A casein kinase I isoform is required for proper cell cycle progression in the fertilized mouse oocyte

Stefan D. Gross1,2, Calvin Simerly3, Gerald Schatten2,3 and Richard A. Anderson1,2,*

1Department of Pharmacology, University of Wisconsin Medical School, 1300 University Avenue, Madison, WI 53706, USA
2Cellular and Molecular Biology Program, University of Wisconsin, Madison, WI 53706, USA
3Departments of Zoology and Obstetrics-Gynecology, University of Wisconsin, 1117 W. Johnson St, Madison, WI 53706, USA

*Author for correspondence (e-mail: randers@fasstaff.wisc.edu)

SUMMARY

Casein kinase I is a family of serine/threonine protein kinases common to all eukaryotes. In yeast, casein kinase I homologues have been linked to the regulation of growth, DNA repair and cell division. In addition, their subcellular localization to membranous structures and the nucleus is essential for function. In higher eukaryotes, there exist seven genetically distinct isoforms: α, β, γ1, γ2, γ3, δ and ε. Casein kinase Iα exhibits a cell cycle-dependent subcellular localization including an association with cytosolic vesicular structures and the nucleus during interphase, and the spindle during mitosis. Casein kinase I has also been shown to modulate critical regulators of growth and DNA synthesis/repair in mammalian cells such as SV40 large T antigen and p53. These results suggest that casein kinase I may be involved in processes similar to those ascribed to the yeast casein kinase I homologues. To define a role for casein kinase Iα in cell cycle regulation, the mouse oocyte was utilized because of its well-defined cell cycle and ease of micromanipulation. Immunofluorescence studies from meiosis I of maturation to the first zygotic cleavage demonstrated that the kinase was associated with structures similar to those previously reported. Microinjection of casein kinase Iα antibodies at metaphase II-arrest and G2 phase, had no effect on the completion of second meiosis or first division. However, microinjection of these antibodies during the early pronucleate phase prior to S-phase onset blocked uptake of the kinase into pronuclei and interfered with proper and timely cell cycle progression to first cleavage. These results suggest that the kinase regulates the progression from interphase to mitosis during the first cell cycle.

Key words: Casein kinase I, Mouse oocyte, Protein kinase, Cell cycle

INTRODUCTION

The growth cycle of eukaryotic cells is divided into four distinct phases termed G1, S, G2 and M. Occurring within each of these phases are specific metabolic processes allowing the cell to attain the appropriate size, replicate its DNA and segregate its genetic material equally among its daughter cells. Naturally, all of these processes are stringently regulated to ensure proper completion before further progression through the cell cycle is allowed. One of the primary players in cell cycle progression regulation is the family of cyclin-dependent kinases or cdks of which eight higher eukaryotic isoforms have been identified so far (Grana and Reddy, 1995; Machlachlan et al., 1995; Nigg, 1996). In yeast, these serine/threonine kinases play direct roles in the regulation of progression through all of the cell cycle phases (Grana and Reddy, 1995; Pines, 1994). However, as the complexities of cell cycle regulation unravel, it is clear that other kinase activities are providing essential functions in these regulatory cascades providing an additional, finer level of control.

One such kinase activity is the casein kinase I (CKI) family of serine/threonine protein kinases common to all eukaryotic systems from yeast to humans (Tuazon and Traugh, 1991). Recent molecular genetic data has shown that in higher eukaryotes there exist at least seven genetically distinct isoforms of CKI termed α, β, γ1, γ2, γ3, δ and ε (Fish et al., 1995; Graves et al., 1993; Rowles et al., 1991; Zhai et al., 1995). As a family, these isoforms occupy their own unique branch on the kinase phylogenetic tree because they are greater than 50% identical to one another and less than 25% identical to any other known kinase (Fish et al., 1995; Graves et al., 1993; Rowles et al., 1991; Zhai et al., 1995). These molecular genetic data subsequently led to the identification of four CKI homologues each in both S. cerevisiae and S. pombe (DeMaggio et al., 1992; Dhillon and Hoekstra, 1994; Hoekstra et al., 1991; Kearney et al., 1994; Robinson et al., 1992; Wang et al., 1992). The budding yeast isoforms HRR25, YCK1, YCK2 and YCK3 (Hoekstra et al., 1991; Robinson et al., 1992; Wang et al., 1996) are the most characterized.

HRR25 was isolated in a screen for mutants with increased sensitivity to the constitutive expression of HO endonuclease, or treatment with alkylating agents or x-irradiation all of which induce DNA strand breakage (Hoekstra et al., 1991). The associated hrr25 phenotype involved defects in a number of processes including meiosis, mitosis, chromosomal segregation as well as a cell cycle delay in G2 (Hoekstra et al., 1991). These defects, it was supposed, were due to aberrations in some checkpoint which interfered with the ability of the mutant to
respond to DNA strand breakage. Sequence analysis of HRR25 established that it was 64% identical to CKIα through the catalytic domain (DeMaggio et al., 1992). In addition, mutations in Hrr25p’s active site lysine, a residue essential for the activity of a number of kinases, resulted in the same phenotype as the null mutant (Hoekstra et al., 1991). A recent report has demonstrated that Hrr25p associates with and phosphorylates SBF, a transcription factor not only involved in the transcriptional response to DNA damage, but also in the transcription of some G1 cyclins (Ho et al., 1997). This evidence suggests that HRR25 may be involved in normal cell cycle progression in addition to the DNA damage response. YCK3, in conjunction with HRR25, comprise an essential gene pair (Wang et al., 1996). The two isoforms are predominantly nuclear and their localization to this compartment is absolutely required for function (Wang et al., 1996).

As for YCK1p and 2, they have been shown to comprise the vast majority of casein kinase activity in S. cerevisiae (Robinson et al., 1993). Knocking out either gene generates no readily apparent phenotype (Robinson et al., 1993). However, interruption of both genes is lethal (Robinson et al., 1992). To date, no direct function has been ascribed to YCK1. On the other hand, YCK2 has been genetically linked to processes involved in bud site selection, bud morphogenesis and cytokinesis (Robinson et al., 1993). In addition, a recent report has linked these CKI isoforms to the regulation of growth resumption after nutrient re-addition as well as some aspect(s) of endo/exocytosis (Wang et al., 1996). Sequence analysis of their respective genes determined that these isoforms are 56% identical to CKIα through the catalytic domain (Graves et al., 1993; Robinson et al., 1993). Furthermore, these isoforms contain an isoprenylation site at their C terminus that is essential for their function (Robinson et al., 1993; Vancura et al., 1994; Wang et al., 1996). Cell fractionation studies indicate that these enzymes partition exclusively in the membrane fraction further supporting the contention that they are covalently attached to the inner leaflet of the plasma membrane (Vancura et al., 1994; Wang et al., 1996).

Two significant observations can be made from these genetic studies in yeast regarding the function(s) of CKI in cells. First, these isoforms are likely involved in the regulation cascades of several cell cycle checkpoints including G0/G1, S/DNA damage, and mitosis. Secondly, the subcellular localization of these isoforms is critical to their functioning. Because of the high degree of conservation between the yeast and higher eukaryotic isoforms, it is likely that these same conclusions will hold for all members of the CKI family of kinases. However, the mechanisms by which the higher eukaryotic isoforms are segregated into specific cellular compartments and the functions that they provide will likely prove more diverse and complex.

As for the higher eukaryotic isoforms, CKIα has been shown to possess a cell cycle-dependent subcellular localization. During interphase, CKIα is associated with cytosolic vesicular structures and the centrosome (Brookman et al., 1992). During mitosis, CKIα is associated with the mitotic spindle (Brookman et al., 1992). In addition, CKIα appears to associate with a distinct subset of cytosolic vesicles, including small synaptic vesicles, and can phosphorylate a specific subset of synaptic vesicle-associated proteins (Gross et al., 1995). These observations suggest that, in higher eukaryotes, CKIα may be performing roles similar to those ascribed to the yeast isoforms. Association with the mitotic spindle suggests a role in mitosis/cytokinesis while a vesicular localization implies a role in vesicular trafficking. In yeast, disruption of both these functions causes cells to exhibit cell cycle-related defects as well (Hoekstra et al., 1991; Robinson et al., 1993; Wang et al., 1996).

In the studies described here, a mouse oocyte developmental system was employed to link CKI both temporally and spatially to a particular function within the context of the cell cycle. This experimental system possesses a number of advantages over established cell lines. First, mouse oocytes are exceptionally large allowing for relative ease of microinjection. Second, at ovulation they are naturally arrested at metaphase of meiosis II thereby allowing for its controlled progression through subsequent phases of the cell cycle by fertilization or parthenogenetic activation. Finally, the mouse oocyte cell cycle is relatively slow, permitting the precise temporal mapping of CKI’s potential involvement in any number of cell cycle-related events. Using the mouse oocyte, we have established that CKIα is associated with cytoplasmic cytasters, microtubule organizing centers structurally and functionally related to centrosomes, and spindles, thereby reconfirming previous observations in established cell lines. We also observed that CKI was exclusively associated with distinct structures within the pronuclei of the one-cell stage embryo. Microinjection of CKI-specific antibodies into living oocytes at various developmental stages from the onset of mitosis of meiosis I through the first zygotic cleavage, established a requirement for CKI in the early pronucleate phase 0-8 hours after fertilization to ensure proper cell cycle progression.

**MATERIALS AND METHODS**

**Materials**

Rabbit polyclonal antibodies to CKIα were synthesized and characterized as previously described (Brockman et al., 1992; Gross et al., 1995; Zhang et al., 1996). Mouse monoclonal antibodies to β-tubulin were obtained from the Iowa Hybridoma Bank (E7; Iowa). Fluorescein and rhodamine-conjugated goat anti-rabbit secondary antibodies were purchased from Zymed Antibodies, CA.

CKI-immunodepleted IgGs were prepared by repeatedly cycling the rabbit immune sera over a CKIα affinity column until no detectable levels of IgG were eluted by the application of 0.1 M glycine, pH 2.5 (5-8 cycles). This depleted sera was then applied to a Protein A-Sepharose column to purify the remaining IgGs by subsequent elution with 0.1 M glycine, pH 2.5.

**Western blotting**

Cell lysates were prepared by the addition of 4 times concentrated SDS-containing Laemmli sample buffer and subsequent boiling. A volume equaling 300 mouse oocytes and approximately 1×10^5 Balb 3T3 cells were then loaded in their respective lanes and run on a 12% SDS-polyacrylamide gel using a Bio-Rad protein II minigel apparatus. The separated proteins were transferred to nitrocellulose and air-dried. Any non-specific binding sites were blocked with a solution containing PBS and 5% (w/v) non-fat dry milk for 18 hours at 4°C. The blot was incubated with CKIα affinity-purified antibodies in a PBS solution containing 3% BSA (w/v) for 2 hours at room temperature. The membrane was subsequently washed three times in PBS with 0.5% Tween-20 and incubated for 1 hour with 125I-Staph A protein (5 μCi/ml) in PBS with 3% BSA (w/v) at 23°C. The membrane was washed overnight in PBS containing Tween-20 as above. After drying, the blot was exposed to film.

**Immunoprecipitation**

Approximately 300 mouse oocytes were lysed for 1 hour at 4°C in a
buffer containing 10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.5% Triton X-100, 2 mM PMSF, 100 µM leupeptin, and 10 µg/ml aprotinin (Stefanovic and Maller, 1988; Gavin and SchorderetSlatkine, 1997). The lysates were then supplemented with a PBS solution containing 3% BSA to a final concentration of 1% BSA. 100 ng of Dynabeads™ conjugated with goat anti-rabbit IgGs were added to each sample and incubated for 1 hour at 4°C with constant mixing by inversion. The beads were then removed based upon their ability to be isolated with the application of a magnetic field. One of the two lysates was then supplemented with 500 ng CKIα antibody and incubated along with the other lysate for 2 hours at 4°C with mixing. Both lysates received 200 µg Dynabeads™ and were incubated for 2 hours with mixing. The beads were then isolated as above and washed 3 times with phosphorylation buffer (50 mM Tris, pH 7.5, 2 mM MgCl2) and used as a source of kinase in the activity assays described below.

Kinase activity assays

The Dynabeads containing kinase or the control were resuspended in 50 µl of phosphorylation buffer containing 40 µg Phosvitin. The two reactions were carried out at room temperature for 30 minutes at room temperature with mixing every 5 minutes. The reactions were quenched with the addition of 4 times concentrated Laemmli SDS sample buffer and the resultant samples were then boiled for 10 minutes. The samples were loaded on a 12% SDS-polyacrylamide gel and separated as described in the western blotting section. The gel was then stained with Coomassie and destained overnight to reduce background noise. The gel was dried down using a Hoefer gel drying apparatus and exposed to film. The bands corresponding to substrate were excised and the amount of radioactivity quantified by scintillation counting.

Animal handling and gamete collection

Superovulation, collection of mature unfertilized oocytes, mating of the stimulated mouse ICR females and collection of immature GV-stage oocytes from ovaries were accomplished as previously described (Simerly et al., 1990). Cumulus cells were removed by treatment with hyaluronidase. The zona was eliminated with a brief incubation in acid Tyrodes, pH 2.5 (Simerly and Schatten, 1993).

Immunofluorescence microscopy

Immunofluorescence procedures were performed essentially as described elsewhere (Simerly and Schatten, 1993). In brief, for detection of CKIα and microtubules (MTs), mouse oocytes and zygotes were prepared for indirect immunofluorescence by permeabilization in a microtubule stabilization buffer containing 50 mM imidazole, pH 6.8, 50 mM KCl, 0.5 mM MgCl2-6H2O, 1 mM EGTA, 0.1 mM EDTA, 1 mM β-mercaptoethanol, 25% glycerol, followed by methanol fixation. Microtubules were labeled with a mouse monoclonal antibody against β-tubulin and casein kinase was detected with a rabbit polyclonal antibody which was raised against the 37.5 kDa CKIα isoform purified from human erythrocyte cytosol. DNA was detected with DAPI in the penultimate PBS rinse. Appropriate secondary antibodies conjugated to fluorescein or rhodamine were used to detect both MTs and CKIα in the same oocytes or zygotes with double-labeling protocols. Conventional epifluorescence microscopy was accomplished using a Zeiss Axioshot equipped with appropriate chromophore filters.

Microinjection of CKIα antibody and immunofluorescence detection

Micropipettes were front-loaded with 7.5 mg/ml affinity-purified CKIα specific antibodies in microinjection buffer (20 mM NaCl, 115 mM KCl, 10 mM Hepes, 1 mM MgCl2; pH 7.0) from a droplet under mineral oil juxtaposed to the culture medium containing the oocytes. Microinjection was performed by puncturing zona-intact oocytes with a 1-2 µm bevelled micropipette, sucking in a small amount of cytoplasm, and expelling the antibody and cytoplasm back into the cell. Typically, ~5% of the egg volume was microinjected into oocytes and zygotes. To localize CKIα in microinjected cells, oocytes or zygotes were labeled with goat anti-rabbit secondary antibody without further primary antibody application. Application of secondary antibodies alone or after microinjection 7.5 mg/ml CKIα Ab-depleted rabbit IgG did not result in MT or CKIα detection.

Parthenogenetic activation of unfertilized oocytes

Meiotic resumption of mature metaphase II-arrested oocytes was accomplished by activation with 7% ethanol in culture medium for 7 minutes (Kaufman, 1983).

RESULTS

CKIα is present and active within mouse oocytes

Mouse oocytes arrested at metaphase II were western blotted with antibodies specific for CKIα to determine whether the kinase was being expressed in this system. The specificity of this antibody for CKIα has been extensively defined for a number of different species, including mouse (Brockman et al., 1992; Gross et al., 1995; Zhang et al., 1996). As shown in Fig. 1, a single immunoreactive band of 37 kDa is evident both in whole cell lysates derived from mouse Balb 3T3 cells and mouse oocytes. This apparent molecular mass is of the size expected for CKIα (Rowles et al., 1991). CKIα was then immunoprecipitated from lysates derived from either metaphase II or fertilized oocytes and analyzed for activity towards phosvitin, a good CKI substrate. Results indicated that the CKIα antibodies immunoprecipitated similar amounts of phosvitin kinase activity from both lysates (Fig. 1B). Fig. 1B depicts measurements of 32P incorporation into phosvitin as described in Materials and Methods.

Indirect immunofluorescence analysis of the subcellular localization of CKIα throughout early mouse development

The subcellular distribution of CKIα within the mouse oocyte from meiosis I of meiotic maturation through metaphase of meiosis II up to the completion of the first zygotic cleavage was then examined to define the development-dependent subcellular distribution of CKIα. Indirect immunofluorescence using antibody probes specific for CKIα on germinal vesicle stage oocytes (Fig. 2A) indicated that the kinase was distributed heterogeneously. CKIα (Fig. 2C) was not detected in the germinal vesicle (Fig. 2B, inset) nor any cytosolic structures including the cytasters as defined by β-tubulin staining (Fig. 2B, arrows). The kinase was, however, clearly associated with the plasma membrane. During meiotic maturation, the kinase was undetectable on the first meiotic spindle apparatus (not shown), however, when the oocytes arrest at second meiotic metaphase (Fig. 2D), CKIα was clearly associated with the spindle (Fig. 2F) as defined by tubulin staining (Fig. 2E). There was no obvious association of the kinase with the plasma membrane except at the microvilli deficient area (MVA, Fig. 2F; arrow) located immediately above the spindle apparatus. When CKIα antibodies were microinjected into metaphase II-arrested oocytes, fixed and prepared for immunofluorescence, the kinase was also observed associated with cytoplasmic cytasters (Fig. 3C, arrow), structurally and functionally similar to centrosomes, in addition to the spindle.

Eight hours after fertilization, CKIα had relocated from the spindle apparatus, MVA and cytasters to the newly formed male and female pronuclei (Fig. 2G-I). CKIα (Fig. 2I) was associated
with discrete pronuclear structures that also contained DNA (Fig. 2H, inset) but was excluded from the nucleoli. The kinase was essentially undetectable on any other structure within the oocyte including the plasma membrane. As the oocytes progressed through their DNA synthesis and G2 phase, CKI remained associated with these pronucleate structures. During first mitosis (Fig. 2J), CKI was again readily apparent on the spindle (Fig. 2L) observed with tubulin antibodies (Fig. 2K). Interestingly, after first cleavage (Fig. 2M), CKI did not immediately relocalize to the daughter nuclei of cleaved zygotes (Fig. 2O) but instead remained distributed throughout the cytosol with an observed accumulation at or around the nuclear envelope.

Microinjection of CKIα antibodies into mouse oocytes interferes with cell cycle progression

To define a role for CKIα in the regulation of early mouse activation and development, CKIα antibodies were microinjected into mouse oocytes or zygotes. The effects of these antibodies on spindle assembly, chromosomal congression and segregation during meiosis and at first zygotic cleavage were then observed. Microinjection of CKIα antibodies into GV-arrested oocytes did not affect spindle formation, chromosomal congression or segregation at first meiosis (not shown). When CKIα antibodies were microinjected into metaphase II-arrested oocytes and observed at 5 hours post-activation, defects in spindle re-orientation, chromosomal sorting, second polar body formation or the appearance of female pronuclei were not apparent (Fig. 3A-F). In addition, when G2 phase (26-28 hours post-hCG) one-cell embryos were microinjected with CKIα or control IgGs and followed out to first cleavage, no defects were observed in spindle formation, chromosome condensation and congression or cytokinesis (not shown).

When fertilized mouse oocytes were microinjected with CKIα antibodies at 20 hours post hCG, at or near the time of sperm incorporation, a defect in subsequent development was readily apparent. These results are depicted in Fig. 4. At 39 hours post-hCG, a time at which more than 80% of the control fertilized oocytes had undergone first cleavage, only 37% of oocytes injected with CKIα antibodies had progressed to the two-cell stage. Those developmentally blocked zygotes did not enter mitosis but rather locked up at the G2/M transition. As an additional negative control, CKIα-immunodepleted IgGs (see Materials and Methods) were injected at the identical concentration. These probes also failed to produce any noticeable aberration in zygotic development in that, like the mock-injected oocytes, more than 80% of the immuno-depleted IgG-injected oocytes had undergone first cleavage at 39 hours post-hCG. When the treated oocytes were analyzed 43 hours post-hCG, >90% of mock-injected and more than 80% of the immuno-depleted controls had undergone first cleavage and were now in the 2-cell stage. On the other hand, only 40% of the oocytes receiving the CKIα antibodies had progressed to the two-cell stage. Those developmentally blocked zygotes did not arrest completed pronuclear formation, apposition and centration correctly and entered into mitosis and completed cleavage normally.

Microinjection of CKIα antibodies prevents the importation of the kinase into the pronuclei

One possible means by which the introduction of CKIα antibodies into early mouse oocytes could be eliciting their effect is by interfering with the kinase’s ability to relocalize to the male and female pronuclei. One could then envision a scenario in which the kinase is prevented from interacting and therefore modulating the activities of substrates critical to proper and timely cell cycle progression. To ascertain whether such a blockade was occurring, mouse oocytes arrested at metaphase II were microinjected with control IgGs or CKIα antibodies and then parthenogenetically activated as described in Materials and Methods. At 9 hours post-activation, the cells were prepared for immunofluorescence. The oocytes were incubated with primary antibodies to CKIα in addition to fluorescently conjugated secondary antibodies. In those oocytes receiving immunodepleted antibodies (Fig. 5A), CKIα was readily apparent in the female pronucleus (Fig. 5B) in a distribution similar to that described previously (Fig. 2I). However, those oocytes microinjected with CKIα antibodies (Fig. 5C) completely lacked any signal for the kinase in the pronuclei (Fig. 5D).
DISCUSSION

Here we have shown that a casein kinase I isoform is present in mouse oocytes using immunoblotting, immunoprecipitation/kinase assays and immunofluorescence techniques. Western blotting results using a CKIα-specific antibody demonstrated that a single immunoreactive band is detected in lysates derived from both mouse oocytes and a mouse cell line. Based upon its observed SDS-PAGE mobility of 37 kDa, which is very similar to the 37.5 kDa calculated molecular mass of CKIα, the phosphokinase activity that immunoprecipitated with the CKIα antibody and the previously defined specificity of the antibody, this isoform is most likely CKIα (Brockman et al., 1992; Gross et al., 1995; Zhang et al., 1996).

Dependent upon the specific developmental stage to which the oocytes progress, there are both significant similarities and fundamental differences in the subcellular distribution of the kinase compared to that previously reported. Within meiosis I stage oocytes, CKI's association with the plasma membrane and absence on the first meiotic spindle seemingly contradicts earlier findings which described an association with cytosolic vesicular structures and the spindle apparatus in established cell lines (Brockman et al., 1992). The kinase is not observed within the germinal vesicle, unlike established cell lines where at least some nuclear signal is always apparent (Brockman et al., 1992). Such differences could be ascribed to the unique nature of the germinal vesicle stage oocyte which is in a state of indefinite arrest, awaiting specific signals to reenter the cell cycle. One possibility is that CKI may associate with these structures in order to help transduce the signals to reenter the cell cycle, or,
alternatively, this association with the plasma membrane may actually prevent the kinase from interacting with and regulating factors essential to further development, including nuclear factors. In agreement with previous reports, CKIα is found on both the spindle of metaphase II-arrested oocytes and zygotes undergoing first cleavage and is readily detectable in the pronuclei of 1-cell embryos (Brockman et al., 1992).

In metaphase II-arrested oocytes, CKIα was also observed to associate with the MVA. This structure is thought to be involved not only in defining oocyte polarity in metaphase II arrest but also in spindle reorientation and polar body formation upon fertilization. Such functions correspond strongly with the cytokinetic defects associated with genetic lesions in the *S. cerevisiae* CKI homologues *YCK1* and 2 (Robinson et al., 1993). However, the exact nature of CKI’s role in cell division in any system remains to be defined. Interestingly, evidence exists linking phosphorylation, particularly via MAP kinase action, to the regulation of these events (Verlhac et al., 1993). In light of the fact that a number of other kinases are linked to MAP kinase-associated signal transduction cascades in other systems, it is likely that other kinases also play a role in the regulation of these developmental events in the mouse oocyte. CKIα is at the very least physically positioned to act in such a pathway.

Another important observation to note here is the nuclear association of the kinase. CKI is not found in germinal vesicles or the nuclei of newly-divided daughter cells of the two-cell embryo. These structures are known to exhibit little, if any, RNA and DNA synthesis activity. There exists evidence linking CKI to the regulation of replication and transcription as well as RNA processing, particularly the addition of poly(A) tails to mRNA (Cegielska and Virshup, 1993; Dahmus, 1981; Hoekstra et al., 1991; Murao et al., 1989, 1990; Rose and Jacob, 1979). Potentially, this evidence could explain the observed differences in nuclear localization. At least for DNA replication and mRNA processing, these processes have been demonstrated to be essential for cell cycle progression in a number of oocyte systems at the one-cell stage (Wormington, 1994).

These mouse oocyte studies were originally initiated to define what role the kinase is performing when associated with mitotic spindles. Studies in *S. cerevisiae* had linked particular isoforms of the kinase to cytokinesis and chromosome segregation (Hoekstra et al., 1991; Robinson et al., 1993). CKIα antibodies were therefore microinjected at metaphase II-arrest and late G2 of the first zygotic cell cycle to determine whether the kinase might be involved in chromosome congression, segregation or cytokinesis. Although no effects were observed, these results do not preclude

Fig. 3. Microinjection of CKIα antibody into mature oocytes. (A, DIC) demonstrates that the kinase (C, CKI) strongly associates with the second meiotic spindle and cytoplasmic cytasters (B, tubulin) but is not apparent on the condensed chromosomes as defined by the DNA-binding dye DAPI (B, inset). Microinjection of CKIα antibodies into living oocytes does not block chromosome segregation (E, DAPI; inset) or second polar body formation (D, DIC) following parthenogenetic activation despite antibody localization to the spindle (F, CKI) as defined by labeling with anti-tubulin antibodies (B and E). Bar, 10 μm.

Fig. 4. Percentage of mouse zygotes undergoing in vitro cleavage following microinjection of CKIα antibodies into pronucleate eggs. Fertilized oocytes were microinjected with either buffer alone (mock) or buffer containing non-specific rabbit IgG (IgG control) or buffer with CKIα antibodies and then visually scored for progress through to the two-cell stage at 39 and 43 hours post-hCG.
activated completely blocks CKIα, which is essential for their function in the regulation of G1/prophase kinase's ability to interact with these nuclear structures. As timely cell cycle progression correlates strongly with the interpretation (Fig. 5D). The observed block in proper and CKIα nuclear import. The complete absence of nuclear signal for CKIα with discrete non-nucleolar pronuclear structures. Microinjection of CKIα antibodies clearly does not have an effect on the association of the kinase with spindles at metaphase II or first mitosis (Fig. 3A-C and not shown) or on subsequent developmental events such as chromosome congression, segregation and in the case of the latter, cytokinesis. Alternatively, other CKI isoforms may have overlapping cell-division functions and compensate for the loss of CKIα.

Microinjection studies using antibodies to CKIα demonstrate that there was a temporal component to the developing oocyte’s sensitivity to addition of this probe. If CKIα antibodies were introduced into oocytes immediately after fertilization at sperm incorporation, progression to first cleavage was significantly impeded. Introduction of these same antibody probes at 8 hours post-fertilization had no effect on subsequent progression to the two-cell stage. In addition, the observed developmental block could very well have been more dramatic if it were not for the inherent developmental variation in the population of oocytes that were microinjected. Such a variation is supported by the fact that a number of the oocytes receiving CKIα antibodies at sperm incorporation were not blocked in development suggesting that the critical temporal window had been missed.

Within this temporal window, the kinase becomes associated with discrete non-nucleolar pronuclear structures. Microinjection of CKIα antibodies into oocytes that are subsequently activated completely blocks CKIα from relocalizing to these intra-nuclear sites most likely by preventing the kinase’s nuclear import. The complete absence of nuclear signal for CKIα in these microinjected oocytes strongly supports this interpretation (Fig. 5D). The observed block in proper and timely cell cycle progression correlates strongly with the kinase’s ability to interact with these nuclear structures. As noted previously, the nuclear localization of Hrr25p and Yck3p is essential for their function in the regulation of G1 progression, DNA replication and repair (Ho et al., 1997; Wang et al., 1996). The temporal window of sensitivity described here is positioned immediately prior to the onset of DNA synthesis. Interestingly, a report by Santos et al. (1996) describes the re-localization of the Drosophila CKIα in early development from the cytosol to the nucleus in response to γ-irradiation, a treatment known to induce double-stranded breaks in DNA. Moreover, recent evidence strongly indicates that the regulation of DNA synthesis and double-stranded break repair employ some of the same regulatory pathways suggesting that CKIα could be involved in these processes (Weaver, 1996), a likelihood already established for the kinase homologues in yeast (Dhillon and Hoekstra, 1994; Hoekstra et al., 1991).

While the phenotypes associated with the yeast CKI homologues demonstrate that they are involved in the regulation of a number of critical cellular events related to cell cycle regulation, few data exist linking higher eukaryotic isoforms of CKI to these events. CKIβ and CKIε can each complement HRR25 function (Fish et al., 1995) and CKIβ partially complements YCK2 (Zhai et al., 1995) suggesting that these isoforms perform parallel functional roles. While this evidence is compelling, γ3 complementation was only partial and required that the gene for this CKI isoform be introduced on a high copy number plasmid (Zhai et al., 1995). In addition, a number of studies have demonstrated that CKI may be involved in the regulation of both G1/S transit and cytokinesis in more complex systems. CKIα exhibits a cell cycle-dependent subcellular localization that includes an association with the mitotic spindle suggesting a potential role in cell division similar to that genetically linked to the YCK1 and 2 isoforms. In addition, CKI has been purified from whole cell lysates based upon its ability to phosphorylate both SV40 large T antigen (Cegielska et al., 1994; Cegielska and Virshup, 1993) and p53 (Milne et al., 1992). In particular, CKIα, when introduced into an in vitro SV40 DNA replication reaction, will phosphorylate SV40 large T on serine residue 123 effecting a significant reduction in this viral protein’s replicative activity (Cegielska et al., 1994; Cegielska and Virshup, 1993; Grasser et al., 1988; Tuazon and Traugh, 1991). It has also been shown that a CKI isoform phosphorylates p53 on serine residue 9 within the transcriptional activation domain (Milne et al., 1992).
Although this residue is phosphorylated in vivo, no modulation of p53 activity has yet been associated with modification of this amino acid (Milne et al., 1992) and recent evidence suggests that this particular modification has no effect on the transcriptional activity of p53 in vivo (Fuchs et al., 1995).

The results outlined in this study are, to our knowledge, the first in vivo evidence indicating that higher eukaryotic isoforms of CKI may be involved in the regulation of cell cycle progression and, in particular, early mammalian development.

The work was funded by the National institutes of Health to G.S. (NIH HD12913) and R.A.A. (NIH GM51968 and GM38906). We than Drs L. Hewistion and S. Zoran for their helpful discussions and figure preparation.

REFERENCES


(Accepted 19 September 1997)