

# Tr-kit-induced resumption of the cell cycle in mouse eggs requires activation of a Src-like kinase

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**Microinjection in mouse eggs of tr-kit, a truncated form of the c-kit tyrosine kinase present in mouse spermatozoa, causes resumption of meiosis through activation of phospholipase C $\gamma$ 1 (PLC $\gamma$ 1) and Ca<sup>2+</sup> mobilization from intracellular stores. We show that the Src-like kinase Fyn phosphorylates Tyr161 in tr-kit and that this residue is essential for tr-kit function. Fyn is localized in the cortex region underneath the plasma membrane in mouse oocytes. Using several approaches, we demonstrate that Fyn associates with tr-kit and that the interaction requires Tyr161. The interaction between tr-kit and Fyn triggers activation of the kinase as monitored by both autophosphorylation and phosphorylation of PLC $\gamma$ 1. Co-injection of tr-kit with the SH2 domain of Fyn, or pre-treatment with a Fyn inhibitor, impairs oocyte activation, suggesting that activation of Fyn by tr-kit also occurs *in vivo*. Finally, microinjection of constitutively active Fyn triggers oocyte activation downstream of tr-kit but still requires PLC activity. We suggest that the mechanism by which tr-kit triggers resumption of meiosis of mouse eggs requires a functional interaction with Fyn and phosphorylation of PLC $\gamma$ 1.**

**Keywords:** Fyn/metaphase II arrest/oocyte activation/  
PLC $\gamma$ 1/tyrosine kinases

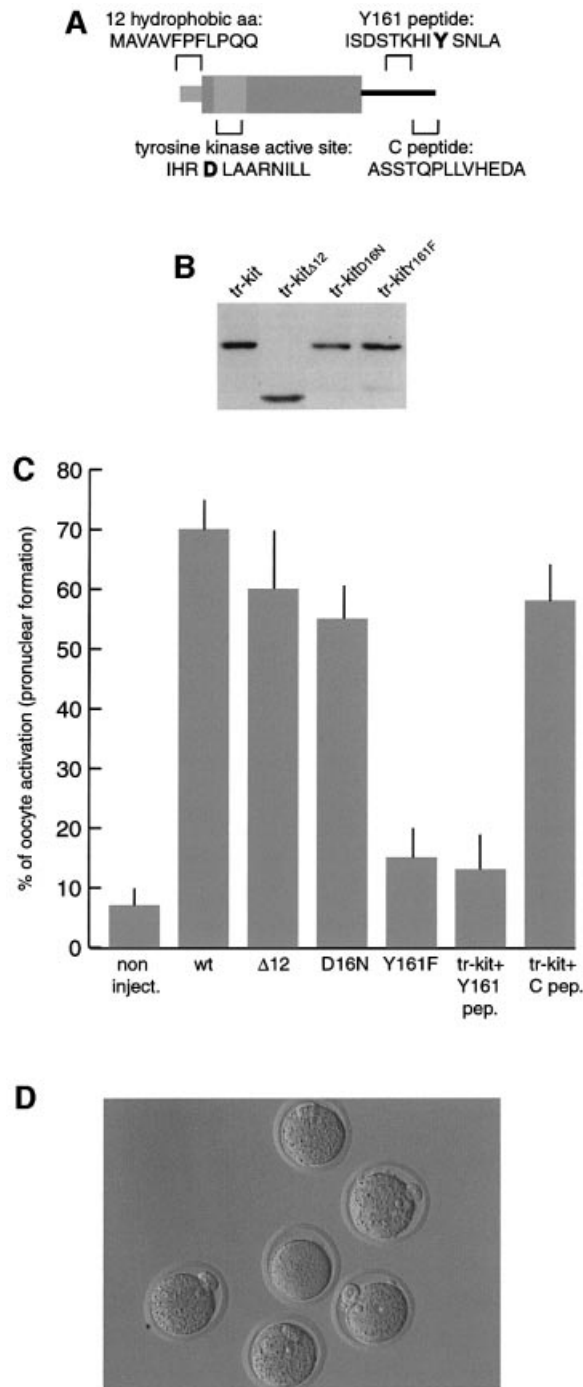
## Introduction

The *c-kit* gene encodes a tyrosine kinase receptor essential for the establishment and the maintenance of the stem cell lineages from which melanocytes, hematopoietic cells and germ cells originate (Sette *et al.*, 2000). In the postnatal mouse testis, *c-kit* is expressed in mitotic spermatogonia and its activation is required for both proliferation and survival of spermatogonia (Blume-Jensen *et al.*, 2000; Kissel *et al.*, 2000; Dolci *et al.*, 2001). Expression of *c-kit* is restricted to proliferating diploid spermatogonia; it decreases in primary spermatocytes and it is absent in post-meiotic cells (Sorrentino *et al.*, 1991). However, round spermatids express an alternative messenger driven by a cell-specific promoter in the 16th intron of the mouse *c-kit* gene (Rossi *et al.*, 1992; Albanesi *et al.*, 1996). The alternative transcript encodes a truncated *c-kit* protein of ~28 kDa, which lacks the whole extracellular and

transmembrane regions, and the ATP-binding site in the intracellular portion of the receptor. The truncated protein, named tr-kit, contains 12 hydrophobic amino acids deriving from translation of intronic sequences, the last 190 amino acids of *c-kit*, accounting for the phosphotransferase catalytic site, and the C-terminal tail, which mediates the interaction of *c-kit* with signaling molecules such as Grb2 and Grb7 (Thommes *et al.*, 1999).

Tr-kit is expressed during differentiation of round spermatids into spermatozoa and it localizes in the mid-piece and subacrosomal region of mature sperm (Sette *et al.*, 1997). Microinjection experiments have shown that recombinant tr-kit is able to trigger the activation of metaphase II (MII)-arrested mouse oocytes following the physiological route described for sperm-induced activation at fertilization: exocytosis of cortical granules; resumption of meiosis and extrusion of the second polar body; inactivation of the MAPK pathway; pronucleus formation; and onset of mitotic embryonic divisions (Sette *et al.*, 1997). As in the case of fertilization, all these events required inositol trisphosphate (IP<sub>3</sub>)-dependent mobilization of Ca<sup>2+</sup> from intracellular stores, since they were suppressed by Ca<sup>2+</sup> chelators or phospholipase C (PLC) inhibitors (Sette *et al.*, 1997). Furthermore, tr-kit was shown to activate PLC $\gamma$ 1 and stimulate IP<sub>3</sub> production in transfected cells. Remarkably, the isolated SH3 domain of the phospholipase inhibited tr-kit function in the oocyte, even though a direct interaction was not observed (Sette *et al.*, 1998).

In mammals, the ovulated oocyte is arrested at metaphase of the second meiotic division. Fusion with the sperm precedes the oscillations of intracellular Ca<sup>2+</sup> levels that are considered the hallmark of a successful fertilization (Lawrence *et al.*, 1997). It is generally accepted that an increase in IP<sub>3</sub> intracellular concentration is responsible for opening of IP<sub>3</sub> receptor-coupled Ca<sup>2+</sup> channels on the endoplasmic reticulum and the onset of the Ca<sup>2+</sup> signal (Yanagimachi, 1994). Indeed, Ca<sup>2+</sup> oscillations are required both for early events, such as exocytosis of cortical granules and the block of polyspermy, and for late events, such as inactivation of the cyclin B-cdc2 complex, known as MPF, and completion of the meiotic cell cycle (Kline and Kline, 1992). Recent evidence, gathered using sperm extracts from different species, indicates that soluble factors released by the sperm could trigger Ca<sup>2+</sup> release and metabolic activation of eggs at fertilization (Stricker, 1999). However, it remains unknown what the sperm factors are that trigger the release of Ca<sup>2+</sup> from the intracellular stores. Since IP<sub>3</sub> is involved, the activation of a PLC, the enzyme that catalyzes the hydrolysis of phosphatidylinositol bisphosphate (PIP<sub>2</sub>) with the production of IP<sub>3</sub> and diacylglycerol (DAG), has been inferred.



**Fig. 1.** Tyr161 is essential for tr-kit-induced egg activation. (A) Schematic representation of tr-kit: the residues affected by site-directed mutagenesis are highlighted in bold. The sequence of the C-terminal regions of tr-kit, mimicked by the synthetic peptides, and of the 12 N-terminal amino acids, encoded by *c-kit* intronic sequences, are reported. (B) Western blot analysis of the expression of wild-type tr-kit and of Δ12, D16N and Y161F tr-kit mutants microinjected into eggs. A 30 μg aliquot of total protein extracts from transfected COS cells was loaded in each lane. (C) Summary of the results of microinjection experiments using 5 pl of tr-kit-transfected COS cell extracts (0.1–0.2 mg/ml) alone or together with 10 mg/ml of C or Y161 peptide. At least 40 eggs were injected for each experimental group. Pronuclear formation was monitored 6–7 h after microinjection. Data are the mean ± SD of at least three separate experiments for each group. (D) A representative example showing that most of the tr-kit-microinjected eggs have extruded the second polar body and formed a parthenogenetic pronucleus.

Studies using echinoderm eggs have pointed to a role for a PLCγ isoform during fertilization (Runft *et al.*, 2002). In the PLCγ subfamily (PLCγ1 and γ2 in mammals), the X and Y catalytic domain are separated by a Src-homology (SH) region containing two SH2 domains, an SH3 domain and a split PH domain. This intervening region seems to regulate the activity of the enzyme negatively. Upon binding of activators to either the SH2 or SH3 domains, and phosphorylation of Tyr783 in the SH region and Tyr1254 near the C-terminus, PLCγ is activated (Carpenter and Ji, 1999). The SH region has been used successfully as a specific dominant-negative inhibitor *in vivo*, probably because it prevents the association of activators with the endogenous enzymes (Roche *et al.*, 1995). Indeed, injection of the tandem SH2 domains of PLCγ into echinoderm eggs blocks or delays the rise in Ca<sup>2+</sup> at fertilization and causes polyspermy, indicating that PLCγ is important in this process (Carroll *et al.*, 1997). Accordingly, it was demonstrated that PLCγ translocates to the plasma membrane and is activated within minutes in response to fertilization of sea urchin eggs (Rongish *et al.*, 1999). More recently, a number of studies have suggested that a Src-like kinase is activated at fertilization in several non-mammalian organisms. Association of this kinase with PLCγ would favor its phosphorylation and activation, with the production of intracellular IP<sub>3</sub>. The consequent release of intracellular Ca<sup>2+</sup> would trail most of the events required for egg activation and the onset of embryogenesis (Runft *et al.*, 2002). However, the mechanisms by which fusion of the gametes induces activation of a Src-like tyrosine kinase remain to be elucidated.

Src is the prototype of a family of soluble tyrosine kinases, which are characterized by a myristoylation site at the N-terminus, followed by a unique region showing most of the heterogeneity in sequence between the members, an SH3 domain that mediates interactions with proteins containing proline-rich sequences, an SH2 domain that interacts with phosphorylated tyrosines in substrates and activators, and a catalytic domain (Thomas and Brugge, 1997). It is known that Src-like kinases are kept in a low activity conformation by phosphorylation of Tyr527 (according to the numbering of the prototype avian c-Src), which is catalyzed by the C-terminus Src kinase Csk. The phosphorylated tyrosine takes part in an intramolecular interaction with the SH2 domain, and this conformational modification stabilizes the binding of the SH3 domain to a polyproline helix in the linker region between the SH2 domain and the catalytic domain (Xu *et al.*, 1997; Young *et al.*, 2001). These intramolecular interactions keep the active site of the kinase poorly accessible to substrates (Sicheri and Kuriyan, 1997). Upon dephosphorylation of Tyr527 or displacement of the SH2 and SH3 intramolecular interactions by other proteins, Src-like kinases acquire a relaxed conformation that allows autophosphorylation of Tyr416 in the catalytic site and stabilizes the high activity state (Young *et al.*, 2001).

In this report, we demonstrate that tr-kit-induced mouse oocyte activation requires a functional interaction with the Src-like tyrosine kinase Fyn. Tr-kit physically interacts with Fyn and this interaction stimulates the ability of Fyn to phosphorylate PLCγ1 both *in vitro* and *in vivo*. These results describe a new mechanism of activation of a Src-

like kinase and suggest a possible pathway for mammalian egg activation at fertilization.

## Results

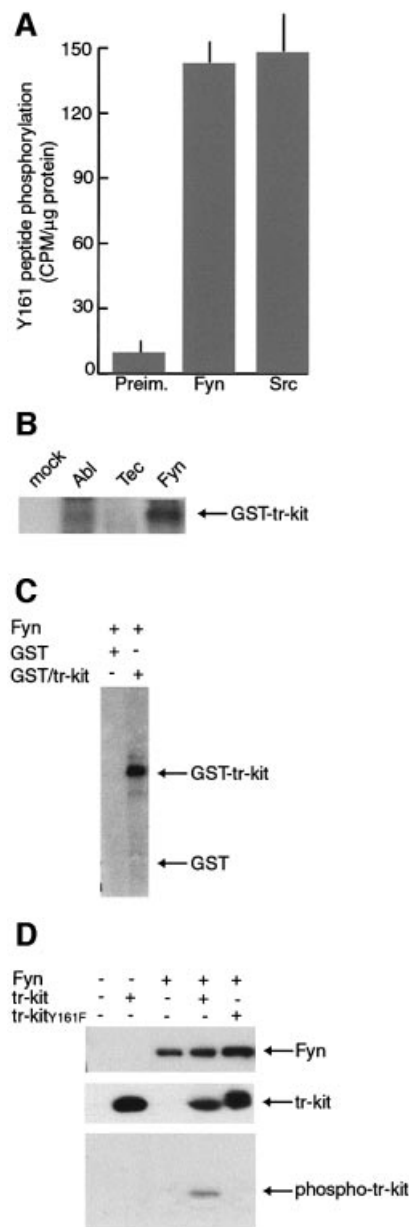
### *Tyr161 in tr-kit is required for oocyte activation*

We have shown previously that a GST-PLC $\gamma$ 1SH3 fusion protein interferes with tr-kit-induced egg activation, despite the fact that a direct interaction with tr-kit does not occur (Sette *et al.*, 1998). To gain more insight into the function of tr-kit, we performed site-specific mutagenesis of regions of possible interest (Figure 1A). The 12 hydrophobic amino acids at the N-terminus, deriving from translation of intronic sequences and unique to tr-kit (Rossi *et al.*, 1992), were deleted because they contain a PFLP motif, a putative SH3-binding site (Kroiher *et al.*, 2001). We mutated the aspartate residue D16 into asparagine in the phosphotransferase domain (tr-kit<sub>D16N</sub>) because this mutation abolishes the catalytic activity of full-length c-kit (Tan *et al.*, 1990). Finally, we substituted Tyr161 into phenylalanine (tr-kit<sub>Y161F</sub>), since the corresponding residue in c-kit mediates the interaction with SH2 domains of the adaptor proteins Grb2 and Grb7 (Thommes *et al.*, 1999).

Wild-type and mutant tr-kit proteins were expressed in COS cells and an amount comparable with 0.5–0.8 sperm equivalents (Figure 1B; see also Sette *et al.*, 1998) was microinjected into mouse MII oocytes. As reported previously (Sette *et al.*, 1997), microinjection of tr-kit caused activation of 60–70% of the oocytes as monitored by both polar body extrusion and pronucleus formation 6–7 h after injection (Figure 1C and D). Interestingly, tr-kit $\Delta$ <sub>12</sub> and tr-kit<sub>D16N</sub> were still able to elicit oocyte activation significantly (Figure 1C), although to a minor extent (~60 and 55%, respectively), indicating that neither the 12 hydrophobic amino acids nor the putative catalytic activity of tr-kit are essential for egg activation. On the contrary, we found that substitution of Tyr161 dramatically inhibited egg activation (15%). Furthermore, we observed that co-injection of tr-kit with a 13 amino acid peptide encompassing Tyr161 strongly decreased the percentage of activated oocytes (13%), whereas co-injection of a control peptide containing the last 13 amino acids of tr-kit had no effect (58%) (Figure 1C). As previously reported (Sette *et al.*, 1997, 1998), non-injected or mock-injected eggs showed a 5–8% spontaneous activation under the culture conditions used. Thus, Tyr161 is critical for tr-kit-induced egg activation.

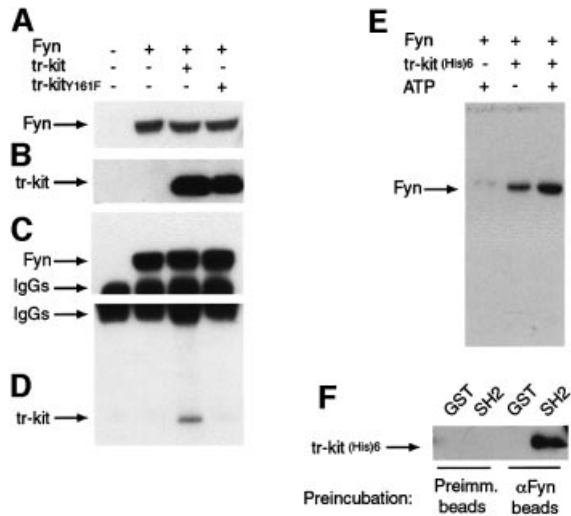
### *Src-like kinases phosphorylate Tyr161 of tr-kit*

Tyr161 in tr-kit lays in a sequence that matches more closely the consensus for phosphorylation by soluble tyrosine kinases rather than by receptor tyrosine kinases (Songyang *et al.*, 1995). Since the involvement of Src-like kinases at fertilization has been hypothesized in both invertebrates and vertebrates (Runft *et al.*, 2002), we investigated whether they could phosphorylate tr-kit on Tyr161. Src and Fyn immunoprecipitated from testis extracts readily phosphorylated the Y161 peptide (Figure 2A). These results were confirmed using immunoprecipitates from Hek 293 cell extracts transfected with Src and Fyn (data not shown). Next, we expressed tyrosine kinases belonging to three different subfamilies, Abl, Tec



**Fig. 2.** Src-like kinases phosphorylate Tyr161 in tr-kit. (A) Mouse testis extracts (1 mg) were immunoprecipitated using 1  $\mu$ g of pre-immune rabbit IgGs, rabbit  $\alpha$ -Fyn or mouse  $\alpha$ -Src antibodies, and the phosphorylating activity of the immunoprecipitates was tested using 10  $\mu$ g of the synthetic of Y161 peptide as substrate (sequence in Figure 1A). Data correspond to c.p.m. incorporated per  $\mu$ g of total protein used for immunoprecipitation. Values are the mean  $\pm$  SD of three independent experiments. (B) Autoradiography of an immunokinase assay using a purified GST-tr-kit (5  $\mu$ g) as substrate for recombinant Abl, Tec and Fyn expressed in Hek 293 cells and immunoprecipitated using specific antibodies. Similar amounts of each kinase were used as judged by western blot analysis of the immunoprecipitates (data not shown). (C) Autoradiography of an immunokinase assay of recombinant Fyn as described in (B) using either purified GST (5  $\mu$ g) or GST-tr-kit (5  $\mu$ g) as substrates. (D) Western blot analysis of Fyn (upper panel) and tr-kit (central panel) expressed in transfected Hek 293 cells, and of tr-kit immunoprecipitated from cell extracts using an anti-phosphotyrosine antibody (lower panel).

and Fyn, in Hek 293 cells and performed an immunokinase assay using purified GST-tr-kit as substrate. We found that Fyn strongly phosphorylates GST-tr-kit, whereas a constitutively active form of Abl phosphorylates it very

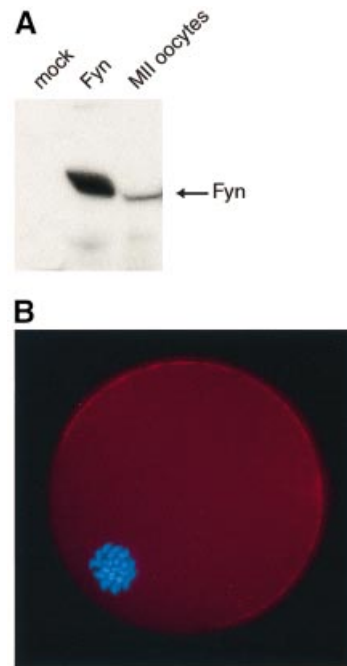


**Fig. 3.** Physical association with Fyn requires Tyr161 in tr-kit. Western blot analysis of extracts (20  $\mu$ g) from transfected Hek 293 cells was performed using the  $\alpha$ -Fyn (A) or the  $\alpha$ -c-kit antibody (B). Fyn was immunoprecipitated from cell extracts (500  $\mu$ g) using 1  $\mu$ g of  $\alpha$ -Fyn, and immunoprecipitated proteins were analyzed by western blot using the  $\alpha$ -Fyn (C) or the  $\alpha$ -c-kit antibody (D). This experiment indicates that tr-kit, but not the Y161F mutant, co-immunoprecipitates with Fyn. (E) Association between Fyn and a bacterially expressed tr-kit protein. Protein A/G-Sepharose beads pre-adsorbed to 1  $\mu$ g of  $\alpha$ -His<sub>6</sub> antibody were incubated for 1 h at 4°C with cell extracts from Hek 293 cells transfected with Fyn in the presence or absence of 100  $\mu$ M ATP and purified tr-kit<sub>His6</sub> (2  $\mu$ g). After three washes with homogenization buffer, proteins bound to the beads were eluted in sample buffer and analyzed by western blotting using the  $\alpha$ -Fyn antibody. (F) Cell extracts from Hek 293 cells transfected with FynY528F were immunoprecipitated with either pre-immune or  $\alpha$ -Fyn IgGs, and immunoprecipitates were incubated with 5  $\mu$ g of tr-kit<sub>His6</sub> for 30 min at room temperature in the presence of 100  $\mu$ M ATP to allow phosphorylation. Then, immunoprecipitates were separated by centrifugation and soluble tr-kit<sub>His6</sub> was incubated with either GST or GST-FynSH2 pre-adsorbed to glutathione-agarose beads. Proteins bound to GST fusions were eluted with 10  $\mu$ M reduced glutathione and analyzed by western blotting using the  $\alpha$ -c-kit antibody.

weakly (Figure 2B). Tec did not phosphorylate GST-tr-kit, even though it could phosphorylate other protein substrates in this assay (see Figure 7). Western blot analysis indicated that similar amounts of kinases were immunoprecipitated selectively from transfected cells (data not shown). Fyn specifically phosphorylates tr-kit in this assay, because GST alone was not phosphorylated (Figure 2C). To examine tr-kit phosphorylation *in vivo* by Fyn and whether Tyr161 in tr-kit is the specific target of the kinase, Hek 293 cells were co-transfected with Fyn and tr-kit wild-type or the Y161F mutant in different combinations, proteins were immunoprecipitated with an anti-phosphotyrosine antibody and the immunoprecipitates tested for the presence of tr-kit. As shown in Figure 2D, wild-type tr-kit, but not tr-kit<sub>Y161F</sub>, was phosphorylated on tyrosines in a Fyn-dependent manner, indicating that Fyn specifically promotes phosphorylation of Tyr161 in tr-kit *in vivo*.

#### Physical interaction between tr-kit and Fyn

To study whether tr-kit and Fyn could physically interact, Hek 293 cells were transfected with the appropriate expression vectors (Figure 3A and B) and cell extracts

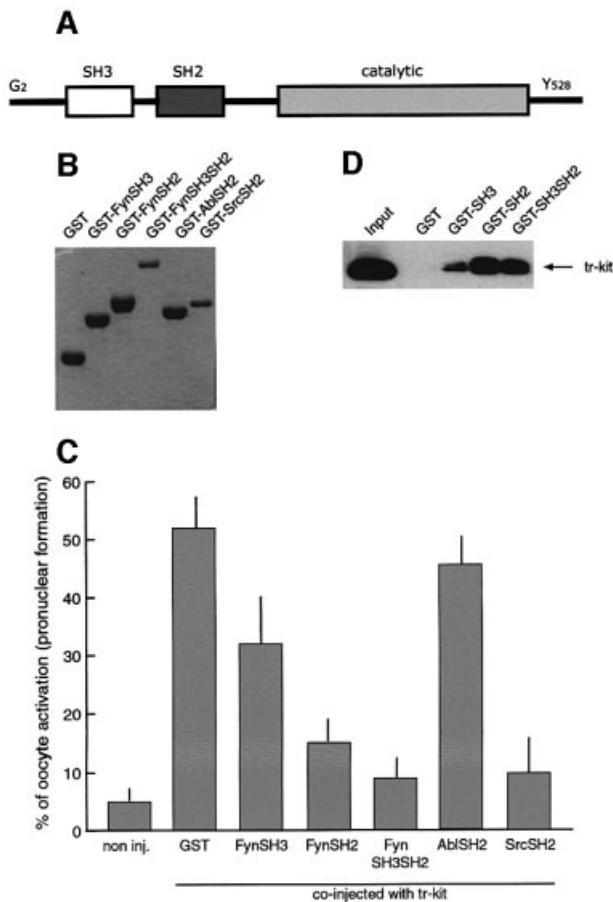


**Fig. 4.** Fyn is expressed in mouse MII oocytes. (A) Western blot analysis of Fyn expressed in 100 mouse ovulated oocytes. For comparison, 30  $\mu$ g of cell extracts from mock- or Fyn-transfected Hek 293 cells were loaded on the gel. (B) Immunofluorescence analysis of Fyn expressed in mouse oocytes: cells were co-stained with  $\alpha$ -Fyn antibody and the DNA dye Hoechst 3332.

were immunoprecipitated with an  $\alpha$ -Fyn antibody (Figure 3C). Western blot analysis of the immunoprecipitates revealed that wild-type tr-kit co-immunoprecipitated with Fyn, whereas tr-kit<sub>Y161F</sub> did not interact with the kinase (Figure 3D). The interaction between tr-kit and Fyn was confirmed with a pull-down experiment using a partially purified tr-kit<sub>His6</sub> protein expressed in bacteria and cell extracts from Hek 293 cells transfected with Fyn in the presence or absence of exogenous ATP. Figure 3E shows that Fyn is pulled down by the  $\alpha$ -His<sub>6</sub> antibody only when the beads had been pre-adsorbed to tr-kit<sub>His6</sub> and that the interaction is improved by exogenous ATP. Next we tested whether the interaction requires phosphorylation of tr-kit and the SH2 domain of Fyn. Extracts expressing recombinant Fyn were immunoprecipitated with either pre-immune or  $\alpha$ -Fyn IgGs and the immunoprecipitates were incubated with purified tr-kit<sub>His6</sub>. Following the incubation, tr-kit<sub>His6</sub> was passed over beads pre-adsorbed to either GST or GST-FynSH2. As shown in Figure 3F, tr-kit<sub>His6</sub> was able to interact directly with GST-FynSH2 only after being pre-incubated with Fyn. These data demonstrate that tr-kit and Fyn physically interact and that Tyr161 is necessary for such interaction, indicating a correlation between tr-kit-Fyn association and biological activity of tr-kit.

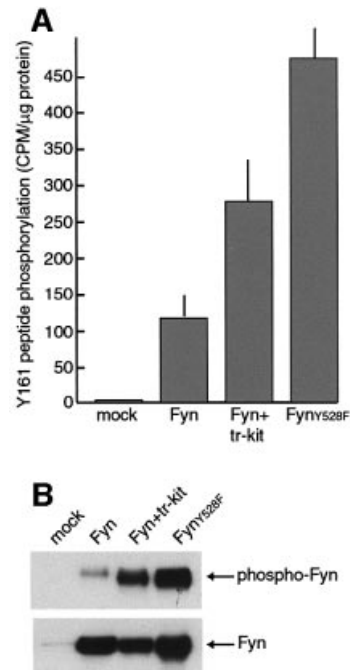
#### The SH region of Fyn inhibits tr-kit-induced oocyte activation

Western blot analysis demonstrates that Fyn is expressed in mouse eggs (Figure 4A), and we found that it localizes in the cortex region (Figure 4B) as described previously in rat eggs (Talmor *et al.*, 1998). We set out to investigate



**Fig. 5.** The SH region of Fyn interferes with tr-kit-induced egg activation. (A) Schematic representation of the organization of the Src-homology domains in Fyn. (B) Coomassie blue staining of an SDS-polyacrylamide gel with purified bacterial fusion proteins containing the Src-homology domains used for the microinjection experiments. (C) Summary of the co-injection experiments using tr-kit and 500  $\mu$ g/ml of GST, or GST-Fyn<sub>SH3</sub>, GST-Fyn<sub>SH2</sub>, GST-Fyn<sub>SH3SH2</sub>, GST-Abl<sub>SH2</sub> or GST-Src<sub>SH2</sub>. Data are the mean  $\pm$  SD of at least three separate experiments for each experimental group (total of at least 30 eggs). (D) A representative pull-down experiment using 4  $\mu$ g of GST-Fyn fusion proteins and cell extracts (500  $\mu$ g) from Hek 293 cells transfected with tr-kit and Fyn.

whether the interaction between Fyn and tr-kit plays a role in oocyte activation. The SH region of Src-like kinases (Figure 5A) plays a crucial role in both autoinhibition of catalytic activity and interaction with activators and substrates (Thomas and Brugge, 1997), and a GST-FynSH3SH2 fusion protein exerts a dominant-negative action on Fyn in live cells (Roche *et al.*, 1995). Interestingly, we observed that co-injection of tr-kit and GST-FynSH3SH2 (Figure 5B) into MII oocytes strongly inhibited resumption of the cell cycle triggered by tr-kit (from 52 to 9% in this set of experiments) (Figure 5C). We next asked whether the SH2 domain is sufficient for inhibition, because purified tr-kit interacts with the SH2 domain of Fyn after being phosphorylated by the kinase on Tyr161. Indeed, the isolated SH2 domain of Fyn acted as a strong inhibitor of tr-kit function in oocytes (Figure 5C), even though a weak inhibition was also exerted by the isolated SH3 domain. Accordingly, pull-down experiments using GST-Fyn fusion proteins indicated that the



**Fig. 6.** Tr-kit stimulates the catalytic activity of Fyn. Fyn activity was measured in an immunokinase assay using extracts from Hek 293 cells that were transfected with wild-type Fyn, Fyn<sub>Y528F</sub> or Fyn and tr-kit. After immunoprecipitation, beads were divided into two equal amounts. (A) Fyn activity was measured in one half of the beads using the Y161 peptide (10  $\mu$ g) and 100  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (0.1  $\mu$ Ci/ $\mu$ l) as substrates, and the assay was carried out as described in Materials and methods. (B) The other half of the beads were incubated as above in 25  $\mu$ l of kinase buffer supplemented with 100  $\mu$ M ATP and, at the end of the incubation, immunoprecipitated proteins were eluted in sample buffer and analyzed by western blot using the anti-phosphotyrosine (upper panel) or the  $\alpha$ -Fyn antibody (lower panel).

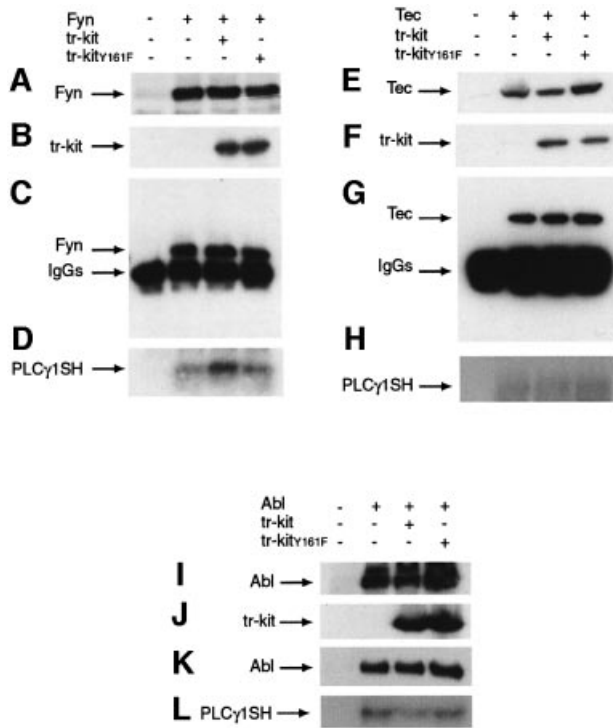
SH2 domain is sufficient for the interaction with tr-kit, albeit that a weak interaction with the SH3 domain was also detected (Figure 5D). The SH2 domain of a related kinase (Src) also inhibited tr-kit action in eggs, whereas that of Abl was ineffective (Figure 5C). Together, these results indicate that a specific interaction between tr-kit and the SH2 domain of a Src-like kinase is necessary for egg activation.

#### **Tr-kit stimulates the catalytic activity of Fyn**

Displacement of the intramolecular interaction between Tyr527 (528 in Fyn) and the SH2 domain by another protein activates Src-like kinases. Interestingly, co-expression of Fyn and tr-kit in Hek 293 cells elicited a 2- to 3-fold stimulation of the catalytic activity of the kinase (Figure 6A). This activation was similar to that achieved by constitutive disruption of the intramolecular interaction between Tyr528 and the SH2 domain in the hyperactive Fyn<sub>Y528F</sub> (Figure 6A). Activation of Fyn by Tyr528 substitution or by tr-kit was also confirmed by looking at the autophosphorylation levels of the kinase (Figure 6B), which correlate with its catalytic activity.

#### **Tr-kit stimulates phosphorylation of PLC $\gamma$ 1 by Fyn**

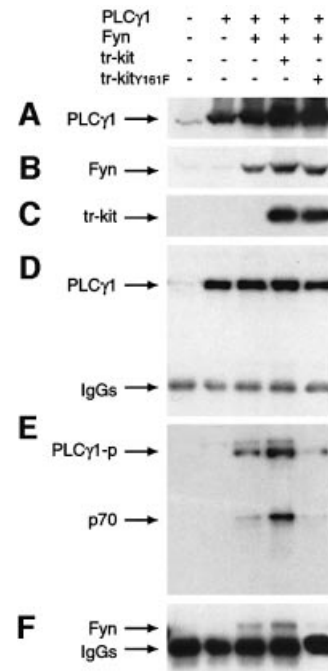
Since tr-kit-induced egg activation requires PLC $\gamma$ 1 (Sette *et al.*, 1998), and PLC $\gamma$ 1 is activated by phosphorylation of Tyr783 in the SH region (Kim *et al.*, 1991), we investigated whether Fyn phosphorylates PLC $\gamma$ 1 and whether the



**Fig. 7.** Tr-kit specifically stimulates phosphorylation of PLC $\gamma$ 1 by Fyn. COS cells were transfected with tr-kit or tr-kit<sup>Y161F</sup> and Fyn (A–D), Tec (E–H) or Abl (I–L) and, after 24 h, cell extracts were immunoprecipitated with the  $\alpha$ -Fyn antibody (C),  $\alpha$ -Tec antibody (G) or  $\alpha$ -Abl antibody (K). Immunoprecipitates were assayed for kinase activity towards the purified SH region of PLC $\gamma$ 1 (D, H and L) as described in Materials and methods. Similar amounts of Fyn (A), Tec (E) and Abl (I), and of tr-kit and tr-kit<sup>Y161F</sup> (B, F and J) were expressed in the cell extracts, and equal amounts of tyrosine kinases were immunoprecipitated and assayed for enzymatic activity (C, G and K).

co-expression of tr-kit affects this reaction. COS cells were co-transfected with the appropriate plasmids and, after immunoprecipitation of cell extracts with the  $\alpha$ -Fyn antibody, the kinase activity was assayed using a GST–PLC $\gamma$ 1SH fusion protein as substrate. Interestingly, we found that Fyn directly phosphorylates GST–PLC $\gamma$ 1SH *in vitro* and that co-expression of tr-kit stimulates PLC $\gamma$ 1 phosphorylation (Figure 7A–D). On the other hand, tr-kit<sup>Y161F</sup>, which is unable to interact with Fyn and to trigger egg activation, did not affect the activity of the kinase (Figure 7A–D). Activation of Fyn by tr-kit was specific, because in similar sets of experiments we demonstrated that tr-kit was unable to activate Tec (Figure 7E–H) or Abl (Figure 7I–L). Interestingly, both Tec and Abl lack the C-terminal tyrosine that plays an autoinhibitory role in Src-like kinases (Smith *et al.*, 2001; Pluk *et al.*, 2002).

Next, we investigated the ability of Fyn to phosphorylate PLC $\gamma$ 1 *in vivo*. To this end, Hek 293 cells were co-transfected in various combinations with PLC $\gamma$ 1, Fyn and tr-kit, PLC $\gamma$ 1 was then immunoprecipitated from cell extracts with a specific antibody, and the immunoprecipitates were analyzed by western blotting. When expressed alone, PLC $\gamma$ 1 (Figure 8D) was weakly phosphorylated on tyrosine residues (Figure 8E) whereas co-expression of Fyn promoted tyrosine phosphorylation of the phospho-

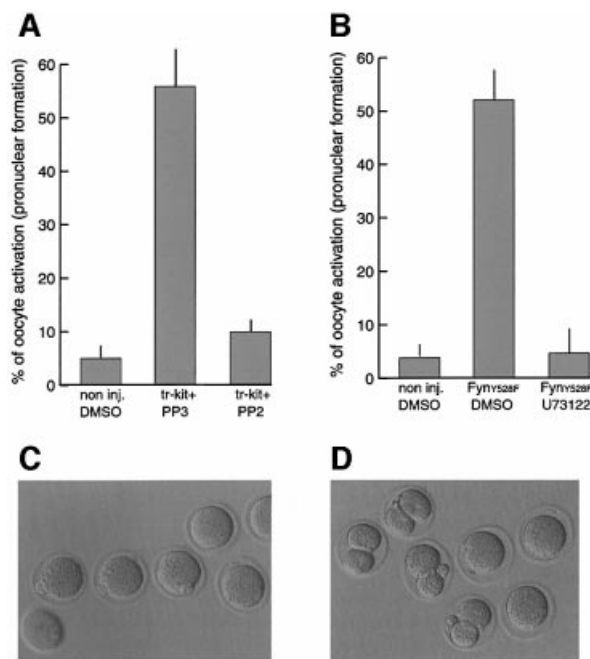


**Fig. 8.** Co-expression of tr-kit stimulates Fyn-dependent phosphorylation of PLC $\gamma$ 1 *in vivo*. Hek 293 cells were transfected with PLC $\gamma$ 1, Fyn, tr-kit and tr-kit<sup>Y161F</sup> in different combinations (A–C). After 24 h, cells were harvested, lysed, and clarified extracts were immunoprecipitated for 2 h using 1  $\mu$ g of  $\alpha$ -PLC $\gamma$ 1 antibody adsorbed to protein A–Sepharose beads. Immunoprecipitated proteins were analyzed by western blotting using the  $\alpha$ -PLC $\gamma$ 1 (D), the  $\alpha$ -phosphotyrosine (E) or the  $\alpha$ -Fyn (F) antibody.

lipase. Moreover, expression of Fyn and tr-kit in the same cells dramatically increased phosphorylation of PLC $\gamma$ 1 (Figure 8E), confirming *in vivo* the effect exerted by tr-kit on the phosphorylation of PLC $\gamma$ 1 by Fyn *in vitro*. Interestingly, a band of  $\sim$ 70 kDa was also detected in the immunoprecipitates, and its phosphorylation was strongly increased in the presence of tr-kit. Furthermore, western blot analysis of the  $\alpha$ -PLC $\gamma$ 1 immunoprecipitates with the  $\alpha$ -Fyn antibody showed that Fyn co-precipitates with PLC $\gamma$ 1 and that tr-kit promotes this association (Figure 8F). Again, tr-kit<sup>Y161F</sup> was unable to stimulate either association of Fyn with PLC $\gamma$ 1 or its level of tyrosine phosphorylation, indicating that an interaction between tr-kit and Fyn through Tyr161 in tr-kit is necessary for such events.

#### Activation of Fyn by tr-kit is sufficient to trigger oocyte activation

We next tested whether tr-kit induces egg activation by sequentially stimulating Fyn and PLC $\gamma$ 1 activation. Oocytes were pre-incubated for 30 min with the Src-like kinase inhibitor PP2 or its inactive analog PP3 before microinjection of tr-kit. PP2 completely suppressed tr-kit action, whereas PP3 was ineffective (Figure 9A), demonstrating the requirement for activation of a Src-like kinase. Furthermore, microinjection of constitutively active Fyn<sup>Y528F</sup> triggered parthenogenetic egg activation mimicking the effect exerted by tr-kit, as demonstrated by pronuclear formation after 6–8 h and cell division after 18–24 h (Figure 9C and D). Furthermore, as in the case of tr-kit-induced egg activation, Fyn<sup>Y528F</sup> action required



**Fig. 9.** Src-like kinase activity is required for tr-kit-induced egg activation. (A) Eggs were pre-incubated with either the vehicle DMSO, 5  $\mu$ M PP2 or 5  $\mu$ M PP3 for 30 min before microinjection with tr-kit. Injected eggs were returned to M16 medium containing the appropriate drug and scored for activation after 6 h. (B) Eggs were pre-incubated with either DMSO or 5  $\mu$ M U73122 for 30 min before microinjection with Fyn<sub>Y528F</sub>. Egg activation was scored after 6 h as pronucleus formation (C) or after 24 h as cell division (D).

PLC activity, since egg activation was inhibited when oocytes were pre-incubated with the PLC inhibitor U73122 (Figure 9B). These results suggest that tr-kit-induced oocyte activation is triggered by the sequential activation of Fyn and PLC $\gamma$ 1.

## Discussion

### **An association between tr-kit and Fyn is required for oocyte activation**

Tr-kit triggers resumption of meiosis and activation of MII-arrested oocytes when microinjected into their cytoplasm. This effect requires activation of PLC $\gamma$ 1 and mobilization of Ca<sup>2+</sup> from intracellular stores (Sette *et al.*, 1997, 1998). Here we demonstrate that activation of PLC $\gamma$ 1 is mediated by a functional interaction between tr-kit and a Src-like tyrosine kinase. Several observations obtained using as prototype the Src-like kinase Fyn, highly expressed in mouse oocytes, support this conclusion. (i) We have identified a mutant, tr-kit<sub>Y161F</sub>, which is unable to associate with Fyn and to induce resumption of oocyte meiosis. (ii) Fyn phosphorylates Tyr161 of tr-kit *in vitro* and in transfected cells, suggesting that it could also promote phosphorylation of this crucial residue in the oocyte. (iii) The SH2 domain of Fyn, which binds *in vitro* to tr-kit, or the Src-like kinase inhibitor PP2, suppress tr-kit-induced egg activation. (iv) Constitutively active Fyn is sufficient to trigger egg activation. Since the SH2 domain of Src also blocks tr-kit-induced egg activation, whereas that of the unrelated tyrosine kinase Abl does not, the interaction appears to be specific for Src-like kinases.

Therefore, we hypothesize that upon entry into the oocyte cytoplasm, tr-kit is phosphorylated by Fyn (or other Src-like kinase) on Tyr161, which promotes its interaction with the kinase, and that this complex triggers oocyte activation. The interaction between tr-kit and Fyn requires phosphorylation of Tyr161 and the SH2 domain of the kinase, as demonstrated by pull-down experiments with purified proteins and cell extracts.

### **Tr-kit acts as a molecular trigger of the catalytic activity of Fyn**

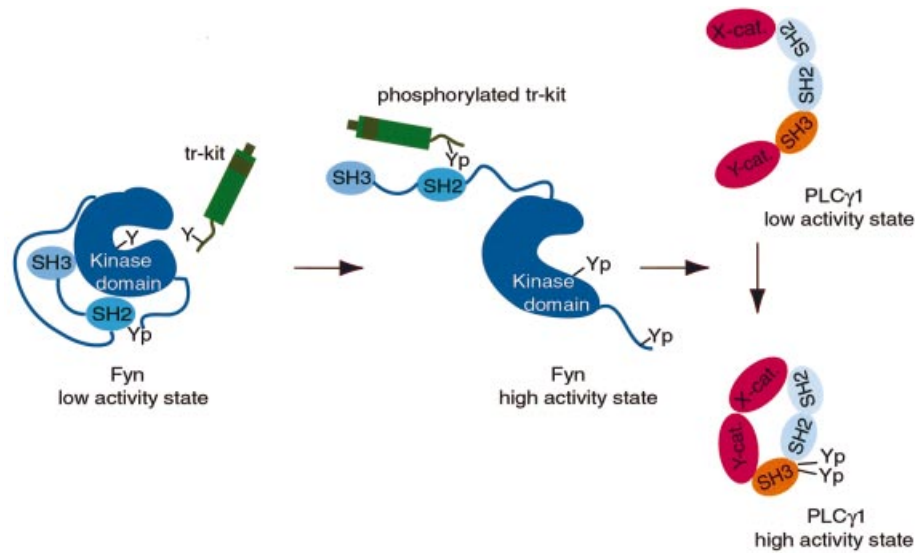
Why is the interaction of tr-kit with Fyn functionally important? We report that co-expression of tr-kit and Fyn causes activation of Fyn as monitored by autophosphorylation of the kinase and phosphorylation of exogenous substrates. Since displacement of the intramolecular interaction between Tyr527 and the SH2 domain activates Src-like kinases (Thomas and Brugge, 1997), we suggest that, once phosphorylated on Tyr161, tr-kit acts as a molecular trigger to change the conformation of Fyn from a low to a high activity state (Figure 10). Indeed, we observed that co-expression of tr-kit stimulates the activity of Fyn 2- to 3-fold, eliciting an activation that is approximately half of that obtained with disruption of the intramolecular interaction in Fyn<sub>Y528F</sub>. Our model is also supported by the observation that tr-kit<sub>Y161F</sub>, which is unable to interact with Fyn, does not induce enzyme activation and that purified tr-kit binds to the SH2 domain of Fyn only after being phosphorylated. Since tr-kit<sub>Y161F</sub> is also unable to trigger oocyte activation, a correlation exists between tr-kit–Fyn interaction and tr-kit biological activity.

### **Tr-kit stimulates phosphorylation of PLC $\gamma$ 1 by Fyn**

Tr-kit induces resumption of meiosis of mouse oocytes by promoting phosphorylation and activation of PLC $\gamma$ 1 (Sette *et al.*, 1998). Here we show that a Src-like kinase mediates activation of PLC $\gamma$ 1 in microinjected eggs. PLC $\gamma$ 1 is activated through phosphorylation of Tyr783 in the SH region (Kim *et al.*, 1991; Carpenter and Ji, 1999). Accordingly, we observed that Fyn directly phosphorylates GST–PLC $\gamma$ 1SH2SH2SH3 and that tr-kit stimulates this reaction. Moreover, co-expression in the same cell of tr-kit, Fyn and PLC $\gamma$ 1 brings about a massive increase in the phosphotyrosine content of PLC $\gamma$ 1. We also observed that tr-kit expression promotes the association of Fyn with PLC $\gamma$ 1. Taken together, our results are consistent with a model in which tr-kit activates Fyn by direct interaction and promotes its association with PLC $\gamma$ 1, and the consequent phosphorylation of the phospholipase brings about its activation (Figure 10). In agreement with such a model, soluble tyrosine kinases such as Tec and Abl, which lack the phosphotyrosine-SH2 domain autoinhibition mechanism typical of Src-like kinases (Smith *et al.*, 2001; Pluk *et al.*, 2002), are not activated by tr-kit.

### **A possible pathway operating at fertilization**

Since tr-kit is expressed specifically in male haploid cells and mature sperm (Sette *et al.*, 1997), it is possible that it participates in egg activation at fertilization. In mammalian oocytes, the signal that triggers resumption of meiosis is provided by oscillatory increases in intracellular Ca<sup>2+</sup> levels triggered by production of IP<sub>3</sub> (Yanagimachi, 1994),



**Fig. 10.** Hypothetical model of tr-kit-induced activation of Fyn. Fyn is maintained in a low activity state by the intramolecular interaction between the SH2 domain and phosphorylated Tyr528 in the C-terminus. Tr-kit is phosphorylated on Tyr161 by the 'low activity' Fyn; this allows interaction with the SH2 domain of Fyn and displacement of the intramolecular inhibition. Activated Fyn autophosphorylates in the activation loop, and interacts with PLCγ1, thereby promoting phosphorylation and activation of the lipase.

and it has been demonstrated that fusion of the gametes precedes the onset of  $\text{Ca}^{2+}$  oscillations (Lawrence *et al.*, 1997). Although it is unknown how the sperm triggers  $\text{Ca}^{2+}$  mobilization, it is plausible that soluble sperm factors are released into the oocyte cytoplasm at fertilization, because microinjection of extracts obtained from sperm of invertebrates and vertebrates, including mammals, is sufficient to elicit  $\text{Ca}^{2+}$  mobilization and egg activation (Stricker, 1999). The 'sperm factor' hypothesis is also supported by the evidence that large molecular weight molecules can transit from the sperm to the oocyte before the onset of  $\text{Ca}^{2+}$  oscillations (Jones *et al.*, 1998). Since  $\text{IP}_3$  production is involved, it is conceivable that a PLC isoform is activated by a such sperm factor(s).

In echinoderm eggs, activation of a Src-like kinase, which associates with the SH2 domains of PLCγ, occurs within minutes after sperm-egg fusion (Kinsey, 1996; Giusti *et al.*, 1999). The kinase involved has not been identified, even though microinjection of active mammalian Src triggers egg activation (Giusti *et al.*, 2000) and the SH2 domain of Src-like kinases can delay or block the  $\text{Ca}^{2+}$  wave at fertilization (see Runft *et al.*, 2002). These data suggest that in echinoderms, the sperm sequentially activates a Src-like kinase and PLCγ, thereby causing an increase in  $\text{IP}_3$ ,  $\text{Ca}^{2+}$  mobilization and egg activation. In the vertebrate *Xenopus laevis*, activation of the Src-like kinase Xyk occurs at fertilization (Sato *et al.*, 1999), and tyrosine phosphorylation and activation of PLCγ are required for  $\text{Ca}^{2+}$  release (Sato *et al.*, 2000). However, activation of PLCγ does not appear to involve the tandem SH2 domains of the enzyme, suggesting the involvement of a different mechanism for PLCγ activation (Runft *et al.*, 1999). Nevertheless, in all the cases reported above, it remains unsolved how the sperm elicits the activation of the Src-like kinase.

Our results in mouse eggs fit well into the incomplete scenario of fertilization. Tr-kit is a sperm protein that interacts with Fyn and causes activation of the kinase and

phosphorylation of PLCγ1, leading to resumption of oocyte meiosis. Since we show that constitutively active Fyn is sufficient to trigger egg activation, a signaling pathway similar to that activated by the sperm in other organisms is potentially functional in mouse oocytes. Similarly to what was observed in *Xenopus* eggs, Mehlmann *et al.* (1998) demonstrated that the tandem SH2 domains of PLCγ1 did not block the onset of  $\text{Ca}^{2+}$  oscillations at fertilization in mouse oocytes. This observation is in agreement with the pathway activated by microinjection of tr-kit because we found that oocyte activation is inhibited by co-injection of the SH3 domain of PLCγ1 but not by the tandem SH2 domains (Sette *et al.*, 1998). One possibility is that the SH3 domain is required for the interaction with a proline-rich adaptor protein that mediates a correct recruitment of PLCγ1 at the plasma membrane (Sette *et al.*, 1998). In support of this hypothesis, we found that a 70 kDa protein phosphorylated at its tyrosine residues co-immunoprecipitated with antibodies directed against PLCγ1 (Figure 8). More interestingly, its phosphotyrosine content was increased dramatically by co-expression of tr-kit and Fyn (Figure 8). Since a phosphotyrosine protein of identical size was also found in Fyn immunoprecipitates in tr-kit-expressing cells (unpublished observations), this 70 kDa phosphoprotein may functionally link PLCγ1, Fyn and tr-kit. Although the nature and function of such protein(s) is still under investigation, it probably represents an additional component taking part to this signaling pathway.

In conclusion, our work demonstrates the presence in mouse sperm of a protein capable of inducing activation of a Src-like kinase in the oocyte. It is noticeable that we have detected a tr-kit homolog in human and rat sperm (M.P. Paronetto and C. Sette, in preparation), suggesting that this mechanism is conserved during evolution. Although there is no indication of tr-kit homologs in frog or lower eukaryotes yet, it is possible that sperm factors with a similar function also operate to trigger Src



activation (i.e. relief of the SH2 domain autoinhibitory constraint) at fertilization in lower organisms.

## Materials and methods

### PCR-based cloning and mutagenesis

Total RNA was isolated from mice thymus and brain using the Trizol reagent (Gibco-BRL) and following the manufacturer's instructions. Mouse Fyn and Src were amplified by RT-PCR using RNA extracted from thymus and brain, respectively, and subcloned into the eukaryotic expression vector pCMV5. pCMV5-Fyn<sup>Y528F</sup> was produced by PCR by inserting the mutation in the 3' oligonucleotide.

Site-directed mutagenesis was carried out by using the PCR-based approach to generate tr-kit $\Delta$ <sub>12</sub>, tr-kit $\Delta$ <sub>D16N</sub> and tr-kit $\Delta$ <sub>Y161F</sub>, and the resulting cDNAs were inserted into pCMV5. All amplifications were performed using *Pfu* polymerase (Stratagene), and the sequence of PCR-derived fragments was confirmed by DNA sequence analysis.

### Expression and purification of GST fusion proteins

pGEX-PLC $\gamma$ <sub>1</sub><sup>SH2SH2SH3</sup> and pGEX-Abl<sup>SH2</sup> were generous gifts of S.Courtneidge (Sugen Inc., Redwood City, CA) and Daniela Barilà (University of Rome 'Tor Vergata'), respectively; DNA sequences encoding SH2, SH3 and SH3SH2 of Fyn and Src were amplified by PCR from the pCMV5-Fyn or pCMV5-Src plasmids using *Pfu* polymerase. Full-length tr-kit was amplified as above. All oligonucleotides used contained the *Bam*HI site at the 5' end and the *Eco*RI site at the 3' end. The PCR products were subcloned in-frame with GST into the pGEX-3X vector (Pharmacia). Plasmids were transformed into the *Escherichia coli* BL21 strain, grown at 30°C in LB medium to an OD<sub>600</sub> = 0.6, and induced with 0.5 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG; Gibco-BRL) for 3 h at the same temperature to produce GST fusion proteins. The recombinant proteins were purified from bacterial lysates and analyzed as reported previously (Sette *et al.*, 1998).

### Expression and purification of His<sub>6</sub>-tagged tr-kit

A tr-kit<sup>His6</sup> construct was prepared by PCR using *Pfu* polymerase. The reverse primer contained the sequence for the six histidines before the stop codon of tr-kit. The resulting cDNA was cloned into the pT77 vector using the *Nde*I and *Eco*RI sites. To express tr-kit<sup>His6</sup>, the plasmid was transformed into the BL21 strain, grown at 30°C in LB medium and induced with IPTG for 3 h at the same temperature. Bacterial lysates were purified on Ni-NTA-agarose (Qiagen) by standard procedures.

### Oocyte collection, microinjection and in vitro culture

MII oocytes were collected from hormonally primed (Hogan *et al.*, 1994) 6- to 7-week-old CD1 female mice (Charles River Italia) 16 h after human corticotropic gonadotrophin injection. Ovulated oocytes were freed from cumulus by incubation with 0.5 mg/ml hyaluronidase (Sigma-Aldrich) in M2 medium (Hogan *et al.*, 1994), washed in M2 medium and then transferred to 50  $\mu$ l drops of M16 (Hogan *et al.*, 1994) under liquid paraffin for 15–20 min before the microinjection. Microinjection manipulations were performed as described previously (Sette *et al.*, 1997). Briefly, we injected into the oocyte cytoplasm 2–5  $\mu$ l of a tr-kit-expressing cell extract diluted to a protein concentration of 0.1–0.2 mg/ml in injection buffer [20 mM HEPES pH 7.4, 120 mM KCl, 100  $\mu$ M EGTA, 10 mM  $\beta$ -glycerophosphate, 1 mM dithiothreitol (DTT), 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml pepstatin]. Quantification of the volume injected was performed as described previously (Sette *et al.*, 1998). Oocytes were transferred to 50  $\mu$ l drops of M2 under mineral oil and microinjected using an Olympus invertoscope (Olympus) equipped with Hoffman modulation contrast optics (Modulation Optics, Inc., Greenvale, NY) and two Leitz mechanical micromanipulators (Leica AG, Heerbrugg, Switzerland). After microinjections, oocytes were returned to M16 medium drops under mineral oil and cultured at 37°C under a humidified atmosphere of 5% CO<sub>2</sub> in air, and scored for pronucleus formation 6–7 h after injection.

### Cell culture and transfections

pCMV5-tr-kit for eukaryotic expression of tr-kit has been described previously (Sette *et al.*, 1997); pSGT-Abl and pSGT-Abl PP, encoding a constitutively active Abl with a double mutation (P242E/P249E) in the linker region, pRK-PLC $\gamma$ <sub>1</sub>, for PLC $\gamma$ <sub>1</sub> expression, and pSR $\alpha$ -Tec, for Tec expression, were generously provided by Dr Superti-Furga (EMBL, Heidelberg, Germany), Dr A.Ullrich (Max Planck Institut, Martinsried,

Germany) and Dr H.Mano (Jichi Medical School, Tochigi, Japan), respectively.

Subconfluent COS or Hek 293 cell monolayers were cultured in 90 mm dishes and processed for CaPO<sub>4</sub> transfection with 10  $\mu$ g of the appropriate plasmid. At 24–48 h post-transfection, cells were harvested in homogenization buffer [50 mM HEPES pH 7.5, 10 mM  $\beta$ -glycerophosphate, 2 mM EGTA, 15 mM MgCl<sub>2</sub>, 0.1 mM sodium orthovanadate, 1 mM DTT, 10  $\mu$ g/ml leupeptin and 10  $\mu$ g/ml pepstatin, 10  $\mu$ g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF)], homogenized with 40 strokes in a glass homogenizer, or lysed by adding 1% Triton X-100 and 120 mM NaCl to the homogenization buffer (lysis buffer). The lysates were centrifuged for 10 min at 10 000 *g* at 4°C and used for further analysis. Protein concentration was determined according to Bradford (1976).

### Pull-down assays

Cell extracts (500  $\mu$ g of total proteins) were added to 2–4  $\mu$ g of GST fusion protein adsorbed on glutathione-agarose (Sigma) in 250  $\mu$ l (final volume) of homogenization buffer supplemented with 0.05% bovine serum albumin (BSA). For binding of tr-kit<sup>His6</sup> to Fyn, protein A/G-Sepharose beads were pre-adsorbed with 1  $\mu$ g of  $\alpha$ -His<sub>6</sub> antibody before incubation with 500  $\mu$ g of cell extracts from Hek 293 cells transfected with Fyn in the absence or presence of 2  $\mu$ g of purified tr-kit<sup>His6</sup> in 250  $\mu$ l of homogenization buffer with or without 100  $\mu$ M ATP for 1 h at 4°C under constant shaking. All beads were washed three times with homogenization buffer, eluted in SDS sample buffer and adsorbed proteins were resolved on a 10% SDS-polyacrylamide gel (Laemmli, 1970) for subsequent western blot analysis.

### Immunoprecipitation and immunokinase assay

Cell extracts (500  $\mu$ g of total proteins) were incubated with 1  $\mu$ g of the specific antibody (described below) for 2 h at 4°C under constant shaking. Immune complexes were collected by adsorption onto protein A-Sepharose (Sigma-Aldrich). To remove non-specifically bound materials, the Sepharose beads were washed three times with homogenization buffer. Beads were either eluted in SDS sample buffer for western blot analysis, or washed once with kinase buffer (homogenization buffer supplemented with 100  $\mu$ M ATP) and used for the enzymatic reaction. The kinase reactions were carried out by incubating the beads with the substrates, 3  $\mu$ g of purified GST-PLC $\gamma$ <sub>1</sub><sup>SH2SH2SH3</sup> or GST-tr-kit, or 10  $\mu$ g of tr-kit Y161 peptide, in 25  $\mu$ l kinase buffer also containing 0.1  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP for 30 min at 30°C, and terminated either by the addition of SDS sample buffer followed by boiling for 5 min (for GST-tr-kit and GST-PLC $\gamma$ <sub>1</sub><sup>SH2SH2SH3</sup>) and separated on SDS-polyacrylamide gels, or by spotting the reaction mixture onto squares of P-81 phosphocellulose paper (Whatman) as previously described (Sette *et al.*, 1997). Radioactivity incorporated was determined by scintillation counting of the paper squares or by autoradiography of the dried gels.

### Western blot analysis

Proteins were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride Immobilon-P membranes (Millipore) using a semi-dry blotting apparatus (Bio-Rad). Membranes were saturated with 5% non-fat dry milk in phosphate-buffered saline (PBS) containing 0.1% Tween-20 for 1 h at room temperature, and incubated with the following first antibodies (1:1000 dilution) overnight at 4°C: rabbit  $\alpha$ -Fyn (SC-16, Santa Cruz Biotechnology); rabbit  $\alpha$ -PLC $\gamma$ <sub>1</sub> (SC-081, Santa Cruz Biotechnology); rabbit  $\alpha$ -Tec (gift of Dr Mano); rabbit  $\alpha$ -c-kit (Albanesi *et al.*, 1996); mouse antiphosphotyrosine PY20 (SC-508); or  $\alpha$ -Abl (SC-23, Santa Cruz Biotechnology). Secondary anti-mouse or anti-rabbit IgGs conjugated to horseradish peroxidase (Amersham) were incubated with the membranes for 1 h at room temperature at a 1:10 000 dilution in PBS containing 0.1% Tween-20. Immunostained bands were detected by a chemiluminescent method (Santa Cruz Biotechnology).

### Immunofluorescence analysis

Mouse MII oocytes were fixed for 15 min in 4% paraformaldehyde and permeabilized for 10 min in 0.1% Triton X-100 in M2 at room temperature. After incubation in blocking solution (M2 supplemented with 5% BSA) for 1 h, oocytes were incubated overnight at 4°C with rabbit polyclonal  $\alpha$ -Fyn (1:200 dilution). Following five washes in M2, oocytes were incubated for 1 h at 37°C with rhodamine-conjugated secondary anti-rabbit IgGs (Calbiochem) and Hoechst dye (0.1 mg/ml, Sigma). Cells were washed five times in M2 drops and analyzed for immunofluorescence with an Olympus invertoscope by using a 40 $\times$

objective. Images were collected with an RT-slider Spot camera (Diagnostic Instruments, Inc.) and digitally recorded using imaging software (IAS 2000) and Photoshop (Adobe Systems, Inc., Mountain View, CA).

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