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S-phase induction and transformation of quiescent NIH 3T3 cells by microinjection of phospholipase C

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ABSTRACT **Two inositol phospholipid-specific phospholipase C (PLC) isozymes (PLC-I and -II) have been purified from bovine brain. When PLC-I or PLC-II was microinjected (100–700 μg/ml) into quiescent NIH 3T3 cells, a time- and dose-dependent induction of DNA synthesis occurred, as demonstrated by [^{3}H]thymidine incorporation into nuclear DNA. In addition, ≈8 hr after PLC injection, NIH 3T3 fibroblasts appeared spindle-shaped, refractile, and highly vacuolated, displaying a morphology similar to transformed cells. The morphologic transformation was apparent for 26–30 hr after which the injected cells reverted back to a normal phenotype. Microinjected PLC at a high concentration (1 mg/ml) was cytotoxic, dissolving the cytoplasmic membrane and leaving behind cellular ghosts. PLC is a key regulatory enzyme involved in cellular membrane signal transduction. Introduction of exogenous PLC into NIH 3T3 cells by microinjection induced a growth and oncogenic potential, as demonstrated by the ability of microinjected PLC (≈10,000 molecules per cell) to override the cellular G_0 block, inducing DNA synthesis and morphologic transformation of growth-arrested fibroblast cells.**

Inositol phospholipid-specific phospholipase C (PLC) (1, 2) is central to the mechanism of membrane signal transduction because it hydrolyses phosphoinositol bisphosphate, generating two second messenger molecules, inositol trisphosphate and diacylglycerol, in response to several receptor-binding polypeptide hormones (3, 4). Diacylglycerol, an integral component of the cytoplasmic membrane, allosterically activates protein kinase C (5, 6), whereas inositol trisphosphate induces the release of Ca^{2+} from intracellular membrane stores (7). The cellular response to an increase in intracellular Ca^{2+} and to activation of protein kinase C is synergistic when both pathways are operating simultaneously (8). The molecular events that regulate transmembrane signaling and elicit the production of diacylglycerol and inositol trisphosphate have not been defined clearly, but several studies have implicated guanine nucleotide-binding regulatory proteins (G proteins) (7, 9). Models of G-protein-regulated signal transduction (10) propose mechanisms in which receptor-mediated hydrolysis of phosphoinositol bisphosphate is coupled through G-protein-dependent activation of PLC (11, 12).

Several research groups have cloned and sequenced isozymes of PLC (13, 14). They reported that PLC-II contains a region that has significant sequence homology to several oncogenes of the nonreceptor tyrosine kinase class, such as *src* (15), *yes*, *fgr*, *abl*, *fps*, and *fes* (16), and a recently identified oncogene *crk* (17). The region of homology is located in the noncatalytic domain that is not essential for tyrosine kinase activity but may be involved in interactions of these isozymes with cellular components that modulate kinase function. PLC and cytoplasmic tyrosine kinases may be regulated by common cellular components, such as G proteins.

Microinjection by using a glass capillary tube is an established method of introducing macromolecules into living mammalian cells to study their intracellular biological effects. Injection of the *ras* gene product p21 demonstrated that this purified oncoprotein was sufficient to override the G_0 block of serum-deprived mouse fibroblast cells, inducing the S phase of the cell cycle. Furthermore, microinjection of ras p21 protein into normal mouse fibroblasts induced a temporary morphologic transformation (18, 19). The c-myc protein, after microinjection into the nuclei of quiescent Swiss 3T3 cells, cooperated with platelet-derived growth factor to stimulate cellular DNA synthesis, suggesting that c-myc may function as an intracellular competence factor (20). Microinjection has proven to be a powerful tool for the study of oncogenes and membrane signal transduction hierarchy (18–22).

Several immunologically distinct isozymes of PLC (2, 23) have been purified from bovine brain, and these enzymes exhibit absolute specificity for inositol phospholipid substrates (1, 2). In the present study, we have microinjected two isozymes of PLC into quiescent NIH 3T3 cells to directly test the growth-promoting and oncogenic potential of these enzymes.

MATERIALS AND METHODS

Cell Culture. NIH 3T3 fibroblast cells were obtained from D. Lowey (National Cancer Institute) and cultured in Dulbecco's modified Eagle's medium with 10% (vol/vol) calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and L-glutamine (4 mM). The cells (25,000 cells per ml) were applied to glass coverslips in 35-mm plates and allowed to grow until near confluency for 1–2 days.

Enzymes. PLC-I and PLC-II were purified from bovine brain as described (1). The catalytic subunit of cAMP-dependent protein kinase A (A kinase) and bovine serum albumin (BSA) were purchased from Sigma.

Microinjection Assay. When NIH 3T3 fibroblast monolayers reached 80% confluency, the normal culture medium was replaced with medium containing 0.5% calf serum. Cultures were starved for 24–36 hr before microinjection of PLC and control solutions. Microinjection assays were performed on double-blind samples. Approximately 200 cells attached to the glass coverslip within an etched circle were injected (10^{-14} liter) with several concentrations of PLC. Injected areas were monitored with time and morphologic transformation was photographed. A 3-hr pulse of [^{3}H]thymidine (5 μCi/ml; 1 Ci = 37 GBq; Amersham) was preformed 10–24 hr

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Abbreviations: PLC, phospholipase C; G protein, guanine nucleotide-binding regulatory protein; BSA, bovine serum albumin.

after injection. Cultures were washed with isotonic phosphate-buffered saline (pH 7.4; PBS) and fixed in 3.7% (vol/vol) formaldehyde.

Autoradiographic Procedure for the Determination of [^{3}H]Thymidine Incorporation into DNA. The fixed cells on the coverslips were mounted onto glass slides, coated with nuclear track emulsion (NTB2; Eastman Kodak), and exposed for 24 hr. Number of cells that had incorporated [^{3}H]thymidine into nuclei were monitored by microscopic observation.

RESULTS

Induction of DNA Synthesis After Microinjection of PLC. Fig. 1 *C–F* illustrates induction of [^{3}H]thymidine incorporation into the nuclei of serum-starved fibroblast cells after microinjection of PLC-I or PLC-II. In contrast, BSA or A kinase (catalytic subunit of the cAMP-dependent protein kinase from rabbit muscle) injection did not induce DNA synthesis (Fig. 1 *A* and *B*). Microinjection of the catalytic subunit of A kinase has been shown (24) to directly induce the expression of fusion genes containing a cAMP-responsive human promoter linked to the bacterial reporter gene and the c-*fos* gene. These genes are regulated by cAMP and the results suggest that protein phosphorylation is involved in the regulation of the expression of these genes (24). A kinase appears to be involved in activation of differentiation process within the cell but not involved in progression of the cell cycle. Microinjection of PLC had little effect at 100 μg/ml, and cytotoxic effects were observed at 940 μg/ml. The injected enzyme literally dissolved the cytoplasmic membrane leaving cell ghosts (Fig. 1*E*). Optimal incorporation of [^{3}H]thymidine into nuclear DNA occurred with injected PLC-I or PLC-II between 200 and 300 μg/ml, inducing 44% and 63%, respectively, of injected cells to enter S phase (Table 1). A PLC concentration of 250 μg/ml is equivalent to microinjecting $\approx$10,000 molecules of enzyme into each cell (injection volume, 10^{-14} liter). Microinjection of purified PLC-II (100–700 μg/ml; specificity activity, 24 μmol per min per mg, ref. 1) resulted in a concentration-dependent dose–response curve (Figs. 1 and 2) for percent injected cells incorporating thymidine into DNA. The concentration of PLC required to induce a 26-fold increase of DNA synthesis in quiescent 3T3 cells is two orders of magnitude less than the concentration of *Escherichia coli*-synthesized ras p21 protein needed to induce DNA synthesis. One million molecules of p21 Ha-ras protein are required to generate a 20-fold induction of DNA synthesis in our microinjection assay (M.R.S. and H.F.K., unpublished data). PLC, purified from bovine brain, is 100 times more effective than *E. coli*-synthesized ras

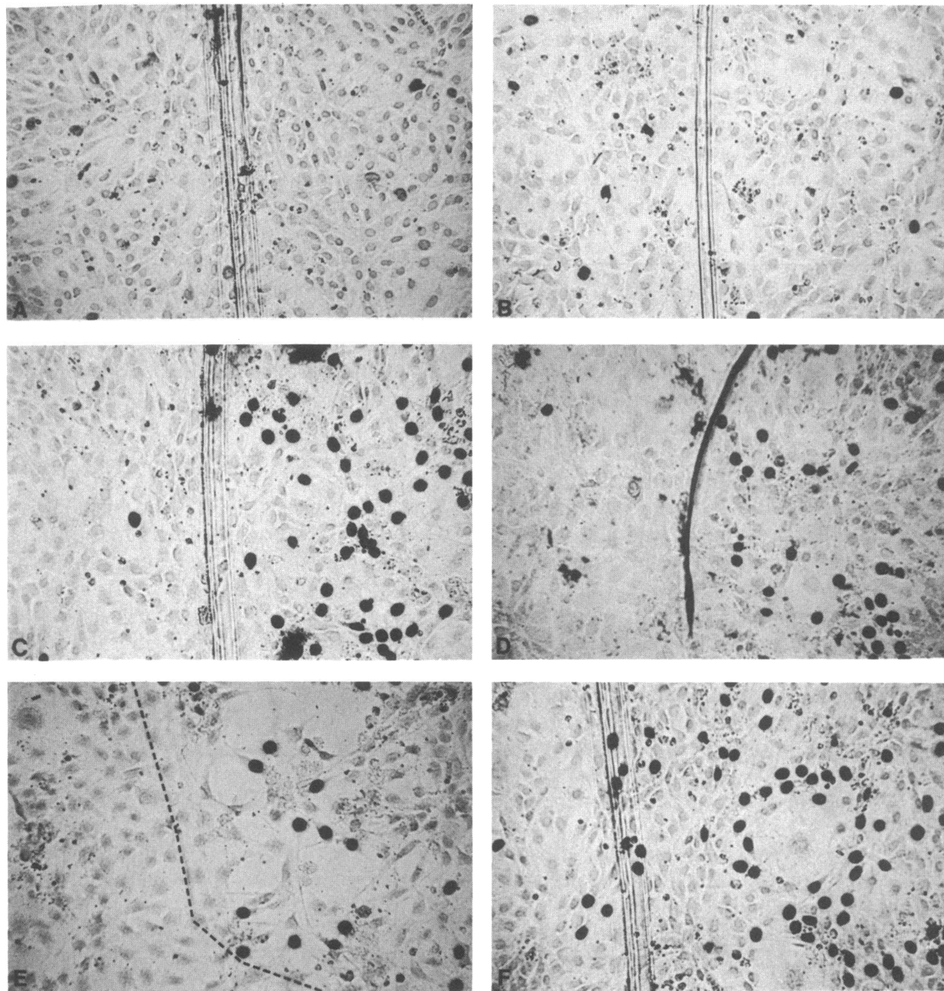

FIG. 1. Microinjection of PLC induces DNA synthesis in quiescent NIH 3T3 fibroblasts. Injected cells are on the right halves of the micrographs, separated from the uninjected cells (left halves) by scratches made on the coverslips. (*A* and *B*) Injection of BSA and A kinase, respectively, into 150–200 cells results in no increase of DNA synthesis. (*C*) Injection of PLC-II (250 μg/ml) results in a 26-fold increase of cells synthesizing DNA. (*D*) Injection of PLC-II (160 μg/ml) results in a 14-fold increase of cells synthesizing DNA. (*E*) Cytotoxic effects of injecting PLC-II (700 μg/ml) are shown but also result in a 10-fold increase of DNA synthesis in the cells that remained. Approximately 50% of the injected cells have died and lifted from the coverslip. (*F*) Injection of PLC-I (250 μg/ml) results in a 18-fold increase of cells entering S phase.

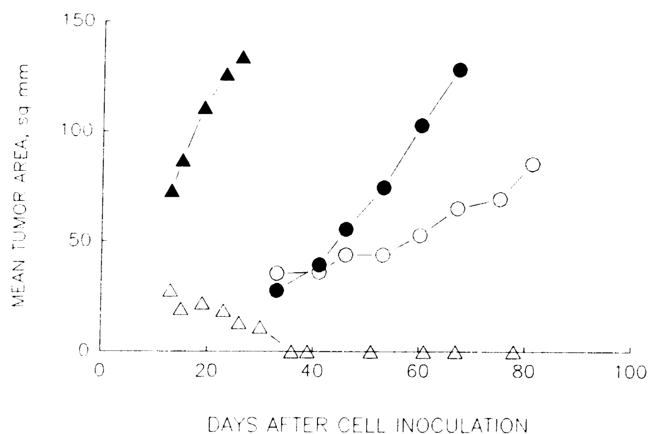

FIG. 1. Growth of MCF-7 and MIII cells in athymic nude mice. Cells (2×10^6) were inoculated into each flank of ovariectomized athymic nude mice with or without E2 supplementation (60-day E2 pellet inoculated subcutaneously at the time of tumor cell inoculation). A total of 24 sites were inoculated for each cell line, there being 12 sites in E2-supplemented animals and 12 sites in non-E2-supplemented animals. Data points represent the mean tumor size; only measurements obtained from growing tumors were included. MIII cells (circles) were passage 12; MCF-7 cells (triangles) were passage >500. Solid symbols represent data obtained from E2-supplemented animals; open symbols represent data from nonsupplemented animals.

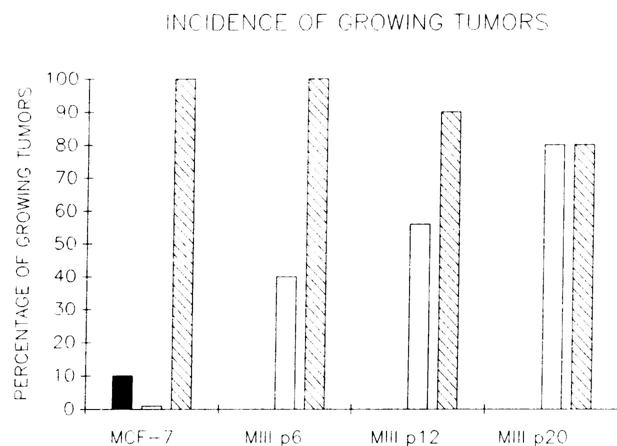

FIG. 2. Effect of time in culture on the tumor incidence of MIII cells. Tumor incidence is defined as the number of proliferating tumors expressed as a percentage of the number of inoculation sites. A total of 24 sites were inoculated for cells at each passage, there being 12 sites in E2-supplemented animals (hatched bars) and 12 sites in non-E2-supplemented animals (open bars). Solid bar represents the tumor incidence observed in the absence of E2 in the original experiment from which the MIII cells were derived.

respond mitogenically to E2 *in vitro*, they respond normally with respect to PGR induction. The basal levels of PGR in MIII cells appear higher than the other cell lines, but this is not significant ($P > 0.05$ for MIII versus MCF-7). MIII, BSK-2, and BSK-3 cells have acquired the ability to form ovarian-independent tumors but the level of EGF-R expression in BSK-2 cells is not substantially different from that in MCF-7 cells. MIII and BSK-3 cells express lower levels of EGF-R when compared with MCF-7 or BSK-2 cells.

Invasive Properties of Cell Lines *in Vivo* and *in Vitro*. The gross morphology of MIII and BSK tumors in either the presence or absence of E2 is similar to the parental tumors (Fig. 3). However, MIII tumors often exhibit considerable invasion into surrounding tissues, leading to occasional intraperitoneal tumor formation. Intraperitoneal tumors occur in both the presence and absence of E2 supplementation. Fig. 3*A* shows an MIII tumor invading through the peritoneal wall. In contrast, MCF-7 and BSK tumors are frequently encapsulated and show no evidence of local invasion into the peritoneal cavity. Fig. 3*B* shows a representative MCF-7 tumor growing subcutaneously but no significant invasion into surrounding skin tissue. While BSK cells possess similar chemoinvasive properties as the parental cell line, MIII cells are significantly more invasive across an artificial basement membrane *in vitro* (Fig. 4). Thus, under conditions in which the chemotactic component of the assay was equivalent for all cell lines, MIII cells show an ≈200% increase in chemoinvasive activity. These observations confirm the apparently more invasive nature of the cells observed *in vivo*.

Table 2. Tumor doubling time in days of cells inoculated into ovariectomized animals with or without E2 supplementation

Cell line	Tumor doubling time, days	
	− E2	+ E2
MCF-7	— (0/12)	13 (8/10)
BSK-2	56 (4/8)	11 (4/8)
BSK-3	54 (3/10)	16 (5/10)
MIII	42 (7/12)	13 (8/10)

Data represent the mean time (days) taken for all the tumors in each group to double in size. Doubling time was determined only for proliferating tumors of 350 mm^3. Numbers in parentheses indicate the number of proliferating tumors included as a fraction of the sites inoculated.

DISCUSSION

Characterization of the Hormone-Independent but Responsive Phenotype. The E2 dependence of MCF-7 cells for tumor formation in ovariectomized mice is widely reported (6, 14, 16, 17) and may reflect, in part, the routine maintenance of these cells in the presence of estrogen. The ability of MIII and BSK cells to form E2-responsive tumors in ovariectomized animals clearly indicates that they have progressed to the intermediate stage of hormone-independent but hormone-responsive growth observed in experimental rodent mammary and prostate cancers (1, 2).

The incidence of MIII tumors in ovariectomized animals reaches the incidence observed in E2-supplemented animals between 12 and 20 passages after establishment *in vitro*. Thus, further adaptation occurred after their initial development *in vivo*, perhaps reflecting either the progressive elimination or adaptation of some subpopulations as implied by the observed alterations in the chromosome number range. Since the BSK and MIII cell lines are not clonal in origin, the ovarian-independent but responsive phenotype could reflect the presence of at least two populations of cells, one ovarian dependent and the other independent.

Ovarian-independent and hormone-unresponsive cell lines and tumors are frequently characterized by a loss of ER and elevated EGF-R expression (18, 19). MIII and BSK-2 cells express similar levels of ER to the parent cell line. EGF-R levels are equivalent in MCF-7 and BSK-2 cells and lower in BSK-3 and MIII cells. The different levels of expression of EGF-R may reflect the presence or absence of estrogenic stimuli, since EGF-R in human breast cancer cells is reported to be increased by treatment with E2 (20). The PR present in BSK-2 cell culture medium could be providing the estrogenic stimulus responsible for the elevated EGF-R levels relative to MIII and BSK-2 cells (9). Acquisition of ovarian-independent and hormone-unresponsive growth in some rat mammary tumors occurs without significant loss of ER and PGR expression (21). Thus, loss of either ER or PGR and/or overexpression of EGF-R may occur in the later stages of

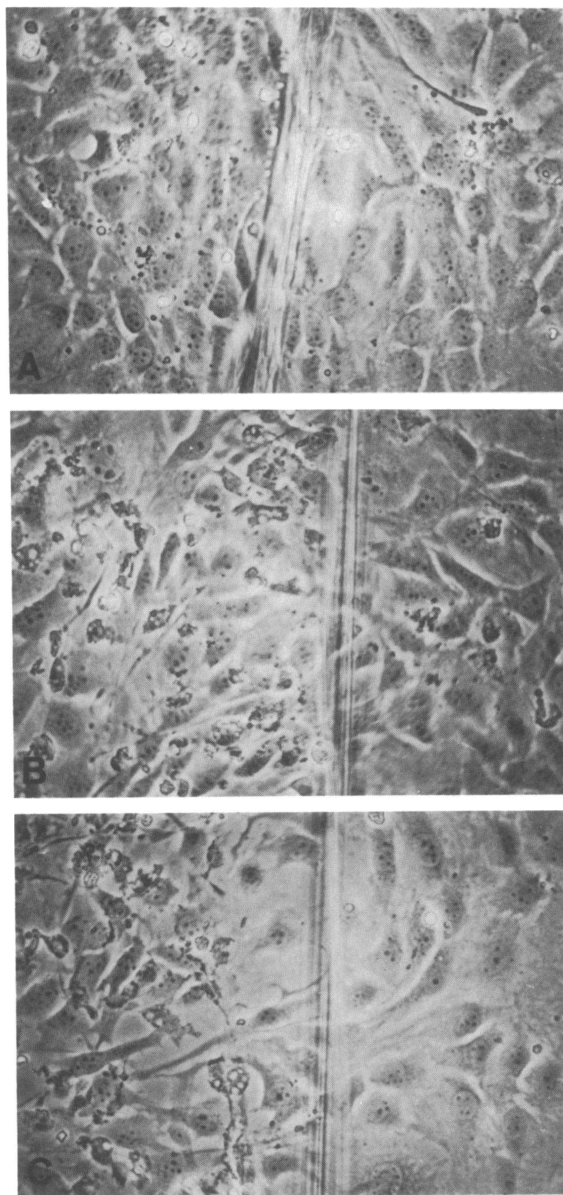

FIG. 3. Morphologic transformation of NIH 3T3 cells microinjected with PLC. Cells on the left side of the center scratch were injected as follows. (*A*) A kinase (250 units/ml). (*B*) PLC-II (160 μg/ml). (*C*) PLC-II (250 μg/ml). Cells were photographed 12 hr later. Cells on the right side of the center scratch are uninjected controls.

DISCUSSION

The experimental results suggest that excess intracellular PLC has a growth-promoting potential: inducing quiescent NIH 3T3 fibroblasts to synthesize DNA in a time-dependent fashion after microinjection of PLC into cells. Microinjection of $\approx$10,000 molecules of PLC-I or PLC-II induced a morphologic alteration similar to that of transformed cells.

The time courses of increased DNA synthesis induced by serum and by microinjection of PLC into quiescent NIH 3T3 cells follow similar patterns of [^{3}H]thymidine incorporation into the cellular DNA. Relatively low levels of labeling were observed in the first 10 hr after induction either by serum or PLC injection, suggesting that the G_0-arrested 3T3 cells were induced to proceed along the cell cycle and enter S phase. The similar pattern of S-phase entry obtained from serum-stimulated and PLC-injected quiescent fibroblasts and the lack of [^{3}H]thymidine uptake during the first 10 hr of induction or after injection suggest that the observed increase in

Table 2. Time course for DNA synthesis induced by serum factors or microinjection of PLC into quiescent NIH 3T3 cells

Microinjected sample	Fold induction			
	0–6 hr	6–12 hr	12–18 hr	18–24 hr
BSA (1 mg/ml)	1.1 (0.3)	1.3 (0.4)	1.2 (0.2)	1.4 (0.4)
PLC-II (220 μg/ml)	1.3 (0.3)	4.4 (2.8)	21 (6.8)	26 (5.8)
Serum*	1.2 (0.4)	7.2 (3.3)	83 (9.3)	96 (4.3)

Fold induction was calculated as in Table 1. Numbers in parentheses are standard deviations from three experiments. Individual cultures of serum-starved NIH 3T3 cells (22–30 hr) were microinjected with BSA (1 mg/ml) or PLC-II (220 μg/ml) and pulse-labeled with [^{3}H]thymidine (5 μCi/ml) during the 6-hr period after microinjection as indicated. Cultures were washed and fixed, and autoradiography was performed.

*Dulbecco's modified Eagle's medium with 10% fetal calf serum was added to serum-starved cultures of NIH 3T3 cells. [^{3}H]Thymidine was added and the cultures were processed in the same manner as the injected cultures.

DNA synthesis is the result of S-phase induction of G_0-arrested cells and not an artifact of DNA excision repair mechanisms.

Intermediates of phosphoinositol metabolism have been shown (25) to be required for the growth-promoting signal transduction of platelet-derived growth factor and bombesin. Matuoka *et al.* (25) microinjected a monoclonal antibody specific for phosphatidylinositol-4,5-bisphosphate into the cytoplasm of NIH 3T3 cells that resulted in blocking DNA synthesis induced by platelet-derived growth factor and bombesin but not by fibroblast growth factor, epidermal growth factor, insulin, or serum. Their experimental results suggest that platelet-derived growth factor and bombesin activate membrane receptors that transduce growth-promoting signals through the inositol phospholipid pathway. This is consistent with our microinjection studies of PLC that demonstrate that inositol phospholipid turnover is involved in cellular proliferation. PLC and cellular oncogenes, such as *src* (15) and *crk* (17), contain regions of sequence homology that are likely to serve a common regulatory function in these signal transduction pathways.

The biochemical role of PLC and the activity of G proteins in inositol phospholipid signal transduction remain obscure. Neutralizing monoclonal antibodies specific for PLC and inositol phospholipid intermediates can be utilized to further study the biochemical role of PLC in normal and transformed cells. The ras protein has been shown to be involved in phosphoinositol turnover (26, 27). In conjunction with the ras-neutralizing monoclonal antibody Y13-259 and various oncogene-transformed NIH 3T3 cells (23), the exact role of PLC and G proteins in the inositol phospholipid signal transduction pathway can be elucidated with the microinjection assay.

The availability of cloned cDNA for PLC (15, 16) will make it possible to pursue structure–function studies of PLC. Utilizing site-directed mutagenesis, mutant PLC protein will be expressed in bacterial or eukaryotic expression systems. Enzymatic activity will be determined and their transforming potential will be assessed using the microinjection assay. The present report has established a bioassay for the intracellular mitogenic activity of PLC.

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