS using these nucleotides at 200 μM under the conditions described in the following section, and then the excess nucleotide was removed by filtration on Amicon-10 concentrators. For analysis of smgGDS activity, Rac2 proteins were pre-exchanged with unlabeled GDP and the binding of [^{35}S]-GTPγS (3.22 μM, 2.5×10^4 cpm/pmol) was determined at 30 °C as described above, in the presence or absence of an equimolar ratio of smgGDS.

[^{3}H]GDP/[^{35}S]GTPγS Dissociation. [^{3}H]GDP-bound forms of wild type and mutant Racs were prepared by incubating Rac2 protein (0.14 μM) with [^{3}H]GDP (2.5 μM at 104 cpm/pmol) at 30 °C in 25 mM Tris-HCl (pH 8.0), 2.1 mM EDTA, 0.2 mM $MgCl_2$ ($[Mg^{2+}_{free}] = 104$ nM), 1 mM DTT, and 0.1 mg/mL bovine serum albumin for various times based upon the binding kinetics observed in preliminary studies. The preloading reaction was stopped by the addition of $MgCl_2$ to 5.14 mM ($[Mg^{2+}_{free}] = 3.0$ mM). Dissociation was initiated by adding a 15% volume of activation solution (294 mM EDTA, and 3.5 mM GTP), thereby decreasing the final concentrations of EDTA to 44 mM, Mg^{2+}_{free} to 100 nM, and unlabeled GTP to ∼500 μM in the reaction mixture. Dissociation of [^{35}S]GTPγS was determined in a like manner after preloading the protein with [^{35}S]GTPγS (Chuang et al., 1994).

Binding Studies. The binding of WT Rac2 and mutants to bead-immobilized smgGDS was carried out essentially as previously described (Chuang et al., 1995). Briefly, the GDP-bound form of each GTPase was prepared as described above with the exception that each protein at 0.6 μM was incubated with 50 μM GDP. The reaction was stopped by raising the $MgCl_2$ concentration to 5.14 mM. A 10-fold excess GST−GDS was added to each GTPase, the protein levels of which were equalized on the basis of the amount of [^{35}S]GTPγS binding to each construct. After incubation at 30 °C for 5 min and at room temperature for 15 min, 100 μL of glutathione−agarose resin pre-equilibrated with TEM was added and incubated at 4 °C for 4 h and at room temperature for 40 min. The resin was pelleted and extensively washed, and then the bound protein was analyzed with 13% SDS−PAGE followed by Western blotting with Rac2-specific antibody R786 (Chuang et al., 1995). Under the conditions of these experiments, we estimated from analysis of both the bound and unbound fractions that at least 50% of the wild type Rac2 bound to the GDS resin, and the levels of protein remaining in the supernatant reflected the level of protein specifically bound to and eluted from the beads.

NADPH Oxidase Assay. Formation of the superoxide anion was determined in the partially recombinant assay system described in Xu et al. (1994) and Heyworth et al. (1993).

RESULTS

GTPase-Deficient Rac Mutations. Mutations consisting of G12 → V (G12V) and Q61 → L (Q61L) modifications were introduced into Rac2. When these same residues are mutated in the Ras protooncogene, the resulting mutant Ras proteins exhibit a GTPase-deficient, constitutively GTP-bound phenotype (Der, 1989; Grant & Owen, 1991; Krengel et al., 1990). As shown in Figure 1, G12V Rac2 and Q61L Rac2 were both unable to hydrolyze GTP in either the presence or absence of a Rac GTPase-activating protein (GAP). Thus, these mutations in Rac2 also result in GTPase-deficient phenotypes and nonresponsiveness to GAPs.

GTP Binding Properties of Rac Mutants. Examination of the ability of the Rac2 mutants to bind [^{35}S]GTPγS at low concentrations of free Mg^{2+} optimal for nucleotide exchange (Knaus et al., 1992) revealed significant differences in the kinetics of nucleotide binding as compared to those of WT Rac2 (Figure 2C). While WT Rac2 rapidly bound [^{35}S]GTPγS to a half-maximal level within 2−4 min at 30 °C, both G12V Rac2 and Q61L Rac2 exchanged nucleotide more slowly, reaching half-maximum by ∼10 and ∼30 min, respectively. These equilibrium binding results suggested that the individual guanine nucleotide binding parameters for these mutant Rac2 proteins differed significantly from those of WT Rac2.

This was examined in more detail by measuring the rates of [^{3}H]GDP and [^{35}S]GTPγS release from the Rac mutants

[1] Abbreviations: GTPγS, guanosine 5′-triphosphate; WT, wild type.

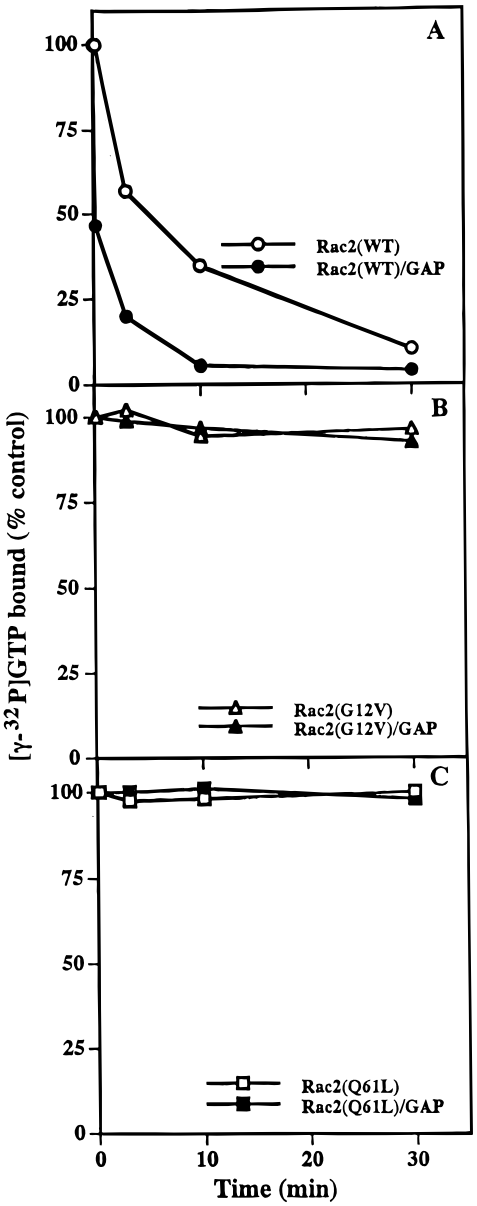

FIGURE 1: Intrinsic GTPase activity and GAP responsiveness of Rac mutants. The GTPase activity of Rac2(WT) (A), Rac2(G12V) (B), and Rac2(Q61L) (C) mutants was determined in the presence (closed symbols) or absence (open symbols) of p190 Rac GAP, as described in Methods. The amount of [γ-^{32}P]GTP bound at $t = 0$ min was set to 100% and was equivalent to 12.5 pmol of [γ-^{32}P]-GTP. Results shown are representative of three or more experiments.

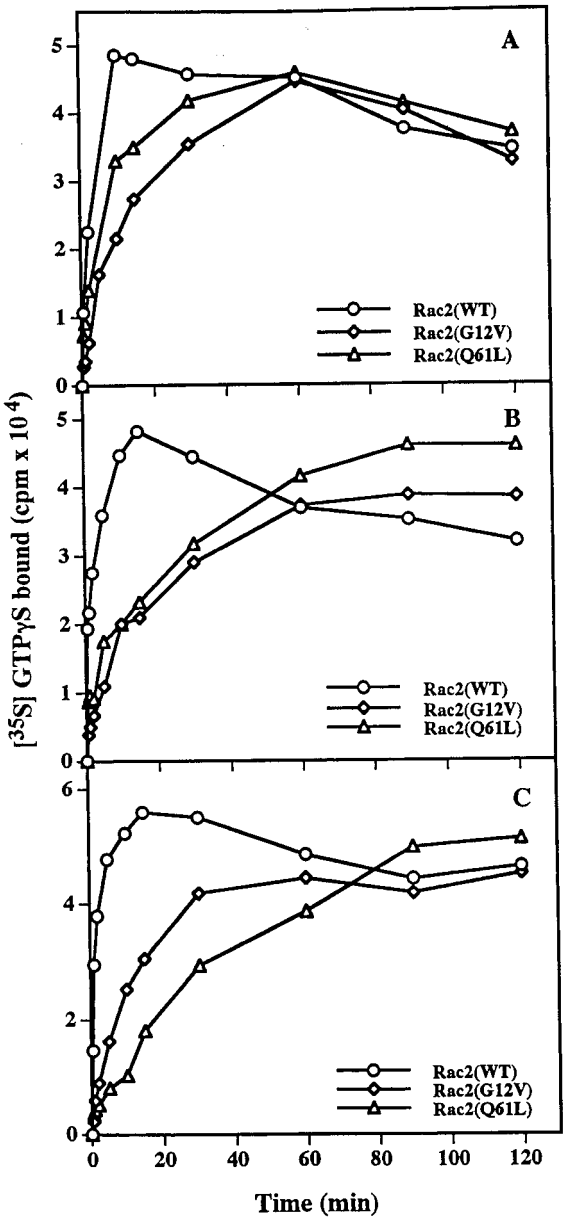

FIGURE 2: Kinetics of guanine nucleotide binding to Rac mutants. The binding of [^{35}S]GTPγS to Rac2(WT), Rac2(G12V), and Rac2-(Q61L) was determined as described in Methods. (A) proteins preloaded with unlabeled GDP prior to assay, (B) proteins preloaded with unlabeled GTPγS prior to assay, and (C) proteins assayed as isolated. The results shown are representative of three or more experiments.

(Figure 3). After preloading with [^{3}H]GDP, we observed that WT Rac2 released bound GDP with a half-time of 2−4 min and G12V Rac2 with a half-time of ∼10 min (Figure 3A), consistent with the rates of exchange seen in the equilibrium binding assay with these proteins. Q61L Rac2 released GDP very rapidly however, with a half-time of 1−4 min in various experiments. This observation was inconsistent with the very slow GTPγS binding observed in Figure 2C.

Dissociation rates for [^{35}S]GTPγS were substantially different than those observed for [^{3}H]GDP (Figure 3B). WT Rac2 had a dissociation half-time of ∼15 min for [^{35}S]-GTPγS. Both G12V and Q61L Rac2 released this nucleotide more slowly, with a half-time of 40−60 min for each.

G12V and Q61L Rac2 both lack intrinsic GTPase activity, and we expect that a portion of these proteins might remain GTP-bound even throughout protein isolation procedures. This bound nucleotide would be likely to influence the GTPγS binding kinetics as observed in Figure 2C. Indeed, when we measured equilibrium binding of [^{35}S]GTPγS after complete preloading of the Rac proteins with either GDP (Figure 2A) or GTPγS (Figure 2B), we obtained binding kinetics consistent with those predicted from the GDP and GTPγS dissociation rates seen in Figure 3. One exception was that binding to GDP-preloaded Q61L Rac2 was still not as rapid as predicted by GDP dissociation rates, suggesting that we were not able to fully exchange GDP for endogenous GTP bound to this mutant. Alternatively, the rapid dissociation of GDP and slow binding of GTP suggest the existence of a stable interim guanine nucleotide-free state of Q61L Rac2. It appears that G12V Rac2 and, particularly, Q61L Rac2 maintain substantial levels of bound GTP even after

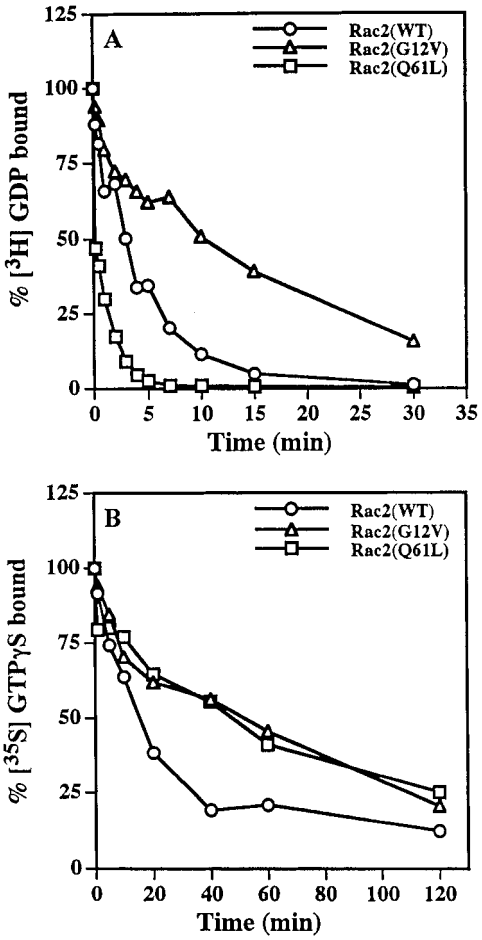

FIGURE 3: Guanine nucleotide dissociation kinetics of Rac2 mutants. The rates of dissociation of [^{3}H]GDP (A) and [^{35}S]GTPγS (B) were measured for Rac2(WT), Rac2(G12V), and Rac2(Q61L) as described in Methods. The results shown are representative of three or more experiments.

undergoing an extensive period of purification and that this mixture of GTP- and GDP-bound proteins accounts for the individual binding kinetics observed in Figure 2C. The high rate of GDP dissociation and the low rate of GTP dissociation we observe predict that Q61L Rac2 would have a high affinity for GTP and would tend to be GTP-bound in intact cells, while G12V Rac2 would have an affinity for GTP somewhere between that of the WT protein and Q61L.

Activation of NADPH Oxidase by Mutant Racs. Consistent with this interpretation, we found that both G12V Rac2 and Q61L Rac2 were active as purified and without addition of exogenous GTP in a cell-free NADPH oxidase assay which we have shown is completely GTP-dependent when WT Rac2 is used (Xu et al., 1994; Heyworth et al., 1993). Both mutants and WT Rac2 elicited a maximal level of oxidant production when added at a concentration of ~20 nM (Figure 4), although we have shown that the Q61L Rac2 mutant appears to have a higher affinity for oxidase targets (Xu et al., 1994; Dorseuil et al., 1996). Q61L Rac2 was inactive when preloaded with GDP, consistent with the need for GTP-bound Rac for activation of NADPH oxidase.

Guanine Nucleotide Binding Characteristics of Rac2 Effector Domain Mutations. Single-point mutations in the region of residues 28−38 of Rac2, as well as double mutants containing both effector domain and activating mutations, were prepared. GTP hydrolysis and the GAP responsiveness of these proteins have been previously described (Xu et al., 1994). The [^{35}S]GTPγS binding properties of these constructs were compared with those of WT Rac2 (Figure 5). Introduction of point mutations V36R and D38A into Rac2 caused little change in the rate of [^{35}S]GTPγS binding. In contrast, mutation F28L markedly increased the rate of nucleotide binding, with the half-time becoming essentially too fast to be accurately measured at 30 °C. The increased rate of exchange results from a marked increase in the dissociation rate of GDP from the F28L mutant protein (data not shown).

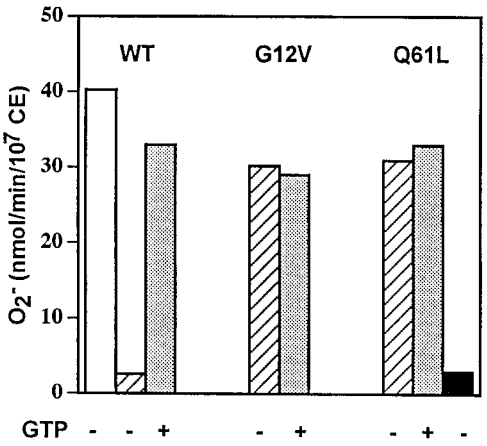

FIGURE 4: Ability of Rac2 mutants to stimulate NADPH oxidase activity. Superoxide anion (O_2^-) formation stimulated by various Rac2 mutants in a recombinant cell-free assay was determined as described in Methods. Activity was determined in the presence (+) or absence (−) of 100 μM GTP. The diagonal striped bars are the indicated Rac protein in the absence of GTP. The shaded bars are in the presence of GTP. The white bar is WT Rac2 preloaded with the nonhydrolyzable GTP analog GTPγS. The black bar is the Q61L Rac2 mutant preloaded with GDP. The results shown are representative of three or more experiments.

As indicated above, the D38A mutation had little effect on nucleotide binding rates when expressed as a single mutation, and introduction of this mutation into a G12V or Q61L background similarly caused little change in the binding parameters (Figure 5). In contrast to this, other mutations in the effector domain (amino acids 32−40) caused significant changes in binding kinetics of G12V and Q61L Rac2 . Both the T35A and V36R mutations markedly increased binding rates of the G12V and Q61R Rac2 mutants, essentially converting them to a wild type rate.

Responsiveness to smgGDS of Rac2 Single-Point Mutations. SmgGDS is one of the few proteins currently known to stimulate nucleotide exchange on Rac proteins (Hiraoka et al., 1992; Chuang et al., 1994). The ability of smgGDS to stimulate the release of GDP and binding of [^{35}S]GTPγS to various Rac2 mutants is summarized in Table 1. As previously reported, smgGDS was effective in promoting GTPγS binding to WT Rac2, stimulating the rate of binding by more than 5-fold under our experimental conditions. G12V Rac2 had a slightly reduced responsiveness to smgGDS (3.5-fold increase), but the Q61L mutation caused a dramatic loss in the ability of smgGDS to induce nucleotide exchange (1.4-fold increase). Mutation of the effector domain also caused decreased sensitivity to GDS, with the V36R mutant only responding with a 2.6-fold stimulation and the D38A mutant essentially nonresponsive (1.2-fold stimulation). Thus, both position 38 and 61 in Rac2 were critical for smgGDS-induced nucleotide exchange.

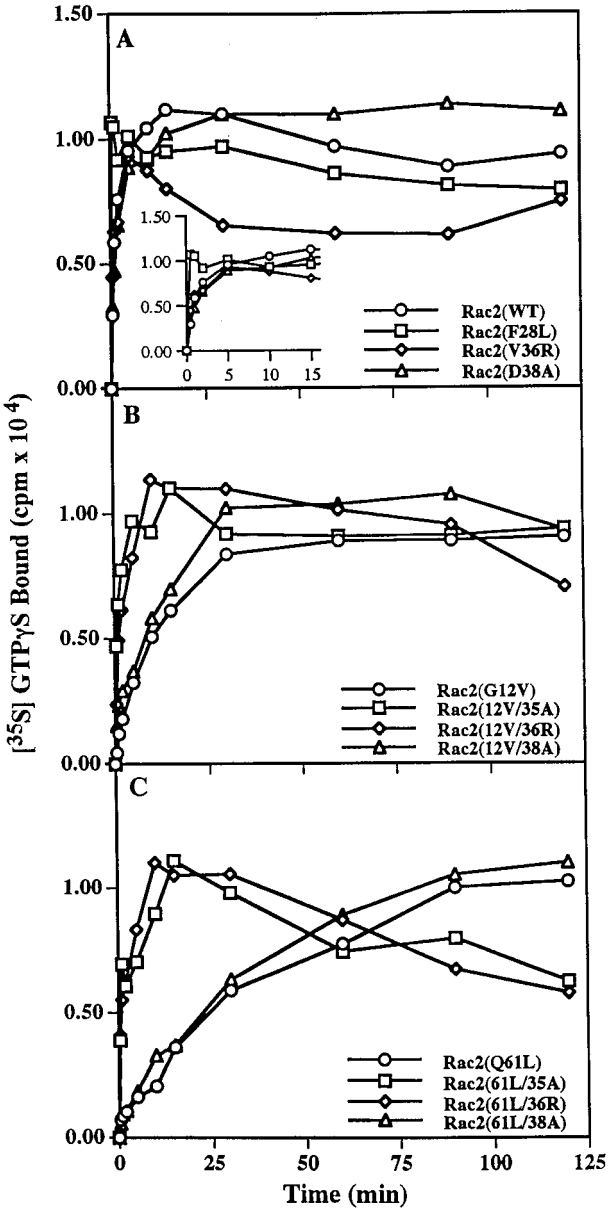

FIGURE 5: Kinetics of [^{35}S]GTPγS binding to various Rac effector domain mutants. The binding of [^{35}S]GTPγS to WT Rac2 and the single effector domain mutants F28L, V36R, and D38A is shown in panel A. A T35A Rac2 single mutation did not produce a functional protein. The inset shows an expanded scale for the early time points of panel A. The influence of the double mutation of G12V (B) or Q61L (C) on the V36R, D38A, or T35A mutations is shown as indicated. Kinetics of binding were determined as described in Methods. The results shown are representative of three or more experiments.

The G12V Mutation Restores the Ability of D38A Rac2 To Respond to smgGDS. While D38A Rac2 failed to respond to the exchange activity of smgGDS (Table 1), we found that introduction of a second mutation, G12V, restored the ability to respond effectively to smgGDS (1.4-fold increase to 3.8-fold). This was not solely due to the presence of an activating mutation, as the Q61L mutation failed to restore sensitivity of D38A Rac2 to smgGDS. Similarly, the sensitivity of the V36R Rac2 mutation to smgGDS was enhanced by addition of a G12V mutation (2.6- to 4.8-fold increase). The increase in stimulation by smgGDS caused by introduction of the G12V mutation appeared to correlate to some extent with a reduction in the basal rate of [^{35}S]-GTPγS binding (Table 1).

Table 1: Response of Rac2 Mutants to smgGDS[a]

protein	−/+	GDS	fold stimulation by GDS	response to GDS[b]
wild type Rac2	−	3.6 ± 0.9	5.6	++
	+	20.0 ± 1.1		
G12V Rac2	−	2.0 ± 0.5	3.5	++
	+	7.0 ± 0.9		
Q6lL Rac2	−	1.8 ± 0.2	1.4	−
	+	2.6 ± 0.1		
F28L Rac2	−	too fast to measure	ND	ND
	+			
V36R Rac2	−	6.0 ± 0.4	2.6	+
	+	16.0 ± 1.7		
D38A Rac2	−	3.9 ± 0.6	1.2	−
	+	4.6 ± 0.8		
12/35 Rac2	−	3.3 ± 0.8	3.8	++
	+	12.6 ± 0.8		
12/36 Rac2	−	1.8 ± 0.6	4.8	++
	+	8.4 ± 0.6		
12/38 Rac2	−	1.6 ± 0.7	3.8	++
	+	6.2 ± 0.4		
61/35 Rac2	−	3.6 ± 0.2	1.1	−
	+	4.1 ± 0.2		
61/36 Rac2	−	5.6 ± 0.9	1.1	−
	+	6.4 ± 0.3		
61/38 Rac2	−	5.4 ± 0.6	1.4	−
	+	7.4 ± 0.6		

[a] The initial rates of binding were determined as described in Methods and are expressed as femtomoles bound per minute and are the means ± SEM of three or more determinations; ND = not determined. [b] Stimulation of more than 3-fold was considered as ++; less than 2-fold was scored as −.

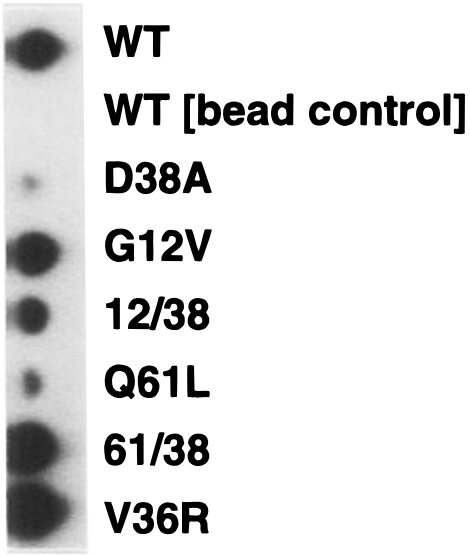

FIGURE 6: Binding of Rac2 mutants to smgGDS. Purified baculovirus-generated Rac2 proteins were assessed for direct binding to the immobilized GST−smgGDS fusion protein, as described in Methods. Equal amounts of each mutant, based on quantitation by binding of [^{35}S]GTPγS, were incubated with the beads. The bound Rac2 was detected with the specific Rac2 antibody R786 (Quinn et al., 1993). Results shown are typical of three or more experiments.

Binding of Mutant Rac2 Proteins to smgGDS. The failure of individual Rac2 mutants to respond to smgGDS could be due either to a loss of intrinsic ability to respond to this exchange protein or to a loss of the ability to effectively bind to smgGDS. We examined the binding of Rac2 mutants to smgGDS expressed as a GST fusion protein (Figure 6). The Rac2 proteins were first preloaded with unlabeled GDP, as Rac should have a higher affinity for the guanine nucleotide exchange factor in this nucleotide-bound state

(Feig & Cooper, 1988). We observed that WT Rac2 bound effectively to smgGDS, as did the G12V and V36R single-point mutants. Each of the latter also responded to smgGDS in the GTPγS binding assay. In contrast, both Q61L and D38A Rac2 exhibited a marked reduction in their ability to bind to smgGDS, suggesting that lack of physical association with smgGDS can account for their inability to respond to this exchange factor in the binding assay.

Of particular interest were the binding results obtained with the Rac2 double-point mutations. Introduction of a G12V mutation into a position 38 mutant markedly enhanced the ability of the D38A mutant to bind to smgGDS. In this case, binding was associated with a restoration of the response to smgGDS. In contrast, we observed that introduction of the Q61L mutation with D38A, while restoring binding to smgGDS, did not restore the ability of this construct to respond to smgGDS activity.

DISCUSSION

The interaction of GTPases such as Rac with proteins able to regulate their guanine nucleotide state appears to be an important mechanism for their activation by *in vivo* signals (Boguski & McCormick, 1993; Habets et al., 1994). Sites on Ras which are required for responsiveness to guanine nucleotide exchange factors have been identified in several studies (Mistou et al., 1992; Verrotti et al., 1992; Hwang et al., 1993; Segal et al., 1993; Mosteller et al., 1994; Jung et al., 1994; Mirisola et al., 1994; Quilliam et al., 1994). However, mutational analysis of the Rab3a protein indicates that different portions of this GTPase are required to respond to a Rab GDS (Brondyk et al., 1993). Similarly, differences in structural requirements for GAP responsiveness of different GTPases have been reported (Brondyk et al., 1993). While previous studies from our laboratory and others have shown that Rac is clearly different from Ras in terms of the structural basis for GAP interactions (Xu et al., 1994; Diekman et al., 1995), the current data indicate that it has a greater similarity to Ras at the level of GDS responsiveness. Mutations at both the 38 position (switch 1) and the 61 position (switch 2) of Rac2 cause the loss of the ability to respond to smgGDS. Similar results have been reported for Ras (Mistou et al., 1992), indicating the importance of both of these conformationally sensitive regions for responsiveness to exchange factors. The D38A and Q61L mutants both have a reduced binding affinity for smgGDS (Figure 6), suggesting that these residues are necessary for efficient binding to smgGDS.

The results we obtained with double mutations in Rac2, while consistently obtained, were less clearly interpreted. The D38A/Q61L double mutant regained the ability to bind smgGDS but still would not respond with an increased rate of guanine nucleotide exchange. It should be noted that, in our previous study (Xu et al., 1994), we observed that the D38A/Q61L double mutation restored the ability of Rac to interact with downstream effectors, presumably due to an overall increase in binding affinity induced by the Q61L mutation. In the current study, the restoration of smgGDS binding activity in the Rac double mutant was surprising since neither mutant alone bound smgGDS effectively. In any case, these results indicate that an intact glutamine at position 61 appears necessary for the catalytic action of smgGDS.

In contrast to the D38A/Q61L mutant, the G12V/D38A double mutant regained both smgGDS binding and smgGDS responsiveness. Modification of position 12 thus had the effect of enhancing binding affinity and restoring sensitivity to the guanine nucleotide-dissociating activity of smgGDS to the effector domain mutation. The molecular basis for this observation is not obvious, since there has been no clear indication that the region surrounding G12 in Ras is involved in interactions with exchange factors. Restoration of the responsiveness to smgGDS was associated with decreases in the basal rates of [^{35}S]GTPγS binding (see Table 1). It is possible that Rac mutations at position 12 somehow modulate the switch 1 and/or switch 2 regions such that binding and response to the exchange factor are enhanced. It is known that mutations at positions 15 and 17 in Ras have marked effects on binding to exchange factors (Feig & Cooper, 1988; Chen et al., 1994), and it is possible that the region from residue 12 to 17 in Rac modulates binding affinity for such regulatory proteins.

We have characterized in detail the guanine nucleotide binding and hydrolysis activities of various Rac mutants. The loss of GTPase activity by the G12V and Q61L mutants predicted by homology with Ras is validated (Figure 1). Surprisingly, we observed that these GTPase-deficient proteins can maintain significant levels of GTP even through prolonged purification procedures. Q61L Rac2 is only active in a biological system when bound with GTP (Figure 4). Conversion of this mutant to the GDP-bound form prevented it from stimulating NADPH oxidase activity in a cell-free assay (Figure 4). These data are inconsistent with the suggestion that Rac can support NADPH oxidase function in its GDP-bound form (Bromberg et al., 1994) and support previous data indicating that Rac is only active when bound to GTP or an active analog (Bokoch, 1995; Heyworth et al., 1993; Abo et al., 1992).

Many studies in the literature have made use of activating mutations and effector domain mutations in Rac and related GTPases. In many of these studies, the proteins were preloaded with guanine nucleotides using identical (short) times of incubation. Our results clearly indicate that the result of such procedures is only partial loading of certain Rac mutant proteins, potentially severely altering the interpretations of the conclusions made in some of these studies. In particular, the G12V and Q61L activating mutations as they are normally isolated exchange nucleotide quite slowly when compared to WT Rac. The use of a typical 5′ to 10′ loading procedure would be inadequate for stoichiometric binding.

A most significant effect on the rate of guanine nucleotide exchange occurred with the F28L Rac2 mutant. Mutation of this residue caused very rapid nucleotide exchange such that the rate was not readily measured at either low or high Mg^{2+} conditions. The lack of responsiveness to smgGDS (Table 1) was probably because the rate of GDP release was no longer rate-limiting in this mutant protein. The Phe residue at position 28 in Ras is highly conserved, and the side chain is involved in an aromatic−aromatic interaction with the guanine ring (Pai et al., 1990; Tong et al., 1989). Mutation of this Phe to Leu has also been reported to enhance nucleotide exchange rates by Ras dramatically (Mistou et al., 1992).

In summary, we have evaluated GTP hydrolysis and intrinsic guanine nucleotide exchange rates of a number of

commonly used Rac mutants. These data provide a rational basis for the *in vitro* loading of these proteins with GTP and/or analogs for biological and biochemical studies. Additionally, we show that responsiveness to the guanine nucleotide exchange-stimulating effect of smgGDS is dependent upon both an intact switch 1 and switch 2 region in Rac.

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