Parthenogenetic activation of mouse eggs by microinjection of a truncated c-kit tyrosine kinase present in spermatozoa

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SUMMARY

A truncated form of the c-kit tyrosine kinase receptor, corresponding to the phosphotransferase portion of the cytoplasmic catalytic domain and the carboxyterminus (tr-kit), is accumulated during late mouse spermiogenesis. Here we report that tr-kit is specifically localized in the residual sperm cytoplasm, with maximal accumulation in the midpiece of the flagellum, suggesting that it can enter the egg during fertilization. Microinjection of extracts from COS cells expressing a recombinant tr-kit protein into metaphase II-arrested mouse oocytes caused complete oocyte activation, including cortical granule exocytosis, completion of the 2nd meiotic division, formation of a parthenogenetic pronucleus and progression through cleavage stages. No activation above background levels was obtained with extracts from mock-transfected COS cells.

Similar results were obtained by microinjection of in vitro synthesized tr-kit mRNA into metaphase II-arrested oocytes. Tr-kit-induced parthenogenetic egg activation was completely inhibited by oocyte preincubation with the Ca²⁺-chelating agent BAPTA-AM or with a specific inhibitor of phospholipase C activity. Tr-kit-induced egg activation was associated with a decrease in activity of mitogen-activated protein kinase, an essential component of the cytosolic factor. These results indicate tr-kit as a putative sperm factor required for triggering activation of mouse eggs at fertilization.

Key words: c-kit, spermatozoa, metaphase II-arrest, egg activation, calcium, tyrosine-kinase, truncated-receptor, MAP kinase, phospholipase C

INTRODUCTION

The 145×10³ Mᵦ c-kit tyrosine kinase receptor (Qiu et al., 1988) is essential for both male and female gametogenesis, since it controls primordial germ cell survival (Dolci et al., 1991), spermatogonial proliferation (Rossi et al., 1993) and oocyte growth (Packer et al., 1994). In the postnatal testis, c-kit expression occurs in spermatogonia and ceases at meiotic stages of spermatogenesis (Sorrentino et al., 1991). In the haploid phase of mouse spermatogenesis, a truncated c-kit gene product, here named ‘tr-kit’, is encoded by an alternative mRNA (Rossi et al., 1992) transcribed under the control of a cell-specific intronic promoter (Albanesi et al., 1996). In transgenic mice, this promoter specifically drives the expression of a reporter lacZ gene in late spermiogenesis (Albanesi et al., 1996).

Tr-kit is a 24×10³ Mᵦ protein, and consists of 202 aminoacids, corresponding to 12 novel hydrophobic residues (encoded by intronic sequences) followed by the 190 carboxyterminal aminoacids of the c-kit open reading frame (ORF) (Rossi et al., 1992) (see Fig. 1A). Tr-kit contains the phosphotransferase catalytic domain of the c-kit cytoplasmic portion, but not the ATP-binding site (Rossi et al., 1992). It also lacks the interkinase domain, including the phosphotyrosine docking site for the interaction with the 85 kDa subunit of inositol 3-phosphate kinase (PI3K) (Serve et al., 1994), but contains the carboxyterminal region relevant for c-kit interaction with phospholipase C γ (PLCγ) (Herbst et al., 1995). Previous western blot analysis indicated that tr-kit is absent in mouse spermatocytes and round spermatids, accumulates in elongating spermatids and is maintained in epididymal spermatozoa (Albanesi et al., 1996). Thus, conceivably, tr-kit may play a role either in the differentiative events of spermiogenesis, or in mature sperm cell functions such as sperm motility, acrosome reaction or egg activation at fertilization.

Although it is widely accepted that sperm-egg fusion induces egg activation by a series of intracellular free Ca²⁺ rises, the molecular mechanisms underlying such Ca²⁺ mobilization within the egg are not fully understood (Whitaker and Swann, 1993; Homa et al., 1993). Two main mechanisms have been proposed (Yanagimachi, 1994). The first suggests that sperm-egg fusion induces activation of an egg-membrane receptor and subsequent PLC activation by either G-proteins or protein tyrosine kinases. The second hypothesis proposes that a soluble sperm factor enters the egg at fertilization and thence elicits Ca²⁺ oscillations. In agreement with the latter possibility, intracytoplasmic injection of soluble sperm extracts...
into metaphase II (MII)-arrested oocytes induces a series of \( \text{Ca}^{2+} \) spikes and complete egg activation (Swann, 1990; Stice and Robl, 1990). One of the ultimate events of the activation is formation of the female pronucleus, which requires a decrease in mitogen-activated protein kinase (MAP kinase) activity (Verlhac et al., 1994; Moos et al., 1995, 1996).

In the present work, we show that tr-kit is specifically localized in the residual sperm cytoplasm, with the highest concentration in the midpiece, and investigate whether sperm tr-kit may play a role in the early events of fertilization after sperm-egg fusion. We report that microinjection of either a recombinant tr-kit protein or a synthetic tr-kit mRNA into MII-arrested oocytes causes complete parthenogenetic activation of mouse eggs and that tr-kit-induced egg activation is inhibited by intracellular \( \text{Ca}^{2+} \)-chelating agents or by specific inhibitors of PLC activity and is associated with a decrease of MAP kinase activity.

**MATERIALS AND METHODS**

**Immunofluorescence analysis**

Spermatozoa from the cauda of epididymis of CD1 mice (Charles River) were collected in Minimum Essential Medium (Hyclone) supplemented with 15 mg/ml bovine serum albumin (BSA) (Sigma). Spermatozoa were spotted on poly-L-lysine-coated glass slides, fixed, permeabilized and processed as previously described (Albanesi et al., 1996). First antibody incubation was carried out for 1 hour at 37°C with either protein A sepharose affinity-purified anti-c-kit, or preimmune IgGs at a final concentration of 20-40 \( \mu \text{g/ml} \) in phosphate-buffered saline (PBS) containing 3% BSA. Control experiments were performed using anti-c-kit IgGs preadsorbed to the mouse c-kit carboxyterminal immunogenic peptide (aa 963-975) (Albanesi et al., 1996). Second antibody incubation was carried out for 45 minutes at 37°C with rhodamine-conjugated goat anti-rabbit IgGs antibody (Calbiochem) diluted 1:75 in PBS containing 3% BSA. Slides were mounted with 50% glycerol in PBS and immediately examined by fluorescence microscopy.

**Expression of recombinant tr-kit protein**

Subclonfluent COS cell monolayers were cultured in 90 mm dishes (Corning) and processed for CaPO\(_4\) transfection either with no DNA (mock) or with 20 \( \mu \text{g} \) of the pCMV5 eukaryotic expression vector containing the tr-kit cDNA as previously described (Albanesi et al., 1996). 48 hours 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 \( \beta \)-glycerophosphate, 10 \( \mu \text{g/ml} \) leupeptin, 10 \( \mu \text{g/ml} \) aprotinin), homogenized and centrifuged for 10 minutes at 14,000 \( \text{g} \) at 4°C. Aliquots of supernatant fractions were immediately frozen at \(-80^\circ\text{C}\). Tr-kit expression was monitored by western blot analysis before microinjection experiments (Fig. 1B).

**In vitro RNA transcription**

Tr-kit cDNA clone 3b (Rossi et al., 1992) was subcloned into the EcoRI site of p-GEM3zf+ (Promega Corporation), thus generating plasmid pSTK3B. Capped tr-kit sense RNA was produced as previously described (Melton et al., 1984), using 1 \( \mu \text{g} \) of BglI-linearized plasmid pSTK3B and SP6 RNA polymerase for 1 hour at 37°C. After ethanol precipitation, synthetic RNA was resuspended in 10 mM Tris-HCl, pH 7.4, containing 0.1 mM EDTA. RNA concentration and integrity were controlled by denaturing agarose gel electrophoresis before microinjection experiments (Fig. 1C).

**Oocyte collection, microinjection and in vitro culture**

MII oocytes were collected from hormonally primed 6-week-old CD1 female mice (Charles River Italia) 15-16 hours after hCG injection. Ovulated oocytes were freed from cumulus cells by a brief incubation in 0.5 mg/ml hyaluronidase (Sigma) in M2 medium (Hogan et al., 1994), washed with M2 medium and immediately processed for microinjection or other experimental procedures. For microinjections, groups of 20 MII oocytes were transferred to 50 \( \mu \text{l} \) drops of M2 under liquid paraffin and subjected to intracytoplasmatic injection with 2-5 pl of the appropriate protein (0.2-2 mg/ml) or RNA (0.05-0.1 mg/ml) solution, using a Nikon invertoscope (Nikon Corp.) equipped with Hoffman modulation contrast optics (Modulation Optics) and two Leitz mechanical micromanipulators (Wild Leica). Following injection, surviving oocytes were cultured at 37°C in M16 (Hogan et al., 1994) under a humidified atmosphere of 5% \( \text{CO}_2 \) in air, for up to 7 hours and scored for pronuclei formation or used for MAP kinase assay as described below. For cortical granule staining, microinjected eggs were fixed for 4 hours and processed as described below. For the experiments using BAPTA-AM (Calbiochem), before microinjection with soluble extracts from COS cells transfected with the tr-kit expression plasmid, oocytes were incubated for 30 minutes at 37°C in M16 medium containing either 10 \( \mu \text{M} \) BAPTA-AM or the solvent alone (0.1% DMSO). For inhibition of PLC activity, mouse eggs were preincubated for 30 minutes with the specific PLC inhibitor U73122 [1-6-(17β-3-methoxyestra-1,3,5(10)-tri-en-17-yl)amino]hexyl]-1H-pyrrolo-2,5-dione] or its inactive analog U73433 [1-6-(17β-3-methoxyestra-1,3,5(10)-tri-en-17-yl) amino]hexyl]-2,5-pyrrolidinedione (Calbiochem) dissolved in 0.3% chloroform in M16 medium (final concentration: 10 \( \mu \text{M} \)). At the end of the incubation, all the oocytes were placed in M2 medium and microinjected with tr-kit-expressing COS cell extracts. After microinjection, eggs were incubated for additional 7 hours in the presence of 5 \( \mu \text{M} \) U73122 or U73443. Ethanol-induced parthenogenetic activation was performed, as previously described (Hogan et al., 1994), by exposing MII oocytes for 5 minutes to a 7% ethanol solution in PBS and in vitro culturing for 7 hours as described above.

**Cortical granules and chromosome staining**

Oocytes, collected 4 hours after microinjection, were freed from the zona pellucida by acidic tyrode solution (Hogan et al., 1994) and fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature. After three washes in M2 medium containing 4 mg/ml BSA (blocking solution), oocytes were treated with 0.1% Triton X-100 in the same medium for 5 minutes and transferred to blocking solution for 60 minutes at room temperature. Oocytes were then treated for 60 minutes at room temperature with 0.1 mg/ml TRITC-labeled lectin (Sigma) in blocking solution (Ducibella et al., 1988), washed four times for 5 minutes in blocking solution, incubated for 5 minutes with 0.1 mg/ml Hoechst dye (Sigma) in blocking solution and washed again. Oocytes were then mounted in 30% glycerol on glass slides with coverslip compression, sealed and analyzed by fluorescence microscopy.

**MAP kinase assay**

6 hours after microinjections, groups of six oocytes were transferred to storage solution (10 mM p-nitrophenyl phosphate, 20 mM \( \beta \)-glycerophosphate, 0.1 mM sodium orthovanadate, 5 mM EGTA, 10 \( \mu \text{g/ml} \) leupeptin and 10 \( \mu \text{g/ml} \) aprotinin), and immediately frozen at \(-80^\circ\text{C}\). MAP kinase assay in oocyte extracts was performed as previously described (Moos et al., 1995) for 30 minutes at 30°C in 20 mM Hepes buffer, pH 7.4, containing 20 mM \( \beta \)-glycerophosphate, 1 mM diithiothreitol, 0.1 mM sodium orthovanadate, 2 mM EGTA, 20 mM MgCl\(_2\), 5 \( \mu \text{g/ml} \) leupeptin, 5 \( \mu \text{g/ml} \) aprotinin, 0.3 mM ATP and 0.2 mM[\( ^{32} \text{P} \)]ATP (Amersham), and using 0.5 mM myelin basic protein (MBP)-derived peptide (Santa Cruz Biotechnology) as substrate. The reaction was carried out for 30 minutes at 30°C, and then stopped by the addition of equal volume of 20% trichloroacetic acid. Bovine serum albumin (2 mg/ml) was added and proteins were precipitated for 10 minutes on ice. Samples were centrifuged at 8,000
Egg activation by a truncated c-kit protein

For detection of recombinant tr-kit protein, extracts from mock or tr-kit-transfected cells were separated on 10% SDS-PAGE, transferred onto nitrocellulose membrane (Amersham) and subjected to western blot analysis as previously described (Albanesi et al., 1996). Briefly, first antibody incubation was carried out with 1:1000 dilution of a polyclonal anti-c-kit antiserum (90 minutes at room temperature), second antibody incubation was carried out with 1:15000 dilution of anti-rabbit IgGs antibody conjugated to horse radish peroxidase (Amersham).

For visualization of MAP kinase electrophoretic mobility, groups of 20 buffer- or tr-kit RNA-injected eggs were placed in SDS sample buffer 6 hours after microinjection and subjected to SDS-PAGE and western blot analysis. Anti-ERK2 polyclonal antibody (Santa Cruz Biotechnology) diluted 1:200 was used as first antibody, and anti-rabbit IgGs antibody conjugated to horse radish peroxidase (Amersham) diluted 1:1000 as second antibody. Reactive bands were detected by chemiluminescent staining (Amersham).

RESULTS

Tr-kit is mainly localized in the residual cytoplasm of mature sperm

The intracellular distribution of the tr-kit protein was investigated in mouse spermatozoa from the cauda of the epididymis by immunofluorescence analysis with an antibody raised against the carboxyterminal portion of the c-kit protein. Intense immunostaining was mainly detected in the midpiece of the flagellum, which, together with the mitochondrial sheath, contains most of the residual sperm cytoplasm (Fig. 2A). With higher exposures, a weak halo of positivity could also be appreciated in the post-acrosomal region at the basis of the sperm head and in the principal piece of the tail, but not in the acrosomal region nor in the connecting piece. The cytoplasmic droplet of the midpiece, when present, showed a very strong staining (Fig. 2B). Together with the previously observed strong positivity of spermatid residual bodies to this antibody (Albanesi et al., 1996), these results suggest accumulation of tr-kit essentially in the residual sperm cytoplasm. Preincubation of the anti-c-kit antibody with the immunogenic peptide almost completely suppressed the staining (Fig. 2C) and immunofluorescence analysis using preimmune IgGs resulted in the complete absence of signal (Fig. 2D), confirming the specificity of the immunostaining. Furthermore, after artificial induction of the acrosome reaction with calcium ionophores in epididymal spermatozoa, no decrease in the amount of the 24 kDa tr-kit protein was detected by western blot analysis with respect to unreacted sperm (data not shown) confirming that tr-kit is not localized in the acrosome. Thus, the subcellular localization of tr-kit is compatible with its transfer into the egg cytoplasm after sperm-egg fusion at fertilization.
Mouse eggs microinjected with a recombinant tr-kit protein complete meiosis, form a parthenogenetic pronucleus and develop to cleavage stages

To investigate whether tr-kit can play a role at fertilization, a recombinant tr-kit protein was expressed in COS cells and used for microinjection experiments into mouse eggs. Western blot analysis of extracts from tr-kit-transfected COS cells showed the presence of an immunoreactive band of the expected size (Fig. 1B). As shown in Table 1, microinjection of extracts from tr-kit-transfected cells into MII oocytes caused complete parthenogenetic activation of 70% of the oocytes, as judged by pronuclear formation. Microinjection of extracts from mock-transfected cells did not induce activation above background levels observed in non-injected eggs (Table 1). Pronuclei were observed between 4 and 7 hours after microinjection, a time approximating that observed during natural fertilization (Hogan et al., 1994). Activated eggs extruded the 2nd polar body, indicating that MII-anaphase transition had occurred and that 2nd meiotic division had been completed. These results were highly reproducible (P<0.0001, by analysis of variance) in twelve separate experiments, using COS cell extracts from six different transfections. Complete parthenogenetic activation of mouse eggs by tr-kit microinjection was further validated by the observation that 90-95% of the activated eggs cultured in vitro for 24 hours underwent normal cleavage and reached the two blastomere stage. Furthermore, at least 30% of the activated eggs developed to later cleavage stages when cultured for 2-4 days.

Microinjection of synthetic tr-kit RNA is sufficient for egg activation

We could not exclude the possibility that egg activation reproducibly observed with extracts from tr-kit-transfected COS cells was due to the action of other factors induced in COS cells by tr-kit expression. To test whether egg activation was due to the direct action of tr-kit, we produced synthetic capped RNA encoding for the tr-kit ORF by in vitro transcription (Fig. 1C), and performed similar microinjection experiments in MII oocytes. Microinjection of synthetic tr-kit RNA induced pronuclear formation in 56% of the oocytes between 4 and 7 hours after microinjection, whereas microinjection of the RNA buffer did not trigger activation above background levels observed in non-injected oocytes (Table 1). Again, these results were highly reproducible (P<0.001) in eight separate experiments, using five different tr-kit RNA preparations. In control experiments, microinjection of in vitro synthesized capped antisense tr-kit RNA did not produce egg activation above background (data not shown). These experiments indicate that the microinjected tr-kit protein is directly responsible for MII-arrested oocyte activation and that posttranslational modifications possibly achieved in a eukaryotic expression environment can be achieved in the MII oocyte cytoplasm after translation of tr-kit RNA.

Tr-kit-dependent egg activation involves Ca\(^{2+}\)-dependent events

To evaluate whether tr-kit-induced egg activation was dependent on Ca\(^{2+}\) mobilization within the cell, oocyte intracellular Ca\(^{2+}\) was chelated by preincubation with the membrane permeable compound BAPTA-AM before microinjection of extracts from tr-kit-transfected COS cells. While preincubation with the vehicle DMSO did not impair egg acti-
vation by tr-kit, BAPTA-AM completely blocked both second polar body extrusion and pronuclear formation (Table 2). This result indicated that activation by tr-kit is dependent on intracellular Ca\(^{2+}\) mobilization.

To further substantiate the involvement of Ca\(^{2+}\)-dependent events, we investigated whether tr-kit was also able to elicit cortical granule exocytosis, a Ca\(^{2+}\)-dependent early sign of oocyte activation that is observed in natural fertilization and is considered essential for the polyspermy block (Yanagimachi, 1994). 4 hours after microinjection, oocytes microinjected with RNA buffer (Fig. 3A) or mock-transfected COS cell extracts (data not shown) maintained the polarized cortical granule distribution typically observed in MII-arrested oocytes (Ducibella et al., 1988) and had the chromosomes blocked in the metaphase plate. By contrast, eggs microinjected with synthetic tr-kit RNA (Fig. 3B) or with extracts from tr-kit-expressing COS cells (not shown) had undergone massive cortical granule release and loss of their polar organization. Furthermore, chromatin staining showed the presence of a pronucleus in activated eggs (Fig. 3B). The pattern of cortical granule exocytosis observed with tr-kit was similar to that observed in parthenogenetic activation of mouse eggs by ethanol (Fig. 3C) and in fertilized mouse eggs (Ducibella et al., 1988).

**Table 2. BAPTA-AM inhibits pronuclear formation induced by tr-kit microinjection in mouse MII oocytes**

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>Number of experiments</th>
<th>Total eggs</th>
<th>Pronuclei formed</th>
<th>(% of activated eggs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>3</td>
<td>22</td>
<td>17</td>
<td>77.27</td>
</tr>
<tr>
<td>10 μM BAPTA-AM</td>
<td>3</td>
<td>31</td>
<td>0</td>
<td>0*</td>
</tr>
</tbody>
</table>

MII oocytes were preincubated for 30 minutes in the presence or absence of 10 μM BAPTA-AM in M16 medium containing 0.1% DMSO as vehicle as described under Materials and Methods. At the end of the incubation, all the oocytes were placed in M2 medium and microinjected with soluble extracts from COS cells transfected with the tr-kit expression plasmid. Pronuclear formation was scored 7 hours after microinjection by phase-contrast microscopy. Statistical analysis as in Table 1 (*P < 0.001).

**Tr-kit-mediated egg activation is blocked by inhibition of PLC activity**

One mechanism by which tr-kit could trigger [Ca\(^{2+}\)]\text{_i}\) elevation within the mouse egg is the activation of PLC\(\gamma\), with consequent production of InsP\(_3\) (Miyazaki et al., 1993). PLC\(\gamma\) is present in ovulated mouse eggs (Dupont et al., 1996), and, as shown in Fig. 1A, it is known that PLC\(\gamma\) can interact with the full-length c-kit receptor, probably through the c-kit carboxyterminal portion (Herbst et al., 1995), which is also present in tr-kit (Rossi et al., 1992).

To test whether tr-kit action inside the egg involves PLC activation, we tested the effect of preincubation of mouse eggs with specific PLC inhibitors before microinjection experiments. We found that pronuclear formation induced by tr-kit mRNA microinjection was completely suppressed by incubation of eggs with the specific PLC inhibitor U73122, but not by incubation with its inactive analog, U73433 (Table 3). These results indicate that egg PLC activity is essential for tr-kit-mediated MII oocyte activation.

**Tr-kit microinjection is associated with a decrease of egg MAP kinase activity**

While inactivation of Ca\(^{2+}\)-sensitive maturation promoting factor (MPF: cdc2/cyclin B1 kinase) through cyclin degradation is important for the exit from MII and 2nd polar body extrusion (Kubiak et al., 1993), high levels of MAP kinase activity are incompatible with pronuclear formation in fertil-

**Table 3. Tr-kit-induced mouse egg activation is blocked by a specific PLC inhibitor**

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Number of experiments</th>
<th>Total eggs</th>
<th>Pronuclei formed</th>
<th>(% of activated eggs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U73433</td>
<td>5</td>
<td>28</td>
<td>16</td>
<td>57.14</td>
</tr>
<tr>
<td>U73122</td>
<td>5</td>
<td>53</td>
<td>3</td>
<td>5.66*</td>
</tr>
</tbody>
</table>

MII oocytes were preincubated for 30 minutes in the presence of U73122 (a specific PLC inhibitor) or U73433 (its inactive analogue) dissolved in 0.3% chloroform in M16 medium (final concentration 10 μM). At the end of the incubation, all the oocytes were placed in M2 medium and microinjected with soluble extracts from COS cells transfected with the tr-kit expression plasmid. After microinjection, eggs were incubated in M16 medium in the presence of 5 μM U73122 or U73433. Pronuclear formation was scored 7 hours after microinjection by phase-contrast microscopy. Statistical analysis as in Table 1 (*P < 0.003).
subjected to western blot analysis using an anti-ERK2 antibody and buffer. MAP kinase activity was measured by using a synthetic MBP (SantaCruz Biotechnology) from intracellular stores (Kline and Kline, 1992; Whitaker and Swann, 1993). Since tr-kit elicits cortical granule exocytosis, a well-defined Ca\(^{2+}\)-dependent event (Kline and Kline, 1992), it appears to activate mouse eggs, at least in part, by elevating [Ca\(^{2+}\)]. Our observation that the Ca\(^{2+}\)-chelating agent BAPTA-AM completely blocks tr-kit effects supports the hypothesis that microinjection of tr-kit into MII oocytes provokes mobilization of Ca\(^{2+}\) from intracellular stores. We also found that preincubation with specific PLC inhibitors suppress tr-kit-mediated parthenogenetic activation of mouse eggs, suggesting that tr-kit-mediated Ca\(^{2+}\) mobilization inside the egg is mediated through PLC activation, with consequent InsP\(_3\) production. This conclusion is supported by the recent finding that the same specific PLC inhibitors that we used can block sperm-induced Ca\(^{2+}\) spiking at fertilization (Dupont et al., 1996). The latter observation suggests that sperm-derived tr-kit could play a physiological role in natural egg activation at fertilization.

Although further experiments are needed to establish which PLC isoform is involved in tr-kit-mediated egg activation, the most likely candidate is PLC\(_{\gamma1}\), which is the only detectable PLC isoform in ovulated mouse oocytes (Dupont et al., 1996). PLC\(_{\gamma1}\) is known to interact with the carboxyterminal portion of the c-kit receptor (Herbst et al., 1995) which is also present in tr-kit (Fig. 1A). How could tr-kit stimulate PLC\(_{\gamma1}\) activity inside the egg cytoplasm? The report that truncated receptors containing only the intracellular kinase domains can interact with and activate tyrosine kinase receptors in a ligand-independent fashion (Chantry, 1995), suggests that a similar mechanism could be involved in tr-kit microinjected mouse eggs even after a normal decline in MPF activity (Moos et al., 1996).

To test whether inhibition of MAP kinase activity occurs in tr-kit-induced mouse eggs at the time of pronuclear formation, we measured the levels of phosphorylation of a specific MAP kinase substrate, which was added in vitro to extracts of oocytes collected 6 hours after tr-kit RNA microinjection. We found that tr-kit RNA microinjection in MII oocytes is associated with a dramatic decrease in MAP kinase activity (Fig. 4A), coupled to a shift to a slightly higher electrophoretic mobility of p42 MAP kinase (ERK2), due to inactivating dephosphorylation (Fig. 4B). Similar results were obtained when eggs were microinjected with extracts from tr-kit-transfected COS cells (not shown). Therefore, tr-kit microinjection reproduces the MAP kinase inhibition observed both in artificially induced egg activation (Verlhac et al., 1994) and in natural fertilization of mouse eggs (Moos et al., 1995).

**DISCUSSION**

Several reports suggest that a soluble sperm factor activates eggs after penetration of the sperm into the cytoplasm both in sea urchin (Dale et al., 1985) and in mammals (Swann, 1990; Stice and Robl, 1990; Dozortsev et al., 1995). The data reported herein indicate that tr-kit is localized mainly in the residual cytoplasm of mouse epididymal spermatozoa, with maximal accumulation in the midpiece, and that microinjection into MII-arrested mouse oocytes of either a recombinant tr-kit protein or synthetic tr-kit RNA is sufficient to trigger the complete set of events associated with egg activation (from cortical granule exocytosis to pronuclear formation and cleavage stages). Our present results suggest that tr-kit could be a sperm factor inducing the early events of natural fertilization. Indeed, its accumulation in the residual sperm cytoplasm indicates that it can be transferred into the egg, where it could participate in the very early events of mouse development. Western blot experiments indicated that the 24×10^3 M\(_{r}\) tr-kit protein in epididymal spermatozoa was more easily detected if sperm samples were directly homogenized in Laemmli sample buffer, instead of homogenization in Triton-containing buffers, and centrifuged prior to electrophoretic separation (unpublished results). These observations suggest that tr-kit may be also partially associated with the Triton-insoluble components of the sperm, such as chromatin, as also indicated by the detection of weak staining in the postacrosomal region of the sperm head in immunofluorescence analysis of permeabilized spermatozoa. The postacrosomal region is the first to be engulfed by the oocyte cytoplasm after sperm-egg fusion (Yanagimachi, 1994). The high concentration of tr-kit in the sperm midpiece is also compatible with an action inside the oocyte after sperm-egg fusion, since it is known that the midpiece penetrates the mammalian egg, to which it supplies paternal centrosome components responsible for aster formation after fertilization in some species (Yanagimachi, 1994).

A universal mechanism used for egg activation at fertilization is considered to be the elevation of [Ca\(^{2+}\)], through release from intracellular stores (Kline and Kline, 1992; Whitaker and Swann, 1993; Homa et al., 1993). Since tr-kit elicits cortical granule exocytosis, a well-defined Ca\(^{2+}\)-dependent event (Yanagimachi, 1994), it appears to activate mouse eggs, at least in part, by elevating [Ca\(^{2+}\)].
action inside the egg cytoplasm. MII oocytes express the full-length c-kit receptor (Manova et al., 1990; Horie et al., 1991; Yoshinaga et al., 1991), and its ligand, stem cell factor (SCF) (Besmer, 1991) has been proposed as one of the factors maintaining the meiotic arrest that occurs throughout oocyte growth (Ismail et al., 1996). We found that treatment with SCF fails to induce cortical granule exocytosis, meiosis resumption and pronuclear formation (unpublished results). Although tr-kit lacks an ATP-binding site, and thus it should lack intrinsic tyrosine kinase activity (Rossi et al., 1992), it might interact with the full-length c-kit receptor present in the egg and produce effects that should be different from those elicited by the extracellular ligand. Alternatively, tr-kit could directly interact with and activate PLCγ with no involvement of tyrosine kinase activity, analogously to the non-catalytic activation of PLCγ observed in vitro in the case of the EGFR receptor (Hernandez-Sotomayor and Carpenter, 1993).

The mechanism responsible for the developmental arrest of mouse oocytes at MII depends on cytoplasmic conditions that cause chromosome condensation to the metaphase stage (Clarke et al., 1988). It is widely accepted that a dephosphorylation-dependent decrease in MAP kinase activity, rather than a decrease in the Ca^{2+}-sensitive MPF activity, is required for modifications of spindle-associated microtubules at anaphase, chromosome decondensation and reconstitution of a nuclear envelope (Verlhac et al., 1993, 1994, 1996; Moos et al., 1995, 1996). In mouse eggs, MAP kinase activity is stimulated by the product of the c-mos protooncogene (Verlhac et al., 1996). c-mos is essential, both in Xenopus (Sagata et al., 1989) and mouse (Colledge et al., 1994; Hashimoto et al., 1994) to maintain MII arrest in the egg (Vande Woude, 1994). Studies on Xenopus egg extracts suggest that the Ca^{2+}-induced release from metaphase arrest, which is mediated by activation of calmodulin-dependent protein kinase II (Lorca et al., 1993), involves inhibition of a complex positive feedback loop between the serine-threonine kinases MPF, c-mos and MAP kinase (Minshull et al., 1994), and that MAP kinase has intrinsic cytosatic factor (CSF) activity (Haccard et al., 1993). Therefore, MAP kinase must be considered the ultimate component of the CSF system in vertebrate eggs. Our finding that tr-kit microinjection into mouse eggs is associated with a dramatic decrease in MAP kinase activity and causes p42 MAP kinase dephosphorylation, indicates that tr-kit follows a physiological route to release the MII arrest and induce pronuclear formation. These effects are likely to be the consequence of the PLC-mediated increase in [Ca^{2+}], triggered by tr-kit, since we found that chelation of [Ca^{2+}], or use of specific PLC inhibitors, block both tr-kit-induced release from MII arrest and pronuclear formation, although other parallel, PLC- and Ca^{2+}-independent actions of tr-kit on some components of the CSF system cannot be excluded.

Recently a sperm-derived protein termed oscillin has been identified and purified from hamster spermatozoa (Parrington et al., 1996). The cDNA for this protein has been cloned from a testis library, and the deduced ORF shows no relation to tr-kit. Oscillin is localized in the cytoplasm of the equatorial region of the sperm head, and it has been proposed as the sperm factor required for intracellular Ca^{2+} oscillations observed during activation of mammalian eggs at fertilization (Swann, 1990; Parrington et al., 1996). However, it is not known whether the protein corresponding to the cloned cDNA can elicit the full complement of events associated with egg activation and/or early embryonic development. It cannot be excluded that, in addition to oscillin, other sperm factors are required for early events of embryonic development.

The results herein reported indicate that the c-kit gene could be considered important not only for the establishment and progression of mouse male and female germ cell lines, but also, albeit through an alternative protein product, for the final function of germ cells at fertilization. The observation that intracytoplasmic injection of round spermatids fails to activate mouse eggs without additional artificial stimulation, whereas testicular spermatozoa are successful (Kimura and Yanagimachi, 1995), indicate that an oocyte-activating factor must be accumulated during the elongation steps of spermigenesis, and this is exactly the case for tr-kit (Albanesi et al., 1996). Although further studies are required to show that tr-kit actually functions during natural egg activation, the elucidation of its mechanism of action inside the oocyte cytoplasm will be an important contribution to the understanding of early events of mouse development.

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