MOLECULAR AND CELLULAR BIOLOGY, Feb. 1986, p. 719–722
0270-7306/86/020719-04$02.00/0

Vol. 6, No. 2

Antibodies to the *ras* Gene Product Inhibit Adenylate Cyclase and Accelerate Progesterone-Induced Cell Division in *Xenopus laevis* Oocytes

SUSAN E. SADLER,[1]* ALAN L. SCHECHTER,[2] CLIFFORD J. TABIN,[2] AND JAMES L. MALLER[1]

Department of Pharmacology, University of Colorado School of Medicine, Denver, Colorado 80262,[1] and Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02139[2]

Received 12 August 1985/Accepted 8 November 1985

Microinjection of monoclonal antibodies (lines 238, 172, and 259) directed against the *ras* gene product, p21, into *Xenopus laevis* oocytes accelerated progesterone-induced germinal vesicle breakdown. Antibody 238 had the greatest effect on the acceleration of progesterone-induced oocyte maturation, and this effect was correlated with in vitro inhibition of adenylate cyclase (EC 4.6.1.1) activity in a concentration-dependent manner. Inhibition of adenylate cyclase by antibody 238 was also measured in membranes prepared from oocytes pretreated with either cholera toxin or pertussis toxin. These results suggest a role for the *ras* gene product in the regulation of vertebrate cell adenylate cyclase activity.

The *ras* genes were first identified as the oncogenes contained within the genomes of the Harvey and Kirsten rat sarcoma viruses (2). Cellular *ras* genes have been identified in most species, and they constitute a family of three genes which are divergent at the nucleic acid level but which encode a remarkably well-conserved protein, p21 (1, 2, 10, 22, 23). The p21 protein has been localized to the inner surface of the plasma membrane in both normal and transformed cells (5, 28), and it is synthesized from a cytosolic precursor protein, pro-21 (21). In vitro, p21 binds GTP (4, 15, 20) and displays intrinsic GTPase activity (13, 25). While the specificity for guanine nucleotide binding is identical for both normal and activated p21, the normal gene product hydrolyzes GTP at a rate that is 8- to 10-fold higher than that of the transforming protein (13, 25). The reduced GTPase activity of the transforming protein is the first identified biochemical parameter that distinguishes the normal from the transforming *ras* gene products.

The role of p21 in controlling cellular proliferation is not known. However, analogies between p21 and the guanine-nucleotide-binding proteins of the adenylate cyclase (EC 4.6.1.1) system have been drawn (6). There is striking resemblance between the guanine-nucleotide-binding proteins of adenylate cyclase (termed N_i and N_s or G_i and G_s) and the *ras* gene product regarding both guanine nucleotide binding and GTPase activity. Furthermore, there is significant sequence homology between the α subunit of bovine G proteins and the *ras* gene product (8, 26). These similarities have suggested that the *ras* proteins play a role in transmembrane signal transduction. Recently, it has been demonstrated that the yeast homolog of p21 has direct effects upon intracellular levels of cyclic AMP and that GTP-dependent adenylate cyclase activity in yeast cells requires a normal *ras* genotype (27).

The potential relationship between adenylate cyclase and p21 is of interest regarding the control of cell division in *Xenopus laevis* oocytes. Progesterone-induced cell division (germinal vesicle breakdown [GVBD]) is mediated, at least in part, by inhibition of the oocyte adenylate cyclase (3, 9, 16) via a mechanism that involves slowing of guanine nucleotide exchange (16, 17, 18). However, in contrast to hormonal inhibition of adenylate cyclase in other cell systems, inhibition of oocyte adenylate cyclase by progesterone is not mediated via the inhibitory guanine-nucleotide-binding subunit, G_i, in the conventional manner (7, 14, 19). To determine whether p21 might be involved in the regulation of oocyte cell division, antibodies to p21 were microinjected into oocytes, and subsequent changes in the time course of progesterone-induced GVBD were monitored.

Monoclonal antibodies against p21 were isolated from hybridoma cells (lines 238, 172, and 259), which were kindly provided by E. Scolnick. The cells were grown in defined medium containing insulin, transferrin, and selenium. After collected medium samples were dialyzed against phosphate-buffered saline, monoclonal antibodies were concentrated by ammonium sulfate precipitation and resuspended in phosphate-buffered saline to a concentration of 1 to 10 mg/ml. In some cases, the antibodies were further purified by anion-exchange chromatography with DE-52 cellulose. By using antibody prepared from line 238, a low level of p21 was detected by immunoprecipitation of extracts from [^{35}S]methionine-labeled oocytes (data not shown).

To investigate the role of p21 in oocyte maturation, each of 20 oocytes was microinjected with 60 nl of solution containing approximately 0.4 μg of antibody 238. After microinjection, the oocytes were incubated in 3 ml of buffer A (83 mM NaCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM KCl, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.9) containing 0.1 μM progesterone (Sigma Chemical Co.). At various times after the addition of the microinjected oocytes to the incubation buffer, the cells were scored for GVBD, which was indicated by the appearance of a white spot on the pigmented animal pole. Oocytes microinjected with a control monoclonal antibody (anti-μ chain) reached 50% GVBD, or $GVBD_{50}$, in 200 min, while oocytes microinjected with anti-p21 achieved $GVBD_{50}$ in 140 min (Fig. 1A). This acceleration of steroid-induced GVBD after the microinjection of antibody 238 was observed in six different experiments with oocytes from different animals.

In view of the evidence that adenylate cyclase activity is inhibited by steroids that induce oocyte maturation and that p21 is homologous with the G subunits of the adenylate

* Corresponding author.

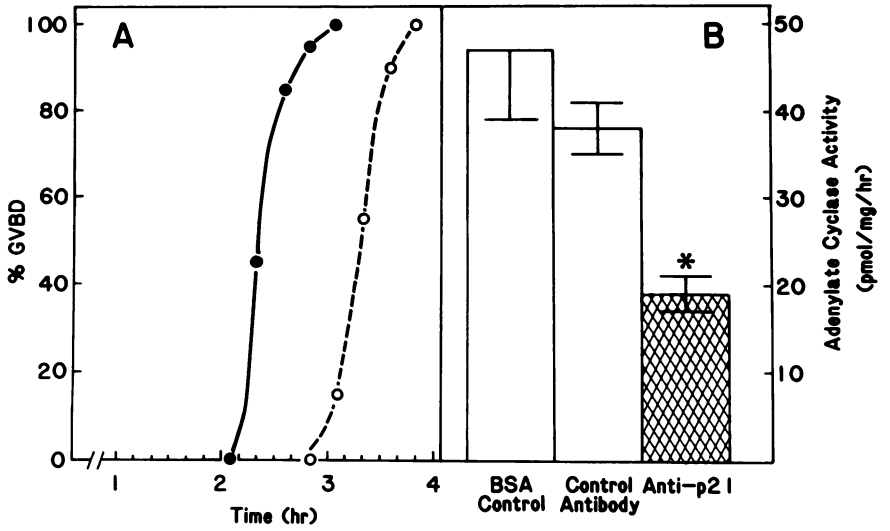

FIG. 1. Effect of anti-p21 on oocyte maturation and adenylate cyclase activity. (A) Acceleration of the rate of GVBD by microinjection of anti-p21. Groups of 20 oocytes were injected with 60 nl of control antibody (anti-μ chain) (○) or anti-p21 (●) before incubation in the presence of 0.1 μM progesterone. (B) Inhibition of oocyte plasma membrane adenylate cyclase by anti-p21. Oocyte membrane samples were prepared and incubated with bovine serum albumin or antibody, as indicated, for 20 min before measurement of enzyme activity in the presence of 0.15 mM Gpp(NH)p. $n = 3$, $\bar{x} \pm$ standard error of the mean. *, Significantly less than control serum; $P \leq 0.025$ by Student's t test.

cyclase system, the effect of anti-p21 on adenylate cyclase activity was examined. Oocyte plasma membrane samples were prepared as previously described (16) and incubated in 50 μl of phosphate-buffered saline, pH 7.4, containing 2 mg of bovine serum albumin per ml, 0.1 μM phenylmethylsulfonyl fluoride, and 16 μg of antibody 238 for 20 min at room temperature. Adenylate cyclase activity was then measured, as previously described (16), in the continued presence of antibody. The assay was initiated by the addition of [α-^{32}P]ATP and guanyl-5′-yl imidodiphosphate [Gpp (NH)p] and terminated after a 1-h incubation at 30°C. Anti-p21 caused significant inhibition of adenylate cyclase activity relative to the control antibody (Fig. 1B).

The abilities of the three anti-p21 monoclonal antibodies (lines 238, 172, and 259) to accelerate progesterone-induced GVBD are compared in Fig. 2. In three separate experiments with different donor frogs, groups of 15 oocytes were injected with serial dilutions of anti-p21 antibody or control antibody. The stock solutions of antibody that were used for these studies contained 10 mg of protein per ml and were diluted into phosphate-buffered saline, pH 7.4, containing 2 mg of bovine serum albumin per ml. Antibody dilutions were calculated on the assumption that the microinjected volume of 60 nl equilibrated with 500 nl of total cell water (24). Antibody 238 had the greatest accelerating effect on GVBD, with the maximum $\Delta GVBD_{50}$ (the decrease in time to $GVBD_{50}$ in anti-p21-injected oocytes relative to control) ranging from 65 to 90 min. The results are expressed as the percentage of the maximal response to antibody 238. In some particularly responsive oocytes, microinjection of antibody 238 was sufficient to induce GVBD without subsequent exposure of the cells to progesterone (data not shown).

The acceleration of steroid-induced oocyte maturation caused by microinjection of antibody 238 directly correlated with the level of in vitro inhibition of adenylate cyclase in a concentration-dependent manner (Fig. 3). Groups of 15 oocytes were microinjected with dilutions of antibody 238; the concentration-dependent acceleration of progesterone-

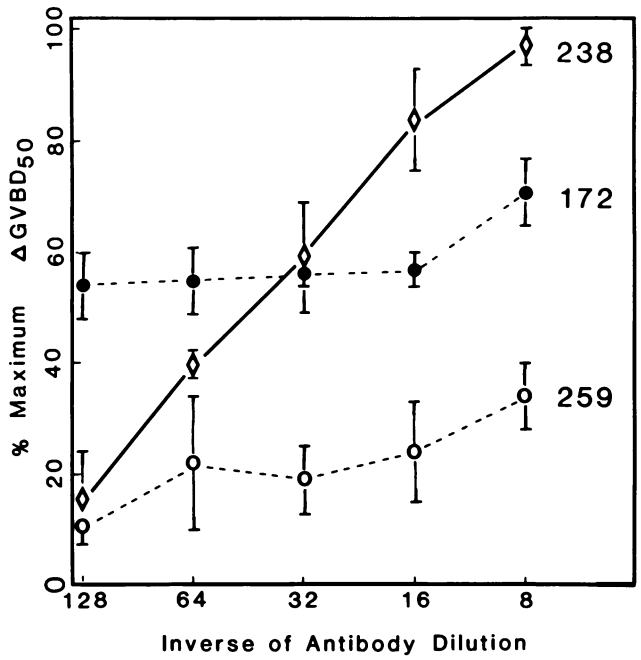

FIG. 2. Dilution curves for acceleration of progesterone-induced GVBD by anti-p21 antibodies. Various dilutions of anti-p21 antibody or control antibody were injected into groups of 15 oocytes, as indicated, and the cells were directly placed in 3-ml volumes of buffer A containing 0.1 μM progesterone. At various times, the oocytes were scored for GVBD as described in the text, and the difference between times required for $GVBD_{50}$ in oocytes injected with anti-p21 and parallel cells injected with control antibody ($\Delta GVBD_{50}$) was determined. In each experiment, the results were evaluated as percent maximal $\Delta GVBD_{50}$ relative to the maximal acceleration of GVBD measured in cells injected with antibody 238. The results from three different experiments were combined and are expressed as $\bar{x} \pm$ standard error of the mean. The final antibody dilutions represent the intracellular concentrations assuming equilibration of injected material with 500 nl of cell water (24).

induced GVBD is expressed as $\Delta GVBD_{50}$ (Fig. 3B). Membrane samples prepared from oocytes isolated from another ovary were preincubated for 20 min in identical antibody solutions before measurement of Gpp(NH)p-stimulated adenylate cyclase activity (Fig. 3A). Consistent with their reduced effect on oocyte maturation (Fig. 2), antibodies 172 and 259 caused significantly less inhibition of oocyte adenylate cyclase activity (data not shown).

Anti-p21 also inhibited adenylate cyclase activity in membrane samples prepared from oocytes that were preincubated with pertussis toxin or cholera toxin (Table 1). Previous studies in this laboratory have shown that the characteristic ADP-ribosylation reactions and functional changes in G_i and G_s are evident in oocyte membranes prepared from toxin-treated cells (16, 18, 19). One group of oocytes was preincubated for 2.5 h in buffer A containing 1 mg of bovine serum albumin and 0.5 μg of pertussis toxin (List Biologicals) per ml, and the other group of oocytes was incubated for 1.5 h in the presence of 0.05 mg of cholera toxin per ml (Schwarz/Mann). Membranes were then isolated, exposed for 20 min to antibody 238 or control antibody, and assayed for adenylate cyclase activity in the presence of Gpp(NH)p; the results are shown in Table 1. The level of inhibition by anti-p21 was approximately 50% both before and after toxin pretreatment (Fig. 1B and Table 1). This level of inhibition is comparable to the maximum level of inhibition previously observed with hormones (18).

TABLE 1. Inhibition of toxin-stimulated oocyte adenylate cyclase activity by anti-p21

Oocyte pretreatment toxin[a]	Antibody[b]	Adenylate cyclase activity (pmol/mg per h)[c]
Cholera	Control	62.2 ± 5.8
	238	30.3 ± 4.1[d]
Pertussis	Control	44.4 ± 2.5
	238	21.3 ± 5.4[d]

[a] Oocytes were preincubated in the presence of 0.05 mg of cholera toxin per ml or 0.5 μg of pertussis toxin per ml before membrane isolation.

[b] Membrane samples were incubated with anti-μ antibody (control) or anti-p21 antibody (238).

[c] Measured in the presence of 0.15 mM Gpp(NH)p. Basal activity = 48.6 ± 5.3.

[d] Significantly less than toxin alone; $P \leq 0.05$ by Student's t test. $n = 3$, $\bar{X}$ ± standard error of the mean.

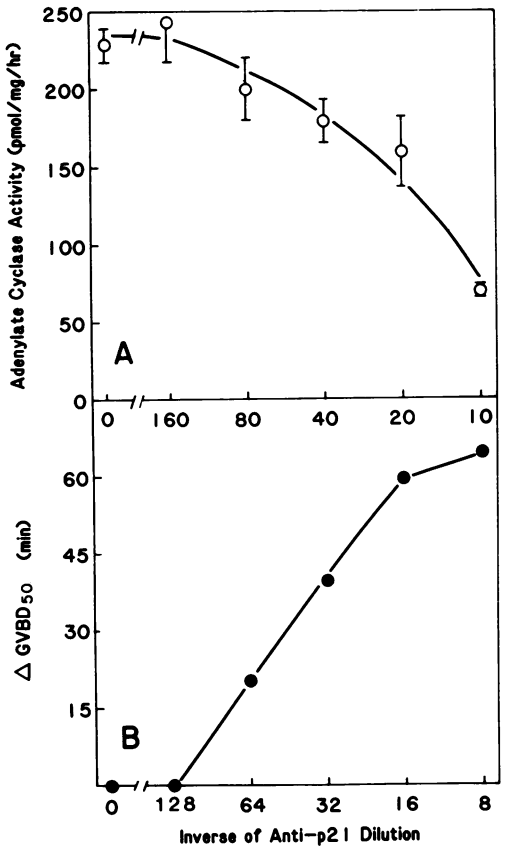

FIG. 3. Correlation of inhibition of adenylate cyclase activity with acceleration of oocyte maturation by anti-p21. (A) Concentration-dependent inhibition of Gpp(NH)p-stimulated adenylate cyclase activity. Membrane samples were treated for 20 min with various dilutions of antibody 238, as indicated, and adenylate cyclase activity was measured as described in the text. $n = 2$, $\bar{x}$ ± half-range. (B) Concentration-dependent acceleration of the rate of oocyte maturation. Various dilutions of antibody 238 or control antibody were injected into groups of 15 oocytes, as indicated, before treatment with 0.1 μM progesterone. The results are expressed as the difference between the time required for $GVBD_{50}$ in oocytes injected with anti-p21 and the time required for control injected oocytes to reach $GVBD_{50}$ ($\Delta GVBD_{50}$). The antibody dilutions represent the intracellular concentrations assuming equilibration with 500 nl of cell water (24).

These results suggest that the p21 protein interacts with the pathway of normal cell division regulated by progesterone. When the p21 protein is affected in vivo by microinjection of anti-p21, progesterone-induced cell division is accelerated (Fig. 1A, 2, and 3B). Although it is possible that microinjected anti-p21 has multiple effects in the intact cell, these data suggest that p21 regulates the rate of oocyte maturation by affecting the oocyte adenylate cyclase system. The possibility also exists that the inhibitory action of the antibodies is due to cross-reaction with the G proteins, but the data suggest that at least part of the in vivo action of anti-p21 involves regulation of adenylate cyclase activity by affecting the *ras* gene product. It is also significant that anti-p21 is able to inhibit adenylate cyclase activity measured in the presence of Gpp(NH)p. Most inhibitory hormones are unable to inhibit adenylate cyclase in the presence of this nonhydrolyzable guanine nucleotide. The only reported exceptions are the inhibition of oocyte adenylate cyclase by progesterone and the inhibitory action of P site adenosine agonists in a number of cell systems, including the amphibian oocyte (11, 12, 17, 18, 29). Previous genetic evidence has implicated *ras* function in regulating yeast adenylate cyclase (27). The results presented in this paper showing inhibition of oocyte adenylate cyclase by anti-p21 suggest that regulation of vertebrate cell division by the *ras* gene product may also involve regulation of adenylate cyclase activity.

This work was supported by Public Health Service grant GM26743 from the National Institutes of Health and grant NP-517A from the American Cancer Society to J.L.M., who is an Established Investigator of the American Heart Association.

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