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Conservation and Divergence of *RAS* Protein Function during Evolution

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The *ras* genes were first isolated as the transforming genes of Harvey and Kirsten sarcoma virus (Ellis et al. 1981). At least three different *ras* genes, Ha-*ras*, Ki-*ras*, and N-*ras*, exist in mammals and code for three very similar 21-kD proteins (Shimizu et al. 1983b). The *ras* proteins are localized in the plasma membrane (Willingham et al. 1980), bind guanine nucleotides (Shih et al. 1980, 1982), and have weak GTPase activity (Gibbs et al. 1984; McGrath et al. 1984; Sweet et al. 1984). A large number of tumor cells contain structurally mutated *ras* genes that are capable of tumorigenic transformation of NIH-3T3 cells upon DNA-mediated gene transfer (Reddy et al. 1982; Tabin et al. 1982; Taparowsky et al. 1982; Capon et al. 1983; Shimizu et al. 1983a; Yuasa et al. 1983). These oncogenic *ras* genes differ from their normal counterparts by single missense mutations which reduce the GTPase activity of the encoded proteins (Gibbs et al. 1984; McGrath et al. 1984; Sweet et al. 1984). Genes homologous to the mammalian *ras* have been identified in virtually every organism investigated, including yeast, slime molds, and fruit flies (Shilo and Weinberg 1981; DeFeo-Jones et al. 1983; Neuman-Silberberg et al. 1984; Powers et al. 1984; Reymond et al. 1984). In mice, the *ras* genes appear to be expressed in all cell types, and in all developmental stages (Mueller et al. 1982, 1983). The *ras* proteins are therefore presumed to be involved in a basic and ubiquitous system controlling cell proliferation.

In the yeast *Saccharomyces cerevisiae* there are two closely related but distinct genes, *RAS1* and *RAS2*, that encode proteins which are highly homologous to the mammalian *ras* proteins (DeFeo-Jones et al. 1983; Dhar et al. 1984; Powers et al. 1984). Although neither *RAS1* nor *RAS2* are by themselves essential genes, some *RAS* function is required for the continued growth and viability of haploid cells (Kataoka et al. 1984, 1985; Tatchell et al. 1984). This observation enabled us to demonstrate that yeast cells expressing the mammalian *ras* protein remain viable even when their endogenous *RAS* genes have been disrupted (Kataoka et al. 1985). Thus, it seems extremely likely that there has been considerable conservation of *ras* function throughout evolution.

RESULTS AND DISCUSSION

We have been examining the function of the yeast *RAS* genes. We have shown by combined biochemical and genetic experiments that activation of yeast adenylate cyclase is one of the essential functions of *RAS* (Toda et al. 1985; Broek et al. 1985). The dependence of yeast adenylate cyclase on *RAS* can also be demonstrated in vitro. Yeast *RAS2* protein, purified from an *Escherichia coli* expression system, will strongly activate the adenylate cyclase present in membranes prepared from yeast cells lacking endogenous *RAS* proteins (Fig. 1a). Human Ha-*ras* protein purified from an *E. coli* expression system will do the same (Fig. 1b), providing further evidence that there has been conservation of the biochemical function of *RAS* during evolution.

It was of considerable interest, therefore, for us to determine if vertebrate *ras* activates vertebrate adenylate cyclase. For this purpose we chose to examine the function of *ras* proteins in *Xenopus laevis* oocytes. Fully grown *Xenopus* oocytes are extemely large cells (diameter 1.1–1.4 mm). They can be easily injected and their size allows biochemical measurements on the injected cells. Moreover, oocytes contain an adenylate cyclase that, as in mammalian cells, is regulated by at least one GTP-binding protein, G_s, which can be ADP-ribosylated in response to exposure to cholera toxin (Olate et al. 1984). In addition, oocyte maturation, that is, the induction of meiosis, is modulated by cAMP. Oocytes, surgically removed from adult *Xenopus* ovaries, are arrested in the prophase of meiosis and can be triggered to undergo meiosis by treatment with progesterone, insulin, IGF-1, and a variety of other agents. Agents, such as cholera toxin or phosphodiesterase inhibitors, which increase cAMP levels, or microinjection of the catalytic subunit of the cAMP-dependent protein kinase (protein kinase A) inhibit maturation (O'Connor and Smith 1976; Maller and Krebs 1977; Schorderet-Slatkine et al. 1978). Injection of proteins that inhibit the activity of the protein kinase A, such as the heat-stabile kinase inhibitor or the regulatory subunit of protein kinase A, induce meiosis. Progesterone, the physiological inducer of maturation, lowers cAMP levels (Maller and Krebs 1977; Speaker and Butcher 1977; Mulner et al. 1979).

Mutant and wild-type human Ha-*ras* proteins were purified from *E. coli* carrying an expression plasmid (Gross et al. 1985). Upon injection of Ha-*ras* protein into *Xenopus* oocytes, maturation was induced, as judged by the appearance of a white spot in the pigmented half of the oocyte, by the breakdown of the germinal vesicle, and by the appearance of a meiotic

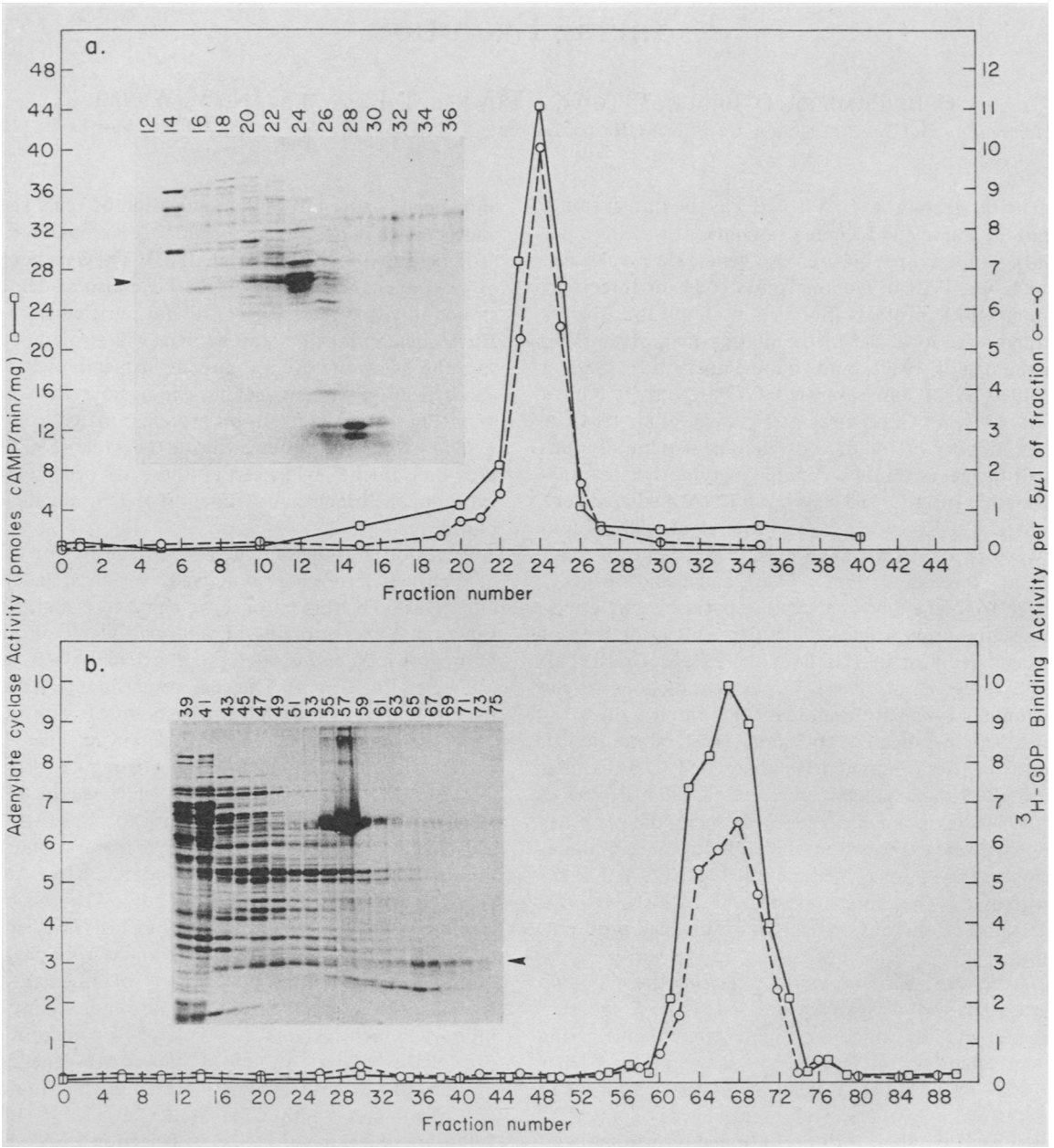

Figure 1. Copurification of *ras* proteins and adenylate cyclase-stimulating activity. (*a*) Partially purified fusion *RAS2* protein (f-*RAS2*) was applied to a Sephacryl S-300 column and 1-ml fractions were collected. [^{3}H]GDP binding activity was determined with 5-μl aliquots of fractions. For reconstitution of adenylate cyclase activity, 5 μl of indicated fractions were incubated with 1 μl of 1 mM Gpp(NH)p for 30 min at 37°C. The samples were chilled and incubated with 30 μg of *bcy1 ras1*$^-$ *ras2*$^-$ membrane proteins at 0°C for 30 min, followed by a 15-min incubation at 27°C in the presence of 0.5 mM ATP. The addition of a mixture containing [α-^{32}P]ATP started the 40-min reaction. Final concentrations of components in the 100-μl reaction mix were 20 units/ml creatine phosphokinase, 20 mM phosphocreatine, 20 mM MES (pH 6.2), 2.5 mM $MgCl_2$, 10 mM theophylline, 1 mM [^{3}H]cAMP (20,000 cpm per reaction), 0.1 mg/ml bovine serum albumin, and 1 mM β-mercaptoethanol. SDS-polyacrylamide gel electrophoresis of Sephacryl S-300 column fractions (*inset*) shows a 42,000 molecular weight protein, identified as f-*RAS2* by immunoprecipitation, eluting at fractions containing maximal GDP binding activity and adenylate cyclase-stimulating activity. Determination of cAMP produced was carried out as previously described (Solomon et al. 1973). See Broek et al. (1985) for details. (*b*) Partially purified Ha-*ras*^{Val12} protein was applied to a Sephadex G-75 gel filtration column (95 cm × 2.5 cm) and 4.5-ml fractions were collected. [^{3}H]GDP binding activity and adenylate cyclase-stimulating activity were determined as described above. SDS-polyacrylamide gel electrophoresis of the Sephadex G-75 column fraction (*inset*) shows a 21,000 molecular weight protein, identified as Ha-*ras*^{Val19} by immunoprecipitation, which elutes at fractions that contain GDP binding activity and a factor that stimulates adenylate cyclase.

spindle. When heat-denatured Ha-*ras* protein was injected, no maturation was observed (Fig. 2). Differences in the biological activity of the mutant and wild-type proteins were immediately apparent. Ha-ras^{Val12} protein, the product of the oncogenic gene with a missense mutation that specifies valine instead of glycine in position 12, induced maturation in 94% of the oocytes when 10 ng of the protein was injected (Fig. 2). The time course of induction of maturation for Ha-ras^{Val12}-injected cells was only slightly slower than for progesterone-treated cells (Fig. 2). The injected wild-type Ha-*ras* protein was much less efficient in the induction of maturation (Fig. 2). At the highest concentration tested, 400 ng of this protein induced maturation in only 55% of the injected oocytes. These experiments indicate that injected Ha-*ras* proteins induce meiosis in *Xenopus* oocytes, and that the *Xenopus* oocytes are much more sensitive to oncogenic Ha-*ras* protein than to wild-type protein.

A very early response of *Xenopus* oocytes to progesterone, the physiological inducer of meiosis, is a drop in cAMP levels, which is possibly the signal for induction of maturation (for review, see Maller 1983). We determined, therefore, whether we could observe a similar drop in *Xenopus* oocytes injected with Ha-ras^{Val12} protein. For this purpose, we injected [α-^{32}P]ATP into oocytes and then determined the amount of radioactivity converted into [^{32}P]cAMP after various treatments. In principle, measurements with this method reflect the equilibrium level of cAMP. Using this method, others have detected decreases in cAMP levels upon progesterone treatment, and increases upon cholera toxin treatment (Mulner et al. 1979). In our experiments, we detect a 70% drop in radioactive cAMP upon progesterone treatment and a sixfold increase upon cholera toxin treatment; however, we failed to detect any significant difference between oocytes injected with Ha-ras^{Val12} protein or injected with bovine serum albumin (Table 1). We conclude from this that the injected Ha-ras^{Val12} protein does not produce a sustained depression or elevation of adenylate cyclase activity in *Xenopus* oocytes. Changes of a transient nature would not be detected in our assay and can therefore not be completely excluded.

Cholera toxin, which raises cAMP production by activating adenylate cyclase, blocks oocyte maturation induced by progesterone (O'Connor and Smith 1976; Schorderet-Slatkine et al. 1978). Therefore, we sought to determine whether all the effects observed upon Ha-ras^{Val12} injections could be blocked by treatment of oocytes with cholera toxin. Cholera toxin inhibited the maturation induced by injected Ha-ras^{Val12} protein, as judged by the absence of germinal vesicle breakdown. However, the appearance of the white spot in the pigmented half of the oocyte could not be totally inhibited by this agent (data not shown). In many of the treated oocytes, we observed that the germinal vesicle had migrated to a position located directly under the white spot. The same concentration of cholera toxin inhibited completely germinal vesicle breakdown, migration, and the appearance of the white spot in progesterone-treated oocytes. These results also suggest that at least some of the effect of Ha-*ras* proteins on oocytes bypass the adenylate cyclase system, and they confirm the results described in the previous paragraph.

Our findings in *Xenopus* oocytes were unexpected in view of our results obtained in the yeast *S. cerevisiae*, where both yeast *RAS* and mammalian *ras* proteins strongly activate adenylate cyclase (Broek et al. 1985; Toda et al. 1985). We are therefore left with an apparent conflict, since yeast *RAS* and mammalian *ras* pro-

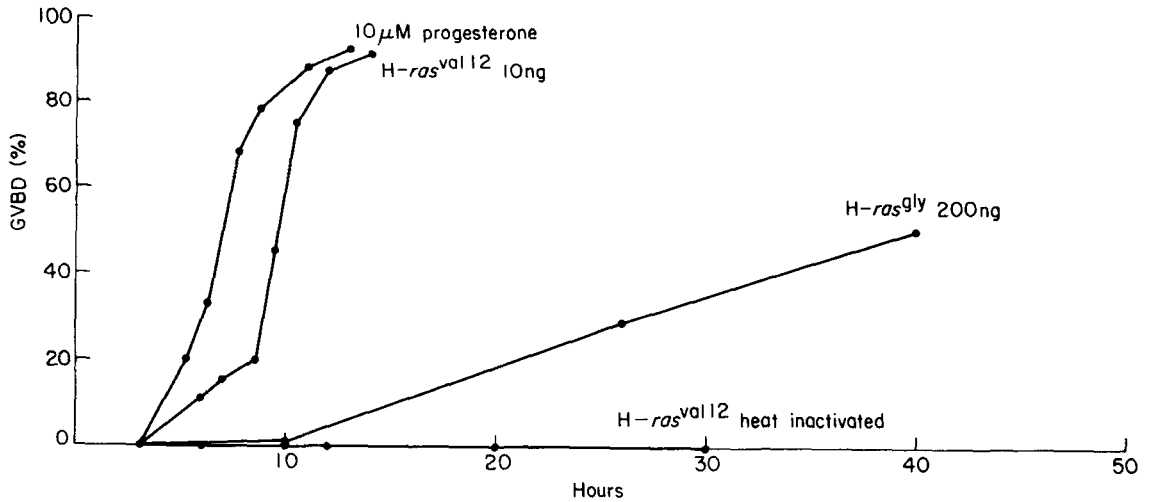

Figure 2. Time course of meiosis induced by injected Ha-*ras* protein. Ha-*ras* protein was purified from *E. coli* (Feramisco et al. 1984; Gross et al. 1985) and injected into the cytoplasm of *Xenopus laevis* oocytes (stage-VI oocytes, according to Dumont 1972). The time course of germinal vesicle breakdown (GVBD) is shown for oocytes injected with 10 ng of Ha-ras^{Val12}, 10 ng of heat-inactivated Ha-ras^{Val12}, 200 ng of Ha-ras^{Gly}, or for oocytes incubated in 10 μM progesterone, as indicated. Oocytes were fixed in 6% trichloroacetic acid after the indicated incubation times and examined for the presence of the germinal vesicle. For each experimental point a minimum of 20 oocytes were analyzed.

Table 1. cAMP Levels in *Xenopus* Oocytes after Various Treatments

Treatment	Time	[^{32}P]cAMP/total ^{32}P-labeled material ($\times 10^5$) experiment 1	experiment 2	experiment 3
BSA injection	15 min	20	29	
	30 min		24	
	45 min		27	
	1 hr	25	26	18
	2 hr	33	26	
	4 hr			
BSA injection + progesterone treatment	1 hr	9	7	
Ha-*ras*^{Val12} protein injection	15 min	23	26	
	30 min		26	
	45 min		24	
	1 hr	28	21	
	2 hr	23	24	
	4 hr	31	24	
BSA injection + cholera toxin treatment	1 hr			109

Xenopus oocytes were injected with 10 ng of protein and 2 μCi [^{32}P]ATP each; after injection, the oocytes were transferred to medium containing 1 μM of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine; in addition, 10 μM progesterone or 50 ng/ml cholera toxin were added as indicated. After the indicated incubation times, the injected oocytes were transferred to 6% trichloroacetic acid, and unlabeled and [^{3}H]cAMP were added as marker and standard for recovery, respectively. The oocytes were homogenized, the insoluble material was removed, and the amount of soluble ^{32}P-labeled material was determined. The amount of [^{32}P]cAMP present in the sample was determined after the following purification steps: Dowex 40W-4X column (Solomon et al. 1973), two-dimensional thin-layer chromatography on polyethyleneimine cellulose plates; solvents for first and second dimension were 60 mM sodium acetate (pH 5.6) and 1.5 M $(NH_4)_2SO_4$, respectively (Boehme and Schultz 1976). After this step, half of the sample was treated with phosphodiesterase from bovine brain, and both aliquots were purified on neutral alumina columns. The amount of hydrolyzable [^{32}P]cAMP was calculated, and this value was adjusted for overall recovery of the [^{3}H]cAMP. The amount of [^{32}P]cAMP present in the total amount of soluble ^{32}P-labeled material was calculated. In experiment 3, oocytes were treated with collagenase to remove follicle cells.

teins appear to have a conserved biochemical function. Two major models can be considered that are consistent with our observations. First, the adenylate cyclase of *S. cerevisiae* may not be directly related to the adenylate cyclase of vertebrate cells. Rather, a domain of a protein which interacts with *RAS* proteins may have been conserved in evolution that has appeared as a regulatory domain on proteins with different function during the course of evolution. In the second model, *RAS* proteins interact with homologous effector proteins in both yeast and vertebrates, but the interactions of these proteins with subsequent cellular signaling systems have been mutated during the course of evolution. At the present time there is no compelling reason to prefer one model over the other. Clearly, we need to know whether the *RAS* proteins interact directly with the *S. cerevisiae* adenylate cyclase, or whether they act through other as yet unidentified proteins.

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