An SH3 domain is required for the mitogenic activity of microinjected phospholipase C-γ1

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Abstract Phospholipase activity is elevated in dividing cells. In response to growth factor stimulation, phospholipase C-γ (PLC-γ) binds to activated tyrosine kinase receptors via SH2 binding domains, resulting in phosphorylation of PLC-γ and activation of its enzyme activity. These observations suggest that PLC-γ participates in the signal transduction pathway employed by growth factors to promote mitogenesis. Consistent with this hypothesis, microinjection of purified bovine PLC-γ into quiescent fibroblasts has been previously reported to initiate a mitogenic response [Smith et al. (1989) Proc. Natl. Acad. Sci. 86, 3659]. We have reproduced this result using recombinant rat PLC-γ protein. Surprisingly, however, a catalytically inactive mutant of PLC-γ, H335Q, also elicited a full mitogenic response. The capacity to induce mitogenesis by microinjection of PLC-γ was mapped to the 'Z' domain of the protein, which contains PLC-γ's SH2 and SH3 motifs. Inactivation of the phosphorylated tyrosine binding properties of both SH2 domains had no effect on the mitogenic activity of the Z-domain peptide. However, deletion of the SH3 domain resulted in a complete loss of activity. These results suggest that PLC-γ's mitogenic properties do not require the enzyme's phospholipase activity, but are instead mediated by a novel pathway for mitogenic stimulation which is dependent upon an intact SH3 domain.

Key words: Phospholipase C; SH2; SH3; Mitogenic stimulation; Microinjection

1. Introduction

Phospholipase C (PLC) generates two second messengers from the hydrolysis of phosphotidylinositol 4,5 bisphosphate: diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). DAG is an activator of protein kinase C, and IP_3 has been shown to regulate intracellular calcium (for reviews, see [2,3]). To date, 9 isoforms of mammalian PLC enzymes have been described, all of which generate the same second messengers. Activation of these enzymes is observed in a variety of signal transduction pathways, wherein different isozymes of PLC are activated by different growth factors. For example, PLC-β is activated by the transforming α subunit of the G_q protein [4,5], and PLC-γ forms 1 and 2 are activated by the tyrosine kinase growth factor receptors of EGF and PDGF [6–12]. Only the γ forms of PLC contain SH2 and SH3 domains. The SH2 domains mediate a physical association between PLC-γ and phosphorylated tyrosines on the activated tyrosine kinase receptors. SH3 domains bind to poly-proline sequences, and at least one protein which specifically associates with PLC-γ through its SH3 domain, dynamin, has been identified [13,14].

The region of PLC-γ which contains the SH2 and SH3 domains has been designated the 'Z' region [15]. This region of the molecule resides between the C-terminal catalytic regions, denoted X and Y (Fig. 1a). The Z domain bears striking similarity to the oncogenic adapter protein, Nck [16–19]. Nck contains two SH2 domains and three SH3 domains, at least one of which shares a common antigenic epitope with PLC-γ SH3. The degree of sequence homology suggests the possibility that PLC-γ, like other proteins involved in signal transduction (e.g. GAP), can act both as an enzyme and an adapter protein during signal transduction.

The data suggesting that overexpression of PLC-γ is sufficient to elicit a mitogenic response in mammalian cells is inconclusive. Microinjection of purified bovine PLC-γ has been shown to activate S-phase DNA synthesis in serum starved NIH 3T3 cells [1]. In addition, rat cells transfected with Z-like peptides expressed under the CMV promoter exhibited shortened cell cycles and increased DNA synthesis [20]. In contrast, however, stable overexpression of full-length PLC-γ using DNA vectors causes no phenotypic changes in 3T3 fibroblasts [21]. The disparity in results between these systems may reflect differences in PLC-γ concentrations, processing, or localization.

In the current study, we have identified the domains of PLC-γ which are necessary for the mitogenic activity resulting from microinjection of recombinant enzyme into quiescent 3T3 fibroblasts. This work demonstrates that while the SH3 domain of PLC-γ is necessary to initiate a mitogenic response, neither its catalytic activity nor its SH2 domains are required.

2. Materials and methods

2.1. Construction of expression plasmids

Cloning of recombinant rat PLC-γ containing a three amino acid epitope tag (EEF) at the carboxy-terminus has been previously described [22]. All subsequent peptides were constructed by PCR amplification of rat PLC-γ or human PLC-γ sequences. All PCR fragments were verified by DNA sequencing (USB; Sequenase). Construction of mutant PLC-γ H335Q was accomplished by two step PCR amplification of mutant DNA oligomers containing a transversion from T to A at nucleic acid position 1036 [23]. Oligonucleotides used to create the mutant were purchased from Midland Reagents, TX, and were as follows:

5′-CTTCCTCGCAAAATACGTATC
5′-GATACGTATTTTGCGAGGAAG

Oligomers homologous to flanking sites in the vector and PLC sequences were used for outside amplification. The 980 bp PCR product was subcloned into the PLC expression construct using unique *Eco*R1

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and *Nco*I sites, and was sequenced to confirm that only one nucleotide was mutated.

The rat PLC Z domain and human PLC Z domains are virtually identical. Only 9 amino acid differences exist between the two proteins, and all of these substitutions are conservative. Human Z-protein fragment was made by digestion of a partial human PLC-γ cDNA clone with *Avr*II and *Bgl*II. The 1071 bp fragment was cloned into the expression vector, T5T, using unique sites [24]. DNA linkers were used to put the EEF tag in frame at the carboxy-terminus:

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5'-CTAGGGGAAGAGTTCTAGTA          -3'
3'-    CCCTTCTCAAGATCATTCGA      -5'
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and to add a methionine to the amino-terminus:

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5'-AATTCGGATCCATTGGAGGATGATTAAATGAA          -3'
3'-    GCCTAGGGTAACCTCCTACTAATTTACTTCTTCTAG  -5'
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Z-protein includes PLC amino acids 516–869. Mutant Z-proteins were constructed by PCR using wild type Z-protein in T5T as a template. Point mutants in each individual SH2 homology were constructed separately using two step PCR amplification, then a double mutant was created by ligation of mutant fragments using the internal *Kpn*I site. The final double Z(SH2−/−) mutant contained two mutations, R→K at R^{586} and R^{694}. Fragment inserts were verified by sequencing. Primers overlapping the R586K mutation were:

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5'-TCCTCGTGAAAGAGAGTGAGACCTTCG
5'-CACTCTCTTTCACGAGGAAGGAGCCGTC
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Primers for the R694K mutation were:

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5'-TCCTGGTGAAGAAGCGGAATGAACCCAAC
5'-TCCGCTTCTTCACCAGGAAGGCCCCATC
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Deletion of the SH3 moiety was accomplished by overlap PCR spanning sequences surrounding amino acids 798–846. DNA oligomers used to create this deletion were:

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5'-GCAGTCAAAGAAGAGATGGTCAACCCCG
5'-CATCTCTTCTTTGACTGCACACTTGAAAG
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To construct the previously described GST–PLC-γ SH3 fusion protein [25], a PCR product including PLC amino acids 796–851 was made in-frame with the *Bam*HI and *Eco*RI sites in pGEX-2T. The oligos used for this amplification were:

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5'-GCTGGATCCGTCAAAGCCCTCTTTGAC
5'-GCAGGAATTCAGTTGACCATCTCTTCCAC
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2.2. Purification of recombinant proteins

Recombinant protein was purified from *E. coli* strain BL21 containing the appropriate expression constructs. Expression and purification of the proteins was performed essentially as previously described [22]. Briefly, expression was induced by IPTG (0.1 mM) for 2–4 h at 25 or 37°C. Cells were centrifuged and soluble PLC-γ protein was isolated with the immobilized YL1/2 monoclonal antibody, eluted with 5 mM AspPhe dipeptide (Sigma) and concentrated, if necessary. Expression of the GST–PLC-γ SH3 fusion was performed as previously described [26]. Mutant Val-12 Ras was obtained from J. Gibbs (Merck). Protein concentrations were determined by Bradford reagent (Biorad). As a negative control, comparable volumes of extracts made from mock-transfected bacteria were also eluted from the antibody column. For injection, these blank lysates were normalized to PLC-γ-containing fractions by bacterial volume. Final purified protein fractions were analyzed on SDS-PAGE by silver staining with a SilverExpress (Novex) development system to assess protein purity. The identity of the purified proteins were verified by Western blotting with the appropriate antisera. Samples were prepared for microinjection by dilution to the final concentration in 0.2 M KCl or phosphate-buffered saline (PBS). Particulates were cleared from the sample immediately prior to injection by centrifugation at 15,000 × *g* for 10 min at 4°C.

2.3. Determination of lipase activity

PLC-γ activity was tested on sonicated lipid vesicles consisting of 90% dimyristoyl-phosphatidylmethanol (DMPM; Avanti) and 10% [^{3}H]phosphatidylinositol-4,5-bisphosphate (PIP_2, 2 μCi/mmol; NEN). Reactions containing 0.3 mM total lipid in 50 mM Tris, pH 8, and 0.1 mM $CaCl_2$ were run at room temperature and terminated by addition of an equal volume of 200 mM $CaCl_2$ in 1 M HCl. Precipitated vesicles were collected on filter plates (Millipore; 96-well hydrophobic Multiscreen DP plates) and inositol-trisphosphate in the flow-through was quantitated by liquid scintillation counting.

2.4. Microinjection of cells

Mouse fibroblast, NIH 3T3 cells (subline obtained from D. Stacy) were grown in 10% CS, 90% DMEM high glucose (Mediatech, Washington, DC), 0.1% pen/strep (Gibco, USA). To prepare cells for microinjection, cells were plated at half-confluence on 175 mm Eppendorf CELLocate gridded coverslips. After reaching confluence (usually 1 day after seeding), cells were growth arrested in media containing 0.5% serum for 24–48 h. Cells were injected in the cytoplasm with an AIS Zeiss automatic microinjector. Approximately 100–125 cells within a 3 × 3 grid were injected at a partial pressure of 65 psi. DNA synthesis was measured using the Cell Proliferation Kit (Amersham) between 8 and 20 h post-injection. Briefly, cells were incubated in fresh media containing 0.5% serum and 40 μM 5-bromo-2′-deoxyuridine (BrdU) and 4 μM 5-fluro-2′-deoxyuridine. At approximately 20 h post-injection, unincorporated BrdU was washed from the cytoplasm and nuclear BrdU incorporation was measured by immunostaining. To show that the incorporation of BrdU reflected S-phase synthesis, DNA labeling was shown to occur only between 8–16 h post-injection (data not shown). Injected cells were located both by markers on the coverslips and by re-examination on the Zeiss microinjector. Injections were monitored in two ways: by parallel co-injections of cells with FITC-dextran (MW 10,000; Molecular Probes, OR), or by immunostaining of parallel injections. PLC-γ, Z-proteins, and GST fusion protein were recognized by anti-PLC antisera (Santa Cruz). To visualize cytoplasmic protein, cells were fixed in 0.5% paraformaldehyde for 45 min, then further solubilized in 0.1% Triton X-100 for 10 min. To block non-specific binding, cells were preincubated in PBS containing 1% BSA (Sigma). Samples were then incubated for 1 h at room temperature in antibody at a concentration of 2 μg/ml. Antibody was identified by binding to FITC-conjugated sheep anti-mouse antisera (Molecular Probes). All incubations were washed 3 times with PBS at room temperature before proceeding to the next step. Using these methods, approximately 70–90% of cells were successfully injected.

3. Results and discussion

Recombinant rat PLC-γ1 (rPLC-γ) containing a three amino acid epitope tag (EEF) at the carboxy-terminus (Fig. 1a) was expressed and purified. A silver stained gel of purified recombinant rPLC-γ is shown in Fig. 1b (lanes 7 and 8). A shorter form of recombinant PLC-γ1 co-purified with both wild type and mutant protein. This smaller fragment contains the carboxy-terminus of PLC-γ, and was a minor component of the preparation. As previously reported for the purified bovine enzyme, rPLC-γ induces a mitogenic response when microinjected into quiescent 3T3 fibroblasts (Fig. 2a). Mitogenic activity was monitored by immunostaining of BrdU incorporation into newly synthesized DNA, and appears as blue-black staining of the nucleus.

Because our recombinant protein was made in *E. coli* and injected into serum starved cells, it is likely that the mitogenic effect was mediated by unphosphorylated PLC-γ. Other laboratories have proposed that tyrosine phosphorylation of PLC-γ is required for activation of the enzyme and subsequent mitogenic stimulation (e.g. [5–7]). However, our recombinant protein was both enzymatically active and capable of inducing mitogenesis. Moreover, a direct comparison between our recombinant PLC-γ and PLC-γ purified from bovine brain showed no difference in specific activity (Huber, data not shown), suggesting that lipase activity of our unphosphorylated protein was equivalent that of consitutive endogenous enzyme.

To determine the role of the catalytic activity of rPLC-γ in eliciting a mitogenic response, an active-site mutant with re-

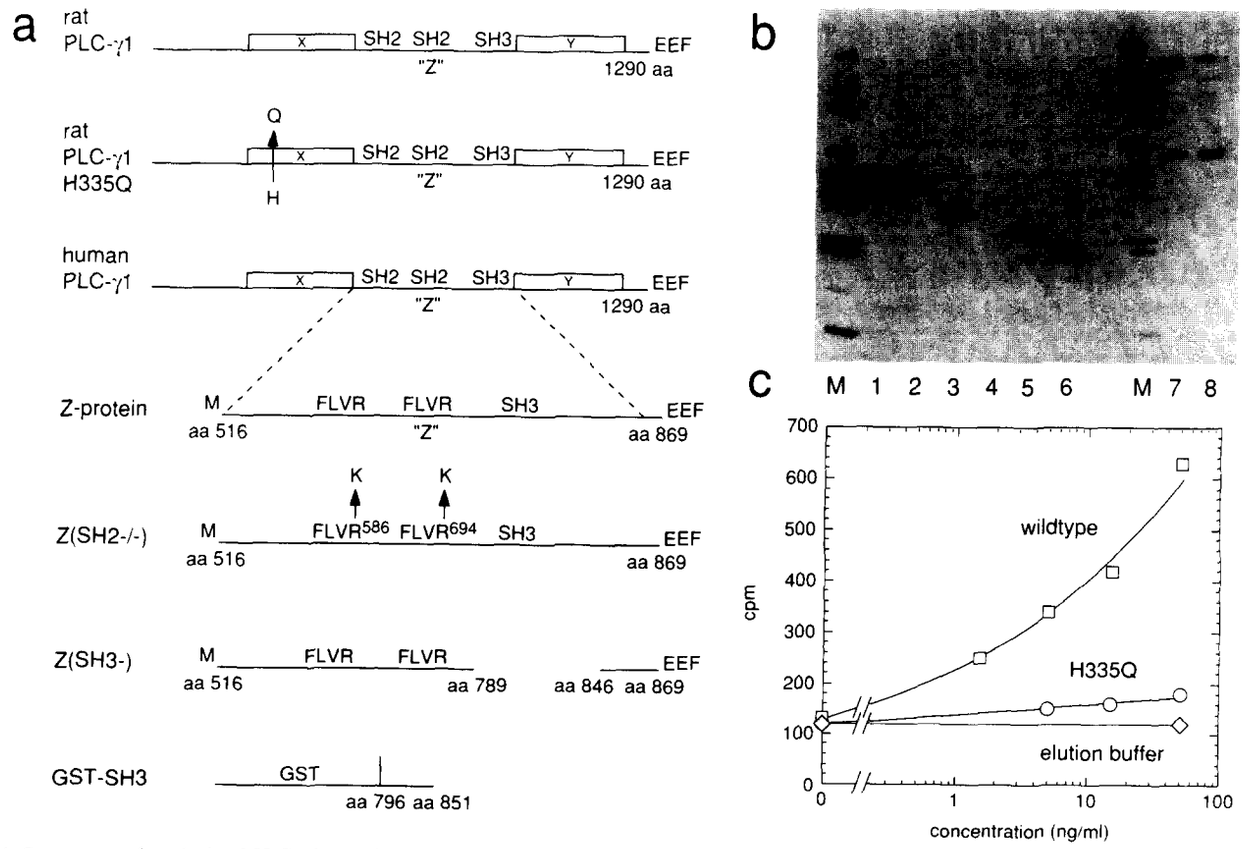

Fig. 1. Sequence and analysis of PLC-γ1 proteins. (a) Schematic diagram of PLC-γ1 proteins. Cloning strategies are described in section 2. (b) Silver stain SDS-PAGE of purified protein used for microinjection studies. Markers shown are prestained, MW in kDa from the top: 170, 116.3, 94, 67, 55.6, 43, 39.2, 30, 26.6, 20.1, 14.4. Sample identification and predicted molecular weight are as follows: lane 1, Z-protein, 41.5 kDa; 2, Z(SH2−/−) 41.5 kDa; 3, Z (SH3−), 35.7 kDa; 4, mock-transfected bacterial lysate purified over the antibody column, normalized for bacterial volume; 5, GST–SH3 fusion, 32.8 kDa; 6, GST, 26.0 kDa; 7, WT rat PLC-γ 149 kDa, approx. 60 kDa; 8, H335Q mutant rat PLC-γ 149 kDa, approx. 60 kDa. The truncated, approximately 60 kDa fragment present in lanes 7 and 8 was determined by Western analysis to contain the carboxy-terminus of the recombinant PLC protein. (c) Enzymatic activity of recombinant rPLC-γ1. Recombinant enzyme was purified and assayed for lipase activity as described in section 2. □, wild type PLC-γ1; ○, H335Q mutant; ◇, elution buffer.

duced lipase activity was generated. A point mutant in the X-catalytic domain of rPLC ($His^{335} \rightarrow Gln$) was reported to be inactive when overexpressed in mammallian cells [27]. A recombinant version of this mutant protein was constructed and tested for lipase activity. As predicted, the mutant rPLC-γ (H335Q) exhibited less than 10% of the enzymatic activity of wild type rPLC (Fig. 1c). Surprisingly, microinjection of this mutant rPLC-γ (H335Q) elicited a mitogenic response comparable to that of wild type rPLC-γ (Fig. 2b). Two-fold dilution of either protein resulted in the total loss of mitogenic activity. As expected, microinjection of a 'null' extract made from non-expressing bacterial lysates did not induce mitogenesis (Fig. 2d). The response of the microinjected cells to PLC proteins was morphologically distinct from that of cells injected with oncogenic Ras. Cells injected with Ras exhibited both DNA synthesis and rounding, as previously described [28]. Cells injected with rPLC-γ were stimulated to synthesize DNA in a time-dependent fashion, like Ras-injected cells, but were not grossly altered in shape compared to uninjected cells.

The mitogenic activity of the mutated, lipase-deficient rPLC-γ (H335Q) suggests that a property other than the enzymatic activity of this enzyme is responsible for the activation of DNA synthesis in quiescent fibroblasts. To map the regions of PLC-γ necessary for mitogenic activity, a number of recombinant PLC-γ fragments containing the Z-domain (PLC-γ amino acids 516–869) were expressed in *E. coli* and purified (Fig. 1a and b.) Fig. 3a–c shows a titration series of microinjected Z-protein into serum starved cells. At concentrations of 240 μg/ml and 120 μg/ml, this molecule was sufficient to induce DNA synthesis. Normalizing for molecular weight, the Z-protein was roughly equipotent to full-length rPLC-γ in eliciting a mitogenic response upon microinjection into quiescent 3T3 fibroblasts, suggesting that the Z-domain is responsible for the mitogenic activity of full length PLC-γ.

To further map the regions of this protein necessary to elicit DNA synthesis, two SH-domain mutants of the Z-protein were generated. The integrity of the proteins' SH2 and SH3 domains was assayed by binding to a phosphotyrosine-containing SH2-binding peptide derived from the EGFR protein (EpYLIP) [29] and to an SH3-binding peptide derived from dynamin (SPTPQRRAPAVPPARPGS, P2 peptide) [13]. The first mutated Z-protein, designated Z(SH2−/−), contains point mutants (Arg→Lys) in the highly conserved FLVR amino acid sequences of the two SH2 domains. As predicted [30], this double point mutant was incapable of binding to the immobilized SH2-binding peptide, but did bind to the SH3-binding peptide [13]. The other mutated Z-protein, designated Z(SH3−), contained a deletion of amino acids 798–846, eliminating the SH3 domain

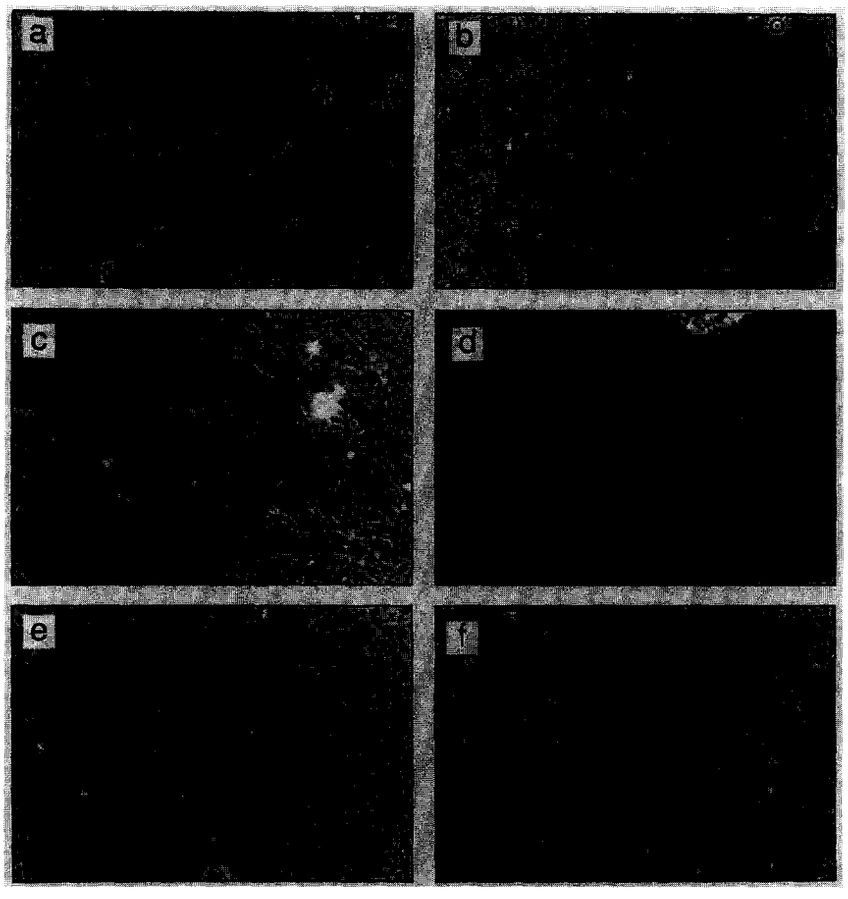

Fig. 2. Microinjection of full length recombinant PLC-γ1 into quiescent 3T3 fibroblasts. BrdU incorporation into the nucleus was determined by immunostaining as described in section 2. (a) Recombinant rat PLC-γ, 150 μg/ml. (b) H335Q rPLC-γ, mutant injected, 106 μg/ml. (c) Val-12 H-Ras injected, 2.2 mg/ml. (d) Blank lysate injected. (e) 10% serum. (f) 0.5% serum.

[31]. This mutant protein did not bind to the dynamin SH3-binding peptide but did bind to the phosphotyrosine-containing peptide. Results from the binding studies are summarized in Table 1.

Microinjection studies were performed with these mutant proteins to assess their ability to induce mitogenesis. The Z(SH2−/−) protein, which was unable to bind to the pY peptide, was fully capable of inducing DNA synthesis in serum starved cells at concentrations similar to wild type Z-protein

Table 1
Summary of microinjection and peptide binding results

Protein	Enzyme activity	Binds E(pY)LIP	Binds dynamin	Mitogenic
rat PLC-γ 1	+	+	+	+
rat PLC-γ 1 H335Q	−	+	+	+
Z-protein	NA	+	+	+
Z (SH2−/−)	NA	−	+	+
Z (SH3−)	NA	+	−	−
GST-SH3	NA	NA	+	−
Val-12 ras	NA	NA	NA	+ + +

NA, not applicable. Mitogenesis key: +++, 50–70% of injected cells positive; ++, 20–40% of injected cells positive; +, 10–15% of injected cells positive. Binding key: +, >50% bound to peptide resin; −, <5% bound to peptide resin. Enzyme activity key: +, specific activity of wild type recombinant enzyme; −, <10% specific activity relative to wild type.

(Fig. 3d–f). Thus, intact SH2 domains were not required for mitogenesis. Deletion of the SH3 domain, however, resulted in a complete loss of mitogenic activity. Injections of Z(SH3−) at concentrations ranging from 536 μg/ml to 107 μg/ml showed no stimulation of DNA synthesis (Fig. 3g–h). Successful microinjection of protein was verified by immunostaining of parallel injections with anti-PLC antisera (in section 2, data not shown).

The results from the previous experiment demonstrate that the SH3 domain was necessary for PLC-γ Z-protein induced mitogenesis in quiescent 3T3 fibroblasts. To assess whether this domain was sufficient to induce mitogenesis, we microinjected a GST–PLC-γ SH3 fusion protein (PLC-γ amino acids 796–851) into quiescent 3T3 fibroblasts. Although this fusion protein was sufficient to bind to the dynamin P2 peptide, it was not able to induce DNA synthesis in our assay (Table 1). These results demonstrate that the PLC-γ Z-protein is not by itself sufficient to initiate a mitogenic response.

SH3 domains can play either positive or negative roles in transformation, depending on their sequence and their context within signal transduction pathways. For example, in the c-Abl and c-Src proteins, SH3 domains are inhibitory for cellular transformation [32–34], while SH3 adapter domains are required for Crk mediated mitogenesis [35]. Our data suggest that the SH3 domain within the Z-region of PLC-γ is responsible for the mitogenic activity of microinjected PLC-γ (Table 1). While an SH3 domain is necessary for induction of mitogenesis

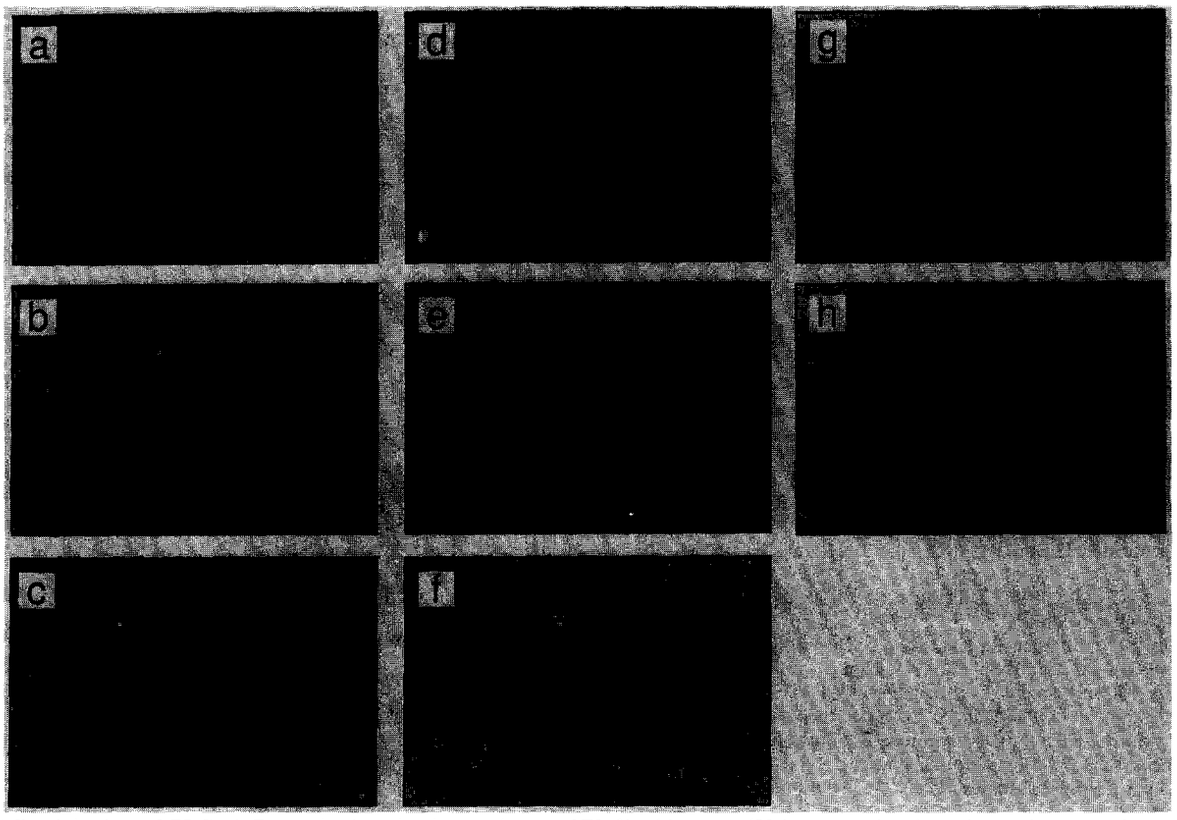

Fig. 3. Microinjection of PLC-γ1 Z-protein domains into quiescent 3T3 fibroblasts. BrdU incorporation into the nucleus was determined by immunostaining as described in section 2. Frames (a–c): Z-protein injections; a, 240 μg/ml; b, 120 μg/ml; c, 60 μg/ml. Frames (d–f): Z(SH2−/−) injections; d, 200 μg/ml; e, 100 μg/ml; f, 50 μg/ml. Frames (g,h): Z (SH3-) injections: g, 574 μg/ml; h, 120 μg/ml.

in this assay, it is not sufficient, as the GST–PLC-γ SH3 fusion protein is unable to induce mitogenesis. We are currently investigating what other sequences in the Z-peptides are required for mitogenic activity.

The PLC-γ Z-region protein also appears to function differently from the adapter proteins, Grb2 and Grb3. Grb2 is not mitogenic by microinjection unless it is co-injected with excess Ras protein [25]. The SH3 domain of Grb3, which is 90% conserved relative to Grb2, has been reported to induce apoptosis when microinjected into serum starved Swiss 3T3 cells [36]. The differences in biological activities between these SH3-domain proteins might be explained by their sequence differences. The SH3 domains of Grb2/3 and PLC-γ have distinct binding preferences: e.g. PLC-γ SH3 binds readily to the P2 dynamin peptide while the Grb2 SH3 does not [13]. Most dramatically, the different SH3 domains can be found in different parts of the cell following microinjection: GST fusions with Grb2-SH3 are found in the membrane ruffles following microinjection, while GST–PLC-γ SH3 fusion proteins can be localized to the actin cytoskeleton [37]. Thus, the disparity in their activities might be explained by their differential avidities for particular binding partners in the cell.

We propose that the high level of overexpression (as determined by injection volumes and immunostaining) achieved by microinjection of these proteins reveals a critical interaction between PLC-γ's SH3 domain and essential components of the cellular mitogenic apparatus. Our data do not indicate whether PLC-γ's SH3 domain induces mitogenesis by activating a cellular mitosis-inducing factor or by inhibiting a cellular mitosis-inhibiting protein. Potential candidates for these activities, cellular SH3-binding proteins, have been identified (e.g. [13,38,39]). The specific proteins which bind to PLC-γ Z-proteins under physiological concentrations, and the relevance of these interactions to the biological activity of PLC-γ, is currently under investigation.

After the completion of the current study, a report was published which also demonstrates that the catalytic activity of PLC is not required for mitogenesis [40]. The results described in that report substantively concur with our results using the catalytic site mutant of PLC-γ and also demonstrates that the catalytic activity of PLC is not required for mitogenesis*.

*The results described in Smith et al. [40] confirm that the H335 active site mutant was still mitogenic when microinjected into quiescent fibroblasts. These authors report that their mutant is only partially active in the mitogenesis assay, however, and that full activity could be restored by co-injections of mutant protein with the enzymatic products, IP_3 and DAG. The differences between our observations and those of Smith et al. might be explained by different sources of the recombinant enzymes: our protein was obtained from *E. coli* and was therefore unphosphorylated, while Smith et al. obtained their protein from a mammalian cell expression system. Thus, differential post-translational processing might account for the different relative mitogenic activities of the wild type and mutant enzymes when microinjected into cells. We are in accord with regard to their fundamental conclusion, however: that a catalytically inactive mutant of PLC-γ retains substantial mitogenic activity. In the current study, we have mapped this activity to the SH3 domain.

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