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Phosphorylation of the *RAS2* gene product by protein kinase A inhibits the activation of yeast adenylyl cyclase

(feedback mechanism/*ras* oncogenes/GTP-binding proteins)

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ABSTRACT **The *RAS2* gene product of *Saccharomyces cerevisiae* expressed in *Escherichia coli* was phosphorylated by protein kinase A *in vitro* to approximately 0.5–0.7 mol of phosphate per mol of protein. Neither protein kinase C nor protein kinase P phosphorylated the RAS2 protein significantly. The RAS2 protein is known to activate, in the presence of either Mg^{2+} and GTP or Mn^{2+}, a yeast membrane preparation with an overexpressed adenylyl cyclase and a deficiency in endogenous RAS1 and RAS2 proteins. When the RAS2 protein was phosphorylated by protein kinase A prior to exposure to the yeast membranes, its capacity to activate the adenylyl cyclase was diminished by 40–60%, while activation by Mn^{2+} remained unaffected. The phosphorylated protein retained, however, its ability to bind GTP. Incubation of protein kinase A with a specific protein kinase A inhibitor prior to phosphorylation prevented the inhibition. Furthermore, the hydrolysis of GTP was not required for the observed inhibition. These data suggest that phosphorylation of the *RAS2* gene product by protein kinase A may function as one mechanism by which the intracellular level of cAMP in yeast is regulated.**

The members of the mammalian *ras* gene family, originally identified as the oncogenes of the Harvey and Kirsten sarcoma viruses (1), contain three distinct genes—*H*, *K*, and *N*—all of which appear to exist in both a protooncogenic (wild type) and an oncogenic (activated) form (2). These genes encode similar but distinct 21-kDa proteins (p21) that localize on the cytoplasmic side of the plasma membrane (3), exhibit guanine nucleotide (GDP and GTP)-binding activity (4), and maintain an intrinsic GTPase activity (5). The protooncogene form of *ras* p21 has been implicated in controlling the process of cell division (6), whereas the activated forms of the protooncogenes are capable of transforming various cells *in vitro* (7, 8).

The gene for p21 has significant sequence similarity to the membrane-associated G proteins (guanine nucleotide-binding regulatory proteins) such as G_s (stimulatory), G_i (inhibitory), G_o (unknown function), and transducin (9). These G proteins have been shown to participate in the transduction of signals across the plasma membrane to specific effector enzymes in response to extracellular stimuli in systems such as adenylyl cyclase, visual transduction, and several others (10). It has not yet been demonstrated that p21, although exhibiting G protein-like properties, can substitute for or act in conjunction with G proteins to transduce a particular signal in mammalian cells.

Saccharomyces cerevisiae contains *RAS1* and *RAS2* genes, which encode proteins that are highly homologous to the mammalian ras proteins. At least one functional yeast *RAS* gene is essential for cell viability and proliferation and each can individually influence the utilization and/or accumulation of various nutrients (11–14). Toda *et al.* (13) and Broek *et al.* (15) have demonstrated both genetically and biochemically that *RAS1* and *RAS2* gene products participate in the yeast adenylyl cyclase system and are involved in controlling the intracellular level of cAMP. Direct experiments have shown that the addition of either the yeast RAS1 or RAS2 proteins or normal or mutant forms of the mammalian p21, all purified from *Escherichia coli* expression systems, are capable of activating yeast adenylyl cyclase in membranes from *S. cerevisiae*. In contrast, this has yet to be demonstrated in any other yeast or mammalian system.

The focus of this paper is to explore further the regulation of adenylyl cyclase by the RAS2 protein in yeast and to establish whether this interaction can be modulated by a specific protein kinase. The following report demonstrates that phosphorylation of the RAS2 protein by protein kinase A (PK-A) prior to incubation with yeast membranes inhibits the ability of the RAS2 protein to activate the adenylyl cyclase.

MATERIALS AND METHODS

Materials. ATP, cAMP, GTP, GDP, dicaproin, casein, and histone IIIS were purchased from Sigma. Phosphatidylserine was obtained from Avanti Polar Lipids. Nitrocellulose filters were from Millipore. [α-^{32}P]ATP, [γ-^{32}P]ATP, and [α-^{32}P]GTP were purchased from Amersham. The catalytic subunit of the cAMP-dependent protein kinase and its inhibitor were both generously provided by E. Fischer (University of Washington, Seattle). Protein kinase P and protein kinase C (PK-P and PK-C) were prepared by the methods of Yanagita *et al.* (16) and Woodgett and Hunter (17), respectively. The *E. coli* strain PNX23 (15) and the *S. cerevisiae* strain TKB111 (18) were gifts from M. Wigler (Cold Spring Harbor Laboratory).

Preparation of the RAS2 Protein and Yeast Membranes. The RAS2 protein was purified from the *E. coli* strain PNX23, which overexpresses the intact *RAS2* gene product controlled by the temperature-inducible λ P_L promoter as described (15). The RAS2 protein was stored in buffer G (20 mM Tris·HCl, pH 7.5/20 mM NaCl/5 mM 2-mercaptoethanol/3 mM $MgCl_2$) plus 50% (vol/vol) glycerol at −20°C. Membranes were prepared from the *S. cerevisiae* strain TKB111, which overexpresses the yeast adenylyl cyclase controlled by the alcohol dehydrogenase promoter (15, 18). Yeast membranes were stored in buffer D (50 mM Na Mes, pH 6.2/1 mM 2-mercaptoethanol/0.1 mM $MgCl_2$/0.1 mM EDTA/10% glycerol) at −80°C.

Protein determinations were by the methods of Bradford (19) and Lowry *et al.* (20).

Protein Kinase Assays. All reactions were carried out in a final volume as described in the figure legends. Reaction

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Abbreviations: PK-A, catalytic subunit of the cAMP-dependent protein kinase, protein kinase A; PK-C, protein kinase C; PK-P, protein kinase P; PK-I, inhibitor of cAMP-dependent protein kinase (peptide 11–30).

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conditions for each enzyme were as follows: (*i*) PK-A: 15 mM NaHepes, pH 6.5/20 mM dithiothreitol/4 mM $MgCl_2$; (*ii*) PK-P: 20 mM NaHepes, pH 7.5/3 mM $MgCl_2$/10 mM thioglycerol/5 μg of histone III; and (*iii*) PK-C: 20 mM Tris·HCl, pH 7.5/5 mM MgOAc/0.5 mM $CaCl_2$/40 μg of phosphatidylserine per ml/50 μM dicaproin. Enzymes and substrates were added as described in the figure legends. Reactions were started by the addition of either 500 μM ATP or [γ-^{32}P]ATP (100–300 cpm/pmol; 3000 Ci/mmol; 1 Ci = 37 GBq) and incubated for 30 min at 30°C. Samples used in adenylyl cyclase or GTP-binding experiments were then treated as described in the figure legends.

For analysis of phosphorylated proteins (see Table 1 and Fig. 2), reactions were terminated by the addition of 15 μl of 4× $NaDodSO_4$/PAGE sample buffer (0.25 M Tris·HCl, pH 6.8/40% glycerol/8% $NaDodSO_4$/2.8 M 2-mercaptoethanol/0.02% bromophenol blue). An aliquot of each sample was spotted on a square of Whatman no. 3 filter paper, washed four times for 15 min in 10% trichloroacetic acid containing 10 mM pyrophosphate with shaking, and dried, and Cerenkov radiation was determined in a Beckman liquid scintillation counter. Additional aliquots, if required, were heated for 3 min at 95°C and analyzed on a 12% $NaDodSO_4$/polyacrylamide gel as described by Laemmli (21). Gels were dried and exposed to Kodak XAR-5 x-ray film for autoradiography (25 hr at −80°C).

GTP Binding Assay. Specific conditions for the treatment of the RAS2 protein are described in the legend of Table 2. The reactions were terminated by the addition of 1 ml of ice-cold buffer G containing 100 μg of bovine serum albumin per ml and samples were passed through a 0.45-μm nitrocellulose filter under suction. Filters were washed three times with 3 ml of ice-cold buffer G, dried, and Cerenkov radiation was determined. Nonspecific binding was determined by adding the ice-cold buffer G immediately after combining the RAS2 protein with GTP.

Adenylyl Cyclase Assay. Specific conditions for the treatment of the RAS2 protein are described in the appropriate figure legends. Thirty micrograms of TKB111 membranes in 10 μl of buffer D was added to each sample followed by 50 μl of a 2× reaction mixture yielding the following final reaction conditions {20 mM Na Mes/12.5 mM $MgCl_2$ (or 2.5 mM $MgCl_2$ and 10 mM $MnCl_2$ where indicated)/1 mM 2-mercaptoethanol/0.1 mg of bovine serum albumin per ml/1.2 mM [α-^{32}P]ATP (50–150 cpm/pmol; 3000 Ci/mmol}. The final ATP concentration reflects a combination of the 1 mM contributed by the reaction mixture and the amount contributed by the preincubation mixture. Reaction mixtures were incubated for 30 min at 30°C and terminated by the addition of 0.9 ml of 0.25% $NaDodSO_4$/1.2 mM ATP/0.2 mM cAMP. cAMP content was quantitated by the method of Salomon *et al.* (22).

RESULTS

Activation of Adenylyl Cyclase by the RAS2 Protein and Manganese. We first verified that the *RAS2* gene product purified from an *E. coli* expression system activates the yeast adenylyl cyclase as described (15) and then standardized the experimental conditions under which the interaction of the RAS2 protein and adenylyl cyclase could be studied. As shown in Fig. 1, exposure of 30 μg of TKB111 yeast membranes (18) to 5 μg of the RAS2 protein preparation (submaximal amount) had no effect on adenylyl cyclase activity in the absence of GTP. When 100 μM GTP was added to the preincubation mixture, the activity increased 12-fold, while GTP alone had no effect. This confirms the observation that the *RAS2* gene product is capable of activating the adenylyl cyclase and that its effect is greatly dependent on the presence of GTP (15). In some experiments, a small stimulation by the RAS2 protein alone was observed and due to either residual GTP present in the membrane preparations or to the regeneration of GTP by some mechanism such as nucleoside-5′-diphosphokinase, an enzyme capable of phosphorylating either a nucleoside diphosphate (i.e., GDP) to a triphosphate (23) or a protein–GDP complex to a protein–GTP complex (24). Manganese, as previously reported, is known to activate directly both mammalian and yeast adenylyl cyclase (25). At a final concentration of 10 mM $MnCl_2$, a stimulation of ≈12-fold was observed, which was unaffected by the addition of GTP. These data reinforce the previous observation that Mn^{2+} activates the adenylyl cyclase and that the stimulation in the presence of Mn^{2+} is independent of both GTP and the RAS2 protein. Generally, the activation of adenylyl cyclase by either the RAS2 protein plus GTP or Mn^{2+} ranged between 6- and 12-fold above the basal activity, with some variation observed between membrane preparations.

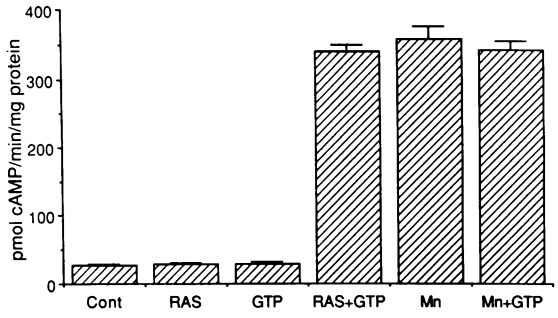

FIG. 1. Activation of adenylyl cyclase by the RAS2 protein or manganese. Five micrograms of the RAS2 protein preparation was combined with 30 μl of buffer D in a final volume of 40 μl containing 4 mM EDTA, 500 μM ATP, and 100 μM GTP (where indicated) and incubated for 30 min at 30°C to facilitate GTP binding. Adenylyl cyclase activity was assayed as described. Results represent mean values of duplicate determinations ± range. Cont, control.

Phosphorylation of the *RAS2* Gene Product by Various Protein Kinases. It was of primary interest to determine whether the activation of adenylyl cyclase by the RAS2 protein could be regulated by a protein kinase. Therefore, three distinct serine/threonine protein kinases were initially chosen to investigate whether the RAS2 protein could serve as a substrate for any of these enzymes. Incubation of the *RAS2* gene product with each of these kinases revealed that PK-A (50 ng to 2 μg) was capable of phosphorylating it effectively, incorporating approximately 0.5–0.7 mol of phosphate per mol of the RAS2 protein (Table 1). During the same time period, PK-A phosphorylated histone III to a level of only 0.2 mol of phosphate per mol of histone, suggesting that the RAS2 protein is a better substrate. A time course revealed that phosphorylation of the *RAS2* gene

Table 1. Phosphorylation of the *RAS2* gene product by various protein kinases

Substrate	Total phosphate incorporated, pmol		
	PK-A	PK-P	PK-C
None	2.4 ± 1.8	0	0
RAS2 protein	67.5 ± 2.8	0	0
Histone III	37.8 ± 1.9	ND	27.8 ± 1.1
Casein	4.6 ± 1.1	23.7 ± 0.4	0.6 ± 0.5

Kinase assays were carried out as described in a final volume of 50 μl at an ATP concentration of 500 μM. All reaction mixtures contained 5 μg of the various substrates and 50 ng of each protein kinase as indicated. [γ-^{32}P]ATP was added to start the reactions and mixtures were incubated for 30 min at 30°C. The RAS2 protein, histone, and casein had overall molecular masses of 35, 31, and 37 kDa, respectively. The results represent the mean values of duplicate determinations ± range. ND, not determined.

product by 50 ng of PK-A in the presence of 500 μM ATP was complete within 15 min (data not shown). The histone-dependent kinase PK-P and the calcium/phospholipid-dependent kinase PK-C, while capable of effectively phosphorylating casein or histone, respectively, were ineffective in phosphorylating the RAS2 protein. Analysis of the PK-A-phosphorylated RAS2 protein by $NaDodSO_4$/PAGE is shown in Fig. 2. The majority of the ^{32}P was incorporated into a 35-kDa protein, the predominant protein as judged by Coomassie blue staining, which corresponds to the RAS2 protein as verified by immunoblot analysis (data not shown) and migrates at a molecular mass similar to that determined by DNA sequence analysis (26). Other phosphorylated bands present may be either degraded or modified *RAS2* gene products or impurities present in the preparation (≈70% purity by $NaDodSO_4$/PAGE).

Phosphorylation of the RAS2 Protein by PK-A Inhibits the Activation of Adenylyl Cyclase. In view of the observation that the RAS2 protein could be phosphorylated by PK-A, it was of interest to determine whether this modification would affect its ability to activate the adenylyl cyclase. Fig. 3 illustrates that 2 μg of PK-A inhibited the RAS2 protein activation of adenylyl cyclase by 50%. PK-A alone had little or no effect on either the basal activity of the membranes or the activation of adenylyl cyclase by 10 mM Mn^{2+}. Prior heat inactivation (95°C for 5 min) of PK-A before use completely abolished the inhibition induced by PK-A.

To further verify whether this phenomenon was due to the phosphorylation of the RAS2 protein specifically and not of any yeast membrane components, the effect of the PK-A inhibitor (PK-I) (27) was explored. Fig. 4 illustrates that phosphorylation of the RAS2 protein by 200 ng of PK-A yielded a 48% reduction in adenylyl cyclase activity (measured for 5 min at 30°C). Prior incubation of PK-A with 100 μM PK-I before phosphorylation completely prevented this inhibition. When PK-I was added after phosphorylation of the RAS2 protein to prevent phosphorylation of any yeast membrane proteins, ≈90% of the original inhibition was retained.

Phosphorylation of the *RAS2* Gene Product Does Not Inhibit GTP Binding. The binding of GTP by the RAS2 protein has been demonstrated as being a critical step in its ability to fully activate the adenylyl cyclase (15). Since it was observed that phosphorylation of the RAS2 protein by PK-A has a substantial effect on its ability to stimulate the adenylyl cyclase, it was critical to test whether an impairment of GTP binding might be responsible for the inhibition. When the *RAS2* gene product was phosphorylated by PK-A and subsequently incubated with 100 μM [α-^{32}P]GTP under two different conditions, no significant changes in the level of GTP binding were observed (Table 2). Binding of GTP under conditions similar to those used for the adenylyl cyclase assay (buffer D, pH 6.2) gave a binding equivalent of 0.6–0.8 pmol of GTP per pmol of RAS2 protein. Similar results were obtained when binding was done under alternative conditions (buffer G, pH 7.5). In both cases, as with the adenylyl cyclase experiments, GTP binding was done in the presence of excess EDTA to chelate free Mg^{2+} ions and facilitate a more rapid GTP exchange as originally described by Hall and Self (28).

The association of a G protein with either GDP or GTP is apparently critical for regulating the transition between an

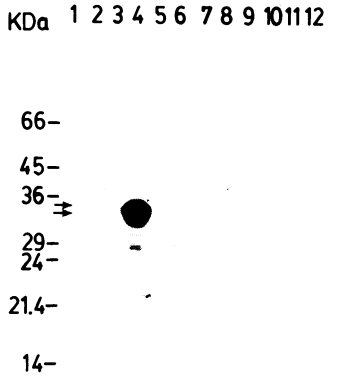

FIG. 2. Analysis of the phosphorylated RAS2 protein by $NaDodSO_4$/PAGE. The RAS2 protein (5 μg) was phosphorylated in a final volume of 50 μl as described in the legend of Table 1 and a 15-μl aliquot of each sample was analyzed by $NaDodSO_4$/PAGE as described in *Materials and Methods*. Arrows indicate bands that correspond to RAS2 proteins as determined by immunoblot analysis. Molecular mass markers are indicated in kDa. Lanes 1–4 (PK-A), 5–8 (PK-P), and 9–12 (PK-C) correspond to the specific reaction conditions for each protein kinase assayed. Each set of four lanes is presented in the following order: reaction mixture alone, RAS2 protein, protein kinase, RAS2 protein plus protein kinase.

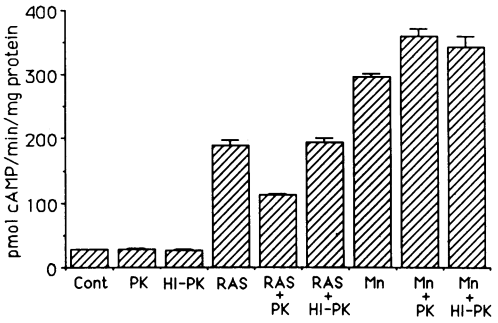

FIG. 3. Inhibition by PK-A of the activation of adenylyl cyclase by the RAS2 protein. Five micrograms of the RAS2 protein preparation was incubated in the absence or presence of 2 μg of PK-A or heat-inactivated PK-A (95°C for 5 min) in a final volume of 20 μl as described. After phosphorylation, 20 μl of buffer D was added to each sample to a final volume of 40 μl containing 4 mM EDTA, 500 μM ATP, and 100 μM GTP and incubated for 30 min at 30°C. Adenylyl cyclase activity was measured as described. Results represent mean values of duplicate determinations ± range. Cont, control; PK, PK-A; HI-PK, heat-inactivated PK-A.

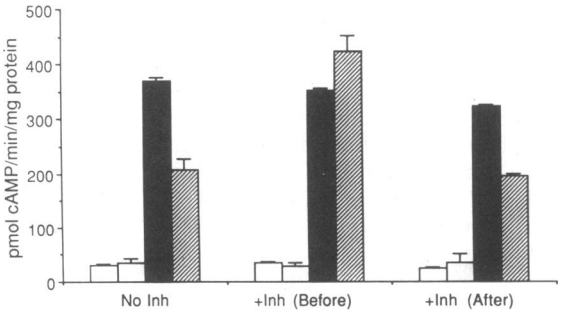

FIG. 4. Blockage of the PK-A effect on the RAS2 protein by PK-I. Three separate sets of samples were prepared where 5 μg of the RAS2 protein preparation was incubated with 200 ng of PK-A (where indicated). PK-I (100 μM; Inh, inhibitor) was added to one set [+Inh (Before)] and then all reaction mixtures were incubated for 5 min at 30°C in a volume of 18 μl as described (without ATP). ATP was added and the mixture (20 μl) was incubated as described. After phosphorylation, 100 μM PK-I was added to a second set [+Inh (After)], while the third set remained unexposed to PK-I (No Inh). Twenty microliters of buffer D was added to each sample to a final volume of 40 μl containing 4 mM EDTA, 500 μM ATP, and 100 μM GTP and incubated for 30 min at 30°C. Adenylyl cyclase activity was measured for 5 min only, but otherwise it was measured as described in *Materials and Methods*. Results represent mean values of duplicate determinations ± range. Open bar, control; stippled bar, PK-A; solid bar, RAS2 protein; hatched bar, RAS2 protein plus PK-A.

Table 2. Effects of phosphorylation of the RAS2 protein on GTP binding

	Total GTP bound, pmol	
	Method 1	Method 2
RAS2 protein	80.6 ± 2.9	85.7 ± 1.0
PK-A	0.6 ± 0.2	0.4 ± 0.1
RAS2 protein + PK-A	84.7 ± 4.2	79.1 ± 10.6
HI/PK-A	0.6 ± 0.3	0.7 ± 0.6
RAS2 protein + HI/PK-A	77.1 ± 4.5	87.3 ± 11.6

Ten micrograms of the RAS2 protein preparation was phosphorylated with 4 μg of PK-A in 20 μl at an ATP concentration of 500 μM as described. For method 1, 10 μl of each sample was combined with 30 μl of buffer D in a final volume of 50 μl containing 4 mM EDTA, 1 mM ATP, and 100 μM [α-^{32}P]GTP. For method 2, 10 μl of each sample was combined with 70 μl of buffer G in a final volume of 100 μl containing 4 mM EDTA and 100 μM [α-^{32}P]GTP (200 cpm/pmol). Reaction mixtures were incubated for 30 min at 30°C and nitrocellulose binding was done as described. The results represent the mean values of duplicate determinations ± range. HI/PK-A, heat-inactivated PK-A.

inactive (GDP) or active (GTP) state of the protein (10, 13). It seemed necessary to test whether the binding of either of these guanine nucleotides would influence the level of phosphorylation by PK-A. When the RAS2 protein was incubated alone or in the presence of 100 μM GDP or GTP for 30 min at 30°C and then exposed to PK-A, no significant change in the level of phosphorylation was observed (data not shown).

GTPase Activity Is Not a Requirement for the PK-A-Induced Inhibition. Another characteristic of RAS proteins is their ability to hydrolyze GTP (GTPase activity) to GDP as part of their regulatory mechanism. To determine whether the reduced ability of the phosphorylated RAS2 protein to activate adenylyl cyclase was due to an alteration in GTPase activity, GTP was compared with GTP[γ-S] and Gpp[NH]p, two nonhydrolyzable GTP analogues. In the presence of 200 μM GTP, a 56% inhibition was observed (Fig. 5). Substitution of GTP with either analogue yielded similar inhibitions of 55–60%. These data indicate that the hydrolysis of GTP was not required for the observed inhibition.

DISCUSSION

Regulation of enzyme activity by phosphorylation has been studied for many years. An early example involves the regulation of glycogen metabolism. Phosphorylase, phosphorylase kinase, and glycogen synthetase, three key enzymes within this cascade, are known to be controlled by a

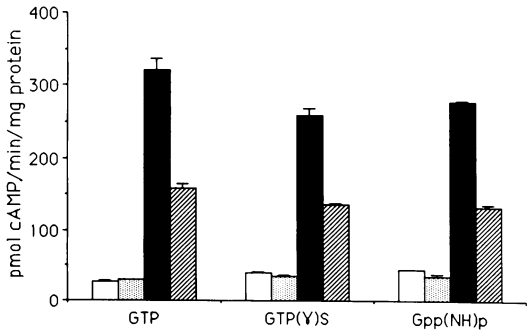

FIG. 5. Effects of nonhydrolyzable GTP analogues on the activation of adenylyl cyclase by the RAS2 protein. Five micrograms of the RAS2 protein preparation was treated as described in the legend of Fig. 3 except that 200 μM GTP, GTP[γ-S], or Gpp[NH]p was used as indicated. Adenylyl cyclase activity was measured as described. Results represent the mean values of duplicate determinations ± range. Open bar, control; stippled bar, PK-A; solid bar, RAS2 protein; hatched bar, RAS2 protein plus PK-A.

phosphorylation/dephosphorylation mechanism (29, 30). Other examples such as the regulation of phosphofructokinase by PK-A (31), of pyruvate dehydrogenase by an intrinsic protein kinase (32), and of the epidermal growth-factor receptor by PK-C (33) or by PK-P (34) have been observed. The β-adrenergic receptor has been recently shown to be phosphorylated by another kinase, the β-adrenergic receptor kinase, which has been implicated in the desensitization of the receptor (35). These examples plus many others (36) illustrate that protein phosphorylation plays a key regulatory role within various biological systems.

The data presented in this paper demonstrate that the RAS2 protein purified from an *E. coli* expression system can be phosphorylated *in vitro* by the catalytic subunit of PK-A to a stoichiometry of approximately 0.5–0.7 mol of phosphate introduced per mol of RAS2 protein. This modification resulted in a 40–60% attenuation of the stimulation of adenylyl cyclase activity induced by the RAS2 protein. PK-A had no effect on either the basal or manganese-stimulated activity. When GTP binding was tested under similar conditions, no significant changes were observed. Furthermore, hydrolysis of GTP was not required for either the activation of the adenylyl cyclase by the RAS2 protein or for the inhibition by PK-A. In contrast, neither PK-P nor PK-C was able to phosphorylate the RAS2 protein to any appreciable level.

One possible explanation for the inability to achieve complete inhibition after exposure of the RAS2 protein to PK-A is that only 50–70% of the protein was phosphorylated under the conditions described. Moreover, the phosphorylated RAS2 protein may still be capable of weakly activating the cyclase (e.g., at 10–20% efficiency). Alternatively, the residual amounts of unphosphorylated RAS2 protein may be superior in binding to the adenylyl cyclase and effectively compete with the phosphorylated protein. Finally, it seems likely for the purpose of regulation that a phosphatase is present in the yeast membrane preparation capable of dephosphorylating the RAS2 protein. When phosphatase inhibitors (e.g., vanadate, zinc, fluoride, and *p*-nitrophenylphosphate) were tested, no significant change in the overall inhibition was observed. Obviously, a phosphatase insensitive to these inhibitors may be present. The presence of a phosphatase may be implicated by the fact that when an increased amount of PK-A (2 μg vs. 50 ng) or a decreased assay time for adenylyl cyclase (5 min vs. 30 min) was used, an increase in the inhibitory response was usually observed (data not shown).

In contrast to the yeast RAS proteins, it has been more difficult to define the role of ras p21 in a mammalian system, although some interesting observations have been recently made. O'Brien *et al.* (37) have reported that either the wild-type or activated forms of N-ras or Ha-ras proteins are capable of interacting with the purified human insulin receptor isolated from placenta, inhibiting the autophosphorylation by its intrinsic tyrosyl kinase without acting as competitive substrates. This effect appears to be a GDP (not GTP)-dependent phenomenon. An inhibition of the autophosphorylation of the platelet-derived growth factor receptor corresponding to the intracellular level of p21 has been observed in fibroblasts transfected or transformed by the mammalian *ras* gene (38). Backer and Weinstein (39) have reported that various p21 proteins purified from *E. coli* expression systems, when combined with isolated rat liver mitochondria, inversely affected the level of phosphorylation of two mitochondrial proteins by endogenous protein kinase activity. A functional change within the isolated mitochondria due to these modifications has yet to be determined.

Two independent observations have been recorded on the *in vivo* and/or *in vitro* phosphorylation of p21. Ballester *et*

al. (40) observed that phorbol 12-myristate 13-acetate (PMA) induced the *in vivo* phosphorylation of c-Ki-ras p21 expressed in the mouse adrenal cortical cell line Y1, but not that of v-Ki-ras p21. Furthermore, PMA was not capable of inducing the phosphorylation of either c-Ha-ras p21 or v-Ha-ras p21 expressed in NIH 3T3 cells. *In vitro*, PK-C was also found to modify only the c-Ki-ras p21 to a level of 0.6–1.4 mol of phosphate per mol of p21. The site of phosphorylation has been localized to a serine residue situated within the c-Ki-ras-specific exon 4B region. Jeng *et al.* (41) reported that the v-Ha-ras p21 was phosphorylated *in vitro* by PK-C but the rate of phosphorylation was ≈270 times slower than with histone as substrate. In both cases, although phosphorylation of p21 was observed, no biological significance has been demonstrated.

The data presented here that phosphorylation of the RAS2 protein by PK-A has a direct effect on its ability to activate the adenylyl cyclase suggest a mechanism by which adenylyl cyclase can be regulated *in vivo* through the action of a PK-A. Activation of adenylyl cyclase by the *RAS2* gene product increases the intracellular level of cAMP, which in turn activates the endogenous cAMP-dependent protein kinase. Subsequently, PK-A can phosphorylate the RAS2 protein, reducing its capacity to activate the adenylyl cyclase. The presence of some as yet unidentified phosphatase must be coupled to this process and responsible for dephosphorylating the RAS2 protein, returning it to a more fully active state. This would continue until the original stimulus that initiated the cycle has dissipated. The specificity of PK-A fits well into this model because it is regulated by the end product of the adenylyl cyclase reaction, which is controlled in part by the RAS2 protein. The decreased efficiency of the phosphorylated RAS2 protein may reflect either steric hindrance due to a conformational change or an electrostatic repulsion due to the addition of the negatively charged functional group. Both possibilities could influence the association of the RAS2 protein with the adenylyl cyclase and, ultimately, the activation process.

Manipulations of genes of the RAS/adenylyl cyclase pathway revealed feedback controls for cAMP production over several orders of magnitude (42). Among others, the RAS2 protein was implicated in this control network. We propose that cAMP itself, in conjunction with PK-A, may participate in this control by phosphorylation of the RAS protein, thereby inhibiting the production of cAMP by the adenylyl cyclase.

In conclusion, further experimentation is required to understand more fully the adenylyl cyclase/RAS protein/protein kinase interaction. It is critical to determine whether the *RAS2* gene product is phosphorylated *in vivo* to assess whether it is a physiologically relevent substrate for PK-A. Ultimately, it is our primary goal to use the yeast system to probe the functions of RAS proteins and to apply this knowledge to the mammalian system. In doing so, we hope to more fully exploit the biochemical relationship between the *ras* oncogenes and cellular transformation.

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