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Inactivation of M-Phase Promoting Factor at Exit from First Embryonic Mitosis in the Rat Is Independent of Cyclin B1 Degradation[1]

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ABSTRACT

Exit from M-phase and completion of cell division requires inactivation of M-phase promoting factor (MPF), a heterodimer composed of the regulatory cyclin B1 and the catalytic p34cdc2 kinase. Inactivation of MPF is associated with cyclin B1 degradation that is brought about by the ubiquitin-proteasome pathway. Our study examined the role of the proteasome in the first mitosis of rat embryos and its participation in the regulation of cyclin B1 degradation and MPF inactivation. We show that in the early zygote the proteasome is evenly distributed in the ooplasm and the nucleus, whereas during mitosis it accumulates on the spindle apparatus. We further demonstrate that inhibition of proteasomal catalytic activity prevents 1-cell embryos from undergoing mitosis. This mitotic arrest is associated with the presence of relatively high amounts of cyclin B1, which unexpectedly does not result in elevated MPF activity. Our findings strongly imply that completion of the first embryonic division depends on proteasomal degradation and that cyclin B1 is included among its target proteins. They also provide the first evidence that MPF inactivation at this stage of development is not solely dependent upon cyclin B1 degradation and is insufficient to allow the formation of the 2-cell embryo.

developmental biology, fertilization, meiosis, oocyte development, ovum

INTRODUCTION

Oocytes released from the ovarian follicles at ovulation are arrested at metaphase of the second meiotic division (MII). Completion of meiosis, characterized by sister chromatid separation and emission of the second polar body (PBII), is triggered by fertilization [1]. The zygote, formed after fertilization, contains two sets of haploid chromosomes surrounded by distinct membranes known as the male and female pronuclei (PN). The haploid pronuclei gradually move toward each other and prepare for the first mitosis by replicating their DNA. The final phase of fertilization, referred to as syngamy, includes the combination of the gametic chromosomes into a single diploid nucleus. Soon after the spindle forms, mitosis is turned on and proceeds through metaphase, anaphase, telophase, and cleavage of the 1-cell zygote to form the 2-cell conceptus [2].

The entry into and the exit from M-phase of the cell cycle are respectively regulated by activation and inactivation of the M-phase promoting factor (MPF). M-phase promoting factor is a heterodimer, composed of the catalytic p34cdc2 kinase and the regulatory cyclin B1. Experiments in *Xenopus* egg extract have demonstrated that in the absence of cyclin B1 synthesis, MPF activation will not take place [3]. However, accumulation of cyclin B1 to a certain threshold level and its association with p34cdc2 forms a pre-MPF complex that is still inactive [4]. Activation of the complex, which occurs upon entry into M-phase, further requires dephosphorylation of p34cdc2 kinase on its Thr-14 and Tyr-15 [5]. The phosphorylation/dephosphorylation of p34cdc2 are regulated by the Wee1/Myt1 kinases and the cdc25 phosphatase, respectively [6, 7]. Exit from mitosis follows cyclin B1 degradation that leads to inactivation of MPF [3, 8]. Cyclin B1 degradation is brought about by the ubiquitin-mediated proteolysis [9, 10]. Further experiments using recombinant goldfish cyclin B1 have shown that cyclin B1 destruction is primarily controlled by the activity of the 26S proteasome [11].

The 26S proteasome is a multisubunit protease complex that exhibits an ATP-dependent proteolytic activity. It is composed of a central catalytic core, the 20S particle, to which two 19S regulatory subunits are attached yielding a dumbbell-shaped complex [12, 13]. The proteasome is responsible for the degradation of a variety of cellular proteins. Most of the short-lived proteins marked for degradation by the proteasome are initially tagged by ubiquitin molecules [14–16]. Ligation of the substrate protein to ubiquitin molecules is brought about by a multienzymatic system consisting of E1, E2, and E3. E1 catalyzes activation of monomeric ubiquitin that is then transferred to one of the several members of a family of E2 or ubiquitin carrier proteins (UBCs). Ligation of the ubiquitin to a specific target protein usually requires a third enzyme, E3 or ubiquitin ligase [17]. The ligation of ubiquitin to cyclin B1 is regulated by a specific E3, a component of the anaphase-promoting complex (APC) [18], or the cyclosome [19].

The general goal of our study was to explore the participation of the proteasome in the regulation of the first mitotic division of rat embryos. We further planned to investigate the involvement of this protease complex in regulating cyclin B1 degradation and MPF inactivation. Our hypothesis was that interference with proteasomal catalytic activity would result in elevated levels of cyclin B1, a sustained high MPF activity, and recession of embryonal mitosis. Our results reinforce the central role of the proteasome in degradation of regulatory proteins that are involved in the control of cell division. They further demonstrate that cyclin B1 is included among the target proteins for proteasomal action. However, MPF inactivation at the first embryonic mitosis is unexpectedly not solely dependent upon cyclin B1 degradation and is insufficient to allow the formation of a 2-cell embryo.

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MATERIALS AND METHODS

Reagents and Antibodies

Leibovitz's L-15 tissue culture medium was purchased from Gibco BRL (Paisley, Scotland). Antibiotics were purchased from Bio-Lab Ltd. (Jerusalem, Israel). Rabbit anti-p34cdc2 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). MG132 (Z-leu-leu-CHO) and lactacystin were purchased from Calbiochem (La Jolla, CA). A calpain II inhibitor, LLmL (*N*-acetyl-L-leu-leu-normethioninal), leupeptin, histone H1 (type III-S), monoclonal mouse anti-β-tubulin antibodies, fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibodies, collagenase, hyaluronidase, bovine albumin, DL-lactic acid, pyruvic acid, and fetal bovine serum were all purchased from Sigma (St. Louis, MO). Cy3-conjugated anti-rabbit antibodies were purchased from Jackson Immuno Research Laboratories Inc. (West Grove, PA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies were purchased from Zymed (San Francisco, CA). [^{32}P]Adenosine 5′-triphosphate (3000 Ci/mmol) and enhanced chemiluminescence (ECL) Western blotting detection reagents were purchased from Amersham (Buckinghamshire, England). Monoclonal mouse anti-cyclin B1 antibodies were a kind gift of Dr. M. Brandeis (The Hebrew University, Jerusalem, Israel). This antibody was raised against residues 160–300 of hamster cyclin B1, which is 99.2% identical to rat cyclin B1 and shares no homology with other rat cyclins. Antisera against rat granulosa 20S proteasomes were a kind gift of Prof. A. Amsterdam (The Weizmann Institute of Sciences, Rehovot, Israel) [20].

Animals

Sexually immature female Wistar rats (23–25 days old) from our departmental colony were injected s.c. with 10 IU of eCG (Sanofi Sante Nutrition Animale, France) in 0.1 ml of 0.9% NaCl for induction of follicular development. Oocytes were recovered at 48 h after the above treatment. Zygotes were recovered from the above rats injected with 10 IU of hCG (Pregnyl; N.V. Organon Oss Holland, Holland) in 0.1 ml of 0.9% NaCl 48–52 h after eCG to induce ovulation. After hCG administration, these female rats were caged overnight with fertile male rats and killed on the following day by cervical dislocation. The study was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy of Science, Bethesda, MD).

Oocyte Recovery and Culture

Ovaries were removed and oocytes were isolated as described previously [21]. Cumulus cells were removed enzymatically by collagenase (50 IU/ml, 30 min), and denuded oocytes were incubated in Leibovitz's L-15 tissue culture medium, supplemented with 5% fetal bovine serum, 100 IU/ml penicillin, 50 μg/ml streptomycin, and 0.25 μg/ml fungizone, at 37°C in a humidified incubator. After an overnight incubation, MII-arrested oocytes were collected.

Embryo Recovery and Culture

Rats were killed and their oviducts were removed 22 h after hCG administration. Fertilized eggs were released into PBS. Cumulus cells were removed enzymatically by hyaluronidase (1 mg/ml, 10 min 37°C), followed by repetitive pipetting. Fertilization was confirmed microscopically by the presence of the attached sperm and the two PN using Nomarski differential interphase contrast (DIC) optics. The 1-cell embryos were then collected and incubated for 30 h in rat fertilization medium (RFM) [22], at 37°C in a 5% CO_2 atmosphere, in the presence or absence of the proteasome inhibitors MG132 or lactacystin as well as the calpain II inhibitor LLmL. For transient exposure, embryos were washed three times after 28–33 h incubation (when the majority of control embryos had divided) and were further incubated in inhibitor-free medium. The embryos were monitored morphologically after an additional 24-h period.

Western Blot Analysis and Immunocytochemistry

At the end of the specified incubation time the oocytes/embryos were lysed in lysis buffer (1% Triton X-100, 50 mM Hepes, pH 7.2, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na-orthovanadate, 10% glycerol, 30 mM NaF, 30 mM Na-pyrophosphate) and subjected to Western blot analysis. The following antibodies were utilized: rabbit antisera against rat granulosa 20S proteasome (1:1000 dilution), rabbit antisera against p34cdc2 (1:1000 dilution), and monoclonal mouse anti-cyclin B1 antibodies (1:750 dilution). The relevant HRP-conjugated secondary antibodies were used and immunoreactive bands were detected by ECL reagents. Densitometric analysis was performed utilizing the PDI 420oe densitometer supported by Quantity One software (PDI, Huntington Station, NY).

For immunocytochemistry, embryos were fixed as described previously [20] and immunostained with rabbit antisera against rat granulosa 20S proteasome (1:200 dilution) and monoclonal mouse anti-β-tubulin antibodies (1:200 dilution). The embryos were further incubated with the secondary antibodies: FITC-conjugated rabbit anti-mouse antibodies (1:200) dilution) and Cy3-conjugated anti-rabbit antibodies (1:250 dilution). Embryos in 50% glycerol/PBS were mounted on silicon-coated glass slides and covered by coverslips resting on a silicone ring containing 100-μm glass beads that served as spacers. The embryos were visualized by both phase-contrast and fluorescent microscopy using an Optiphot-2 microscope (Nikon Co., Tokyo, Japan) equipped with BP546/455 filters.

Histone H1 Kinase Assay

Histone H1 kinase activity was determined in lysates of 25 oocytes/embryos, prepared by freezing and thawing in 10 μl kinase buffer (15 mM morpholinepropanesulfonic acid, 80 mM β-glycerophosphate, 10 mM EGTA, 15 mM $MgCl_2$, 0.1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml PKI, a cAMP-dependent protein kinase inhibitor peptide). Kinase reactions were initiated by the addition of 5 μl of substrate buffer (4 mg/ml histone H1, 4 mM dithiothreitol, 10 μCi [^{32}P]ATP), and the reactions were carried on at 30°C for 30 min. Kinase reaction products were subjected to SDS-PAGE and autoradiographed. Densitometric analysis was performed utilizing the Fujix BAS1000 phosphoimager supported by MacBas software. Quantitation analysis was performed by computerized densitometer analysis (Computing analysis, PDI 420oe).

Statistical Analysis

Statistical analysis was performed using SAS software for windows 6.12. Data from the H1 kinase assay of 1-cell,

2-cell, and MG132-treated embryos were analyzed as a randomized block design, where the different experiments were the blocks: 1-cell, 2-cell, and MG132-treated embryos were the treatments. The analysis was followed by ANOVA; then by Fisher's least significance difference (LSD). Data from the H1 kinase assay of MII-arrested unfertilized oocytes and early 1-cell embryos was analyzed using Student's *t*-test.

RESULTS

The first mitotic cell cycle in our rat experimental model was temporally characterized by morphological examination of the embryos from zygote formation up to the first embryonic cleavage. Because the time of sperm penetration varies among individual oocytes, the kinetics of the different events is expressed in reference to the time of hCG administration. As mentioned in the *Materials and Methods,* the rats were killed at 22 h after hCG administration. The fertilized eggs released from the oviducts at this time point were characterized by the presence of an attached sperm. A maximal fraction of these zygotes (65%) exhibited distinct male and female pronuclei (2PN) at 28 h. Syngamy occurred at around 48 h with 60% of the embryos completing the first embryonic cleavage at 52 h after hCG administration (Fig. 1). Based on the above timing, we denominated 1-cell embryos that were collected 22 h post-hCG as early 1-cell, whereas those collected 45–49 h after hCG, as late 1-cell embryos.

Proteasome Translocates to the Mitotic Spindle

Our initial experiment was designed in order to analyze fertilized rat eggs undergoing the first embryonic mitosis for subcellular localization of their proteasomes. Embryos at different developmental stages were examined by double immunofluorescent staining using antibodies generated against the 20S proteasomes and anti-β-tubulin antibodies. We found that in the 2PN embryos the proteasome was evenly distributed in the ooplasm with some tendency to accumulate around the PN (Fig. 2, A1–A3). This pattern of spatial organization did not change at syngamy (Fig. 2, B1–B3). However, at the first mitotic metaphase, the cytoplasmic concentrations of the proteasomes diminished, and it translocated to the spindle apparatus (Fig. 2, C1–C3). The accumulation of the proteasome on the spindle apparatus was even more dramatic after the first cleavage upon the formation of the 2-cell embryo (Fig. 2, D1–D3). This cell cycle-dependent translocation suggests a role for the proteasomal system in degrading specific cellular substrates that are associated with the mitotic spindle at this stage of the cell cycle.

Proteasome Inhibitors Block the First Embryonic Cleavage

In order to further elucidate the role of the proteasome at the first mitosis, the effect of selective proteasome inhibitors on early embryogenesis was analyzed. Zygotes (1-cell embryos) were incubated in the presence or absence of proteasome inhibitors and monitored morphologically for the first cleavage as manifested by the formation of 2-cell embryos. Two potent and highly selective agents, lactacystin and MG132, that are irreversible and reversible inhibitors of proteasomal proteolytic activity, respectively, were utilized. As shown in Figure 3 both inhibitors prevented the first embryonic cleavage in a dose-dependent manner (lactacystin, ED_{50} = 0.3 μM, maximal effective dose = 10 μM; MG132, ED_{50} = 0.3 μM, maximal effective dose = 1.1 μM). Reversibility of the MG132 effect was demonstrated in 1-cell embryos extensively washed after 28–32 h of exposure and further incubated in an inhibitor-free medium for 24 h. Recovery was observed in 70.3 ± 2.7% (n = 107) of the MG132-treated embryos (2 μM) that proceeded through mitosis and reached the 2-cell stage. In contrast, 1-cell embryos transiently treated with lactacystin did not proceed through mitosis exhibiting a high rate of degeneration. The specificity of the proteasome inhibitors was demonstrated by examining a calpain II inhibitor, LLmL, that had no effect on embryonic cleavage (Fig. 3).

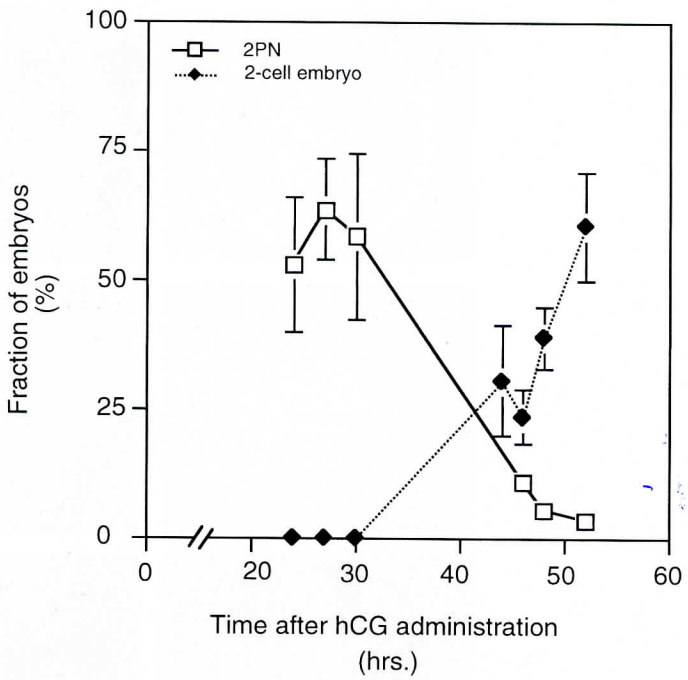

FIG. 1. The kinetics of male and female PN (2PN) formation and the first embryonic cell division. Zygotes (1-cell embryos) were isolated from the oviducts of fertilized female rats, 22 h after hCG administration. The embryos were incubated and monitored morphologically at the indicated time points using DIC microscopy as described in the *Materials and Methods.* The fraction of either 2PN or 2-cell embryos out of the total embryos examined at each time point is presented. The experiment was repeated three times, with at least 30 embryos examined at each time point. The bars represent SEM.

Cyclin B1 Is Degraded by the Proteasome

The fact that the first embryonic division was prevented by the proteasome inhibitors and the information that cyclin B1 is subjected to proteasomal degradation led us to examine further the MG132-treated embryos for their content of cyclin B1. Our initial experiment was undertaken for documentation of the cyclin B1 pattern of expression in early embryos of rat. The cyclic pattern of cyclin B1 expression was demonstrated previously for early embryos of clam [23], mouse [24], starfish [25], newt [26], and *Drosophila* [27]. We found a sharp decrease in the amount of cyclin B1 in fertilized rat eggs (early 1-cell embryos) at their exit from the meiotic MII arrest, as compared to ovulated unfertilized oocytes (Fig. 4A). This was followed by a dramatic accumulation of cyclin B1 in late 1-cell embryos, upon entry into the first mitosis (Fig. 4A). The newly formed 2-cell embryos that exited the first mitotic M-phase again exhibited a reduced amount of cyclin B1 that was substantially lower than that found in late 1-cell embryos

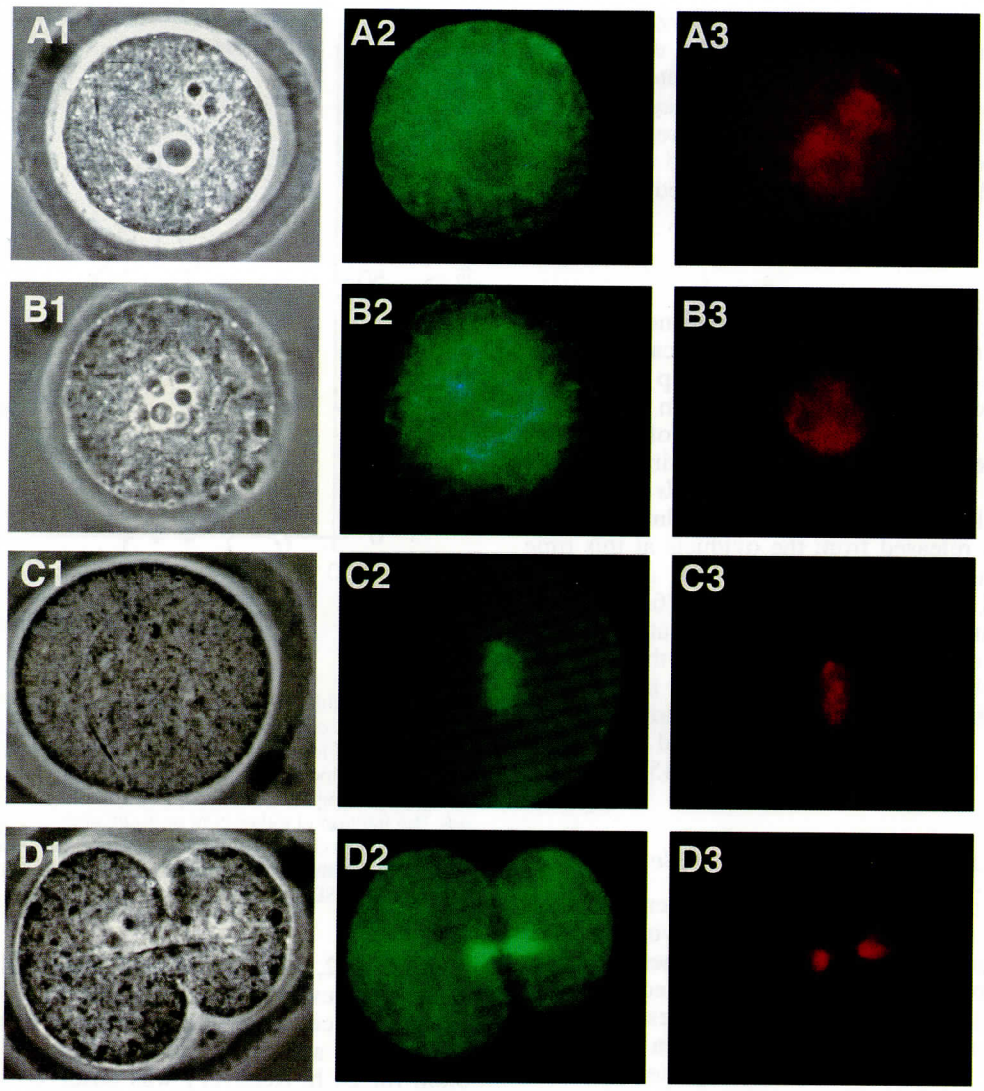

FIG. 2. Subcellular localization of the proteasome in rat embryos during the first embryonic mitosis. Rat embryos were fixed at various stages of mitosis and immunostained with anti-20S proteasome antibodies (red) and anti-β-tubulin antibodies (fluorescent yellow-green). A 2PN embryo (**A1**), that has not yet assembled a spindle apparatus (**A2**), showing even distribution of proteasomes in the ooplasm with some tendency to accumulate around the PN (**A3**). An embryo at syngamy (**B1**), demonstrating aster-like organization of microtubules (**B2**), while the proteasomes accumulate around the newly formed nucleus (**B3**). The first embryonic metaphase (**C1**), with an organized spindle apparatus (**C2**), clearly stained by the anti-proteasome antibodies (**C3**). Cytokinesis and formation of a 2-cell embryo (**D1**). The proteasome remains colocalized on the spindle apparatus that is located at the bridge of the 2-cell embryo (**D2**, **D3**). The experiment was repeated three times, with at least 20 embryos examined at each experimental point. Original magnification ×400 (**A1**–**D3**).

before their first division (Fig. 4A). As expected in MG132-treated embryos, cyclin B1 degradation was prevented (Fig. 4B). The MG132-treated embryos contained a considerably higher amount of cyclin B1 than control embryos that formed 2-cell embryos at the same time point of incubation.

Proteasome Inhibitor Fails to Maintain High Levels of MPF Activity

In order to examine the relationship between cyclin B1 accumulation and MPF activation we further employed the histone H1 kinase assay, an assay that is routinely used for monitoring p34cdc2 kinase activity. Histone H1 kinase activity was measured at the same time points corresponding to the examination of cyclin B1 expression. This experiment showed that similar to the starfish [25] and the mouse [28, 29], MPF activity is high in MII-arrested rat oocytes and drops significantly (1.7-fold, $P < 0.05$) after fertilization, in early 1-cell embryos (Fig. 5A). It rises prior to the first mitosis in late 1-cell embryos and declines again by 2.4-fold at the formation of the 2-cell embryos (Fig. 5B). Unexpectedly, the accumulation of cyclin B1 in MG132-treated embryos did not result in elevated MPF activity (Fig. 5B). In fact the MPF activity dropped similarly to the drop in MPF activity occurring upon the completion of the first embryonic mitosis. Statistical analysis of variance shows a significant difference among 1-cell, 2-cell, and MG132-treated embryos ($P < 0.01$). The analysis was followed by Fisher's LSD. Results show that the group of 1-cell embryos is significantly different than both 2-cell and MG132-treated embryos, which are not significantly different from each other ($\alpha = 0.05$). It was therefore surprising that in spite of MPF inactivation the MG132-treated embryos failed to complete the first mitotic division. The apparent controversy between the low activity of MPF in the presence of the relatively high amounts of cyclin B1 was clarified by Western blot analysis using anti-p34cdc2 antibodies. Similar to previous findings in mice [28, 29]

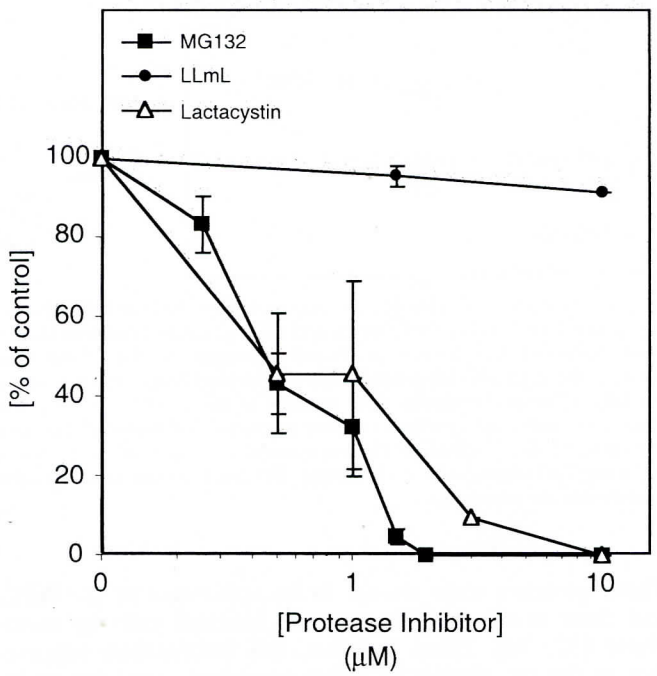

FIG. 3. Dose-dependent effect of proteasome inhibitors on the first mitosis in rat embryos. Zygotes (1-cell embryos) were incubated for 30 h in the presence or absence of the proteasome inhibitors either MG132 or lactacystin. The effect of the calpain II inhibitor LLmL was also tested. The fraction of 2-cell embryos out of the total embryos examined is presented. Results of the average of four experiments including at least 90 embryos at each experimental point is presented. The bars represent SEM.

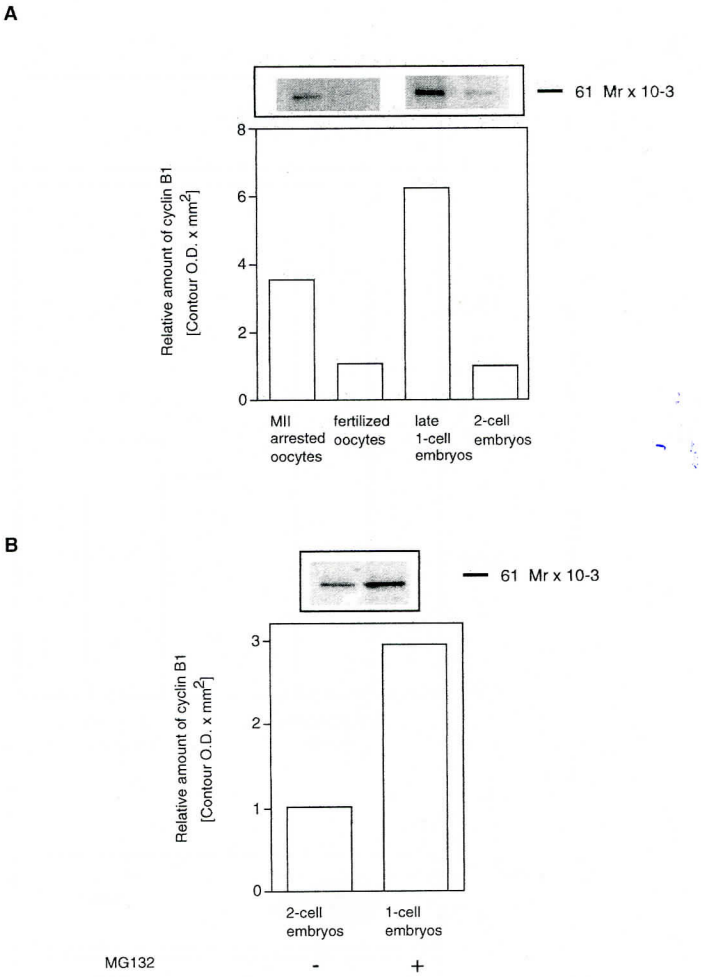

FIG. 4. Expression of cyclin B1 in MII-arrested oocytes and early embryos: effect of MG132. **A**) Control MII-arrested oocytes, freshly isolated fertilized oocytes (early 1-cell embryos), late 1-cell embryos, and 2-cell embryos (after in vitro incubation) were examined. **B**) Embryos were examined after in vitro incubation in the presence or absence of MG132 (2 μM). In the absence of MG132, the zygotes completed mitosis and formed 2-cell embryos, while the MG132-treated embryos were arrested at the 1-cell stage. The oocytes/embryos were extracted and their proteins separated and immunoblotted using anti-cyclin B1 antibodies. The experiment was repeated three times, including 100 embryos for each sample. The results of one representative experiment are presented. The lower panel depicts the densitometric analysis of this experiment.

and rat [30], we herein demonstrate that the expression profile of p34cdc2 in rat embryos presents three different migrating forms of the enzyme, in conjunction with its phosphorylation. The phosphorylated forms of p34cdc2 represent the inactive enzyme, while the appearance of a dephosphorylated form directly correlates with the activation of MPF, as measured by histone H1 kinase activity. In accordance with the observed active MPF, late 1-cell rat embryos exhibited a nonphosphorylated p34cdc2 (Fig. 6). Upon the completion of the first cell cycle and formation of the 2-cell embryo, p34cdc2 appeared rephosphorylated at an intermediate level, conveying an inactive state of MPF. The MG132-treated embryos, arrested at the 1-cell stage, possess a phosphorylated p34cdc2 (Fig. 6) in conjunction with an inactive MPF.

DISCUSSION

Our study provides clear evidence that the proteasome regulates the first embryonic division. In addition, we show for the first time that MPF inactivation is insufficient to allow the formation of the 2-cell embryo. We further suggest that inactivation of MPF upon exit of the 1-cell embryos from M-phase is not solely dependent on cyclin B1 degradation and is also regulated by p34cdc2 rephosphorylation.

Cyclins were first identified in sea urchin as embryonic proteins that oscillate during the cell cycle [31]. This and later reports have shown that cyclin B1 accumulation takes place upon entry into M-phase and that its degradation occurs at exit from mitosis [3, 8, 32]. Studies demonstrating that a truncated form of this protein, which is resistant to proteolysis, arrests cells at the exit from M-phase established the notion that cyclin B1 degradation is essential for completion of mitosis [8, 9, 33, 34]. However, demonstrations that the nondegradable cyclin B1 arrests cell division in telophase rather than metaphase [33] seem to suggest the existence of noncyclin substrates that are degraded via APC-mediated proteolysis and inhibit anaphase.

In full agreement with this idea, we herein demonstrate for the first time that inhibition of proteasomal action prevents the completion of the first embryonic mitosis in spite of the drop in MPF activity. Our findings suggest that cyclin B1 degradation and/or MPF inactivation is insufficient to allow the formation of the 2-cell embryo. They support the idea that proteasomal degradation of protein/s other than cyclin B1 is required for completion of mitosis.

The other candidates to be degraded by the ubiquitin-proteasome dependent pathway could possibly include the recently identified securins, a family of proteins that inhibit separation of sister chromatids [35, 36]. This family includes Pds1 (*Saccharomyces cerevisiae*), Cut2p (*Saccharomyces pombe*) and the vertebrate orthologue, vSecurin.

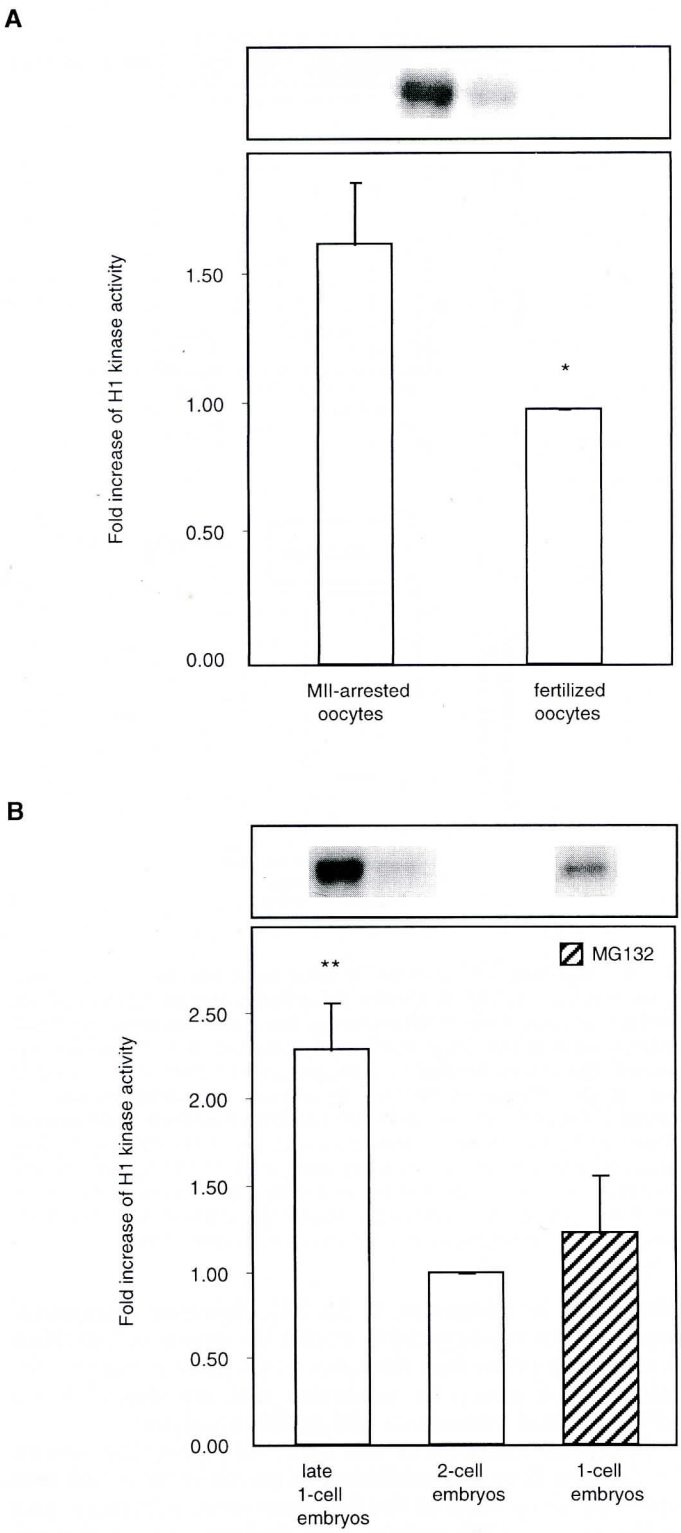

FIG. 5. Histone H1 kinase activity in MII-arrested oocytes and early embryos: effect of MG132. **A**) Control MII-arrested oocytes and freshly isolated fertilized oocytes (early 1-cell) were examined. **B**) Late 1-cell embryos, 2-cell embryos, and MG132-arrested embryos (incubated overnight in the presence or absence of 2 μM MG132) were examined. The oocytes/embryos (25/sample) were extracted and assayed for histone H1 kinase activity. The results of one representative experiment are presented in the upper panel. The lower panel depicts the fold increase in MPF activity of MII-arrested oocytes/fertilized oocytes (**A**) and fold increase of late 1-cell embryos/2-cell embryos and MG132 arrested 1-cell/2-cell embryos. **B**) Statistical analysis was performed on pooled results from six individual experiments. $*P < 0.05$; $**P < 0.01$.

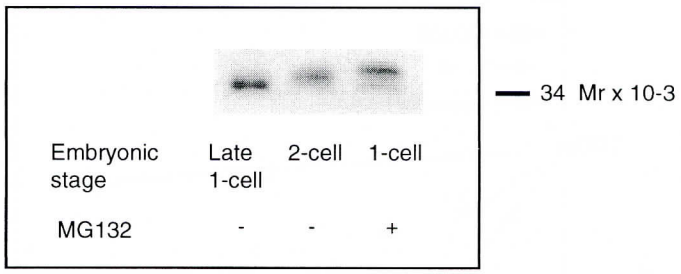

FIG. 6. Expression of p34cdc2 in early embryos: effect of MG132. Embryos: late 1-cell and 2-cell embryos and MG132-treated embryos (2 μM) were collected and further incubated overnight. In the absence of MG132, the zygotes completed mitosis and formed 2-cell embryos, while the MG132-treated embryos were arrested at the 1-cell stage. The embryos were extracted and their proteins separated and immunobloted using anti-p34cdc2 antibodies. The experiment was repeated three times, including 100 embryos for each sample. The results of one representative experiment are presented.

These proteins were shown to be substrates of the APC, and their nondegradable mutants blocked cells in metaphase [37, 38]. Taken together, this information suggests that in the rat, similar to lower organisms, proteins maintaining sister chromatid cohesion may also be subjected to degradation by the ubiquitin-proteasome machinery near the end of mitosis. Interference with degradation of such proteins by the proteasome inhibitors employed in our study could possibly explain the failure of the embryos to complete their mitosis.

The role of the proteasome in regulation of the first embryonic division was initially suggested by our findings that at metaphase the proteasomes translocate to the spindle apparatus. Translocation of the proteasome to the spindle at metaphase of embryonic cells undergoing mitosis has been demonstrated previously only in lower organisms such as ascidian *Halocynthia roretzi* [39] and *Drosophila* embryos [27]. Colocalization of proteasomes with spindle microtubules has also been shown in mitotically dividing nonembryonic cell [20]. A recent report from our laboratory shows that the proteasome accumulates on the metaphase spindle also in rat oocytes that resume meiosis [40]. Translocation of the proteasome that is observed during the mitotic as well as meiotic cell cycle may represent a mechanism for regulation of its action. Its colocalization with the spindle apparatus further suggests a role for the proteasome in degrading specific cellular substrates such as cyclin B1 [32] that are associated with the metaphase spindle. The securins that are positioned between sister chromatids perfectly meet these requirements as well. Strong evidence for this idea is gained by an immunogold electron microscopy study that localized the proteasome in mitosis around the chromosomes [41].

The cell cycle-associated pattern of proteasome translocation demonstrated in our study indeed proposes that completion of the first mitosis is a cellular event regulated by proteolysis. This idea is strongly supported by our demonstration that inhibition of proteasomal catalytic activity arrests the embryos at the 1-cell stage. The two inhibitors employed in this experiment were fully effective in preventing the first embryonic mitosis. The effect of the reversible inhibitor, MG132, was indeed transient. The high level of recovery of its action eliminates possible toxic influence of this compound. Specificity of these inhibitors toward the proteasome is evidenced by the failure of a calpain inhibitor to elicit a similar effect in this system. Cal-

pain, a calcium-activated protease was reported to be modified following fertilization in rat eggs [42]. Furthermore, fertilization triggers a wave of oscillation in intracellular concentration of calcium [43]. Our results regarding calpain reinforce the findings reported in *Xenopus* egg extracts, whereby the calcium-mediated cyclin B1 degradation, immediately after fertilization is not due to calpain action [44, 45].

A previous study in our laboratory demonstrated that inhibition of proteasomal catalytic activity that arrested oocytes at the first metaphase of meiosis was associated with accumulation of cyclin B1 that resulted in a high level of MPF activity [40]. Surprisingly, in our present study inhibition of cyclin B1 degradation did not result in a sustained elevated activity of MPF. This last finding provides evidence for the intriguing possibility that MPF inactivation at completion of the first embryonic division is regulated by an additional mechanism that is independent of cyclin B1 degradation. The alternative mechanism for modulation of MPF activity that bypasses cyclin B1 availability could possibly involve kinases that phosphorylate the p34cdc2/cyclin B1 complex resulting in a reduction of its activity. Monophosphorylation of p34cdc2 on Tyr-15 that is catalyzed by Wee1 [46], and dual phosphorylation of Tyr-15 and Thr-14 by Myt1 have been reported in *Xenopus* egg extracts [6, 7, 47]. Mouse oocytes were shown to display a developmentally regulated expression of Wee1 [48], but the activity of this kinase in mammalian oocyte has not been studied as yet. The presence of the phosphorylated form of p34cdc2 demonstrated herein provides strong evidence that the mechanism of MPF inactivation upon completion of the first embryonic mitosis in rat embryos involves rephosphorylation of the p34cdc2 kinase. The presence of phosphorylated forms of p34cdc2 was also found in mouse embryos that exhibit a profound 2-cell block [49–51]. The investigators associate this sustained phosphorylation with the inability of the embryos to progress beyond the 2-cell stage of development. In contrast to the mouse, the presence of rephosphorylated p34cdc2 in 2-cell embryos was not related to 2-cell block and may therefore represent a normal profile of the enzyme [30]. The mechanism responsible for p34cdc2 kinase rephosphorylation at this mitotic cell cycle requires further elucidation.

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