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PLCγ1 Src Homology Domain Induces Mitogenesis in Quiescent NIH 3T3 Fibroblasts[1]

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Previously, we demonstrated that microinjection of phosphoinositide-specific phospholipase Cγ1 (PLCγ1) and lipase-defective mutants of PLCγ1 induced G_0 growth arrested NIH 3T3 fibroblasts to enter S phase of the cell cycle. These experiments suggested that regions other than the catalytic domain of PLCγ1 may be responsible for inducing mitogenesis. To test other regions of PLCγ1 for DNA synthesis inducing activity, cDNA fragments encoding Src homology (SH) and pleckstrin homology (PH) domains were subcloned into the bacterial expression plasmid pGEX-2TK, and the GST fusion proteins were purified. The complete PLCγ1 SH domain peptide was found to induce DNA synthesis after microinjection into growth arrested fibroblasts. Peptides containing a single SH3 domain or two SH2 domains induced a partial response that was restored to full activity if they were co-injected. The PH domain peptide did not induce DNA synthesis. Thus, both SH3 and SH2 activity combine to give maximum DNA synthesis induction, demonstrating that non-catalytic structural domains of PLCγ1 have pronounced effects on mitogenic signaling pathways. © 1996 Academic Press, Inc.

In higher eukaryotic cells, growth and metabolism are tightly regulated, one mechanism utilized to achieve this regulation is the formation of transient signaling complexes that are recruited to the activated cytoplasmic region of a ligand-bound receptor. Proteins commonly found to participate in the formation of these complexes contain a protein-binding motif called Src homology 2 and 3 (SH2 and SH3) domains (1–3). Proteins that contain SH domains can be subdivided into two groups. The first group consists of those containing an enzymatic activity necessary for downstream signaling, such as members of the Src-related family of tyrosine kinases, the phosphotyrosine phosphatase Syp, Ras GAP, and PLCγ. The second group of SH domain proteins is void of enzymatic activity and act as adapters for complex protein associations. Members of this group include the p85 subunit of phosphoinositol 3-kinase (PI3K), Crk, Nck, Shc, and members of the Sem-5/Grb2/drk family (4,5). Information pertaining to the specific associations between this family of SH containing proteins is incomplete.

Previously published results had shown that overexpression of PLCγ1 in quiescent fibroblasts, via direct microinjection, induces DNA synthesis (5) and alters the growth rate and morphology of transfected cells (6). Surprisingly, PLCγ1 lipase-defective mutants also induced DNA synthesis (7,8), suggesting that regions other than the catalytic domain may be responsible for the mitogenic response. Further experimental evidence suggesting that PLCγ1 SH domains play a critical role in mitogenic signaling came from injection studies with a mixture of monoclonal antibodies raised against PLCγ1. These antibodies were found to inhibit serum- and Ras-induced mitogenesis (9).

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From this mixture of PLCγ1-specific monoclonal antibodies it was determined that one, αB16-5, precipitates in addition to PLCγ1, Nck (10,11), suggesting that PLCγ1-specific monoclonal antibodies cross react with other SH domain proteins. In addition to inhibiting serum- and Ras-induced mitogenesis, PLCγ1 monoclonal antibody, B16-5, inhibited lipase-defective PLCγ1 mutant H335F-mediated DNA synthesis induction (7). In the present study, we demonstrate that the PLCγ1 SH domain activates mitogenesis in G_0 arrested fibroblasts, while the PH domain did not exhibit this activity.

MATERIALS AND METHODS

Cell culture. Murine NIH 3T3 fibroblasts were grown in Dulbecco's modified essential medium (DMEM; Life Technologies) supplemented with 10% calf serum. Cells were seeded on etched glass coverslips in 35 mm dishes at 1×10^5 cells/ml and incubated at 37°C for 1–3 days until confluent. The cultures were made quiescent by treatment with DMEM supplemented with 0.5% fetal calf serum for 24–30 hrs in preparation for microinjection experiments.

Construction of PLCγ1 SH and PH peptides. Fusion proteins comprising various domains of PLCγ1 were fused with glutathione-S-transferase (GST) (Figure 1A). For each fusion protein [denoted SH3, SH22, SH223, and PH(NC)], polymerase chain reaction products flanked by BamHI and EcoRI linkers were inserted into the BamHI and EcoRI site of the pGEX-2TK vector (Pharmacia). The pleckstrin-homology (PH) domain of PLCγ1 is split and the segments are located at the amino (N) and carboxyl (C) termini in relation to the SH domains of the protein (Figure 1A). cDNA fragments containing sequences from the split PH domains were amplified by PCR. The PH(N) domain corresponding to the amino acid sequence Ser482-Thr523 and the PH(C) domain corresponding to the amino acids Asn865-Arg936 were fused with a Glycine insertion. The 5′- and 3′-primers for PH(N) contained BamHI and SmaI sites, respectively. The 5′- and 3′-primers for PH(C) contained SmaI and EcoRI sites, respectively. Thus, the combined PH(NC) construct had BamHI, SmaI, and EcoRI sites sequentially. The GST fusion vector pGEX-2T was used for the expression of the combined PH domain. The cDNA fragment of PH(NC) was ligated into the BamHI and EcoRI-digested pGEX-2T (Figure 1A). The fusion vectors were used to transform *Escherichia coli* competent cells.

Purification of GST fusion proteins. After growing the bacteria at 30°C, expression of the GST fusion protein was induced with 0.1 mM isoproyl-β-D-thiogalacto-pyranoside (IPTG), and the cells were collected by low speed centrifugation (2000 × g for 15 min). The cells were sonicated in PBS and centrifuged at 5000 × g for 15 min. The supernatant was mixed with a 50% slurry of glutathione sepharose 4B equilibrated with PBS at room temperature for 30 min. After centrifugation, the supernatant was removed. The glutathione sepharose 4B pellet was washed with 10 bed volumes of PBS and the peptides were eluted from the sepharose beads by addition of thrombin (10 μg/ml) at room temperature for 6 hrs. This fraction contained the protein of interest, while GST remained bound to the matrix. The peptides were further purified by Mono Q FPLC (25 mM ethanolamine, pH 8.8, 1 mM EDTA, 0–150 mM NaCl gradient, 25 min, 1 ml/min) to remove thrombin. Concentration of the purified proteins was assessed with Bio-Rad protein reagent and standardized on 12% polyacrylamide gels (Figure 1B).

Microinjection assay. NIH 3T3 fibroblasts were treated with low serum DMEM (0.5% fetal calf serum) for at least 24 hrs before microinjection of coded samples of PLCγ1 peptides. Samples (50–250 μg/ml) were loaded into glass capillary micropipets pulled with an automatic P80/PC micropipet puller (Sutter Instrument Co.) and injected (approximately 0.1–1.0 $\times 10^{-1}$ ml/cell) into designated cells on a coverslip with an Eppendorf microinjection system (microinjector 5242 and a micromanipulator 5171). Injected cultures were maintained in low serum DMEM for 16–18 hrs, pulsed with [^{3}H]thymidine (0.5 μCi/ml; 1 Ci = 37 GBq; Amersham) for 4 hrs, washed with isotonic PBS, and fixed with 2.5% (v/v) glutaraldehyde in PBS.

Determination of [^{3}H]thymidine incorporation into DNA. The fixed cells on the coverslips were mounted onto glass slides, coated with nuclear tracking emulsion (NTB2; Eastman Kodak), and exposed in the dark for 36–48 hrs. The autoradiography was processed in D-19 developer and rapid fixer (Eastman Kodak). The cells were stained with Giemsa and the injected and background areas were scored and photographed with a Wild Flourautomat MPS45 camera (Leitz). The number of cells that incorporated [^{3}H] thymidine into DNA was monitored by microscopic observation with a Leitz Laborlux 12 microscope.

RESULTS AND DISCUSSION

Previously, PLCγ1 and PLCγ1 mutants without catalytic lipase activity were shown to induce mitogenesis in quiescent NIH 3T3 fibroblasts (5,7,8), suggesting that a non-catalytic region may be responsible for the mitogenic activity. To test other regions of PLCγ1 for mitogenic activity, bacterial plasmids expressing peptides from several PLCγ1 domains were constructed (Figure 1). Portions of the PLCγ1 gene coding for various domains were fused to the glutathione-S-transferase

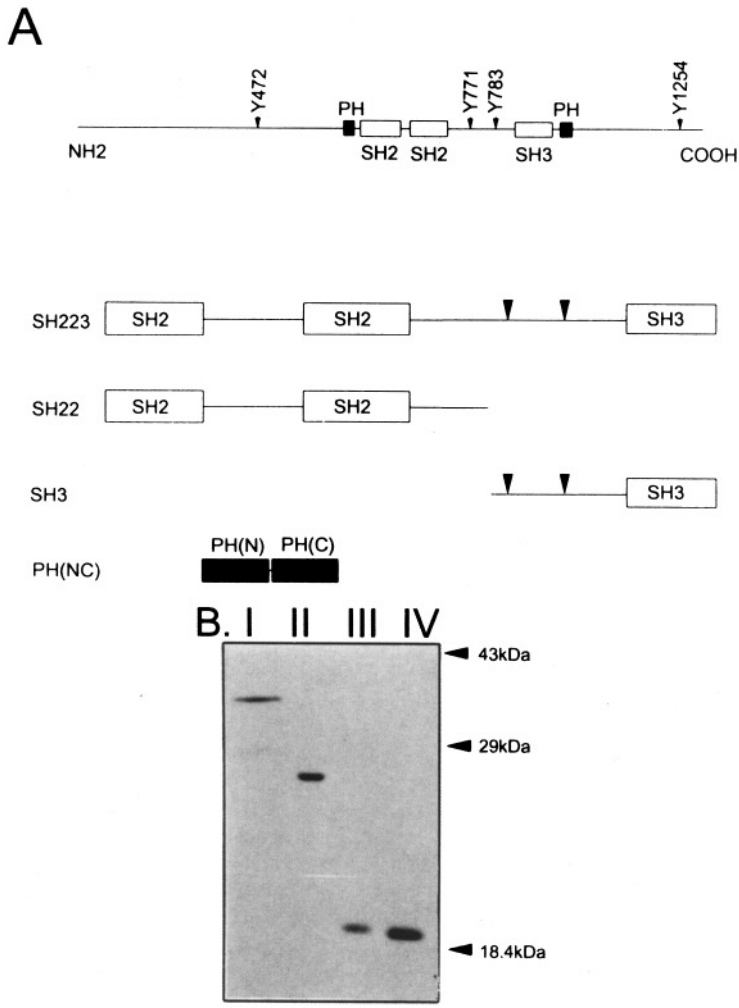
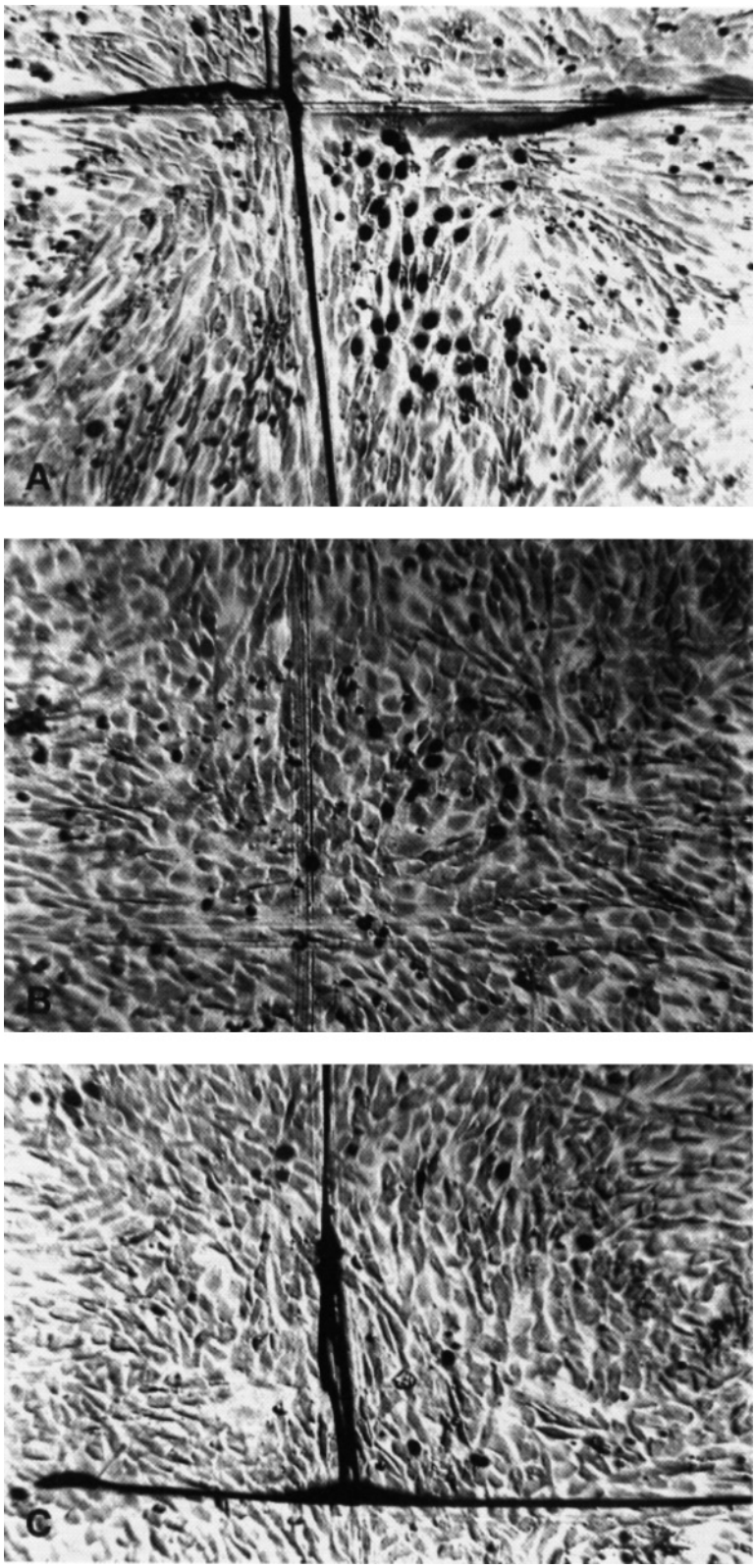


FIG. 1. Construction and analysis of PH- and SH2/SH3-containing PLCγ1 proteins. (A). Schematic representation of PH- and SH2/SH3-containing constructs of PLCγ1. Portions of the PLCγ1 gene were engineered and replicated using the bacterial expression plasmid, pGEX-2TK, construct SH223 encompasses nucleotides 1597–2553, construct SH22 encompasses nucleotides 1597–2274, construct SH3 encompasses nucleotides 2275–2553. The PH(N) and PH(C) domains were cut out and spliced together. Arrows indicate critical tyrosine phosphorylation sites at amino acids 472, 771, 783, and 1254. The numbering of the PLCγ1 gene is based on a previously reported sequence (24). (B) Recombinant fusion proteins produced from each of the expression constructs shown in (A) using the pGEX-2TK expression system. Purified proteins were run on a polyacrylamide gel. Lane I is SH223, lane II is SH22, lane III is SH3, and lane IV is the PH domain. Molecular weight markers are indicated by arrows.

(GST) gene using the pGEX-2TK vector (Figure 1A). Fusion proteins were purified from transformed bacteria using glutathione sepharose 4B affinity chromatography. Purity was assessed by running the proteins on polyacrylamide gels (Figure 1B).

Purified proteins containing different regions of PLCγ1 were microinjected into quiescent NIH

FIG. 2. The PLCγ1 SH domain induced DNA synthesis after microinjection into quiescent NIH 3T3 cells. Approximately 100 quiescent cells to the right of the vertical scratch were microinjected with the complete PLCγ1 SH domain, SH223 (A), a pair of PLCγ1 SH2 regions, SH22 (B), or the pleckstrin homology (PH) domain, PH(NC) (C). Cultures were incubated at 37°C for 18 hrs, pulsed with [^{3}H]thymidine (0.5 μCi/ml) for 4 hrs, fixed with 2.5% glutaraldehyde and autoradiography was performed in nuclear tracking emulsion. Cells were stained with Giemsa and the injection areas scored and photographed at 100× (original magnification).


A
B
C

3T3 cells. It was determined that 50–100 μg/ml gave a maximum induction of DNA synthesis, 250 μg/ml showed signs of cytotoxicity (55% determined by propidium iodide exclusion, 12; data not shown). Microinjection of the complete PLCγ1 SH domain (SH223) resulted in an approximate 28-fold induction of DNA synthesis (Figure 2A and Table 1), while SH domain fragments, SH22 (Figure 2B and Table 1) and SH3 (Table 1) were scored at approximately 10-fold induction of DNA synthesis. However, when SH3 and SH22 fragments were co-injected into cells, DNA synthesis inducing activity was restored to levels seen after injection of the entire SH domain, with approximately 25-fold induction (Table 1). This result suggests that the SH3 and SH22 fragments each have an effect on DNA synthesis induction and further demonstrates that the distinct biological function of each structural region contributes to give an additive response in the assay. Bovine serum albumin gave a value of 3-fold induction of DNA synthesis, similar to the activity seen after injection of the PH domain (Table 1).

Pleckstrin homology (PH) domains, a PLCγ1 region that has not been extensively characterized, have been identified in several proteins that are intermediates in the Ras activation pathway (examples include Btk/Tec, PLCγ, p120 GAP, Sos, and GAP1^m). The function of this structural motif is not known, but it may participate in anchoring proteins to membranes, and/or facilitating complex associations with other membrane proximal molecules (13–15). Microinjection of this region into quiescent cells had no effect (Figure 2C and Table 1), suggesting that the PH domain plays no direct role to induce mitogenesis.

PLCγ1 is a large multifunctional protein with a catalytic lipase domain and several other structural/regulatory domains (16). Considering the enzymatic activity of PLCγ1, it would naturally be assumed that a major function of the enzyme during mitogenic signaling would result from the enzymatic hydrolysis of PIP_2 forming IP_3 and DAG (Figure 3). Recently, two independent groups have shown that lipase-defective PLCγ1 mutants still induce DNA synthesis after microinjection into quiescent fibroblasts (7,8), suggesting that another region of PLCγ1 is responsible for inducing mitogenesis. We observed that lipase-defective PLCγ1 mutants induce DNA synthesis in

TABLE 1
Functional Analysis of PLCγ1 SH and PH Domains: Microinjection of the PLCγ1 SH Domain Induces DNA Synthesis in Quiescent Fibroblasts

Injected sample	% + INJ cells	Fold induction DNA synthesis	# cells injected
BSA	4 (2)	2.8 (1.7)	500
PKC	7 (2)	4.2 (1.8)	850
SH3	28 (7)	14.1 (4.7)	2250
SH22	18 (6)	7.8 (5.3)	1650
SH223	58 (12)	27.5 (6.4)	2250
SH22 + SH3	49 (9)	25.0 (6.2)	1050
PH(NC)	5 (2)	3.2 (1.3)	980

Note. Murine NIH 3T3 cells were plated on glass coverslips and grown to confluence before serum starvation for 22–30 hrs in DMEM supplemented with 0.5% fetal calf serum. Coded samples of protein were microinjected at 50–150 μg/ml (injection of $0.1–1.0 \times 10^{-11}$ ml of sample introduced between 1,000–8,000 molecules, peptide concentrations were adjusted so that similar numbers of molecules were injected) and the cultures were incubated at 37°C for approximately 18 hrs, pulsed with [^{3}H]thymidine (0.5 μCi/ml) for 4 hrs, fixed in 2.5% glutaraldehyde, and autoradiography was performed with nuclear tracking emulsion for 2 days. Addition of 10% fetal calf serum DMEM to the serum-starved cells resulted in approximately 65% of the cells in the culture incorporating [^{3}H]thymidine into DNA. Injected and background areas were scored by microscopic observation. Fold induction of DNA synthesis is a measure of the sample's ability to drive the growth-arrested G_0 fibroblasts into S phase. It is defined as the ratio of injected cells that incorporate [^{3}H]thymidine into DNA divided by the ratio of uninjected cells near the injected area that incorporate [^{3}H]thymidine into DNA. This ratio is corrected for background induction of DNA synthesis, which is less than 2%. The mean and standard deviation, in parenthesis, were determined from at least 5 separate experiments.

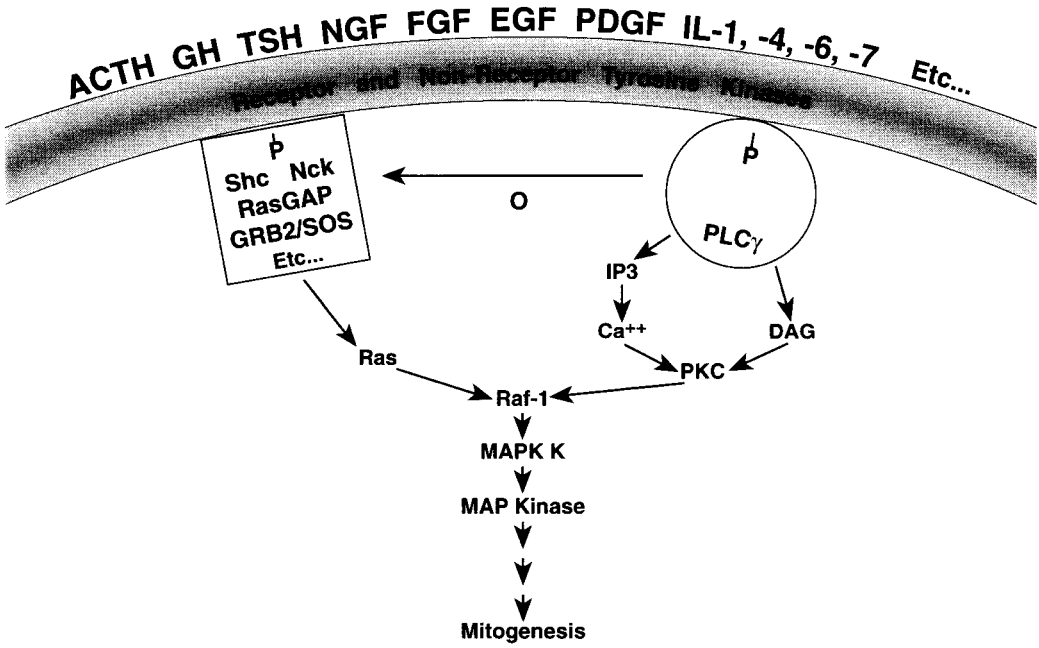


FIG. 3. A molecular model depicting the role of PLCγ1 during growth factor, cytokine, and neurotransmitter induction of mitogenic signal transduction. At least two pathways are used to transduce signals from receptor and nonreceptor tyrosine kinases, one Ras-dependent and another Ras-independent. Raf-1 is the focal point of the two pathways and activates mitogenesis through MAP kinase. During PLCγ1 overexpression (O) both pathways may be activated through PLCγ catalytic activity and by PLCγ SH structural domains. The PLCγ1 SH domain may interact with other SH-containing proteins to activate basal levels of Ras, which leads to Ras-specific phosphorylation of sites on Raf-1. The result of disrupting this family of protein adapters sends a positive mitogenic signal.

15–20% of the injected cells and that wild-type PLCγ1 induces 35–45%. If IP_3 and DAG were co-injected with mutant PLCγ1 H335F full activity was restored (7). Under these experimental conditions, the catalytic activity of PLCγ1 potentiated the mitogenic response. Interestingly, injected PKC does not induce mitogenesis (Table 1). PKC has been shown to phosphorylate specific sites on Raf-1 distinct from the Ras-mediated phosphorylation sites (17) and to differentially activate MAP kinase. Results from Huang et al. (17) also suggest that both the Ras and PLCγ1 components of the FGF receptor signaling system can separately induce mitogenesis, yet during normal signaling the PLCγ1 branch of the pathway is responsible for approximately 60% of the signal and the Ras branch is responsible for 40% of the signal. Our data suggest that overexpression of PLCγ can induce mitogenesis through activation of both branches of the signaling cascade used by growth factors, cytokines, and neurotransmitters (Figure 3). Excess PLCγ1 SH domain may disrupt the equilibrium that exists in the family of SH-containing adaptor proteins in such a way as to transmit a mitogenic signal.

In this report, we demonstrate that the complete PLCγ1 SH domain can induce mitogenesis after microinjection into quiescent cells. Two SH2 regions (SH22) and the isolated SH3 region induce only one third of the response seen from the complete SH domain (SH223) while the PH domain did not have any effect. The partial activity seen after injection of the SH fragments, SH22 and SH3, gave a result clearly different from the background induction seen after BSA or PH domain injection. The entire SH domain was required to achieve maximum levels of DNA synthesis induction. However, co-injection of the SH fragments SH3 and SH22 also resulted in complete induction of DNA synthesis. The report by Huang et al. (8) confirm that microinjection of PLCγ1 and mutant PLCγ1 with defective lipase activity induce DNA synthesis in quiescent fibroblasts,

and further demonstrated a requirement for the SH3 domain during mitogenic signaling with no activity reported for the SH2 region. Their report did not demonstrate any direct effect for the isolated SH3 and SH2-SH2 regions. The inactivity may have resulted from inclusion of the GST portion of the GST-SH3 fusion protein during analysis. We cleaved and separated the region of interest from the GST tag. Furthermore, when they introduced point mutations into the SH2-/- construct (i.e. mutation of FLVR to FLVK), it no longer recognized the Tyr-P binding sites. The lack of SH2 binding affinity may have contributed to the lack of additive mitogenic activity with this construct.

The proteins that associate to form signaling complexes at the inner cytoplasmic surface play a critical role in determining the specificity of the signal that is transmitted into the nucleus and SH-containing proteins act to facilitate the assembly of these signaling complexes. Perhaps, the overexpression of the PLCγ SH domain, achieved by microinjection or transfection, interferes with other SH family members and disrupts the signaling pathway in a way that activates Ras and mitogenesis. Selective interactions within the SH domain family of proteins has been demonstrated (18,19), although the role played by SH domains during receptor complex assembly and the induction of DNA synthesis is not completely understood. SH3 domain containing proteins can have both positive or negative roles during cellular transformation depending upon their amino acid sequence and the location of the protein within a signaling pathway. The SH3 domains of c-Src and c-Abl act to protect against transformation (20–22), while Crk SH3 domains act to promote mitogenesis and transformation (23). Thus, the PLCγ1 SH domain or proteins docking at this domain are responsible for the induction of DNA synthesis observed after microinjection of PLCγ1 into quiescent fibroblasts. The SH3 and PH domains may function to localize PLCγ1 to specific membrane constituents or receptor complexes during signaling. Events that disrupt this tightly regulated equilibrium of proteins in the PLCγ SH domain family act to send positive growth signals and contribute to neoplastic transformation.

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