Involvement of Phospholipase Cγ1 in Mouse Egg Activation Induced by a Truncated Form of the C-kit Tyrosine Kinase Present in Spermatozoa

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Abstract. Microinjection of a truncated form of the c-kit tyrosine kinase present in mouse spermatozoa (tr-kit) activates mouse eggs parthenogenetically, and tr-kit–induced egg activation is inhibited by preincubation with an inhibitor of phospholipase C (PLC) (Sette, C., A. Bevilacqua, A. Bianchini, F. Mangia, R. Geremia, and P. Rossi. 1997. *Development [Camb.]*. 124:2267–2274). Co-injection of glutathione-S-transferase (GST) fusion proteins containing the src-homology (SH) domains of the γ1 isoform of PLC (PLCγ1) competitively inhibits tr-kit–induced egg activation. A GST fusion protein containing the SH3 domain of PLCγ1 inhibits egg activation as efficiently as the whole SH region, while a GST fusion protein containing the two SH2 domains is much less effective. A GST fusion protein containing the SH3 domain of the Grb2 adaptor protein does not inhibit tr-kit–induced egg activation, showing that the effect of the SH3 domain of PLCγ1 is specific. Tr-kit–induced egg activation is also suppressed by co-injection of antibodies raised against the PLCγ1 SH domains, but not against the PLCγ1 COOH-terminal region. In transfected COS cells, coexpression of PLCγ1 and tr-kit increases diacylglycerol and inositol phosphate production, and the phosphotyrosine content of PLCγ1 with respect to cells expressing PLCγ1 alone. These data indicate that tr-kit activates PLCγ1, and that the SH3 domain of PLCγ1 is essential for tr-kit–induced egg activation.

Key words: truncated-c-kit • spermatozoa • egg-activation • phospholipase-C-γ1 • src-homology-domains

After sperm–egg fusion, sperm cytosolic factors are released into the egg cytoplasm, and recent evidence obtained in a number of animal systems suggests that such a factor may trigger the series of events culminating in cell cycle resumption and the first mitotic division of the zygote (Stice and Robl, 1990; Swann, 1990; Homa and Swann, 1994; Dozortsev et al., 1995; Wu et al., 1997; Stricker, 1997). In many species, a series of Ca^{2+} transients is the early event triggered by the sperm at fertilization (Whitaker and Swann, 1993), and the increase in intracellular Ca^{2+} is required for several of the events that accompany egg activation (Kline and Kline, 1992). In the mouse, it has been shown that sperm–egg fusion precedes the onset of these Ca^{2+} oscillations (Lawrence et al., 1997), suggesting that a factor released by the sperm is responsible for the fertilization-associated Ca^{2+} mobilization. However, the nature of such factor in mammals is still uncertain. A possible candidate is oscillin, a glucosamine 6-phosphate deaminase that has been localized in the equatorial segment of the hamster sperm head (Parrington et al., 1996). However, whereas the protein fraction containing oscillin induces Ca^{2+} transients when microinjected into mouse eggs (Parrington et al., 1996), neither recombinant nor highly purified oscillin has oscillogenic activity, even though they maintain glucosamine 6-phosphate deaminase activity (Wolosker et al., 1998). Thus, it is possible that either oscillin requires additional factors to elicit egg activation or a different protein of the sperm is responsible for such function.

An additional candidate for a soluble sperm factor inducing the early events of fertilization is tr-kit, an alternative product of the *c-kit* gene (Sette et al., 1997). Tr-kit is encoded by an mRNA specifically expressed in the haploid phase of mouse spermatogenesis (Sorrentino et al., 1991; Rossi et al., 1992). Tr-kit mRNA is transcribed in late spermiogenesis under the control of an intronic promoter, as demonstrated by the tr-kit promoter driven expression of a reporter gene in transgenic mice (Albanesi

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et al., 1996). The open reading frame (ORF)[1] of tr-kit encodes a 23-kD protein that contains only part of the cytoplasmic portion of the c-kit receptor tyrosine kinase (Rossi et al., 1992). This region corresponds to the c-kit phosphotransferase catalytic domain, but lacks the inter-kinase region, the ATP-binding site, the transmembrane and the extracellular domains. The tr-kit protein has an apparent molecular size of 24–28 kD, is expressed in elongating spermatids (Albanesi et al., 1996), and immunofluorescence experiments indicate that it is localized in the residual cytoplasm of mouse epididymal spermatozoa (Sette et al., 1997). We have previously reported that microinjection of either lysates from cells expressing a recombinant tr-kit protein or synthetic tr-kit RNA into metaphase II (MII)-arrested mouse oocytes triggers the set of events associated with egg activation, from cortical granule exocytosis to pronuclear formation and progression through cleavage stages (Sette et al., 1997).

Tr-kit action is blocked by chelation of egg intracellular Ca^{2+} or by preincubation of eggs with an inhibitor of phospholipase C (PLC) (Sette et al., 1997), suggesting that tr-kit mediates Ca^{2+} mobilization through activation of a PLC isoform(s). PLCs are a family of enzymes that catalyze hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP_2), with production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($InsP_3$) (Berridge, 1993). DAG is a powerful stimulator of various protein kinase C (PKC) isoforms, and it has been suggested that PKC activity is required for sperm-induced egg activation (Colonna et al., 1997; Gallicano et al., 1997*a,b*). On the other hand, $InsP_3$ binds to receptors coupled to channels responsible for the release of Ca^{2+} from intracellular stores (Berridge, 1993). An increase in $InsP_3$ production is required for the Ca^{2+} wave at fertilization in *Xenopus* oocytes (Nuccitelli et al., 1993) and $InsP_3$ receptors have been found to play an essential role in mammalian egg activation at fertilization (Miyazaki et al., 1992, 1993; Xu et al., 1994; Berridge, 1996). Furthermore, the involvement of $InsP_3$ produced by PLC in mammalian fertilization is also supported by the observation that a PLC inhibitor can block the sperm-induced Ca^{2+} spiking at fertilization in mouse eggs (Dupont et al., 1996).

PLCγ1 may represent the most likely PLC isoform involved in tr-kit action inside the egg for the following reasons: (*a*) PLCγ1 has been shown by immunoblotting of ovulated mouse oocytes (Dupont et al., 1996); (*b*) PLCγ1 is activated after physical interaction with tyrosine kinases (Lee and Rhee, 1995; Kamat and Carpenter, 1997; Rhee and Bae, 1997), and it has been found to interact with the activated c-kit receptor (Herbst et al., 1991; Lev et al., 1991; Rottapel et al., 1991); (*c*) mutation of a tyrosine residue (Y936) of the COOH-terminal portion of the human c-kit receptor impairs association with PLCγ1 (Herbst et al., 1995), and the homologous residue is also present in the mouse c-kit receptor (Y934) and in tr-kit (Y161); and (*d*) it has been recently reported that PLCγ is essential for the sperm-induced Ca^{2+} mobilization at fertilization in starfish eggs (Carroll et al., 1997).

Both physical interaction with tyrosine kinases and tyrosine phosphorylation of PLCγ1 correlate with PLCγ1 activation and subsequent stimulation of PIP_2 hydrolysis (Lee and Rhee, 1995; Kamat and Carpenter, 1997; Rhee and Bae, 1997). In addition to catalytic domains, PLCγ1 contains several regulatory regions, and in particular src-homology 2 (SH2) and SH3 domains, which mediate its interaction with upstream and downstream effectors (Cohen et al., 1995; Pawson, 1995). The SH2 domains of the protein directly bind specific phosphotyrosine residues present in cytoplasmic domains of receptor tyrosine kinases (RTKs) (Mohammadi et al., 1991), whereas the targets of the SH3 domain are proline-rich sequences present in proteins such as the microtubule-associated GTPase dynamin (Gout et al., 1993).

In the present study, we demonstrate that PLCγ1 is actually involved in tr-kit–induced parthenogenetic egg activation and that the SH3 domain of PLCγ1 is essential for this process. Using biochemical approaches in transfected COS cells, we also show that coexpression of PLCγ1 and tr-kit stimulates an increase in tyrosine phosphorylation of PLCγ1, together with production of DAG and inositol phosphates (InsPs). These data strongly suggest that the mechanism of mouse egg activation triggered by tr-kit microinjection involves PLCγ1-mediated hydrolysis of PIP_2.

Materials and Methods

Expression of Recombinant Tr-kit Protein

Subconfluent COS cell monolayers were cultured in 90-mm dishes (Corning Glass Works, Corning, NY) and processed for $CaPO_4$ transfection with either 20 μg of the pCMV5 eukaryotic expression vector containing the tr-kit cDNA (pCMV5-tr-kit) or no DNA (mock) as previously described (Albanesi et al., 1996). 48 h after transfection, mock- and tr-kit–transfected COS cells were harvested in microinjection buffer (20 mM Hepes, pH 7.5, 120 mM KCl, 0.1 mM EGTA, 10 mM β-glycerophosphate, 10 μg/ml leupeptin, 10 μg/ml aprotinin), homogenized, and then centrifuged for 10 min at 14,000 *g* at 4°C. Aliquots of supernatant fractions were immediately frozen at −80°C. Tr-kit expression was monitored by Western blot analysis before microinjection experiments.

Quantification of Tr-kit in Mouse Spermatozoa and in COS Cell Extracts

Spermatozoa from the cauda of the epididymis of 12- to 15-wk-old CD1 mice were collected in MEM (GIBCO BRL, Gaithersburg, MD) supplemented with 30 mg/ml BSA (Sigma Chemical Co., St. Louis, MO). After a 2-h incubation at 37°C, spermatozoa were collected by centrifugation at 3,000 *g* at 4°C, washed twice with PBS, and then lysed in SDS-PAGE sample buffer. Lysates were sonicated, for three cycles of 20 s at 4°C, boiled for 5 min, and then centrifuged for 10 min at 10,000 *g* at 4°C. Soluble material was analyzed by Western blot.

Cell lysate from 3×10^6 spermatozoa and 50 μg of proteins from mock- and tr-kit–transfected COS cell extracts were separated on a 10% SDS-PAGE gel under denaturing conditions, blotted onto a nitrocellulose membrane, and then analyzed by Western blot using an anti–c-kit antibody as described below. Intensity of the bands corresponding to tr-kit were quantified by optical densitometry using the Molecular Analyst program and a GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA). 50 μg of tr-kit–transfected COS cell extracts contained an amount of tr-kit threefold higher than that present in 3×10^6 spermatozoa (see Fig. 1). In microinjection experiments we injected 5 pl of a 0.2–0.4 μg/μl

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[1] *Abbreviations used in this paper*: DAG, diacylglycerol; GST, glutathione-S-transferase; InsP, inositol phosphate; $InsP_3$, inositol 1,4,5-trisphosphate; MII, metaphase II; NRTK, non-receptor tyrosine kinase; PIP_2, phosphatidylinositol-4,5-bisphosphate; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; PKC, protein kinase C; PLC, phospholipase C; RTK, receptor tyrosine kinase; SCF, stem cell factor; SH, src-homology; SH2, src-homology 2; SH3, src-homology 3; tr-kit, truncated c-kit.

solution of tr-kit cell extracts (1–2 pg of proteins), an amount corresponding to 0.2–0.4 sperm equivalents of tr-kit.

Oocyte Collection, Microinjection and In Vitro Culture

MII-arrested oocytes were collected from hormonally primed (Hogan et al., 1994) 4- to 6-wk-old CD1 female mice (Charles River, Calco, Italia) 15 h after hCG (Sigma Chemical Co.) injection. Ovulated oocytes were freed from cumulus cells by a brief incubation in 0.5 mg/ml hyaluronidase (Sigma Chemical Co.) in M2 medium (Hogan et al., 1994), washed with M2 medium, and then immediately processed for microinjection as described (Sette et al., 1997). Groups of 20 MII oocytes were transferred to 50-μl drops of M2 under mineral oil (Sigma Chemical Co.) and subjected to intracytoplasmic injections using a Nikon invertoscope (Nikon Corp., Tokyo, Japan) equipped with Hoffman modulation contrast optics (Modulation Optics, Inc., Greenvale, NY) and two Leitz mechanical micromanipulators (Leica AG, Heerbrugg, Switzerland). A quantification of the approximate volume of solution microinjected into a single oocyte, was performed in repeated experiments as follows: a known amount of injection solution (usually 100 pl) was drawn in the injection pipette and used completely for a series of microinjections under the same routinary conditions. The average number of oocytes microinjected with 100 pl of solution was 17. Considering the loss of small amounts of solution between injections, the injected volume per oocyte was ~5 pl. After injection, surviving oocytes were cultured at 37°C in M16 medium (Hogan et al., 1994) under a humidified atmosphere of 5% CO_2 in air for ≤7 h, and then scored for pronuclei formation by phase-contrast microscopy. To confirm the score, in most experiments eggs were fixed in 4% PFA in PBS 7 h after the injection, and stained with 10 μg/ml Hoechst 33342 (Sigma Chemical Co.) for 5 min. After five washes in M2, eggs were mounted in 30% glycerol in PBS on glass slides with coverslip compression, sealed, and then analyzed by fluorescence microscopy.

For cortical granule staining, microinjected eggs were fixed after 1–4 h, and processed as described below.

Cortical Granule and Chromosome Staining

1–4 h after microinjection, cultured oocytes were freed from the zona pellucida by acidic tyrode solution (Hogan et al., 1994) and fixed in 4% PFA in PBS for 30 min at room temperature. After three washes in M2 (blocking solution), oocytes were incubated with 0.1% Triton X-100 in the same medium for 5 min and transferred to blocking solution for 60 min at room temperature. Oocytes were then treated for 60 min at room temperature with 0.1 mg/ml TRITC-labeled lectin from Lens Culinaris (Sigma Chemical Co.) in blocking solution (Ducibella et al., 1988), washed four times for 5 min in blocking solution, incubated for 5 min with 10 μg/ml Hoechst 33342 dye in blocking solution, and washed again. Oocytes were then mounted in 30% glycerol in PBS as described above and analyzed by fluorescence microscopy.

Glutathione-S-Transferase–PLCγ1 Fusion Proteins and Antibodies Used in Microinjection Experiments

The glutathione-S-transferase (GST)-encoding plasmid pGEX3X was obtained from Pharmacia Biotech, Inc. (Piscataway, NJ). Plasmid DNAs encoding for GST fusion proteins of bovine PLCγ1 SH2–SH2 and human PLCγ1 SH3 (see Fig. 3) in pGEX2T′6 were a kind gift from S. Courtneidge (Sugen, Inc., Redwood City, CA).

Affinity-purified GST-PLCγ1 SH region fusion protein (GST-PLCγ1-SH2SH2SH3) (No. sc4019), and GST-Grb2-SH3 (residues 156–199; No. sc4036) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Control GST protein, GST-PLCγ1 SH2-SH2, and GST-PLCγ1 SH3 fusion proteins were produced as described by Gish et al. (1995), affinity purified on glutathione Sepharose, extensively dialyzed against PBS, and concentrated to 10 mg/ml using a 10-kD cutoff Centricon (Millipore Corp., Bedford, MA). Protein concentration was determined according to Bradford (1976) using BSA as a standard.

The anti-PLCγ1bd antibody (No. 426; Santa Cruz Biotechnology) consists of affinity-purified rabbit polyclonal IgGs directed against the SH region of rat PLCγ1 ("binding domain"; amino acids 530–850). The anti-PLCγ1ct antibody (No. 81; Santa Cruz Biotechnology) consists of affinity-purified rabbit polyclonal IgGs directed against a peptide in the COOH terminus of bovine PLCγ1 (amino acids 1,249–1,262). Both antibodies recognize mouse PLCγ1 in Western blot and immunoprecipitation and do not cross-react with other PLC isoforms. As a control, affinity-purified normal rabbit IgGs were used.

For tr-kit co-injection experiments in mouse eggs, GST fusion proteins were diluted in the injected solution to 500 μg/ml, whereas affinity-purified rabbit polyclonal IgGs were diluted to 10 μg/ml. Since we injected 5 pl of protein solution in the oocytes, considering the average volume of mouse eggs equal to 270 pl, the final concentration inside the injected eggs of all microinjected proteins was ~50-fold lower (10 μg/ml for the GST fusion proteins and 0.2 μg/ml for the antibodies), unless otherwise specified in the Results section. Control experiments using the Santa Cruz glycerol buffer (vehicle of GST fusion proteins) in addition to tr-kit did not interfere with egg activation (Sette, C., and A. Bevilacqua, unpublished observations).

Measurement of DAG and InsP Production in COS Cells

For measurement of DAG production, subconfluent COS cell monolayers in 90-mm dishes were processed for $CaPO_4$ transfection with either 20 μg pRK-PLCγ1 (expression vector for PLCγ1, a generous gift from Dr. A. Ullrich, Max-Planck Institut, Martinsried, Germany) alone, or with 20 μg pRK-PLCγ1 and 20 μg pCMV5-tr-kit (see Albanesi et al., 1996). 18 h after transfection, cells were washed with PBS and cultured for additional 2 h in DME containing 10% FCS (GIBCO-BRL) and 0.5 mCi/ml [^{3}H]arachidonic acid. At the end of the incubation, cells were washed twice with cold PBS and harvested in 0.5 ml PBS/dish. The pH of the cell suspensions was lowered to 2–3 by addition of HCl (30 mM final concentration). Lipids were extracted by addition of 4 vol of chloroform/methanol (1:2) in glass tubes according to the method of Boukhchache and Lagarde (1982). Neutral lipids were separated by thin layer chromatography on silicagel plates (Merck, Darmstadt, Germany) using a solution of hexane/diethyl ether/acetic acid (50:50:1) for the migration. Plates were stained with 0.3 mg/ml Coomassie brilliant blue R250 (Bio-Rad Laboratories) in 0.15 M NaCl containing 20% methanol. The DAG bands were identified on the plates based on the migration of known lipid standards (Sigma Chemical Co.), scraped off, mixed with Picofluor (Packard), and their radioactivity was determined by liquid scintillation counting. DAG-associated radioactivity was expressed as cpm incorporated in DAG versus 10^3 CPM incorporated in total lipids (neutral lipids bands plus phospholipids at the origin of the chromatogram). DAG-associated radioactivity ranged between 1,200 and 3,000 cpm; the average amount of CPM in total lipids was ~200,000.

For measurement of InsPs production, subconfluent COS cell monolayers in 35-mm dishes were transfected with either 4 μg pRK-PLCγ1 alone, or 4 μg pRK-PLCγ1 and 4 μg pCMV5-tr-kit. Immediately after transfection cells were transferred to DME containing 10% FCS and 5 μCi/ml D-*myo*-[^{3}H]inositol, and cultured for additional 12–24 h. We selected two time points after transfection (12 and 24 h) to investigate whether cells had reached steady state of phosphoinositides labeling and InsPs accumulation. The tr-kit–induced InsPs accumulation measured at 24 h is only slightly higher than that observed at 12 h, suggesting that cells had reached steady-state levels. During the final 60 min of incubation, 10 mM LiCl was added to the medium. Incubation was stopped by washing three times with PBS and adding ice-cold 10% TCA to the cells. [^{3}H]Inositol-labeled InsPs were extracted, separated by ion exchange chromatography on Dowex 1×8-200 and counted as described by Adamo et al. (1985). InsPs were expressed as cpm incorporated in InsPs fractions per mg of total protein resuspended after TCA precipitation.

Immunoprecipitation and GST-PLC Coprecipitation Experiments

Subconfluent COS cell monolayers in 90-mm dishes were processed for $CaPO_4$ transfection with the appropriate plasmids as described above. Cells transfected with pCMV5-c-kit (obtained by subcloning c-kit cDNA from pCDM8-c-kit [a generous gift from Dr. P. Besmer, Sloan Kettering Cancer Center, New York, NY] into pCMV5) were treated for 10 min with or without 100 ng/ml stem cell factor (SCF) in the presence of 250 μM sodium orthovanadate in complete medium before harvesting. 24 h after transfection, cells were rinsed with PBS, harvested in lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 15 mM $MgCl_2$, 15 mM EGTA, 250 μM sodium orthovanadate, 10% glycerol, 1% Triton X-100, 0.1% SDS, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin), and incubated on ice for 5 min. Detergent-soluble extracts were separated by 10-min centrifugation at 15,000 *g* at 4°C.

For immunoprecipitation experiments, either a rabbit anti-kit antiserum raised against the 13 COOH-terminal amino acids common to the mouse c-kit and tr-kit proteins (1:100 dilution; Albanesi et al., 1996) or a mixture of 1 μg anti-PLCγ1bd and 1 μg anti-PLCγ1ct IgGs were preincubated for 60 min with protein A–Sepharose beads (Sigma Chemical Co.). At the end of the incubation, the beads were washed once with 20 mM Tris-HCl, pH 7.8, containing 0.5 M NaCl, twice with 20 mM Tris-HCl, pH 7.8, and then incubated for 90 min at 4°C with the detergent-soluble extracts under constant shaking. Protein A–Sepharose–bound immunocomplexes were rinsed three times with PBS containing 0.05% BSA, twice with PBS, and finally eluted in SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% (wt/vol) SDS, 0.7 M 2-mercaptoethanol, and 0.0025% (wt/vol) bromophenol blue). For GST-PLC coprecipitation experiments, 10 μg of affinity-purified GST-PLC (SH2-SH2-SH3) protein were added to detergent-soluble extracts. After 30 min, samples were incubated with glutathione–agarose beads for additional 90 min at 4°C under constant shaking. At the end of the incubation, glutathione-agarose–bound protein complexes were rinsed three times with PBS before elution in 50 mM Tris-HCl, pH 8.0, containing 5 mM reduced glutathione. Eluted proteins were diluted in SDS-PAGE sample buffer for Western-blot analysis.

Western Blot Analysis

For detection of recombinant proteins, samples from transfected cells, from immunoprecipitation, and from coprecipitation with GST-PLC protein, were separated on 10% SDS-PAGE, transferred onto nitrocellulose membrane (Amersham) and subjected to Western blot analysis with different antibodies as previously described (Albanesi et al., 1996). In brief, first antibody incubation (90 minutes at room temperature) was carried out with 1:1,000 dilution of a polyclonal anti-kit antiserum (Albanesi et al., 1996), or affinity-purified polyclonal anti-PLCγ1bd IgGs described above, or affinity-purified mouse anti-phosphotyrosine mAb (No. 508, Santa Cruz Biotechnology). Second antibody incubation was carried out with 1:10,000 dilution of either anti-rabbit or anti-mouse IgGs antibody conjugated to horseradish peroxidase (Amersham Corp., Arlington Heights, IL). Immunostained bands were detected by the enhanced chemiluminescence method (Amersham Corp.). Tyrosine phosphorylation of immunoprecipitated PLCγ1 was quantified as the ratio of the optical density detected by the anti-phosphotyrosine antibody (αPY) versus that detected by the anti-PLCγ1 antibody (αPLCγ1) (mean ± SD of six separate experiments). Optical Densitometry was performed using the Molecular Analyst program and a GS-700 Imaging Densitometer (Bio-Rad Laboratories).

Results

Kinetics of Tr-kit–induced Parthenogenetic Activation of Mouse Eggs

We have previously shown that microinjection of either cell extracts expressing recombinant tr-kit or synthetic tr-kit mRNA is able to induce complete parthenogenetic activation of mouse eggs and cleavage to the two-cell stage (Sette et al., 1997). To compare the amount of recombinant tr-kit capable of activating mouse eggs with the amount carried by a single mouse sperm, we performed Western blot analysis (Fig. 1). Densitometric analysis of immunoreactive bands indicated that the amount of tr-kit protein obtained from extracts of 3×10^6 sperm was nearly threefold smaller than that from 50 μg of extracts of tr-kit–expressing COS cells (see also Materials and Methods). As shown in Fig. 2 and Table I, injection of ∼0.2–0.4 sperm equivalents of recombinant tr-kit is sufficient to exert activation of 60–70% mouse eggs. Injection of ∼0.1 sperm equivalents of tr-kit reduced the activation rate to 40–50%, and injection of ∼0.01 sperm equivalents did not result in significant activation of mouse eggs above the background reported in Table I (not shown). Although the timing of egg activation triggered by microinjection of recombinant tr-kit is not completely synchronous, the typical time course and pattern of the cell cycle events (Fig. 2) closely resemble those observed at fertilization of the mouse eggs (Mori et al., 1988). 1 h after injection, 60–70% of the eggs underwent metaphase–anaphase transition and initiated polar body extrusion (Fig. 2, *A* and *B*). At 4 h, the polar body was extruded in all activated eggs, chromosome decondensation had begun and an incipient pronucleus was evident (Fig. 2 *C*). The size of the pronucleus progressively increased from the time of appearance to reach its full size after 6–7 h from the injection in all the activated eggs (Fig. 2 *D*).

A GST Fusion Protein Containing the PLCγ1-SH3 Domain Inhibits Tr-kit–induced Parthenogenetic Activation of Mouse Eggs

To test the hypothesis that PLCγ1 is involved in tr-kit–induced parthenogenetic activation of mouse eggs, we subjected MII-arrested oocytes, which express PLCγ1 (Fig. 3 *A*; Dupont et al., 1996), to co-injection of extracts from COS cells expressing a recombinant tr-kit protein, and a purified GST fusion protein containing the entire SH2-SH2-SH3 region of PLCγ1 (GST-PLCγ1-SH2SH2SH3) (Fig. 3 *B*). The SH region mediates interaction of PLCγ1 with proteins involved in enzyme regulation (Lee and Rhee, 1995; Kamat and Carpenter, 1997; Rhee and Bae, 1997), and competition to this region of PLCγ1 has been shown to prevent enzyme activation (Chen et al., 1994). Injection of recombinant tr-kit alone, or co-injection of tr-kit together with a control GST protein resulted in activation of 63–70% of the eggs, as monitored by formation of a pronucleus 4–7 h after injection (Table I). Co-injection of tr-kit and GST-PLCγ1-SH2SH2SH3 significantly reduced the activation rate to 15%, suggesting a role of PLCγ1 in tr-kit action in the egg cytoplasm. Only 5–8% of spontaneous activation was obtained in either non-injected eggs (Table I) or in eggs injected with extract from mock-transfected cells (data not shown; Sette et al., 1997).

To further investigate which SH domain of PLCγ1 is involved in tr-kit–induced egg activation, we co-injected tr-kit with GST fusion proteins containing either the tandem SH2 domains (GST-PLCγ1-SH2SH2) or the SH3 domain (GST-PLCγ1-SH3) of the protein (Fig. 3). Co-injection of GST-PLCγ1-SH2SH2 only slightly reduced tr-kit–induced egg activation (53% versus 70%; Table I). However, co-injection of GST-PLCγ1-SH3 inhibited egg activation as efficiently as the entire SH2-SH2-SH3 region, reducing the activation rate to 14% (Table I). Since we have previously reported that tr-kit–induced egg activation is also associ-

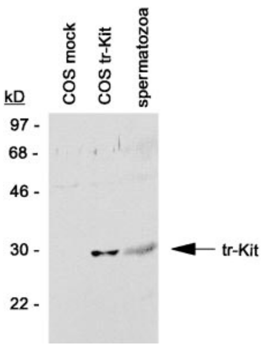

Figure 1. Quantitative analysis of recombinant tr-kit microinjected into MII-arrested mouse oocytes. Western blot analysis with an anti–c-kit antibody was performed on 25 μl (50 μg of total proteins) of extracts from COS cells expressing recombinant tr-kit or on extracts from 3×10^6 sperms. The expected major immunoreactive band of ∼28 kD was present in both samples in a 3:1 ratio (see Materials and Methods). Since in our microinjection experiments we inject into mouse eggs ∼1–2 pg of proteins from extracts of tr-kit–expressing COS cells, we can calculate that we inject between 0.2 and 0.4 sperm equivalents of recombinant tr-kit. This experiment was performed twice with similar results.

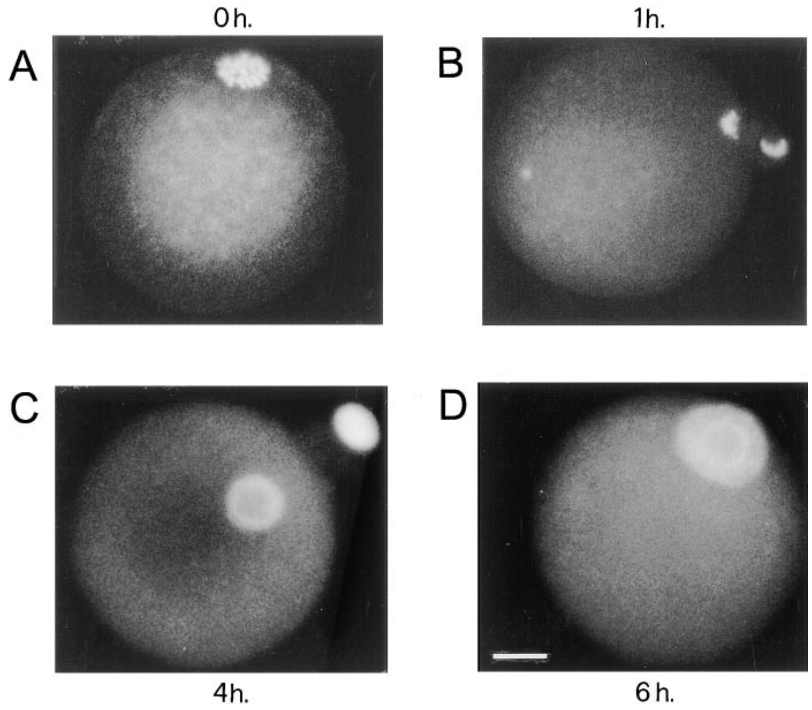

Figure 2. Time-course of the cell cycle events induced by microinjection of recombinant tr-kit into MII-arrested mouse oocytes. This pattern was constantly observed in the tr-kit–activated oocytes. Eggs were fixed and stained with Hoechst (10 μg/ml) either before microinjection (*A*), or after increasing times from microinjection of extracts from COS cells expressing tr-kit (200 μg/ml), 1 h (*B*), 4 h (*C*), and 6 h (*D*). Bar, 30 μm.

ated with early events of egg activation, such as the Ca^{2+}-dependent cortical granules (CGs) exocytosis (Sette et al., 1997), the effect of the GST-PLCγ1 SH2 and SH3 fusion proteins on CGs release was investigated. Co-injection of GST-PLCγ1-SH3 inhibited both cortical granule release (Fig. 4 *A*) and polar body extrusion (not shown) with a rate similar to that observed for pronuclear formation (Fig. 4 *A*; see Table I for rate of inhibition) while the GST-PLCγ1-SH2SH2 protein was much less effective (Fig. 4 *B*; see Table I for rate of inhibition). Co-injection of 10-fold diluted GST-PLCγ1-SH3, at a final concentration in the egg of ∼1 μg/ml, resulted in an almost equally efficient inhibition of tr-kit–induced pronuclear formation (Table I). To test for the specificity of the SH3 domain of PLCγ1, we co-injected tr-kit together with a GST fusion protein containing the SH3 domain of the adaptor protein Grb2. Although the SH3 domains of Grb2 and PLCγ1 can bind to common targets, such as dynamin (Gout et al., 1993), they have been reported to direct the corresponding GST fusion proteins to different cell compartments when microinjected in NIH3T3 fibroblasts (Bar-Sagi et al., 1993), implying that the Grb2 and PLCγ1 SH3 domains can also recognize different targets. As shown in Table I, co-injection of GST-Grb2-SH3 was not able to affect tr-kit–induced egg activation. These data indicate that competition for targets of the SH3 domain specific to PLCγ1 impairs tr-kit–induced egg activation.

Table I. A GST-PLCγl-SH3 Domain Fusion Protein Specifically Inhibits Tr-kit–induced Parthenogenetic Activation of Mouse Eggs

Sample	No. of experiments	Total eggs	Pronuclei formed	Percent Activated eggs
Non-injected	9	80	4	5***
Tr-kit	16	118	75	63.5
Tr-kit+GST	4	20	14	70.0*
Tr-kit+PLCγ1SH2SH2SH3	4	46	7	15.2***
Tr-kit+PLCγ1SH2SH2	6	41	21	53.8**
Tr-kit+PLCγ1SH3	6	56	8	14.2***
Tr-kit+PLCγ1SH3 (1 μg/ml)	3	35	7	20.0***
Tr-kit+Grb2SH3	3	24	16	66.6*

Summary of microinjection experiments using 5 pl of extracts (200–400 μg/ml) from tr-kit–transfected COS cells (*tr-kit*) alone or plus GST (*tr-kit+GST*), or plus GST-PLCγ1-SH2SH2SH3 (*tr-kit+PLCγ1SH2SH2SH3*), or plus GST-PLCγ1-SH2SH2 (*tr-kit+PLCγ1SH2SH2*), or plus GST-PLCγ1-SH3 (*tr-kit+PLCγ1SH3*), or plus GST-Grb2-SH3 (*tr-kit-Grb2SH3*). Final concentration of all co-injected GST fusion proteins inside the egg was 10 μg/ml. GST-PLCγ1-SH3 was also tested at a 10-fold lower concentration (1 μg/ml). Pronuclear formation was scored 4–7 h after microinjection by phase-contrast microscopy. In most experiments pronuclear formation was confirmed by Hoechst staining. Statistical analysis (ANOVA test) was performed using program PSI-plot 3.0 from Polysoftware International (P values vs. tr-kit: $*P > 0.5$; $**P < 0.05$; $***P < 0.0001$).

An Antibody Directed Against the SH2-SH2-SH3 Region of PLCγ1 Blocks Tr-kit–induced Activation of Mouse Eggs

The role of the SH region of PLCγ1 in tr-kit–mediated egg activation was also investigated by microinjection experiments using antibodies raised against different regions of PLCγ1. The anti-PLCγ1bd antibody is directed against the SH region of PLCγ1 and we hypothesized that its binding would prevent PLCγ1 interaction with effector proteins. The anti-PLCγ1ct antibody is directed against the COOH terminus of PLCγ1, a region of the enzyme that is not known to be involved in catalytic activity and/or interaction of PLCγ1 with other proteins (Lee and Rhee, 1995). These antibodies are specific for PLCγ1, and they do not cross-react with other PLC isoenzymes. The anti-PLCγ1bd (Fig. 3 *A*) and the anti-PLCγ1ct antibodies (not shown) recognize PLCγ1 in Western blots from extracts of both PLCγ1-overexpressing COS cells and of MII-arrested mouse oocytes. Microinjection experiments showed that the anti-PLCγ1bd antibody almost completely suppresses

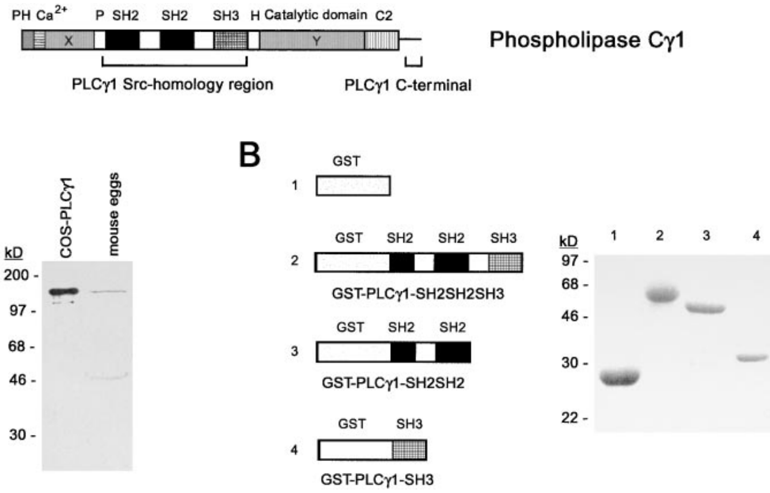

Figure 3. Expression of PLCγ1 in MII-arrested mouse eggs, and schematic representation of the bacterial fusion proteins containing different PLCγ1 domains. In the schematic representation of PLCγ1 on the top of this figure, brackets identify the regions of PLCγ1 recognized by the two antibodies used for microinjection experiments shown in Table II. *PH*, pleckstrin homology domain; *Ca*$^{2+}$, EF-hand domain (calcium binding motif); *X* and *Y*, split catalytic domain; *P* and *H*, split pleckstrin homology domain; *SH2* and *SH3*, src-homology domains; *C2*, Ca^{2+}-dependent lipid binding domain. (*A*) Western blot from extracts of COS cells transfected with a PLCγ1 expression vector (50 μg), and from extracts of 50 mouse eggs, probed with the anti-PLCγ1bd antibody, described in the Materials and Methods section, and used for the microinjection experiments shown in Table II. (*B*) Bacterial fusion proteins containing different PLCγ1 domains used for the experiments shown in Table I. The Coomassie blue staining of a 10% SDS-PAGE gel loaded with affinity-purified GST fusion proteins is shown on the right: lane *1*, GST; lane *2*, GST-PLCγ1-SH2SH2SH3; lane *3*, GST-PLCγ1-SH2SH2; lane *4*, GST-PLCγ1-SH3.

tr-kit–induced mouse egg activation, resembling the effect obtained by co-injection of tr-kit and either the GST-PLCγ1-SH2SH2SH3 or the GST-PLCγ1-SH3 fusion proteins, whereas nonimmune antibodies at the same concentration are ineffective (Table II). On the other hand, co-injection of recombinant tr-kit and the anti-PLCγ1ct antibody did not significantly inhibit egg activation, showing that binding of antibodies to the COOH terminus of PLCγ1 does not impair interaction with factors required for egg activation (Table II). The inhibition obtained with the anti-PLCγ1bd antibody confirms that PLCγ1 is involved in parthenogenetic egg activation triggered by tr-kit and that an essential role in such pathway is played by the SH region of PLCγ1.

Tr-kit Stimulates PIP$_2$ Hydrolysis in Transfected COS Cells

The ability of tr-kit to stimulate the catalytic activity of PLCγ1 was investigated by cotransfection experiments in COS cells. Cells were transfected with a tr-kit expression

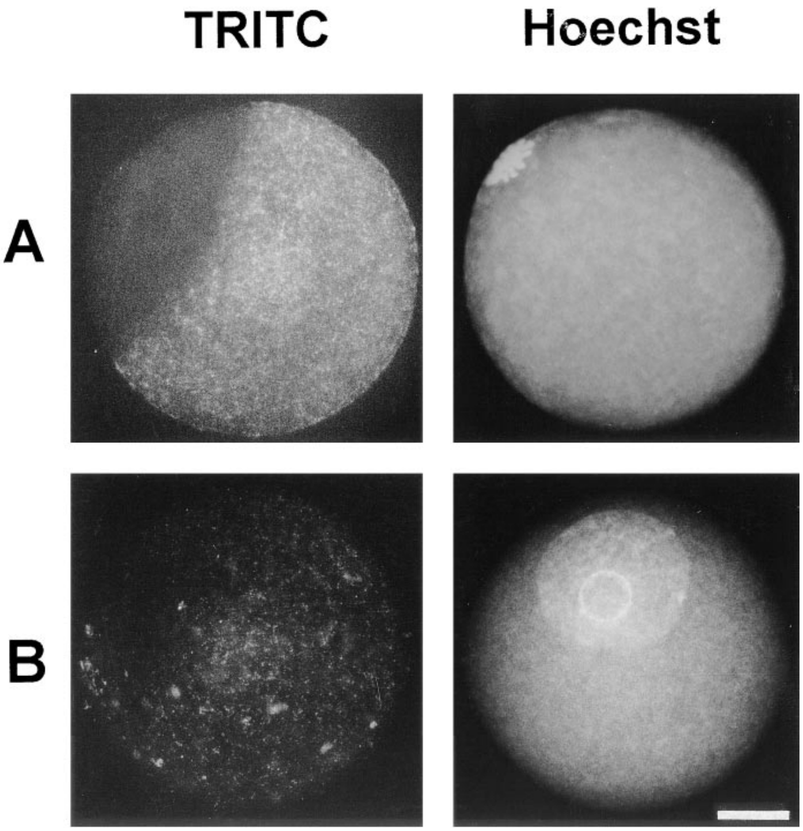

Figure 4. The SH3 domain, and not the SH2 domains, of PLCγ1 inhibits tr-kit–induced cortical granules exocytosis and pronuclear formation in mouse eggs. Eggs were co-injected with cell extracts (200 μg/ml) containing recombinant tr-kit and 500 μg/ml (final concentration in the egg: ~10 μg/ml) of either GST-PLCγ1-SH3 (*A*) or GST-PLCγ1-SH2SH2 (*B*) and fixed 4 h after injection. Tr-kit–induced cortical granule reaction was inhibited by co-injection of GST-PLCγ1-SH3, but not by GST-PLCγ1-SH2SH2, with a similar rate as for pronuclear formation (see Table I) in three separate experiments. Double staining of chromatin (Hoechst dye) and cortical granules (TRITC-labeled lectin) was performed as described under Materials and Methods. Bar, 30 μm.

Table II. Antibodies Against the SH Region of PLCγ1 Inhibit Tr-kit–induced Parthenogenetic Activation of Mouse Eggs

Sample	No. of experiments	Total eggs	Pronuclei formed	Percent activated eggs
Non-injected	3	30	2	6.6**
Tr-kit	3	23	15	65.2
Tr-kit+IgGs	3	33	19	57.5*
Tr-kit+anti-PLCγ1bd	4	40	5	12.5**
Tr-kit+anti-PLCγ1ct	4	23	12	52.1*

Summary of microinjection experiments using 5 pl of extracts from tr-kit transfected COS cells (*tr-kit*) (200–400 μg/ml) plus nonimmune rabbit IgGs (*IgGs*), or plus an antibody directed against the entire SH region of PLCγ1 (*anti-PLCγ1bd*) or plus an antibody directed against the COOH terminus of PLCγ1 (*anti-PLCγ1ct*) (all the antibodies were at a 10 μg/ml concentration in the injected solution, final concentration inside the egg was ~0.2 μg/ml). Pronuclear formation was scored 4–7 h after microinjection by phase-contrast microscopy, and confirmed by Hoechst staining. Statistical analysis (ANOVA test) was performed using program PSI-plot 3.0 Polysoftware International (P values vs. tr-kit: $*P > 0.2$; $**P < 0.0001$).

vector, or with a PLCγ1 expression vector, or cotransfected with both plasmids, labeled with either [^{3}H]arachidonic acid or [^{3}H]inositol, and processed for assays of diacylglycerol production or InsP production, respectively.

As shown in Fig. 5 *A*, when cells were transfected with tr-kit alone, a slight increase in DAG production was observed, likely indicating activation of endogenous PLCs. As expected, an increase in DAG production was also observed in cells transfected with PLCγ1 versus mock-transfected cells. Coexpression of tr-kit and PLCγ1 was reproducibly accompanied by a much higher activation of DAG production, suggesting that, in cotransfection experiments, tr-kit is able to stimulate DAG production by activating PLCγ1. Cotransfection of PLCγ1 with the full-length c-kit receptor did not induce any increase in DAG production with respect to cells transfected with PLCγ1 alone. Moreover, stimulation of c-kit–transfected cells with the c-kit ligand (SCF) did not induce any increase in DAG production with respect to both unstimulated cells and cells transfected with PLCγ1 alone (Fig. 5 *A*), even though PLCγ1 associates with c-kit after SCF stimulation (see below, Fig. 7 *A*). The inability of autophosphorylated c-kit to activate PLCγ1 is in agreement with our previous observation that SCF treatment fails to activate MII-arrested oocytes (Sette et al., 1997), which express the c-kit receptor (Manova et al., 1990; Horie et al., 1991; Yoshinaga et al., 1991). As shown for the closely related PDGFβ receptor (Valius et al., 1995), the simultaneous binding to the c-kit receptor to a particular blend of other signal transduction molecules might interfere with PLCγ1 activation.

Since DAG can be produced by other metabolic routes, such as phosphatidylcholine hydrolysis by phospholipase D and conversion of the resulting phosphatidic acid into DAG by a specific phosphatidate phosphohydrolase (Exton, 1997), we set out to measure the activity of PLCγ1 by assaying InsPs production, a more specific marker of PIP$_2$ hydrolysis. In agreement with the increase in DAG production, we found that coexpression of tr-kit and PLCγ1 in COS cells induced a 2.5-fold increase in InsPs production compared with cells transfected with PLCγ1 alone (Fig. 5 *B*). Similar to DAG production, transfection of tr-kit alone induced only a slight increase in InsPs production versus mock-transfected cells (Fig. 5 *B*), however, cotransfection with PLCγ1 amplifies tr-kit–induced InsPs production. Western blot analysis of extracts from these cells indicated that similar amounts of PLCγ1 were expressed in the PLCγ1 transfected cells (Fig. 5 *C*). Therefore, the concomitant stimulation of DAG and InsPs production in COS cells coexpressing tr-kit and PLCγ1 is likely due to posttranslational activation, rather than increased expression, of PLCγ1. These results indicate that in COS cells tr-kit is able to induce activation of PLCγ1.

Tr-kit Stimulates Tyrosine Phosphorylation of PLCγ1 in Transfected COS Cells

Since tyrosine phosphorylation is often associated with activation of PLCγ1 (Rhee and Bae, 1997), we tested whether an increase in phosphotyrosine content of PLCγ1 is detectable in tr-kit–expressing cells. In cells transfected with a PLCγ1 expression vector alone, PLCγ1 was found to be

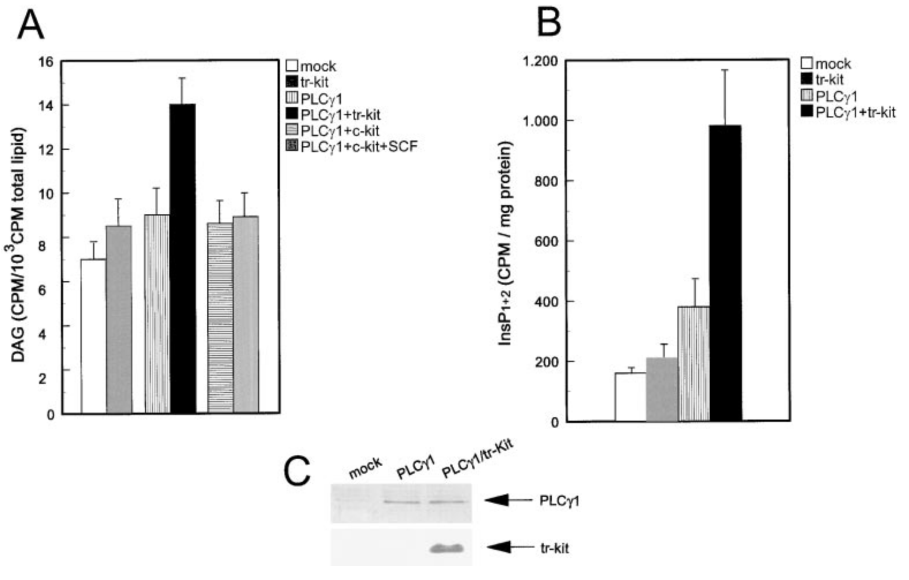

Figure 5. Tr-kit stimulates DAG and InsP production in COS cells coexpressing PLCγ1. Cells were transfected with the indicated expression vectors and labeled with either [^{3}H]arachidonic acid (*A*) or [^{3}H]inositol (*B*) and processed as described under Materials and Methods. (*A*) DAG content was measured as cpm incorporated in DAG versus 10^3 cpm incorporated in total lipids. (*B*) InsPs content was measured as cpm incorporated into InsPs per mg protein 24 h after transfection. The data represent the mean ± SD of three separate experiments, each performed in triplicate. (*C*) Pellets obtained after TCA precipitation of representative samples analyzed for InsP production were resuspended in SDS-PAGE sample buffer and analyzed in Western blot (50 μg in each lane) by using either anti-PLCγ1bd or anti-kit antibodies.

already tyrosine-phosphorylated, but a significant increase in its phosphotyrosine content was observed in tr-kit/PLCγ1 cotransfected cells (Fig. 6 *A*, *right panel*). We routinely observed an increase in immunoprecipitated PLCγ1 from PLCγ1/tr-kit cotransfected cells (Fig. 6 *A*, *left panel*), but this does not reflect higher PLCγ1 expression in these cells as shown by the Western-blot analysis of total cell extracts (Fig. 6 *B*, *left panel*; see also Fig. 5 *C*). Densitometric analysis indicated that the tyrosine phosphorylation of PLCγ1 (normalized for PLCγ1 content of the immunoprecipitates) was approximately threefold higher in tr-kit cotransfected cells, with respect to cells transfected with PLCγ1 alone (not shown). This effect was selective since tr-kit expression did not induce a general increase in the tyrosine phosphorylation pattern of total cell extracts (Fig. 6 *B*, *right panel*).

PLCγ1 Activation Does Not Require a Stable Association with Tr-kit

Tr-kit shares with c-kit the 190 COOH-terminal residues (Rossi et al., 1992), a region thought to mediate the interaction of activated c-kit with PLCγ1. Indeed, mutation of tyrosine 936 to phenylalanine in the human c-kit receptor impairs docking of PLCγ1 (Herbst et al., 1995), and this residue is conserved in mouse tr-kit (tyr161). SCF-induced autophosphorylation of the c-kit receptor creates docking sites for several signaling proteins (Herbst et al., 1991; Lev et al., 1991; Rottapel et al., 1991; Koike et al., 1993; Blume-Jensen et al., 1994; Herbst et al., 1995), and presumably, phosphorylation of tyrosine 936 creates a binding site for the SH2 domains of PLCγ1 or of intercalated adaptor proteins. It is therefore conceivable that also tr-kit, if phosphorylated on tyr161, can bind to PLCγ1.

To test this hypothesis we expressed tr-kit in COS cells and purified the cell extracts on a GST-PLCγ1-SH2SH2SH3 fusion protein bound to glutathione–agarose beads. We could not detect any binding of tr-kit to the GST-PLCγ1 (Fig. 7 *A*), indicating that tr-kit does not interact directly, or at least does not stably associate, with PLCγ1. Under these conditions, although tr-kit was efficiently precipitated by the anti–c-kit antibody (not shown), no tyrosine phosphorylation of tr-kit was detected (Fig. 7, *B* and *C*), even though we cannot rule out the possibility that this was due to the sensitivity limits of the assay conditions. As a control for the coprecipitation experiment shown in Fig. 7 *A*, we used cell extracts from c-kit–expressing COS cells that had been previously incubated for 10 min with or without SCF to induce autophosphorylation of the receptor. As expected, SCF induced tyrosine phosphorylation of the c-kit receptor (Fig. 7, *B* and *C*), and c-kit was copurified with the GST-PLCγ1 fusion protein only after SCF treatment, while almost no receptor was bound in its resting state (Fig. 7 *A*) indicating that tyrosine phosphorylated c-kit binds to PLCγ1. Similar results were obtained by immunoprecipitation of cell extracts with anti-kit or anti-PLCγ1 antibodies followed by Western blot analysis using anti-PLCγ1 or anti-kit antibodies, respectively (not shown). Thus, cotransfection experiments in COS cells indicate that tr-kit stimulates tyrosine phosphorylation and activation of PLCγ1 in the absence of a direct association with it.

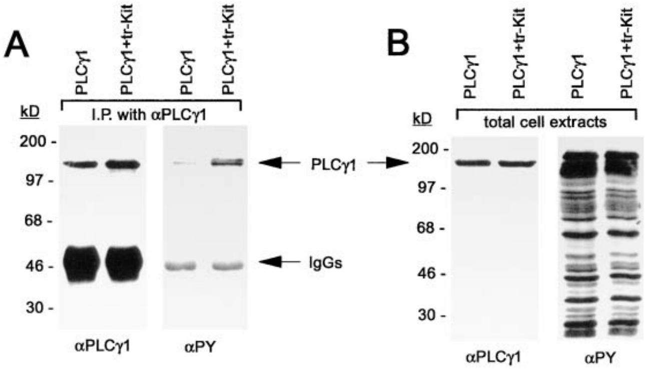

Figure 6. Tr-kit stimulates tyrosine phosphorylation of PLCγ1 in transfected COS cells. Cells were transfected with a PLCγ1 expression vector, either alone or together with the tr-kit expression vector. (*A*) Cell extracts were immunoprecipitated using a mixture of anti-PLCγ1 antibodies (see Materials and Methods). Immunoprecipitated proteins were analyzed in Western blot using either the anti-PLCγ1bd antibody (*αPLCγ1*, *left panel*) or an anti-phosphotyrosine antibody (*αPY*, *right panel*). These images are representative of six separate experiments, which gave similar results. (*B*) Western blot analysis with anti-PLCγ1 antibody (*αPLCγ1*) and anti-phosphotyrosine antibody (*αPY*) of total cell extracts (50 μg in each lane) from PLCγ1- and PLCγ1/tr-kit–transfected COS cells.

Discussion

Entrance of a sperm factor(s) into the egg cytoplasm after sperm–egg fusion is thought to trigger a series of events starting with Ca^{2+} release from intracellular stores and culminating in completion of the meiotic cell cycle and the onset of embryonic development (Whitaker and Swann, 1993). Recently, it has been reported that activation of PLCγ is required for the sperm-induced Ca^{2+} rise observed in starfish eggs at fertilization, and that the two SH2 domains of PLCγ and their ability to bind phosphotyrosines are important for PLCγ activation and the onset of Ca^{2+} rise (Carroll et al., 1997). Here we report that PLCγ1 mediates the parthenogenetic activation of mouse eggs induced by microinjection of recombinant tr-kit, a protein present in the residual cytoplasm of mouse spermatozoa. The SH3 domain of PLCγ1 plays a fundamental role in tr-kit–induced egg activation, being required for both cortical granule exocytosis and cell cycle resumption. This role is specific since the SH3 domain of the Grb2 adaptor protein does not inhibit tr-kit action inside the egg. We also show that tr-kit is able to activate recombinant PLCγ1 when coexpressed in a heterologous system.

Activation of PLC results in hydrolysis of PIP$_2$ with production of DAG and InsP$_3$ (Berridge, 1993), and both these second messengers are likely to play a major role in mammalian egg activation at fertilization. DAG and/or synthetic PKC activators are able to trigger resumption of cell cycle in MII-arrested oocytes (Colonna et al., 1997; Gallicano et al., 1997*a,b*). Microinjection of InsP$_3$ into mammalian eggs is able to trigger Ca^{2+} transients, cortical granule exocytosis, and pronuclear formation, and microinjection of antibodies directed against the InsP$_3$ receptor blocks sperm-induced egg activation (Miyazaki et al., 1992, 1993; Xu et al., 1994; Berridge, 1996). Furthermore, inhibition of PIP$_2$ hydrolysis with the specific PLC inhibi-

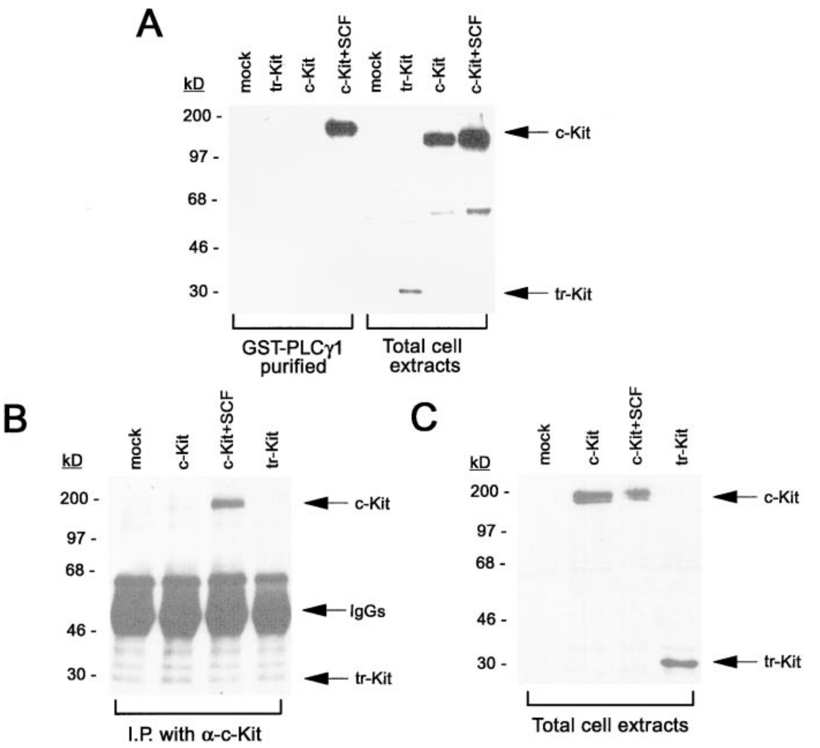

Figure 7. Tr-kit does not stably associate with PLCγ1. (*A*) COS cells were transfected with no DNA (*mock*), or 20 μg/dish pCMV5-c-kit (*c-kit*), or 20 μg/dish pCMV5-tr-kit (*tr-kit*). C-kit–transfected cells were incubated for the final 10 min with or without 100 ng/ml SCF. Cell extracts were either analyzed immediately in Western blot (50 μg in each lane) with an anti-kit antibody (*right side of the panel*), or incubated for 2 h with a GST-PLCγ1-SH2SH2SH3 fusion protein linked to glutathione–agarose beads. Proteins bound to the beads were eluted as described under Materials and Methods and analyzed in Western blot using an anti-kit antibody (*left side of the panel*). (*B*) Cells were transfected as described in *A* with tr-kit or c-kit expression vectors. Cell extracts were immunoprecipitated using an anti-kit antibody preadsorbed to protein A–Sepharose beads. Immunoprecipitated proteins were analyzed in Western blot using an anti-phosphotyrosine antibody. The band recognized by the anti-phosphotyrosine antibody with a molecular size similar to the one expected for tr-kit is present in all the samples, regardless of tr-kit presence, indicating that this band is due to a different tyrosine-phosphorylated protein present in the anti-kit immunoprecipitates from COS cells. (*C*) Cell extracts (50 μg) from the same samples shown in *B* were analyzed in Western blot using an anti-kit antibody. All panels are representative of at least three separate experiments.

tor U73122 blocks the sperm-induced Ca^{2+} spiking at fertilization in mouse eggs (Dupont et al., 1996). The present data, showing that tr-kit acts through activation of PLCγ1, are in agreement with the previous observation that U73122 blocks parthenogenetic egg activation triggered by tr-kit (Sette et al., 1997). The whole of these data indicates that tr-sit is a sperm factor that might play a physiological role in triggering early mouse embryonic development. Further support to this hypothesis is the observation that activation of mouse eggs is elicited by microinjection of an amount of recombinant tr-kit comparable to that carried by a single mouse sperm.

PLCγ1 is activated by tyrosine kinase–dependent pathways (Lee and Rhee, 1995; Kamat and Carpenter, 1997; Rhee and Bae, 1997) and data suggest that tyrosine kinase activity is involved in egg activation in different species. Stimulation of artificially expressed RTKs can initiate egg activation in *Xenopus* (Yim et al., 1994) and in starfish eggs (Shilling et al., 1994). Moreover, endogenous soluble src-related tyrosine kinases are activated shortly after fertilization both in *Xenopus* and in sea urchin (Sato et al., 1996; Kinsey, 1996). In addition, a membrane-associated c-abl–related tyrosine kinase is also activated at fertilization in sea urchin eggs (Moore and Kinsey, 1994). Although the activation of these tyrosine kinases does not always precede the Ca^{2+} rise, these data indicate that tyrosine phosphorylation is involved in the early events of fertilization. Furthermore, the involvement of PLCγ in Ca^{2+} rise at fertilization in starfish eggs (Carroll et al., 1997) suggests that one or more tyrosine kinases play a role in the upstream signaling pathway at fertilization in several species. Indeed, experiments with specific inhibitors have shown that tyrosine kinase activity is important for both block of polyspermy and late events of starfish egg activation (Moore and Kinsey, 1995). In the mouse, it has been shown that inhibitors of both tyrosine kinases and PLC can impair very early events, such as sperm-induced Ca^{2+} spiking, associated with egg activation at fertilization (Dupont et al., 1996).

Although tr-kit lacks an ATP-binding site, and thus it should not present intrinsic tyrosine kinase activity (Rossi et al., 1992), the possibility exists that tr-kit interacts with either RTKs or non-receptor tyrosine kinases (NRTKs) present in the egg, which in turn phosphorylate tr-kit itself, or other proteins mediating PLCγ1 activation. The full-length c-kit RTK is present in ovulated mouse oocytes (Manova et al., 1990; Horie et al., 1991; Yoshinaga et al., 1991); however, we have previously shown that SCF fails to induce cortical granule exocytosis, meiosis resumption and pronuclear formation in MII-arrested oocytes (Sette et al., 1997). In agreement with those observations, we show here that the SCF-stimulated c-kit receptor binds PLCγ1 but does not stimulate its enzymatic activity in transfected COS cells, as previously reported in other cellular systems (Lev et al., 1991; Koike et al., 1993; Blume-Jensen et al., 1994, Kozawa et al., 1997). The results herein presented also indicate that tr-kit is able to stimulate both DAG and InsPs production when coexpressed with PLCγ1 in COS cells. Since activation of PIP_2 hydrolysis does not seem to require a stable physical interaction between tr-kit and PLCγ1, intercalated proteins may mediate the activation of PLCγ1.

The SH region of PLCγ1 plays an essential role in tr-kit–mediated activation of mouse eggs, as shown by direct competition experiments with either a GST-PLCγ1-SH2SH2SH3 fusion protein or an antibody specifically directed against this region of the enzyme. Since a GST-PLCγ1-SH2SH2 fusion protein inhibits sperm-induced activation of starfish eggs (Carroll et al., 1997), our results suggest that SH-mediated activation of PLCγ1 is an evolutionary conserved mechanism of egg activation. On the other hand, a GST-PLCγ1-SH3 fusion protein is much more effective than a GST-PLCγ1-SH2SH2 fusion protein in inhibiting tr-kit action in mouse eggs. These results are somehow surprising, since the interaction of the SH2 domains of PLCγ1 with phosphotyrosine residues present in activated RTKs or NRTKs is thought to be an essential step for tyrosine phosphorylation, translocation, and activation of PLCγ1 (Lee and Rhee, 1995; Kamat and Carpenter, 1997; Rhee and Bae, 1997).

Tyrosine phosphorylation of PLCγ1 has been shown to correlate with activation of the enzyme (Kim et al., 1991). We found that, although PLCγ1 is already tyrosine-phosphorylated when overexpressed in COS cells, coexpression of tr-kit induces an increase in PLCγ1 phosphotyrosine content together with activation of PIP_2 hydrolysis. Since we have observed that the SH3 domain instead of the SH2 domains competes for PLCγ1 activation in mouse eggs, it is possible that additional mechanisms, beside tyrosine phosphorylation of PLCγ1, are involved in the regulation of the activity of the enzyme by tr-kit. Indeed, alternative routes of PLCγ1 activation have been described (Rhee and Bae, 1997). For instance, activation of tyrosine phosphorylated PLCγ1 by PDGF in a fibroblast cell line requires interaction of the pleckstrin homology (PH) domain of the enzyme with phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (Falasca et al., 1998). Moreover, activation of the T cell receptor causes phosphorylation of PLCγ1, but enzyme activation also requires tyrosine phosphorylation of Grb2-associated proteins (Motto et al., 1996). Ultimately, cytosolic PLCγ1 has to reach the particulate compartments of the cell to exert its enzymatic function. Translocation of PLCγ1 to the membrane and/or the cytoskeleton might bring the enzyme in close proximity to other agents, such as phosphatidic acid (Jones and Carpenter, 1993), arachidonic acid in concert with microtubule-associated tau proteins (Hwang et al., 1996), and PIP_3 (Bae et al., 1998; Falasca et al., 1998), which have been reported to stimulate its hydrolytic activity also independently from tyrosine phosphorylation. The SH3 domain of PLCγ1 has been shown to direct the enzyme to the cytoskeleton in proximity of the plasma membrane (Bar-Sagi et al., 1993), whereas the PH domain is required for the stable interaction of PLCγ1 with membrane lipids (Falasca et al., 1998). According to this model, our data suggest that tr-kit triggers activation of PLCγ1 by allowing its interaction with effector proteins in the particulate compartment of the egg via the SH3 domain.

The SH3 domain might also be directly involved in the modulation of PLCγ1 enzymatic activity. Indeed, microinjection of a catalytically inactive PLCγ1 into quiescent NIH3T3 fibroblasts induces a mitogenic response, and the SH3 domain of the protein is required for this effect (Huang et al., 1995), suggesting that the SH3 domain of PLCγ1 is the target of inhibitory proteins. Titration of these proteins with exogenous PLCγ1-SH3 domains might allow activation of endogenous PLCγ1, leading to the mitogenic response. Deletion experiments suggest that the SH region of PLCγ1 exerts an inhibitory role on the enzyme, probably impairing the correct folding of the two X and Y catalytic domains (Horstman et al., 1996). Presumably, tyrosine phosphorylation of the enzyme produces a conformational modification and relieves this negative influence (Kamat and Carpenter, 1997). However, it is possible that other interactions within the SH region, such as binding of proteins to the SH3 domain, are able to induce similar modifications and derepress PLCγ1 enzyme activity. Intercalated proteins might mediate the interaction between tr-kit and PLCγ1 causing the consequent activation of the enzyme. Indeed, it is known that SH2-containing, tyrosine-phosphorylated, adaptor proteins, such as the Syp tyrosine phosphatase, can indirectly couple other signaling proteins to tyrosine-phosphorylated RTKs (Li et al., 1994). Tyrosine phosphorylation induced by tr-kit interaction with a kinase present in the egg cytoplasm might create docking sites for intercalated adaptor proteins, which in turn may activate PLCγ1 by association with its SH3 domain.

Recent findings highlight the importance of SH3 domains in cell signaling. In *Xenopus* oocytes, the ras-GAP pathway is involved in germinal vesicle breakdown, and it has been shown that both an antibody directed against the SH3 domain of GAP, or peptides encompassing this region of the enzyme, are able to block germinal vesicle breakdown induced by oncogenic ras (Duchesne et al., 1993). The role of SH3 domains in regulating enzyme activity has been demonstrated in the case of some NRTKs. Interaction of proline-rich targets with the SH3 domain of src-related kinases results in enzyme activation, as demonstrated for Nef-mediated activation of Hck (Moarefi et al., 1997). Furthermore, the SH3 domain of Itk (a Tec-related kinase) interacts with a proline-rich region of the enzyme resulting in intramolecular inhibition, suggesting that binding of other proline-rich proteins to this SH3 domain might result in Itk activation (Andreotti et al., 1997).

Experiments are underway to identify proteins possibly interacting with tr-kit and PLCγ1 inside the egg cytoplasm and to investigate the physiological role played by tr-kit at fertilization. Mutagenesis experiments will clarify whether the phosphotransferase domain, or discrete tyrosine residues, or other structural elements present in tr-kit are involved in PLCγ1 stimulation and consequent egg activation.

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