Fyn kinase–tubulin interaction during meiosis of rat eggs

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Abstract

Prior to fertilization, the spindle of vertebrate eggs must remain stable and well organized during the second meiotic metaphase arrest (MII). In a previous study we have determined that the completion of meiosis is a Src family kinase (SFK)-dependent event. In the current study we have used the SFK inhibitors, SU6656 and PP2, and demonstrated that inhibition of SFKs caused the formation of a disorganized spindle. The observation that proper organization of an MII spindle is an SFK-dependent process, combined with our previous finding that Fyn kinase is localized at the microtubules (MTs), prompted us to examine the potential role of Fyn in MT signaling. Our results show an association between Fyn and tubulin, the ability of Fyn to phosphorylate tubulin *in vitro* and stimulation of meiosis completion by injection of a constitutively active form of Fyn (CAF).

We suggested that SFKs mediate significant functions during the organization of the MII spindle. In view of CAF injection experiments, and of the pronounced concentration of Fyn kinase at the spindle, we propose that Fyn may play an important role in some aspects of the spindle functions, possibly those involving the MTs.

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Introduction

Ovulated rat eggs arrest at the metaphase of the second meiotic division (MII). During the arrest phase, the chromosomes remain aligned at the metaphase plate, awaiting sperm-induced egg activation that would trigger their segregation, among other processes. The meiotic spindle which originates from the microtubule organizing centers (MTOCs), is barrel shaped, and is devoid of astral microtubules (MTs). Not much is known about the precise mechanism that enables the spindle of an MII-arrested mammalian egg to remain correctly organized. The MII arrest is mediated by the mitogen-activated protein kinase (MOS/.../MAPK) pathway and has been extensively studied, especially in relation to the aspect of the M-phase-promoting factor (MPF; a complex of cdc2 and cyclin B). Indeed, during the MII arrest, MPF levels remain high, owing to the cytostatic factor (CSF) activity generated by the MOS/.../MAPK pathway that controls the organization of MTs as well (Terret *et al.* 2003). The achievement of a normally developed embryo – as a result of a successful fertilization process – relies, among other things, on the proper organization of MTs in MII eggs.

Src family protein tyrosine kinases (SFKs) are implicated in a variety of cellular processes. SFKs are cytoplasmic enzymes, linked to the cell membrane via amino-terminal

fatty acids. They are composed of one src homology 2 (SH2) domain, one src homology 3 (SH3) domain, a catalytic domain and a carboxy-terminal regulatory sequence (Superti-Furga & Courtneidge 1995). Their function at fertilization is currently under investigation. There is increasing evidence that SFKs play an important role in regulating mitotic events. Fyn, c-Src and c-Yes are activated in response to various growth factors during the transition from the G0 to the G1 phase of the cell cycle (Kypta *et al.* 1990, Courtneidge *et al.* 1993, Rongish & Kinsey 2000). The three SFKs are also activated in fibroblasts during the late G2 phase and during the entry into mitosis (Roche *et al.* 1995a). Furthermore, microinjection of antibodies which neutralize c-Src, c-Yes and Fyn in NIH3T3 cells, inhibits entry into mitosis, suggesting that the catalytic activity of SFKs is necessary for initiation of mitosis (Roche *et al.* 1995b). Progression of the cell cycle is blocked by inhibition of SFKs (Moasser *et al.* 1999). These studies demonstrate the requirement of SFKs for entry into mitosis, suggesting that interactions among the SH2 domain of the SFKs, the signaling-tyrosine-phosphorylating (STP) proteins and the cytoskeletal proteins, are necessary for mitotic cell division in fibroblasts.

Changes in activity and localization of c-Src during mitosis, suggest a mitotic function (David-Pfeuty & Nouvian-Dooghe 1990, 1995, Bagrodia *et al.* 1991, Zhao

et al. 1992, Taylor & Shalloway 1993, David-Pfeuty *et al.* 1993). An increase in the tyrosine phosphorylation of a 68 kDa protein is observed during mitosis, concomitant with an increase in c-Src activity (Fumagalli *et al.* 1994, Taylor & Shalloway 1994). Localization of c-Src to fibroblastic endosomes and to MT structures, implies c-Src involvement in protein trafficking and in mitotic centriol organization (David-Pfeuty & Nouvian-Dooghe 1990, Kaplan *et al.* 1992). Fyn kinase was found to be associated (Katagiri *et al.* 1993, Marie-Cardine *et al.* 1995) or co-localized (Ley *et al.* 1994, Yasunaga *et al.* 1996) with the spindle MTs during the M-phase of various cells.

Although the function of the specific SFKs is unclear at present, their subcellular localization has provided some clues. Localization of c-Src, c-Yes and Fyn to different compartments in the rat egg indicates that these proteins may have different functions within the egg (Talmor *et al.* 1998, Talmor-Cohen *et al.* 2004). The localization of Fyn (but not of c-Src or c-Yes) to the MTs suggests that Fyn may have a role in directing the MTs' action. The use of SFK inhibitors enabled us to inhibit the completion of the cell cycle (Talmor-Cohen *et al.* 2004). We may infer that SFKs in general and Fyn in particular, could control MT organization. The objective of the present study is to analyze the association of SFKs with the spindle.

Materials and Methods

Oocytes and eggs

Wistar-derived rats, 23–25 days old, were primed with 10 IU pregnant mares serum gonadotropin (PMSG; Syncro-Part, France) and with 10 IU human chorionic gonadotropin (hCG, Sigma). Cumulus-enclosed oocytes, either at the germinal vesicle breakdown (GVBD) or at the metaphase I (MI) stage were isolated from the large follicles 4–8 h after hCG administration; MII eggs, on the other hand, were isolated from the oviducts, 14 h after hCG administration into culture medium (TH medium; Talmor *et al.* 1998). Cumulus cells were removed by a brief exposure to hyaluronidase (400 IU/ml, H-3631 Sigma). MII-ovulated eggs were parthenogenetically activated by 2 μ M ionomycin (Calbiochem, San Diego, CA, USA).

Antibodies and drugs

Anti-Fyn-3 peptide polyclonal antibody (pAb; Santa Cruz Biotechnology, Santa Cruz, CA, USA), was chemically coupled to protein A-sepharose to prepare an immobilized antibody affinity reagent for immunoprecipitation (Talmor *et al.* 1998). Anti- β tubulin monoclonal antibody (mAb) and nocodazole were purchased from Sigma. Stock solution of 2 mg/ml of nocodazole was prepared in DMSO and diluted in TH medium before use to a final concentration of 10 μ g/ml. Stock solutions of 13 mM 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2), PP3 and of 10 mM SU6656 (Calbiochem-Novabiochem, CA, USA) were prepared in DMSO and diluted before use in TH

medium to final concentrations of either 10–100 μ M (PP2, PP3) or 1–5 μ M (SU6656).

Immunoprecipitation and immunoblotting

Batches of 400 eggs were lysed in 100 μ l NP-40 lysis buffer and immunoprecipitated with anti-Fyn-3 peptide pAb, as described previously (Talmor *et al.* 1998). Western blot analysis was performed with an anti- β tubulin mAb (1:200). Bound antibody was recognized by horseradish peroxidase conjugated to anti-mouse antibody (1:5000; Sigma). Detection was performed by ECL (Pierce, Rockford, IL, USA). Approximate molecular masses were determined by comparison with the migration of prestained protein standards (Amersham).

CAF construction

In order to create a form of Fyn kinase that would be constitutively active (CAF), we modified its C-terminal phosphorylation site to a non-phosphorylatable residue. A mutation was induced in the Fyn sequence of *Xenopus* that substituted Tyr 532 by Phe. This was done by PCR, using a sense primer 5' TAG AAT TCG ATA ATG GGC TGT GTG CAA T3' and an antisense primer 5' TAG CTC GAG GTT GTC TCC AGG CTG AAA TT 3'. The PCR product was cloned into pGEX4T3 and the sequence GST-Fyn^{Y532F} was confirmed. The fusion protein was expressed in *Escherichia coli* and purified by affinity chromatography (GST Technical Manual; Pharmacia Biotech, Piscataway, NJ, USA). Further purification was accomplished by chromatography on a diethylaminoethyl (DEAE) column followed by elution by a 50–500 mM KCl gradient. Fractions containing protein tyrosine kinase (PTK) activity were pooled, concentrated and dialyzed to KPS buffer (150 mM KCl, 3 mM NaCl, 10 mM KH₂PO₄, pH 7.2). Catalytic activity was confirmed by a kinase assay using a synthetic peptide substrate.

Microinjection

CAF was microinjected into unfertilized eggs (~10 pl per oocyte). Control eggs were microinjected with KPS buffer alone or with a boiled CAF protein. The estimated concentration of CAF that would be within the egg cytoplasm is indicated in the Results section. Microinjections (Narishige Micromanipulators, Japan) were performed under differential interference contrast (DIC; inverted microscope TE-200, Nikon, Japan), at $\times 20$ (objective) magnification, by intracytoplasmic injection micropipettes (Perry, Medical Instrumental Development Ltd, Ra'anana, Israel). All microinjection experiments were performed on a warm stage (30 $\pm$ 0.5 $^{\circ}$ C) in TH medium supplemented with 10% fetal calf serum (FCS; Biological Industries, Beit-Haemek, Israel). The microinjected eggs were allowed 1 h of recovery in TH medium at 37 $^{\circ}$ C, 5% CO₂ in air, prior to fixation (3% paraformaldehyde and 0.01% glutaraldehyde in Dulbecco's phosphate-buffered saline (DPBS)) and

to zona pellucida (ZP) removal. Eggs were monitored for morphological criteria that indicate progression through the activation process (the chromosomal stage was detected by a DNA-specific fluorochrome (Hoechst 33342, Sigma); cortical granules exocytosis (CGE) was detected by lens culinaris aectin (LCA) and Texas-red streptavidin (Vector; Eliyahu & Shalgi 2002). Data are expressed as a percentage: the number of treated eggs that resumed meiosis successfully, or demonstrated CGE, per total number of injected eggs.

Immunofluorescent staining and laser-scanning confocal microscopy

Oocytes/eggs at various stages of meiosis were fixed and permeabilized (Talmor *et al.* 1998), then incubated in the presence of two primary antibodies: either anti-Fyn-3 peptide pAb (1:10) or anti- β tubulin mAb (1:5000), followed by an incubation with Cy-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA): donkey anti-rabbit IgG-Cy2 (1:500) or donkey anti-mouse IgG-Cy3 (1:500) respectively. Data collected from three experiments were pooled. Groups of three to four oocytes from each experiment were recorded.

DNA was detected by Hoechst 33342. Stained oocytes/eggs were visualized and photographed using a Zeiss confocal laser scanning microscope (CLSM; Rufas *et al.* 2000). The dye intensity was measured using the corrected mean density values obtained by the LSM software.

In vitro phosphorylation of tubulin

Samples of purified bovine brain tubulin (1 μ g) were incubated with 10 ng GST-Fyn^{Y532F} bound to glutathione-sepharose (Pharmacia Biotech) in kinase buffer (15 mM HEPES, 10 mM MgCl₂, 1 mM 2-mercaptoethanol, 1 mM [³²P]ATP (3 mCi/ μ mol); pH 7.2) for 10 min at 25°C. The reaction was stopped by an addition of 2 $\times$ Laemmli sample buffer and the products were resolved by SDS-PAGE. The gel was treated with 1 M KOH for 1 h at 50°C to hydrolyze any P-Ser and P-Thr, then dried and analyzed by autoradiography.

Statistical analysis

The significance of differences between experimental groups was determined by Fisher's exact probability test or by one-way ANOVA test, combined with Tukey's method for multiple comparisons; $P < 0.05$ was considered significant.

Results

Effect of SFK inhibitors on tubulin organization within rat eggs

Immunofluorescence confocal analysis was performed to determine the effect of SFK inhibitors (PP2, SU6656) on the organization of MTs within the egg. MII eggs were

cultured in the presence of 5 μ g/ml SU6656, 100 μ g/ml PP2 or of 10 μ g/ml nocodazole for 30 min; they were washed, fixed and labeled with anti-tubulin and Hoechst dye. The chromosomes of MII eggs were aligned on the equatorial plane of the spindle, while the MTs constituted the meiotic spindle, which was oriented radially to the cell cortex (Fig. 1A'). Exposure of MII eggs to 5 μ M SU6656 caused a disintegration of the barrel-shaped spindle and appearance of dispersed tubulin fibers

Figure 1 Effect of Src inhibitors, PP2 and SU6656, on tubulin organization. MII eggs were labeled with anti- β -tubulin antibody (1:5000) detected by fluorescent-labeled Cy secondary antibody (1:2500). DNA was labeled by Hoechst (33342, 2 μ g/ml). The localization of β -tubulin (red), and DNA (blue) was imaged using confocal microscopy. Light microscopy (A–D), confocal microscopy (A'–D'). Unfertilized (MII) egg (A, A'); MII egg incubated in the presence of 5 μ g/ml SU6656 (B, B'), 100 μ g/ml PP2 (C, C') or 10 μ g/ml nocodazole (D, D') for 30 min. Scale bar, 10 μ m.

throughout the cytoplasm. Moreover, the chromosomes were no longer aligned on a metaphase plate (Fig. 1B'). PP2 did not have such a dramatic effect on the metaphase plate but caused a minor disorganization of the spindle (Fig. 1C'). SFK inhibitors did not cause depolymerization of the MTs in MII eggs, as was the case for nocodazole (Fig. 1D'), which suggests a different mechanism of action.

Fyn-tubulin association during meiosis

In a recent study we were able to show that Fyn kinase is localized to the meiotic spindle (Talmor-Cohen *et al.* 2004). Immunofluorescence confocal microscopy demonstrated colocalization of a subpopulation of Fyn kinase with MTs, already at the GVBD stage. Oocytes from a representative experiment are shown in Fig. 2, demonstrating short MTs surrounding the chromosomes of an oocyte undergoing GVBD (Fig. 2A'). A bipolar spindle was observed 6 h post hCG injection, and the chromosomes gathered at the equatorial plane (Fig. 2B'), as in the MII stage (Fig. 2C'). As a specificity control for each experiment, a synthetic peptide antigen was coinubated with the primary antibody to act as a competitive inhibitor. The antibody staining was completely blocked by the synthetic peptide (data not shown). Our findings indicate that localization of Fyn kinase at the spindle MT is a general phenomenon, observed, in the present study, during oocyte maturation (Fig. 2A, B and C), and as shown in a previous work, during fertilization in both the meiotic and the mitotic spindles (Talmor *et al.* 1998).

The colocalization of Fyn kinase with the MT fibers during all stages of meiosis and mitosis could imply an association with tubulin; this was tested by the use of 10 µg/ml nocodazole. As could be seen by immunofluorescence confocal microscopy, the depolymerization of

Figure 2 Localization of Fyn kinase and tubulin during meiosis. Oocytes at various stages of meiosis were double-labeled with anti-Fyn-3 peptide antibody (1:10; A, B and C) and anti-β-tubulin antibody (1:5000; A', B' and C'). Primary antibodies were detected by fluorescent-labeled Cy secondary antibodies (1:500). DNA was labeled by Hoechst (33342, 2 µg/ml; A', B' and C'). The localization of Fyn (green), β-tubulin (red) and DNA (blue) was imaged using confocal microscopy. (A, A') GVBD oocyte (4 h after hCG); (B, B') MI oocyte (6–8 h after hCG); (C, C') MII egg (14 h after hCG). Scale bar, 10 µm.

MTs that occurred after incubation of the eggs in the presence of the drug, abolished the tubulin staining without affecting the chromosomal metaphase (Fig. 3B'). Nocodazole treatment caused dispersal of Fyn staining from the spindle, and was observed throughout the cytoplasm of all eggs tested (Fig. 3B); it also caused abolishment of tubulin staining (Fig. 3B'). After washing off the drug most of the eggs (69/75) re-exhibited a fully recovered spindle (Fig. 3C') accompanied by Fyn staining (Fig. 3C). These results suggest that the association of Fyn with the spindle MTs is dynamic and can be disrupted and re-established when MTs are allowed to depolymerize and repolymerize respectively.

The interaction between Fyn and the spindle MTs was also examined by coimmunoprecipitation of Fyn and tubulin. Fyn was immunoprecipitated from detergent extracts of batches of 400 eggs equivalent to ~13 µg protein. Tubulin was detected, by Western blot analysis, in samples of unfertilized and of ionomycin-activated eggs using an anti-β-tubulin antibody (Fig. 4). We were able to detect a weak tubulin band when Fyn was immunoprecipitated from lysates of non-activated MII eggs (Fig. 4, lane a). The tubulin band became more prominent after activation by ionomycin (Fig. 4, lane b). Control samples containing only culture media were devoid of any band (Fig. 4, lane c) as were samples immunoprecipitated by a non-related control antibody (data not shown). The detected β-tubulin band served as an indicator for the presence of the tubulin heterodimer in the precipitate.

We tested the ability of *Xenopus* Fyn^{y532f} (CAF), expressed as a GST fusion protein, to phosphorylate bovine brain tubulin *in vitro*, and were able to show that purified tubulin from bovine brain acts as a substrate for

Figure 3 Effect of nocodazole on Fyn kinase localization. MII eggs were double-labeled with anti-Fyn-3 peptide antibody (1:10; A, B and C) and anti-β-tubulin antibody (1:5000; A', B' and C'). Primary antibodies were detected by fluorescent-labeled Cy secondary antibodies (1:500). DNA was labeled by Hoechst (33342, 2 µg/ml; A', B' and C'). The localization of Fyn (green), β-tubulin (red), and DNA (blue) was imaged using confocal microscopy. MII egg (A, A'); MII egg cultured in the presence of 10 µg/ml nocodazole for 1 h and fixed immediately (B, B') or 30 min after removal of the drug (C, C'). Scale bar, 10 µm.

Figure 4 Association of Fyn kinase with tubulin during egg activation. Batches of 400 eggs were lysed: MII eggs (lane a), eggs activated by a 3-min ionomycin treatment (lane b), IP buffer (control; lane c). Lysates were immunoprecipitated with anti-Fyn 3 peptide antibody (IP = Fyn), subjected to SDS-PAGE analysis and transferred onto a PVDF membrane. The blot was probed with anti- β -tubulin antibody (blot = β -tubulin; 1:200). Bound antibody was localized by protein A conjugated to horseradish peroxidase and revealed by ECL. The results of a representative experiment demonstrate a β -tubulin band at 55 kDa (arrow) as calculated from the migration of protein standards, with known molecular mass.

Fyn kinase. As seen in Fig. 5, the kinase reaction containing GST-Fyn^{y532f} resulted in a single 85 kDa band representing the autophosphorylated kinase, although some lower molecular weight contaminants or proteolytic fragments are also phosphorylated. Tubulin alone had no kinase activity (Fig. 5, lane B). Addition of tubulin to the kinase reaction resulted in phosphorylation of a doublet (approx 45 kDa) representing α - and β -tubulin (Fig. 5, lane C). While tubulin is similar in M_r to some of the faint bands in lane A, the 45 kDa doublet is phosphorylated at a higher level relative to other contaminants and is therefore unlikely to represent a phosphorylated contaminant.

The results obtained so far, demonstrate the interaction between Fyn and tubulin during meiosis. To determine whether Fyn is involved in cell cycle resumption, we microinjected CAF into MII eggs. Microinjection of CAF caused completion of meiosis and PBII extrusion (Fig. 6Ac), whereas most of the eggs injected with boiled CAF remained at the MII stage (Fig. 6Ab and B (gray bars)). Completion of meiosis in response to injection of CAF occurred in a dose-dependent fashion (Fig. 6B, black bars). Some 77% of eggs injected with CAF at a concentration of 555 μ g/ml (calculated as final concentration

Figure 5 *In vitro* phosphorylation of tubulin. Kinase reactions containing GST-Fyn^{y532f} (lane A), tubulin (lane B), or both (lane C), were incubated in the presence of 15 mM HEPES, 10 mM MgCl₂, 1 mM 2-mercaptoethanol, 1 mM [³²P]ATP (3 mCi/ μ mol); pH 7.2 for 10 min at 25 °C. The reactions were transferred into gel, dried and analyzed by autoradiography. The resulting autoradiograph demonstrates the phosphorylation of tubulin in lane C (arrow).

within the egg of 6.4 μ M), resumed meiosis, whereas only 28% resumed meiosis at concentration of 37 μ g/ml (within the egg 0.4 μ M). Only 21% of MII eggs injected with boiled CAF, at any injected dose, resumed meiosis (Fig. 6B, gray bars), compared with 11% of MII eggs that resumed meiosis after being injected with KPS buffer alone (data not shown).

Discussion

Are SFKs involved in spindle stability?

We are still at the early stages of understanding the fine regulation of mitotic events. However, the importance of protein phosphorylation in general, and of tyrosine phosphorylation and dephosphorylation in particular, has become evident in the last years, and work is under way to identify and study the mitotic phosphoproteins. There is increasing evidence indicating that Src kinases play an important role in regulating mitotic events. Src kinases may be involved in phosphorylation of one or more of these mitotic phosphoproteins (Stukenberg *et al.* 1997, Whitaker & Larman 2001).

In a previous study we were able to show that completion of meiosis is an SFK-dependent process (Talmor-Cohen *et al.* 2004). In the present study we demonstrated that inhibition of SFKs caused the disintegration of the spindle and dispersion of tubulin fibers in the cytoplasm. Moreover, as a result of this treatment the chromosomes were not aligned on the metaphase plate. For a successful

Figure 6 Completion of meiosis after CAF injection into MII rat eggs. Eggs were microinjected with CAF or with boiled CAF proteins, fixed and stained for DNA. (A) Light microscopy: MII egg (panel a); MII egg injected with boiled CAF (panel b); PBII extrusion by an MII egg injected with CAF (panel c; PBII, gray arrow). DNA was labeled by Hoechst (33342, 2 μg/ml, dense white). The final concentration of CAF (boiled and unboiled) inside the egg's cytoplasm was 555 μg/ml. Each image was taken at the egg's equatorial plane. Scale bar, 10 μm. (B) Dose-dependent completion of meiosis triggered by CAF. Eggs were microinjected with CAF (black bars) or with boiled CAF (gray bars) at various final intra-egg concentrations: 37 μg/ml (a); 222 μg/ml (b); 555 μg/ml (c). Data are expressed as the percentage of injected eggs that resumed meiosis successfully. Each bar represents eggs of, at least, three independent experiments. The numbers at the top of each bar are the number of injected eggs. Fisher's exact probability test was used to determine the significance of difference between the effect of CAF and of boiled CAF on completion of meiosis. a and b, not significantly different; c, significantly different ($P < 0.001$) from a and b.

fertilization, the spindle of vertebrate eggs must remain stable and correctly organized during the second meiotic metaphase arrest (Terret *et al.* 2003, Wassmann *et al.* 2003). Thus, we have suggested that SFKs mediate some functions during the organization of a proper MII spindle. However, we have not yet determined whether the SFK function during fertilization controls meiotic arrest either by maintaining MPF stability or by ensuring that the spindle is properly organized during the arrest.

Potential function of Fyn in microtubule signaling

Fyn kinase is the only one of the three SFKs studied that is localized to meiotic and mitotic spindles (Talmor *et al.* 1998, Talmor-Cohen *et al.* 2004). Our findings that the proper organization of the MII spindle is an SFK-dependent process, and that Fyn kinase is localized to the MTs, lead us to investigate the involvement of Fyn in microtubule function.

Fyn kinase was found to be associated with (Katagiri *et al.* 1993, Marie-Cardine *et al.* 1995) or localized to (Ley *et al.* 1994, Yasunaga *et al.* 1996) the spindle MTs during the M-phase of various cells. In the present study,

localization of Fyn to the egg's spindle MTs was observed during various stages of oocyte maturation, and during the M-phase of the meiotic division.

Our results demonstrate an association of Fyn with tubulin. The band of tubulin which has been coimmunoprecipitated by Fyn kinase appears more intense in activated eggs than in MII-arrested eggs. We would like to suggest that during the M-phase, Fyn kinase interacts, directly or indirectly, with only a small number of tubulin units, while after egg activation, either the number of interacting units increases or the affinity between tubulin and Fyn increases, hence allowing a more efficient coimmunoprecipitation. Studies involving other cell systems have shown that mitogenic stimuli induce phosphorylation of the tyrosine residues of α -tubulin and that the phosphorylated α -tubulin is capable of binding the Fyn SH2 domain (Katagiri *et al.* 1993, Ley *et al.* 1994, Marie-Cardine *et al.* 1995). The spindle is a complex structure that is known to associate with a variety of proteins, including motor proteins such as kinesin (Neighbors *et al.* 1988) and dynein (Pfarr *et al.* 1990), MT-associated proteins (MAPs; Sherline & Mascaro 1982) and Ca^{2+} calmodulin kinase II (Ohta *et al.* 1990). It has also been shown that Fyn binds MAP-2c (Zamora-Leon *et al.* 2001) and dynein complex (Campbell *et al.* 1998). The present study allows us to conclude that Fyn kinase is not merely colocalized to, but is also associated with tubulin, before and during egg activation induced by ionomycin, as demonstrated by the use of nocodazole and by coimmunoprecipitation. Fyn can phosphorylate tubulin *in vitro*, as implied by our *in vitro* phosphorylation experiment. The question whether Fyn interacts with tubulin directly or indirectly remains open. Fyn, like other SFKs, is commonly involved in multi-protein complexes involving interaction with the SH2 and SH3 domains. It is therefore possible that the localization of Fyn to the microtubules involves other MT-associated proteins.

Our findings demonstrated that CAF stimulates the completion of meiosis in rat eggs. This result should be taken with reservation because the constructed CAF is cytosolic, and as such it is incapable of bonding with a myristic acid chain. This diminished its efficiency in finding its suitable substrate, although it did not hinder its kinase activity. As a result, we were forced to employ a relatively high concentration of the activated kinase.

The CAF injection result is supported by another study concerning SFK inhibitors (Talmor-Cohen *et al.* 2004) and is in accordance with yet another study, performed with mouse eggs (Sette *et al.* 2002). In view of the CAF injection experiment and of the pronounced concentration of Fyn kinase at the spindle, we suggest that Fyn may play an important role in some aspects of spindle functions, possibly those involving the MTs themselves.

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