perature often varies erratically, particularly in the intertidal zone, the bloom may be a more reliable signal of impending favorable temperatures than temperatures at the time of spawning. Density of zooplankton is at an annual minimum at the onset of the spring phytoplankton outburst (*17, 18*); therefore, larval mortality due to zooplankton predators may be minimized. In addition, during the bloom when many species are spawning, larval mortality is likely reduced because feeding by predators has attained a maximum (*19*). In conclusion, a major advantage of phytoplankton as a spawning cue is that it integrates various environmental parameters indicating favorable conditions for larval success. In many species mechanisms may have evolved that directly couple their larval phase with phytoplankton abundance.

REFERENCES AND NOTES

1. G. Thorson, *Meddr. Kommn. Danm. Fisk. Plankton* **4**, 1 (1946).
2. A. C. Giese and H. Kanatani, in *Reproduction of Marine Invertebrates*, A. C. Giese, J. S. Pearse, V. B. Pearse, Eds. (Blackwell Scientific, Palo Alto, CA/ Boxwood Press, Pacific Grove, CA, 1987), vol. 9, pp. 251–329.
3. J. H. Himmelman, *J. Exp. Mar. Biol. Ecol.* **20**, 199 (1975).
4. J. R. Smith and D. R. Strehlow, *Int. J. Invertebr. Reprod.* **6**, 129 (1983).
5. H. Barnes, *Ann. Biol.* **33**, 67 (1957); D. J. Crisp and C. P. Spencer, *Proc. R. Soc. London Ser. B* **148**, 278 (1958).
6. In temperate seas, the spring bloom begins with small diatoms, such as *Skeletonema*; larger diatoms, flagellates, and dinoflagellates follow [D. H. Cushing, *J. Plankton Res.* **11**, 1 (1989)].
7. In all experiments there was virtually no mortality.
8. R. R. L. Guillard and J. H. Ryther, *Can. J. Microbiol.* **8**, 229 (1962).
9. Throughout our experiments, neuromuscular coordination of urchins as measured by righting times [J. A. Percy, *Physiol. Zool.* **46**, 129 (1973)] remained normal, indicating that experimental conditions did not cause a deterioration in their physiological condition.
10. Chlorophyll a concentrations were determined by the fluorimetric or spectrophotometric methods [T. R. Parsons, Y. Maita, C. Lalli, Eds., *A Manual of Chemical and Biological Methods for Seawater Analysis* (Pergamon, Oxford, 1984)]. In temperate seas chlorophyll a concentrations of 8 to 22 mg per cubic meter are usually attained during the spring phytoplankton increase [(*3*); references in (*18*)].
11. B. L. Bayne, R. J. Thompson, J. Widdows, in *Marine Mussels: Their Ecology and Physiology*, B. L. Bayne, Ed. (Cambridge Univ. Press, Cambridge, 1976), pp. 121–206.
12. M. Starr, J. H. Himmelman, J.-C. Theriault, unpublished data.
13. Diatom cultures were filtered through GF/C Whatmann filters by means of a vacuum of <0.5 atm. This technique causes little cell damage [T. R. Parsons *et al.* in (*10*)].
14. J. S. Lucas, *J. Exp. Mar. Biol. Ecol.* **65**, 173 (1982); G. Paulay, L. Boring, R. R. Strahmann, *ibid.* **93**, 1 (1985).
15. L. Legendre and S. Demers, *Can. J. Fish. Aquat. Sci.* **41**, 2 (1984).
16. H. U. Sverdrup, *J. Cons. Perm. Int. Explor. Mer* **18**, 287 (1953).
17. A. K. Heinrich, *ibid.* **27**, 15 (1962).
18. D. H. Cushing, *Marine Ecology and Fishes* (Cambridge Univ. Press, Cambridge, 1975).
19. E. D. S. Corner, R. N. Head, C. C. Kilvington, L. Pennycuick, *J. Mar. Biol. Assoc. U.K.* **56**, 345 (1976).

20. We are indebted to S. Gosselin, J. Côté, P. DeKoninck, and T. Duchêne for aid in the laboratory, and to J. McNeil, H. Guderley, and L. Druehl for comments on the manuscript. This research was funded by National Sciences and Engineering Research Council (NSERC) and Ministère de l'Agriculture, des Pêcheries, et de l'Alimentation du Québec grants to J.H.H., by Department of Fisheries and Oceans operating funds to J.-C.T., and by Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR) and NSERC funding to GIROQ. M.S. was supported by scholarships from NSERC and FCAR.

7 July 1989; accepted 1 December 1989

Inhibition of Serum- and Ras-Stimulated DNA Synthesis by Antibodies to Phospholipase C

MARK R. SMITH, YA-LUN LIU, HYUN KIM, SUE GOO RHEE, HSIANG-FU KUNG

Several immunologically distinct isozymes of inositol phospholipid–specific phospholipase C (PLC) have been purified from bovine brain. Murine NIH 3T3 fibroblasts were found to express PLC-γ, but the expression of PLC-β was barely detectable by radioimmunoassay or protein immunoblot. A mixture of monoclonal antibodies was identified that neutralizes the biological activity of both endogenous and injected purified PLC-γ. When co-injected with oncogenic Ras protein or PLC-γ, this mixture of antibodies inhibited the induction of DNA synthesis that characteristically results from the injection of these proteins into quiescent 3T3 cells. However, when oncogenic Ras protein or PLC-γ was co-injected with a neutralizing monoclonal antibody to Ras, only the DNA synthesis induced by the Ras protein was inhibited—that induced by PLC was unaffected. These results suggest that the Ras protein is an upstream effector of PLC activity in phosphoinositide-specific signal transduction and that PLC-γ activity is necessary for Ras-mediated induction of DNA synthesis.

ACTIVATED *ras* GENES ARE FOUND IN a significant fraction of malignant human tumors (*1*), and it has been postulated that these genes are responsible for tumor development (*2*). Genetic mutations that transform the *ras* proto-oncogene into a gene that actively induces tumors result from amino acid substitutions at critical positions, such as Gly^{12} (*3*) and Gln^{61} (*4, 5*), in the primary sequence of the Ras protein. All members of the Ras protein family bind guanine nucleotides (*6, 7*), have intrinsic guanosine triphosphatase (GTPase) activity (*4, 8*) that is enhanced by interaction with GTPase-activating protein (GAP) (*9*), and have sequence homology to signal-transducing G proteins (*10*). These observations suggest that the biological role of Ras proteins may be to couple cell surface receptor activation to a variety of intracellular enzymes and ion channels that modulate cellular activity (*11*). Microinjection of bacterially synthesized oncogenic Ras proteins (*12, 13*) or inositol phospholipid–specific phospholipase C (PLC) purified from bovine brain (*14*) into quiescent NIH 3T3 fibroblasts induces morphologic transformation and DNA synthesis. Induction of DNA synthesis in resting fibroblast cells is also a property of serum growth factors. Thus, PLC and Ras proteins may be intermediates in the biochemical pathways used by serum growth factors to activate proliferation of cells.

Several growth factors have been shown to require a functional Ras protein for activity by microinjection experiments with neutralizing antibodies to Ras (anti-Ras) (*15*). The position of Ras participation in signal-transduction pathways was partially identified by injection of anti-Ras into various oncogene-transformed 3T3 cells (*16*). Injection of the anti-Ras monoclonal antibody Y13-259 blocked serum-induced cell division in NIH 3T3 cells (*15*) and caused the morphological phenotype of *ras*-transformed 3T3 cells to revert to that of the parental cell line (*17, 18*). These results suggested an absolute requirement for Ras proteins for 3T3 cell growth.

Bombesin (*19*) and platelet-derived growth factor (PDGF) (*20*) increase the intracellular concentration of several inositol phospholipids. PLC-γ is directly phosphorylated on tyrosine and serine residues by the PDGF receptor after PDGF treatment of quiescent 3T3 mouse fibroblasts (*21*), and further, injection of a monoclonal antibody specific for phosphatidylinositol 4,5-bis-

M. R. Smith and Y.-l. Liu, Biological Carcinogenesis and Development Program, Program Resources Inc., National Cancer Institute–Frederick Cancer Research Facility, Frederick, MD 21701.
H. Kim and S. G. Rhee, Laboratory of Biochemistry, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892.
H.-f. Kung, Laboratory of Biochemical Physiology, Biological Response Modifiers Program, Division of Cancer Treatment, National Cancer Institute—Frederick Cancer Research Facility, Frederick, MD 21701.

Table 1. Comparison and quantitation of PLC isozymes in bovine brain and established murine cell lines by radioimmunoassay. Bovine brain and murine cells were homogenized in 20 m*M* tris-HCl (*p*H 7.4) containing 0.2% sodium deoxycholate. The homogenates were centrifuged at 120,000*g*, and radioimmunoassay was performed on the supernatants as described (*27*). One PLC-specific antibody was absorbed onto 96-well plates and blocked with bovine serum albumin after which PLC standards, crude homogenates, or buffer was added, and the plate was incubated for 2 hours. A different PLC-specific ^{125}I-labeled antibody was then added and the plate was incubated for a further 2 hours. The plate was finally washed and the bound ^{125}I-radioactivity of each well was measured. C6 Bul is a continuous rat glioma cell line; PC-12 is a mouse neuroblastoma cell line; and NIH 3T3 fibroblasts are from mouse. Values are means ± SD from triplicate determinations.

Sample	Amount of PLC isozyme (ng/mg protein)	
	PLC-β	PLC-γ
Brain	1500 ±106	640 ± 58
C6 Bul	13 ± 2	250 ± 28
PC-12	15 ± 2	230 ± 21
3T3 fibroblasts	4 ± 1	220 ± 17

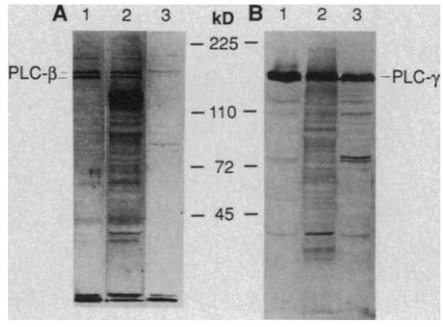

Fig. 1. Protein immunoblot showing the expression of PLC-β (**A**) and PLC-γ (**B**) in established cell lines (Table 1). Lane 1, C6 Bul; lane 2, PC-12; and lane 3, NIH 3T3 fibroblasts. The positions of PLC-β and PLC-γ are indicated. Cell suspensions were disrupted by ultrasound and centrifuged at 12,000*g*, and 200 μg of lysate was loaded on 8% polyacrylamide gels. Purified PLC-β and PLC-γ have been used to standardize the immunoblots and show that PLC-β and PLC-γ present in the lysates migrate at approximately the same rate as pure enzyme. Mixtures of antibodies (10 μg/ml of each) with different epitopic specifities were used for immunoblotting: anti–PLC-β antibodies included K32-3, K82-3, and K92-3 and anti–PLC-γ antibodies included B64, B23, D77-3, B2-5, E8-4, and E9-4 (*27*). Antibodies of the same immunoglobulin (Ig) subclass as anti–PLC-β and anti–PLC-γ (IgG1 and IgG2a) were used to control for specificity in the immunoblots. In addition, PLC-γ from 3T3 cells does not cross-react with antibodies specific for PLC-β, as previously described (*27*).

phosphate (PIP_2) blocks bombesin- and PDGF-induced mitogenesis (*22*). Similarly, in *ras*-transformed cells the rate of inositol phospholipid turnover is three times as fast as in untransformed cells (*23*). NIH 3T3 cells expressing mutant *ras* genes have increased basal rates of inositol phosphate (*23, 24*) or diacylglycerol (*25*) production, implying a chronic stimulation of PLC-dependent PIP_2 turnover (*23, 24*).

It is possible to synchronize cultures of NIH 3T3 fibroblasts in the G_0 phase of the cell cycle by depriving the cells of serum growth factors. After 24 hours, only 2 to 5% of the cells incorporate [^{3}H]thymidine into nuclear DNA during a 3-hour labeling period. Microinjection of activated Ras protein (*12*) or PLC (*14*) induces resting cells to enter the S phase of the cell cycle and incorporate [^{3}H]thymidine into their nuclear DNA. Induction of [^{3}H]thymidine uptake into nuclear DNA after microinjection of putative oncogenic proteins has become an established method of identifying the oncogenic potential of a protein (*26*). Because proteins that induce DNA synthesis after injection usually also morphologically transform NIH 3T3 cells (*12–14*), activation of DNA synthesis is a reliable indicator of proliferative responses and may suggest oncogenicity.

The hybridoma cell lines that produced the monoclonal antibodies used in this study were derived from spleen cells of BALB/c mice immunized with PLC-β or PLC-γ purified from bovine brain (*27, 28*). Each antibody binds to a different epitope on the PLC molecules, and antibodies made to PLC-β and PLC-γ do not cross-react with each other. A specific and sensitive tandom radioimmunoassay for quantitating PLC-β and PLC-γ was developed with these antibodies (*27*) (Table 1). On the basis of the ability of an antibody to inhibit the in vitro bioactivity of inositol phospholipid–specific PLC (*27*), several monoclonal antibodies with different epitopic specificities were mixed to maximize inhibition. We have used microinjection to evaluate the biological significance of adding PLC to a resting cell and to examine the result of depleting intracellular PLC with neutralizing antibody during growth activation by serum factors. PLC is thought to be constitutively expressed and to participate in the promotion of growth signals in cultured cells (*24*) and brain tissue (*29*).

Bovine brain was found by radioimmunoassay to express large amounts of PLC-β and PLC-γ, whereas several established cell lines express lesser amounts of

Table 2. Effect of antibodies to PLC and Ras on the stimulation of DNA synthesis in quiescent NIH 3T3 fibroblasts induced by microinjection of PLC. NIH 3T3 cells (4×10^6) were plated on glass cover slips in 35-mm culture dishes and allowed to grow to confluency for 1 to 2 days in Dulbecco's modified essential medium (DMEM) with 10% calf serum. The medium was then replaced with DMEM containing 0.5% calf serum, and the cells were incubated for 24 hours before microinjection (10^{-11} ml) of coded samples into 150 to 200 cells within a defined area on the cover slips. Injected cells were maintained in DMEM with 0.5% calf serum; [^{3}H]thymidine (1 μCi/ml; Amersham) was added for the period 18 to 24 hours after injection. Cultures were washed with isotonic phosphate-buffered saline (*p*H 7.4) and fixed in 3.7% (v/v) formaldehyde. Cover slips were mounted onto glass microscope slides, coated with nuclear track emulsion (NTB2; Eastman Kodak), and autoradiographed for 24 hours. Slides were stained with Geimsa and photographed, and labeled cells were counted. Background thymidine uptake was calculated by dividing the number of cells that had incorporated [^{3}H]thymidine in an area of uninjected cells by the total number cells in that area. Thymidine uptake was similarly calculated for the injected cells in a defined area. Fold induction (mean ± SD; $n > 3$) was calculated by dividing the percentage thymidine uptake of injected cells by that of uninjected cells. PLC-β and PLC-γ were purified from bovine brain (*28*). Mixtures of anti–PLC-β and anti–PCL-γ antibodies were as described in the legend to Fig. 1. Anti-Ras monoclonal antibodies Y13-259 and Y13-238 are specific for Ras protein (*17*): Y13-259 neutralizes intracellular Ras activity after microinjection but Y13-238 does not (*15, 16*). BSA, bovine serum albumin; H-Ras, Harvey Ras protein. Concentrations: BSA, 2 mg/ml; PLC-β, 225 μg/ml; antibodies 5 mg/ml; PLC-γ, 225 μg/ml; and H-Ras, 2.5 mg/ml.

Injected sample	Thymidine uptake (%)		
	Uninjected cells	Injected cells	Fold induction
BSA	5.2	4.1	1.3 ± 0.4
PLC-β	2.4	55.8	23.3 ± 4.2
PLC-β + anti–PLC-β	2.1	7.6	3.6 ± 2.1
PLC-β + anti–αPLC-γ	3.0	60.5	21.5 ± 5.1
PLC-γ	2.3	51.5	22.4 ± 4.8
PLC-γ + anti–PLC-γ	2.6	3.4	1.3 ± 0.6
PLC-γ + Y13-259	2.5	59.0	23.6 ± 3.7
PLC-γ + Y13-238	2.3	55.9	24.3 ± 4.0
PLC-γ + anti–PLC-β	2.2	57.8	26.3 ± 5.2
H-Ras	2.6	45.8	17.6 ± 3.6
H-Ras + anti–PLC-γ	3.2	4.2	1.3 ± 0.6
H-Ras + Y13-259	3.4	4.1	1.2 ± 0.7
H-Ras + Y13-238	2.8	51.0	18.2 ± 3.3

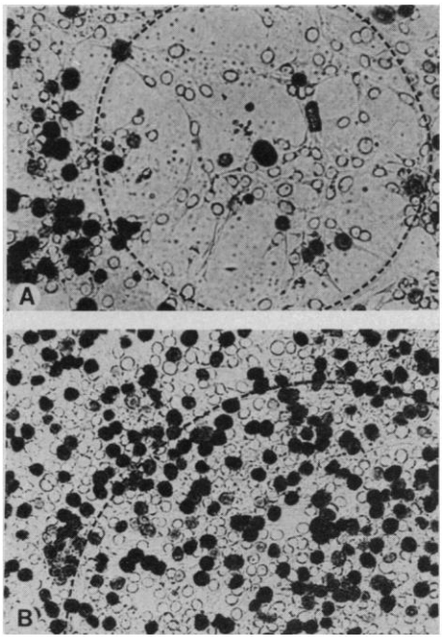

Fig. 2. Requirement for PLC-γ in serum-stimulated DNA synthesis in NIH 3T3 fibroblasts. Monoclonal antibodies to PLC-γ (**A**) or PLC-β (**B**) were microinjected into every quiescent NIH 3T3 cell contained within the circle. Antibody mixtures were the same as described in Fig. 1. After injection, cultures were incubated with serum for 18 hours, labeled with [^{3}H]thymidine (1 μCi/ml; Amersham) for 3 hours, and subjected to autoradiography for 24 hours.

PLC-γ and very little PLC-β, with 3T3 fibroblasts expressing PLC-β in amounts slightly higher than background (Table 1). Protein immunoblotting also revealed that the expression of PLC-β was low in glioma and neuroblastoma cell lines and was barely detectable in NIH 3T3 fibroblasts (Fig. 1A). Large amounts of PLC-γ expression were detected in rat glioma, mouse neuroblastoma, and mouse NIH 3T3 fibroblast cell lines (Fig. 1B). The PLC-γ present in the cell line extracts migrated at a position corresponding to a size of ~145 kD on SDS-polyacrylamide gels. PLC-β from glioma and neuroblastoma cell lines migrated as a doublet on immunoblots, with the top band migrating at ~150 kD and a smaller degradation product being evident below it.

Mixtures of PLC-β and PLC-γ monoclonal antibodies were found to inhibit the in vitro biochemical activity of inositol phospholipid–specific PLC by 75 and 89%, respectively. Combined inhibition is greater than that caused by any one antibody alone. We have determined that the PLC-γ–specific mixture of monoclonal antibodies neutralizes the biological activity of both cellular and purified PLC-γ. Microinjection of this mixture of antibodies into the cytoplasm of proliferating 3T3 cells inhibited DNA synthesis (Fig. 2). The majority of cells injected with antibodies to PLC-γ (anti–PLC-γ) were inhibited from synthesizing DNA (Fig. 2A), in contrast to the uninjected cells. Anti–PLC-β–specific monoclonal antibodies did not inhibit the growth of NIH 3T3 cells after microinjection (Fig. 2B). Perhaps the reason that anti–PLC-β has no effect on serum-induced 3T3 cells is that these cells do not contain PLC-β (Table 1 and Fig. 1).

Results from several microinjection experiments are summarized in Table 2. Injection of a control nonspecific protein, bovine serum albumin, did not induce DNA synthesis in serum-starved NIH 3T3 cells. Injection of PLC-β or PLC-γ into resting cells resulted in a 23-fold average increase in the number of cells synthesizing DNA (54% of injected cells incorporated [^{3}H]thymidine into DNA compared to 2% in control injected or uninjected cells). Injection of Ras protein resulted in an 18-fold increase in DNA synthesis. When PLC-γ or Ras protein was co-injected with neutralizing anti–PLC-γ monoclonal antibodies, an antibody-dependent reduction of DNA synthesis was observed (Fig. 3, A and B). When PLC-γ or Ras protein was co-injected with the neutralizing anti-Ras monoclonal antibody Y13-259, PLC-γ–induced DNA synthesis was not inhibited (Fig. 3C); the Ras protein–induced DNA synthesis was reduced to background levels, as previously described (*17*). Co-injection of PLC-γ or Ras protein with a nonneutralizing anti-Ras monoclonal antibody, Y13-238, had no effect on induced DNA synthesis in either case (Fig. 3D). Injection of PLC-β resulted in the activation of DNA synthesis in resting 3T3 cells (*14*), even though it is not normally expressed in this cell (Table 1 and Fig. 1), and co-injection of PLC-β with anti–PLC-β monoclonal antibodies blocked PLC-β–induced DNA synthesis (Table 2). Specificity of the inhibition of DNA synthesis by the anti–PLC-γ antibody mixture was validated by co-injecting quiescent cells with either PLC-γ plus anti–PLC-β–specific monoclonal antibodies or PLC-β plus anti–PLC-γ–specific monoclonal antibodies: no inhibition of the DNA synthesis induced by PLC-β or PLC-γ was observed (Table 2).

After it was first shown that microinjection of a neutralizing monoclonal antibody to Ras could block serum-stimulated growth of fibroblast cells (*15*), several studies have used antibodies to oncogene products (*16*) and signal-transduction intermediates (*22, 30*) to dissect the molecular events of membrane signal transduction. The binding site of the Y13-259 antibody to Ras has been mapped to a highly conserved region (amino acids 63 to 73) of the protein (*31*); antibody binding does not directly interfere with known in vitro biochemical functions, such as guanosine triphosphate (GTP) binding, GTPase activity, or autophosphorylation activity (*32*). The Y13-259 antibody does interfere with the nucleotide-exchange

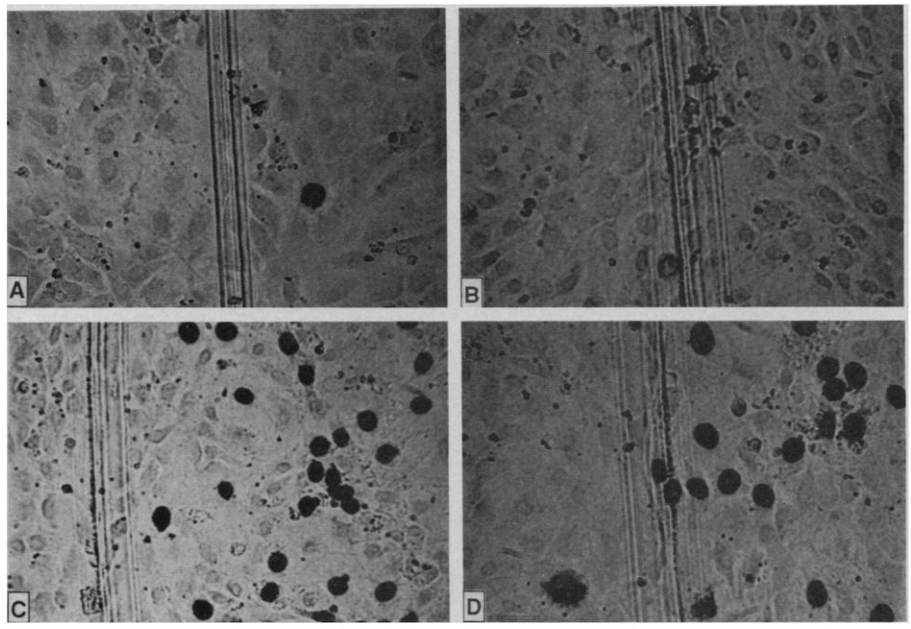

Fig. 3. Effect of co-injection of H-Ras protein or PLC-γ together with monoclonal antibodies on DNA synthesis in NIH 3T3 fibroblasts. Microinjection of PLC-γ plus anti–PLC-γ monoclonal antibodies (**A**); transforming H-Ras protein plus anti–PLC-γ monoclonal antibodies (**B**); PLC-γ plus Y13-259 neutralizing anti-Ras monoclonal antibody (**C**); and H-Ras plus Y13-238 anti-Ras monoclonal antibody (**D**). Cells on the right side of each photomicrograph were injected with oncoprotein plus antibody, and cells on the left side of the scratch were uninjected controls. The microinjection assay was performed as described in the legend to Table 2.

reaction between bound and exogenous guanine nucleotides (*33*) and blocks GAP interaction with ras (*34*), which may explain why this antibody blocks serum-induced growth in 3T3 cells (*15*). Microinjection of this antibody showed that the transforming signals generated by several growth-factor receptor–like oncogenes (*fms*, *fes*, and *src*) were dependent on the cellular Ras protein. In contrast, the transforming signal generated by cytoplasmic oncogenes (*raf* and *mos*) were not dependent on Ras protein for activity because induction of DNA synthesis by these oncogenes was not blocked by the Y13-259 antibody (*16*). These injection experiments established a hierarchy within signal-transducing biochemical pathways for the oncogenes and showed that a downstream block could stop the transforming signal of an upstream oncogene. The antibody to PIP_2 that inhibits bombesin- and PDGF-induced DNA synthesis (*22*) also blocks mitogenesis when injected into *ras*, *src*, and *erb*B oncogene–transformed NIH 3T3 cells, whereas *myc*-transformed cells are not affected (*30*). In summary, our data indicate that PLC-γ activity is necessary for Ras-mediated induction of DNA synthesis in NIH 3T3 fibroblasts and that Ras may function as a G protein–like molecule in inositol phospholipid signal transduction.

REFERENCES AND NOTES

1. J. L. Bos *et al.*, *Nature* **327**, 293 (1987); L. F. Parada, C. J. Tabin, C. Shih, R. A. Weinberg, *ibid.* **297**, 474 (1982); E. Santos, S. R. Tronick, S. A. Aaronson, S. Pulciani, M. Barbacid, *ibid.* **298**, 343 (1982).
2. M. Barbacid, *Annu. Rev. Biochem.* **56**, 779 (1987).
3. P. H. Seeburg, W. W. Colby, D. J. Capon, D. V. Goeddel, A. D. Levinson, *Nature* **312**, 71 (1984); D. J. Clanton, Y. Lu, D. G. Blair, T. Y. Shih, *Mol. Cell. Biol.* **7**, 3092 (1987).
4. J. P. McGarth, D. J. Capon, D. V. Goeddel, A. D. Levinson, *Nature* **310**, 644 (1984).
5. C. J. Der, T. Finkel, G. M. Cooper, *Cell* **44**, 167 (1986).
6. V. Manne, S. Yamazaki, H.-F. Kung, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 376 (1985).
7. T. Y. Shih, A. G. Papageorge, P. E. Stokes, M. O. Weeks, E. M. Scolnick, *Nature* **287**, 686 (1980).
8. R. W. Sweet *et al.*, *ibid.* **311**, 273 (1984).
9. M. Trahey and F. McCormick, *Science* **238**, 542 (1987).
10. J. B. Hurley, M. I. Simon, D. B. Teplow, J. D. Robishaw, A. G. Gilman, *ibid.* **226**, 860 (1984).
11. E. J. Neer and D. E. Clapham, *Nature* **333**, 129 (1988).
12. D. W. Stacey and H.-F. Kung, *ibid.* **310**, 508 (1984).
13. J. R. Feramisco, M. Gross, T. Kamata, M. Rosenberg, R. W. Sweet, *Cell* **38**, 109 (1984).
14. M. R. Smith, S.-H. Ryu, P.-G. Suh, S.-G. Rhee, H.-f. Kung, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3659 (1989).
15. L. S. Mulcahy, M. R. Smith, D. W. Stacey, *Nature* **313**, 241 (1985).
16. M. R. Smith, S. J. DeGudicibus, D. W. Stacey, *ibid.* **320**, 540 (1986).
17. H.-F. Kung, M. R. Smith, E. Bekesi, V. Manne, D. W. Stacey, *Exp. Cell Res.* **162**, 363 (1986).
18. J. R. Feramisco *et al.*, *Nature* **314**, 639 (1985).
19. M. J. O. Wakelam *et al.*, *ibid.* **323**, 173 (1986).
20. T. Kamata and H.-f. Kung, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5799 (1988).
21. J. Meisenhelder, P.-G. Suh, S. G. Rhee, T. Hunter, *Cell* **57**, 1109 (1989).
22. K. Matuoka, K. Fukami, O. Nakanishi, S. Kawai, T. Takenawa, *Science* **239**, 640 (1988).
23. L. F. Fleischman, S. B. Chahwala, L. Cantley, *ibid.* **231**, 407 (1986).
24. M. J. Berridge and R. F. Irvine, *Nature* **312**, 315 (1984); Y. Nishizuka, *ibid.* **308**, 693 (1984); J. F. Hancock *et al.*, *Oncogene* **3**, 187 (1988).
25. J. C. Lacal, J. Moscat, S. A. Aaronson, *Nature* **330**, 269 (1987); A. Wolfman and I. G. Macara, *ibid.* **325**, 359 (1987).
26. L. Kaczmarek, J. K. Hyland, R. Watt, M. Rosenberg, R. Baserga, *Science* **228**, 1313 (1985).
27. P.-G. Suh, S.-H. Ryu, W. C. Choi, K.-Y. Lee, S. G. Rhee, *J. Biol. Chem.* **263**, 14497 (1988).
28. S. H. Ryu, P.-G. Suh, K. S. Cho, K.-Y. Lee, S. G. Rhee, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6649 (1987).
29. S. G. Rhee, P.-G. Suh, S.-H. Ryu, S. Y. Lee, *Science* **244**, 546 (1989).
30. K. Fukami *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9057 (1988).
31. I. S. Sigal, J. B. Gibbs, J. S. D'Alonzo, E. M. Scolnick, *ibid.* **83**, 4725 (1986).
32. J. C. Lacal and S. A. Aaronson, *Mol. Cell. Biol.* **6**, 1002 (1986).
33. S. Hattori *et al.*, *ibid.* **7**, 1999 (1987).
34. S. K. Srivastava, A. DiDonato, J. C. Lacal, *ibid.* **9**, 1779 (1989).

35. We thank D. Longo, J. Rossio, H. Young, and C. Faltynek for critical review of this manuscript and C. Hemp and T. Hardy for assistance in preparation of this manuscript. Funded in part by the Department of Health and Human Services under contract number N01-CO-74102 with Program Resources, Inc.

20 September 1989; accepted 11 January 1990

Translation Initiation and Ribosomal Biogenesis: Involvement of a Putative rRNA Helicase and RPL46

A. B. SACHS* AND R. W. DAVIS

Cold-sensitive mutations in the *SPB* genes (*spb1–spb7*) of *Saccharomyces cerevisiae* suppress the inhibition of translation initiation resulting from deletion of the poly(A)-binding protein gene (*PAB1*). The SPB4 protein belongs to a family of adenosine triphosphate (ATP)–dependent RNA helicases. The aberrant production of 25*S* ribosomal RNA (rRNA) occurring in *spb4-1* mutants or the deletion of *SPB2* (*RPL46*) permits the deletion of *PAB1*. These data suggest that mutations affecting different steps of 60*S* subunit formation can allow PAB-independent translation, and they indicate that further characterization of the *spb* mutations could lend insight into the biogenesis of the ribosome.

THE FUNCTION OF PROTEINS NECESSARY for the processing and assembly of ribosomal components can be explored through mutational studies. Mutations in these proteins could result in an improperly assembled ribosome capable of bypassing steps normally required for translation to be initiated. The *spb* genes (*spb1* through *spb7*) represent one class of such mutations since they suppress the requirement for the poly(A)-binding protein (PAB) in translation initiation (*1, 2*). This requirement could prevent the efficient translation of partially degraded mRNAs lacking their poly(A) tail and its associated PAB. The 60*S* ribosomal subunit probably mediates the PAB requirement because the *spb* mutations affect the amounts of this particle, and one of them (*SPB2*) encodes ribosomal protein L46 (*2*). The identification of the site of action of these mutations suggested that different alterations in the native form of the 60*S* subunit could lead to a basal level of PAB-independent translation initiation. Here we report that these alterations can result from the aberrant production of 25*S* rRNA or the deletion of *RPL46*.

The gene encoding SPB4 (suppressor of *pab1-F364L*) was identified and isolated from a yeast genomic library (*2, 3*) by its ability to rescue the recessive cold-sensitive phenotype of a *spb4-1* mutant (YAS165) (*2*), and the nucleotide sequence of the 2349-bp fragment containing the complementing activity was determined (Fig. 1). Genetic analysis showed tight linkage between this fragment and *spb4-1*. DNA and RNA blot analysis revealed *SPB4* to be a single-copy gene encoding an mRNA of approximately 2.1 kb (Fig. 2, lanes 1 to 4). A diploid strain heterozygous for a replacement of *SPB4* with *TRP1* and homozygous for *trp1* (YAS325) produced up to 50% viable spores (compared with >95% for controls), and all such spores were tryptophan auxotrophs. Furthermore, a haploid strain containing this genomic replacement could only grow in the presence of *SPB4* on a plasmid (YAS356). These experiments show that *SPB4* is essential for growth, and they imply that at the nonrestrictive temperature *spb4-1* encodes a partially functional protein.

SPB4 is a 69,430-dalton basic protein

Department of Biochemistry, Stanford Medical Center, Stanford, CA 94305.

*To whom correspondence should be addressed. Present address: Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142.