Guanine nucleotide binding properties of *rap1* purified from human neutrophils

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The guanine nucleotide binding properties of *rap1* protein purified from human neutrophils were examined using both the protein kinase A-phosphorylated and the non-phosphorylated forms of the protein. Binding of GTP[S] (guanosine 5'-[γ -thio]triphosphate) or GDP was found to be slow in the presence of free Mg^{2+} , but very rapid in the absence of Mg^{2+} . The binding of guanine nucleotides was found to correlate with the loss of endogenous nucleotide from the *rap1* protein, which was rapid in the absence of Mg^{2+} . The relative affinities of GTP and GDP for the binding site on *rap1* were modulated by the presence of Mg^{2+} , with a preferential affinity (approx. 15-fold) for GTP observed only in the absence of this bivalent cation. The dissociation of GDP from *rap1* was not affected by the G-protein β/γ -subunit complex. Phosphorylation of *rap1* *in vitro* by protein kinase A did not modify any of the observed nucleotide-binding parameters. Furthermore, the ability of a cytosolic *rap1* GTPase-activating protein to stimulate neutrophil *rap1* GTP hydrolysis was not modified by phosphorylation. These data suggest that the activation of *rap* *in vivo* may be regulated by the release of endogenous GDP, but that phosphorylation by protein kinase A does not affect guanine nucleotide binding or hydrolysis.

INTRODUCTION

The existence of a group of GTP-binding proteins (G-proteins) with M_r values of 20000–28000 has been demonstrated during the past few years. These include the *ras* [1], *rho* [2], *ral* [3], *Rras* [4], *rab* [5], *rap* [6], *rac* [7], smg p25 [8] and ADP-ribosylation factor (ARF) [9] gene products identified by molecular cloning. Only a limited number of these proteins has actually been purified from mammalian tissues, and there has been little characterization of their biochemical properties. The *rap1* protein, also designated *Krev1* [10], has recently been purified from bovine brain and human platelets as smg p21 [11,12] and has been shown to bind guanine nucleotides and to hydrolyse GTP. *rap1* can also serve as a substrate for protein kinase A (PKA) *in vitro* [13]. However, the regulation of the nucleotide-binding properties of this low- M_r G-protein has not been studied in any detail, nor have the effects of PKA-catalysed phosphorylation upon these biochemical parameters been rigorously examined.

The modes of activation of *rap1* and the other low- M_r G-proteins are unknown at present. The *ras* proteins exist as GDP-bound inactive and GTP-bound active forms [1]. The decrease of GTPase activity caused by point mutations in *ras* increases the biological activities of the modified proteins [1]. Because of these observations and other analogies with the receptor-associated oligomeric G-proteins, it has been postulated that there exists a protein(s) which can promote the exchange of guanine nucleotides in order to stimulate *ras* function, and an additional 'effector' protein which can be modulated by the active form of *ras*. Consistent with this hypothesis is the demonstration of the existence of the *ras* GTPase-activating protein (GAP) [14]. This protein stimulates *ras* GTPase activity 100–200-fold [14], interacts with the putative effector region of *ras* [15] and requires a GTP-bound form of *ras* for effective interactions to occur [16]. Distinct GAPs have also been reported for both *rap1* [17] and the *rho* [18] proteins. The CDC25 gene product identified in yeast appears to serve as a guanine nucleotide exchange factor for the yeast *Ras*

[19], and a similar guanine nucleotide exchange factor for smg p25 has recently been described in bovine brain cytosol [20].

The normal cellular functions of *rap1* and other low- M_r G-proteins have yet to be identified. However, *rap1* has an amino acid sequence identical to that of *ras* in the putative effector domain (amino acids 32–40) that has been shown to be the site of interaction with the *ras* GAP [14,15]. The *rap1* (or *Krev1*) protein has been reported to act as an anti-oncogene, suppressing the *ras*-induced transformation of the DT cell line and suggesting that *rap* might compete with *ras* for the *ras* GAP [10]. In addition, there are indications that, in the neutrophil, low- M_r G-proteins may participate in regulating cell activation, including stimulation of secretion and NADPH oxidase function [21]. We have previously identified a number of low- M_r G-proteins in human neutrophils, including a novel botulinum C_3 ADP-ribosyltransferase substrate and *rap1* [7,22–24]. Evidence for the co-purification of *rap1* and the cytochrome *b* component of the neutrophil NADPH oxidase system has been obtained [25], and the recently cloned p47 protein involved in oxidase activation has been reported to exhibit limited sequence similarity with the *ras* GAP [26].

Because of the intense interest in *rap1* as a cellular regulatory protein in neutrophils, and as an anti-oncogene in other cells, we have examined in detail the properties and kinetics of guanine nucleotide binding to purified *rap1*. We demonstrate that the dissociation of endogenous GDP is a rate-limiting step for binding of GTP to the *rap1* protein. In addition, we examine the effects of PKA-stimulated phosphorylation of *rap1* upon its intrinsic nucleotide binding properties and its ability to interact with the *rap* GAP in human neutrophil cytosol.

EXPERIMENTAL

Purification of *rap1*

Neutrophil G-proteins were purified essentially as described in [23], with the following modifications. (1) Membrane extractions

Abbreviations used: G-protein, GTP-binding protein; PKA, protein kinase A (cyclic AMP-dependent protein kinase); GTP[S], guanosine 5'-[γ -thio]triphosphate; GAP, GTPase-activating protein; G_{12} , the major pertussis toxin substrate of human neutrophils; ARF, ADP-ribosylation factor; AMF, 20 μ M-AlCl₃/10 mM-MgCl₂/10 mM-NaF.

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were done in the presence of 10 mM-NaCl, with AMF (20 μ M- AlCl_3 /10 mM- MgCl_2 /10 mM-NaF) present in the extraction buffer. (2) The initial DEAE-Sephacel column was larger (~ 110 ml) and was washed with 1 column vol. of equilibration buffer prior to elution with a 300 ml linear gradient of 0–250 mM-NaCl. Fractions of 4.5 ml were collected. (3) Heptylamine-Sephacel chromatography of *rap1* was performed in the absence of AMF, with only 10 mM- MgCl_2 included in the buffers. *rap1* obtained from the heptylamine-Sephacel column was approx. 70% pure as judged by silver staining and was completely resolved from other neutrophil low- M_r G-proteins, including the botulinum C_3 toxin substrate (G. M. Bokoch, H. Mueller, C. J. Der, L. A. Sklar & L. A. Quilliam, unpublished work). Further resolution by Mono Q ion-exchange f.p.l.c. using the procedure described by Kawata *et al.* [11] resulted in a symmetrical peak of guanine nucleotide binding activity which exhibited a single silver-stained band at M_r 22000 on SDS/PAGE ($> 90\%$ pure). This was identified as *rap1* by (1) N-terminal amino acid sequence analysis; (2) recognition by specific anti-*rap1* antibodies; and (3) stoichiometric phosphorylation by PKA *in vitro*.

Guanine nucleotide binding analysis

The time course of [^{35}S]GTP[S] or [^3H]GDP binding to *rap1* was performed as follows. A 250 ng sample of *rap1* was incubated in a total volume of 500 μ l with either 1 μ M-[^{35}S]GTP[S] (10000 c.p.m./pmol) or [^3H]GDP (3000 c.p.m./pmol) in 50 mM-Hepes (pH 8.0)/1 mM-dithiothreitol/0.1% Lubrol/5 mM-EDTA, containing either sufficient excess of MgSO_4 to yield 1–25 mM free Mg^{2+} over the EDTA concentration, or water alone (zero Mg^{2+}). Samples were removed at the indicated times and added to 2 ml portions of ice-cold 25 mM-Tris/HCl (pH 8.0)/100 mM-NaCl/30 mM- MgCl_2 , and binding was quantified by vacuum filtration on BA-85 nitrocellulose filters [27].

The dissociation of [^3H]GDP from *rap1* was determined by prelabelling of 1 μ g of *rap1* with [^3H]GDP (3000 c.p.m./pmol) during a 10 min incubation at 30 $^\circ\text{C}$ in the absence of free Mg^{2+} , and then adding a 1 mM excess of MgSO_4 over the EDTA to 'lock' the bound GDP on to the protein. Samples of [^3H]GDP-prebound *rap1* were then added to separate incubation tubes containing an excess (100 μ M) of unlabelled GTP and G-protein β/γ -subunits, or various concentrations of EDTA, MgSO_4 or buffer to give the indicated final concentrations of each. Bound nucleotide was assessed as described [27].

Competition by various unlabelled nucleotides for [^{35}S]GTP[S] binding was determined as follows. The binding of [^{35}S]GTP[S] was performed at a concentration of 5×10^{-8} M, which was the approximate K_d of GTP[S] for *rap1* as determined in preliminary experiments (results not shown). Binding reactions contained various concentrations of unlabelled nucleotides from 2×10^{-9} M to 1×10^{-3} M, and incubations were for either 2 min ($-\text{Mg}^{2+}$) or 30 min ($+\text{Mg}^{2+}$) at 30 $^\circ\text{C}$.

Assay of GAP activity

GAP activity for *rap1* was assessed by pre-equilibrating *rap1* with [γ - ^{32}P]GTP and then quantifying the decrease in radiolabelled *rap*-GTP complex following incubation with neutrophil cytosol. Cytosol was obtained during the course of neutrophil membrane preparation [28], filtered over a Sephadex G-25 column equilibrated with 25 mM-Tris/HCl (pH 7.5)/1 mM-EDTA/5 mM- MgCl_2 /0.5 mM-dithiothreitol/100 mM-KCl, and subsequently concentrated 4-fold on a Centricon 10 microconcentrator. *rap1* was phosphorylated as described below, and both control and phosphorylated proteins were pre-equilibrated with [γ - ^{32}P]GTP as described for [^3H]GDP, except that 0.5 μ M-[γ - ^{32}P]GTP (10–50 Ci/mmol) was used. A standard GAP assay was performed for 5 min at room temperature in a

final volume of 50 μ l containing 25 mM-Hepes (pH 7.5)/5 mM- MgCl_2 /1 mM-EDTA/50 μ g of bovine serum albumin/0.2 mM-GTP/25 ng of *rap1* (approx. 10^5 c.p.m. of prebound [γ - ^{32}P]GTP) plus cytosol preparation or G25 column buffer. Reactions were terminated by addition of 2 ml of ice-cold 25 mM-Tris/HCl (pH 8)/100 mM-NaCl/30 mM- MgCl_2 /2 mM-dithiothreitol/1 mg of bovine serum albumin/ml, and bound *rap*-GTP was analysed by filtration on nitrocellulose [27]. Release of γ - ^{32}P from *rap*-GTP was not observed with a purified preparation of *ras* GAP (results not shown), indicating that release was due to a *rap*-specific GAP activity. Conversion of GTP to GDP was confirmed by t.l.c. analysis [14].

Phosphorylation by PKA

rap1 was phosphorylated to a stoichiometry of greater than 0.9 pmol/pmol of GTP[S] binding by incubation in 50 mM-Tris/HCl (pH 7.5)/1 mM-EDTA/10 mM- MgCl_2 /50 μ M-ATP/PKA catalytic subunit (50 units/ml) for 30 min at 30 $^\circ\text{C}$. Stoichiometry was assessed in parallel incubations in the presence of [γ - ^{32}P]ATP (1000–5000 c.p.m./pmol) and by analysis by trichloroacetic acid precipitation and filtration over BA-85 nitrocellulose filters, as in [29].

Miscellaneous

All data shown are indicative of at least two and usually more than three experiments, with individual data points determined in duplicate. PKA catalytic subunit was purchased from Sigma (product number 2645). [^{35}S]GTP[S], [γ - ^{32}P]GTP and [^3H]GDP were from New England Nuclear. Protein determinations were by the Amido Black assay [30].

RESULTS

Effect of Mg^{2+} on guanine nucleotide binding to *rap1*

The kinetics of GTP[S] and GDP binding to *rap1* were examined (Fig. 1). Both nucleotides bound very slowly to *rap1* in the presence of 1–25 mM- MgCl_2 , with binding only approaching saturation after more than 120 min. In marked contrast, in the absence of Mg^{2+} (i.e. excess EDTA), binding was very rapid, reaching a maximum within 2–5 min at 30 $^\circ\text{C}$. Thereafter, the level of nucleotide bound gradually declined to a level similar to that obtained in the presence of Mg^{2+} . The maximum level of binding obtained at early times in the absence of Mg^{2+} can be maintained by addition of excess Mg^{2+} , as indicated by the arrow at $t = 9$ min in Fig. 1(a).

The observation that Mg^{2+} decreased the dissociation rate of guanine nucleotide from both $\text{G}_i\alpha$ [31] and N-*ras* [32] suggested that the binding kinetics that we observed might be explained by the prior occupation of the purified *rap1* nucleotide binding site by endogenous GDP. Both *ras* [33] and the oligomeric G-proteins [31] have been found to purify with prebound GDP, and the presence of Mg^{2+} in our *rap* purification procedure would prevent loss of GDP during the purification.

In order to assess this hypothesis, we examined the dissociation rate of [^3H]GDP from the protein under various conditions (Fig. 2). In the presence of free Mg^{2+} , the dissociation rate of GDP was slow, with a half-time of greater than 60 min. In the absence of Mg^{2+} , the GDP dissociation rate was very rapid, with essentially all of the prebound [^3H]GDP dissociated within 5 min. This is consistent with the observed kinetics of GTP[S] and GDP binding to *rap1*, and suggests that an endogenous nucleotide (likely to be GDP) occupies the guanine nucleotide binding site. This interpretation is supported further by the observation of binding kinetics which are linearly dependent upon the concentration of guanine nucleotide present in the absence of Mg^{2+} , consistent with a simple bimolecular reaction under this condition (results not shown).

Fig. 1. Time course of guanine nucleotide binding to neutrophil *rap1*

(a) Binding of $[^{35}\text{S}]\text{GTP}[\text{S}]$; (b) binding of $[^3\text{H}]\text{GDP}$. ●, Control *rap1*; ▲, phosphorylated *rap1* (both in the absence of Mg^{2+}); ○, control *rap1*; △, phosphorylated *rap1* (both in the presence of 1 mM- Mg^{2+}); □, control *rap1* in the absence of Mg^{2+} until $t = 9$ min (indicated by arrow), when 20 mM- Mg^{2+} was added. Analysis was as described in the Experimental section.

Lack of interaction with G-protein β/γ -subunits

We examined the effect of G-protein β/γ -subunit complex on the GDP dissociation rate kinetics of *rap1* in order to assess any interaction of β/γ -subunit with *rap1*. β/γ -Subunit has been shown to decrease the dissociation rate of GDP from the receptor-linked (oligomeric) G-proteins [34], and this parameter would probably be affected for *rap1* if it too is able to interact with these G-protein subunits. In the absence of free Mg^{2+} , the condition likely to be most favourable for association of β/γ -subunit with a $G\alpha$ -subunit [29,35], we saw no effect of a 5-fold molar excess of β/γ -subunit on the GDP dissociation rate. The dissociation kinetics were also examined in the presence of micromolar levels of free Mg^{2+} . This condition produced an intermediate GDP dissociation rate, with an apparent half-time of approx. 40 min. The addition of β/γ -subunit again had no significant effect upon this dissociation rate. Thus the G-protein β/γ -subunit complex did not appear to interact with *rap1* as assessed by this parameter. It has largely been assumed in the literature that the low- M_r G-proteins, including *rap1*, do not interact with G-protein β/γ -subunits because they do not co-purify with these proteins. We have also found that neutrophil

Fig. 2. Dissociation of prebound $[^3\text{H}]\text{GDP}$ from *rap1*

The dissociation of prebound $[^3\text{H}]\text{GDP}$ from *rap1* was assessed as described in the Experimental section, under the conditions indicated in the Figure. ●, Results in the presence of 5 mM-EDTA plus 6 mM- MgSO_4 . The triangles show results from assays in the presence of 5 mM-EDTA and 5 mM- MgSO_4 , in the absence (△) and in the presence (▲) of G-protein β/γ -subunit complex. The squares show assays in the presence of 5 mM-EDTA alone, with (□) or without (■) β/γ -subunit complex added.

rap1 does not co-purify with associated β/γ -subunits, even when purified under non-activating conditions (i.e. no AMF). Two other experimental approaches were used to assess such possible interactions. We determined the sedimentation rate of *rap1* on sucrose density gradients in the presence or absence of 5-fold stoichiometric levels of bovine brain β/γ -subunits under conditions [35] which result in association of β/γ with $G\alpha$. In two experiments, we saw no change in the apparent sedimentation coefficient of *rap1* (detected by $[^{35}\text{S}]\text{GTP}[\text{S}]$ binding) in the presence of β/γ -subunits ($s_{20,w} = 2.4 \pm 0.15$ S). We also examined the ability of *rap1* to compete with $G_n \alpha$ -subunit for β/γ -subunits in a pertussis toxin ADP-ribosylation assay [29], and saw no competition with levels of *rap1* in 5-fold excess over $G_n \alpha$ -subunits. In total, these data all support the conclusion that *rap1* does not interact with G-protein β/γ -subunits with an affinity approaching that of the oligomeric G-protein α -subunits.

Binding of various nucleotides to *rap1*

The ability of various nucleotides to bind to the *rap1* protein was examined, and apparent K_d values for the interaction are shown in Table 1. Binding was specific for guanine versus adenine nucleotides, but ITP ($K_d 2 \times 10^{-5}$ M) as well as CTP ($K_d 5 \times 10^{-4}$ M) and UTP ($K_d 2 \times 10^{-4}$ M) were also able to compete for GTP[S] binding. We have not ruled out the possibility that competition by the latter was due to minor contamination of these commercial nucleotide preparations by GTP or GDP. We examined in more detail the relative affinities of GTP and GDP for *rap1* in the presence and absence of Mg^{2+} (Fig. 3). In the presence of Mg^{2+} , both nucleotides bound to *rap1* with similar

Table 1. Competition by various nucleotides for [³⁵S]GTP[S] binding to *rap*

N.C. = did not compete up to 1×10^{-3} M. *GP*₄, guanosine tetraphosphate; GDP[S], guanosine 5'-[β-thio]diphosphate; p[NH]ppA, adenosine 5'-[βγ-imido]triphosphate.

Nucleotide	Apparent K_d (M)
GTP[S]	4×10^{-8}
<i>GP</i> ₄	2×10^{-4}
GMP	5×10^{-4}
GDP[S]	3×10^{-6}
Cyclic GMP	N.C.
ITP	2×10^{-5}
IDP	5×10^{-5}
CTP	5×10^{-4}
UTP	2×10^{-4}
ATP	N.C.
p[NH]ppA	N.C.
ADP	N.C.
AMP	N.C.

Table 2. Effect of phosphorylation of *rap1* on neutrophil cytosol GAP activity

Results are the means of duplicate determinations and are representative of three separate experiments. Cytosol filtrate concentration = 10 mg/ml.

Cytosol (μl)	[γ- ³² P]GTP bound (c.p.m.)	
	Control <i>rap</i>	Phosphorylated <i>rap</i>
0	80000	78900
0.5	73200	—
2	52100	51200
5	29600	34100
10	16500	—
2 (5 min, 100 °C)	75800	—

Fig. 3. Competition for [³⁵S]GTP[S] binding by GTP and GDP in the presence and absence of Mg^{2+}

Competition assays were performed as described in the Experimental section, using either non-phosphorylated (○, □) or phosphorylated (●, ■) *rap1* protein in the presence of 25 mM- $MgSO_4$ (a) and in the absence of $MgSO_4$ (5 mM-EDTA) (b). GTP competition curves (○, ●) and GDP competition curves (□, ■) are shown.

affinity, exhibiting an apparent K_d of approx. 1×10^{-7} M. In contrast, in the absence of Mg^{2+} the affinity of GTP increased by approx. 5-fold (apparent K_d 2×10^{-8} M), whereas it decreased 3-fold for GDP (apparent K_d 3×10^{-7} M). Thus, in the absence of bivalent cation, there was a 15-fold difference in the relative affinities of GTP and GDP for binding to *rap1*.

Effect of *rap1* phosphorylation on biochemical function

rap1 protein was stoichiometrically (> 90 %) phosphorylated under the conditions described in the Experimental section and analysed for potential changes in guanine nucleotide binding or hydrolysis activities. We observed no differences in either the time course of GTP[S] binding (Fig. 1) or the affinity of GTP[S] binding ($K_d \sim 40$ nM; results not shown). Similarly, there were no significant differences in the affinities of GTP or GDP for the phosphorylated *rap* in either the presence or the absence of Mg^{2+} (Fig. 3).

Neutrophil *rap1* exhibits an intrinsic GTPase activity (0.01 pmol/min per pmol of GTP[S] bound), and this activity was not changed significantly in the phosphorylated protein (results not shown). This finding has been reported for the protein purified from human platelets as well [13]. Since the GTPase activity of many of the low- M_r G-proteins, including *rap* [17], is regulated by GAPs, we postulated that this interaction might be altered in the phosphorylated protein. *rap* GAP activity was detected in neutrophil cytosol, as shown in Table 2. This activity was not mimicked by purified *ras* GAP, although *ras* GAP activity was also present in neutrophil cytosol (results not shown). Under conditions where GAP activity was limiting in the assay (i.e. the assay was not saturated with excess GAP and was still linear with regard to time), we observed no change in the ability of the neutrophil cytosol *rap* GAP to stimulate GTP hydrolysis by *rap1* when it was phosphorylated by PKA.

DISCUSSION

The guanine nucleotide binding properties of most of the recently identified low- M_r G-proteins have not yet been evaluated in any detail, particularly using the native, fully processed forms of the proteins. We undertook more detailed studies characterizing nucleotide binding to neutrophil *rap1* in order to gain insight into how the activation of this protein by guanine nucleotides might be regulated at a biochemical level. These studies show that the binding of active GTP analogues to *rap1* is dependent upon the dissociation of GDP from the nucleotide-binding site of the protein. Nucleotide exchange is slow in the presence of Mg^{2+} concentrations as low as 0.1 mM, but very rapid in the absence of the divalent cation. Additionally, in the absence of free Mg^{2+} , the *rap1* protein exhibits an increased affinity for GTP as compared with GDP, with the relative affinities changing such that the Mg^{2+} -free protein is approx. 15 times more likely to bind GTP rather than GDP. Mg^{2+} thus appears to regulate the conformation of the *rap1* nucleotide-binding site. These properties are similar to those previously reported for the N-*ras* protein by Hall & Self [32]. These authors suggested that the *ras* guanine

nucleotide binding characteristics observed in the absence of Mg^{2+} might reflect changes induced *in vivo* by a guanine nucleotide exchange protein or receptor. Our data extend this possibility to the *rap1* protein and indicate a potential regulatory role for Mg^{2+} in *rap1* activation. The CDC25 protein has been suggested to function as a GDP-GTP exchange factor in yeast [19] and an exchange protein has recently been reported for brain smg p25 [20]. The existence of such a regulatory protein for human neutrophil *rap1* is indicated.

Because of similarities in the guanine nucleotide binding characteristics of *ras* and *rap1* and the conservation of their nucleotide binding domains at the amino acid level, our findings suggest that mutations in *rap1* analogous to those which have been reported to regulate *ras* activities by modulating guanine nucleotide binding/hydrolysis activities might also produce similar modifications in *rap1* function. Such activated forms of *rap1* would be useful for elucidation of biological activities of *rap1* in intact cells.

Examination of the effect of the phosphorylation of *rap1* *in vitro* upon its intrinsic biochemical properties indicated that neither the rate of GDP exchange in the presence or absence of Mg^{2+} nor the relative affinities for GTP versus GDP in the presence or absence of Mg^{2+} were substantially affected. In addition, phosphorylation did not modify the intrinsic GTP hydrolysis rate of *rap1*, nor its ability to interact with a *rap* GAP activity present in neutrophil cytosol. It is still possible that the interaction of *rap1* with other receptor or effector molecules may be modified. It has recently been reported that the elevation of cyclic AMP in platelets stimulates the phosphorylation of an M_r -22000 membrane protein [36]. Associated with the phosphorylation of this protein is release of the phosphoprotein to the cytosol. We have determined that *rap1* is the major M_r -22000 substrate for PKA in human neutrophils (G. M. Bokoch, H. Mueller, C. J. Der, L. A. Sklar & L. A. Quilliam, unpublished work) and that neutrophil *rap1* is also released from the plasma membrane upon phosphorylation (G. M. Bokoch, unpublished work). Such translocation could play an important role in the regulation of *rap1* function in the neutrophil by influencing the ability of *rap1* to interact with receptor/effector molecules and/or GAP at the plasma membrane. Because *rap1* has been suggested to interact with a component of the neutrophil NADPH oxidase system [25] and superoxide generation is particularly sensitive to the cyclic AMP inhibitory pathway in neutrophils [37,38], these possibilities are worth investigating.

In summary, we have demonstrated that (1) *rap1* exhibits a number of guanine nucleotide binding properties which are similar to those reported for *ras*, and Mg^{2+} appears to regulate the conformation of the protein relative to the nucleotide-binding site; (2) *rap1* does not effectively interact with G-protein β/γ -subunits; and (3) *rap1* phosphorylation by PKA does not affect several biochemical properties of the protein, including stimulation by *rap* GAP activity. A goal of future studies will be to identify cellular functions of *rap1*, and to relate the regulation of these functions to the biochemical properties of the protein described here.

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