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Degradation of the proto-oncogene product p39^{mos} is not necessary for cyclin proteolysis and exit from meiotic metaphase: requirement for a Ca^{2+} – calmodulin dependent event

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Exit from M phase, which requires cyclin degradation, is prevented from occurring in unfertilized eggs of vertebrates arrested at second meiotic metaphase due to a cytostatic factor recently identified as p39^{mos}, the product of the proto-oncogene *c-mos*. Calpain can destroy both p39^{mos} and cyclin *in vitro* in extracts prepared from metaphase-arrested *Xenopus* eggs, but only when free Ca^{2+} concentration is raised to the millimolar range. When free Ca^{2+} concentration is raised for only 30 s to the micromolar range, as occurs in physiological conditions after fertilization, cyclin degradation is induced, but p39^{mos} is not degraded. Cyclin proteolysis at micromolar free Ca^{2+} is not inhibited by calpastatin, and therefore does not involve calpain. A cyclin mutant modified in the destruction box is found to be resistant at micromolar, but not millimolar free Ca^{2+}, suggesting that the ubiquitin pathway mediates cyclin degradation at micromolar Ca^{2+} concentration whereas calpain is involved at the millimolar level. A synthetic peptide which binds Ca^{2+} – calmodulin with high affinity suppresses cyclin degradation at micromolar but not millimolar free Ca^{2+}, and this only when it is present in the extract during the first 30 s after raising free Ca^{2+} concentration. The inhibition of the cyclin degradation pathway by the Ca^{2+} – calmodulin binding peptide can be overcome by adding calmodulin. These results strongly suggest that a Ca^{2+} – calmodulin process is required as an early event following fertilization to release the cyclin degradation pathway from inhibition in metaphase-arrested eggs. In contrast, p39^{mos} degradation is not required.

Key words: Ca^{2+} – calmodulin/calpain/cell cycle/cyclin/p39^{mos} proto-oncogene/*Xenopus* eggs

Introduction

Exit from M phase requires inactivation of MPF (maturation promoting factor), a universal inducer of M phase (meiosis and mitosis) recently identified as a stoichiometric complex between a *cdc2*$^{+}$-encoded protein kinase and cyclin B (Labbé *et al.*, 1989; Gautier *et al.*, 1990). MPF inactivation requires cyclin proteolysis (Murray *et al.*, 1989), which begins at the end of metaphase and is completed within a very short window of the cell cycle. It has ben shown that addition of active cdc2 kinase triggers cyclin degradation in cell-free extracts of interphase *Xenopus* eggs (Felix *et al.*, 1990), providing evidence of a negative feedback loop to terminate metaphase. Cyclin degradation on exit of the mitotic metaphase has been shown to be accompanied by the formation of cyclin – ubiquitin conjugates (Glotzer *et al.*, 1991). Moreover, a mutation within cyclin that inhibited ubiquitin conjugation also inhibited degradation. This strongly suggested that cdc2 kinase regulates the conjugation of ubiquitin to cyclin, leading to the rapid destruction of cyclin by the ubiquitin-dependent proteolytic system.

In vertebrates, unfertilized eggs are prevented from exiting metaphase of meiosis II, due to the presence of a cytostatic factor (CSF). Although CSF has never been purified, p39^{mos}, the product of the proto-oncogene *c-mos*, has been shown to be intimately associated with this activity. Indeed, microinjection of synthetic *mos* mRNA into two-cell embryos induces cleavage arrest at metaphase. By contrast, cytosol extracts of eggs, when immunodepleted of endogenous p39^{mos}, lose their cleavage-arresting activity in injected embryos (Sagata *et al.*, 1989). In CSF-arrested cells, cyclin is stable even though cdc2 kinase activity is high, suggesting that a p39^{mos}-catalysed phosphorylation event either makes cyclin resistant to proteolysis or inhibits some enzyme required for cyclin degradation. The disappearance of CSF activity at fertilization is believed to be caused by a transient increase in cytoplasmic free calcium upon fertilization, and is indeed calcium-sensitive *in vitro* (Meyerhof and Masui, 1977; Shibuya and Masui, 1988). It has been proposed that disappearance of CSF activity at fertilization is due to degradation of p39^{mos} by calpain II (Watanabe *et al.*, 1989), but this seems unlikely for the following reasons. First, p39^{mos} was reported to disappear relatively late after fertilization or parthenogenetic activation (Sagata *et al.*, 1989), whereas CSF inactivation would be expected to precede cdc2 kinase inactivation, which occurs quickly after parthenogenetic activation *in vivo* (Capony *et al.*, 1986; Labbé *et al.*, 1988). Second, *in vitro* degradation of p39^{mos} by calpain was observed when free Ca^{2+} was made higher than 1 mM, whereas it never exceeds 1.5 μM in intact eggs following fertilization (Busa and Nucitelli, 1985; Kubota *et al.*, 1987; Cross, 1981; Jaffe, 1983).

In the present work, we report that increasing free Ca^{2+} only transiently to the micromolar range is sufficient to induce cyclin degradation in extracts prepared from metaphase II-arrested *Xenopus* eggs, and even in the presence of calpastatin. At this free Ca^{2+} concentration, p39^{mos} does not undergo proteolysis, even in the absence of calpastatin. We also report that at micromolar Ca^{2+}, cyclin degradation can be suppressed by adding MLCK(488–511), a synthetic peptide corresponding to the auto-inhibitory domain of chicken gizzard myosin light chain kinase, which binds calmodulin with high affinity (Kemp *et al.*, 1987). This suggests that the burst of Ca^{2+} at fertilization releases the cyclin degradation pathway from CSF inhibition by first activating a still unidentified Ca^{2+} – calmodulin-dependent enzyme.

Results

Calpain can destroy both p39^{mos} and full-length cyclin in vitro

Addition of millimolar amounts of $CaCl_2$ to cytosol prepared from metaphase II-arrested oocytes has been shown to trigger p39^{mos} degradation by calpain (Watanabe *et al.*, 1989). Since cyclin degradation was not tested in those experiments, we performed similar experiments and monitored either p39^{mos} or cyclin B for calcium-dependent and calpastatin-inhibited degradation. Oocytes were crushed by centrifugation in a buffer containing 5 mM EGTA to prepare 15 000 *g* supernatants. [^{35}S]methionine-labelled *Xenopus* p39^{mos} or starfish cyclin B, both produced in reticulocyte lysates, were added to these extracts. The degradation reaction was started by adding $CaCl_2$ to 6 mM (total amount). As shown in Figure 1A, Ca^{2+} was found to induce proteolysis of both p39^{mos} (Figure 1A, panel b,

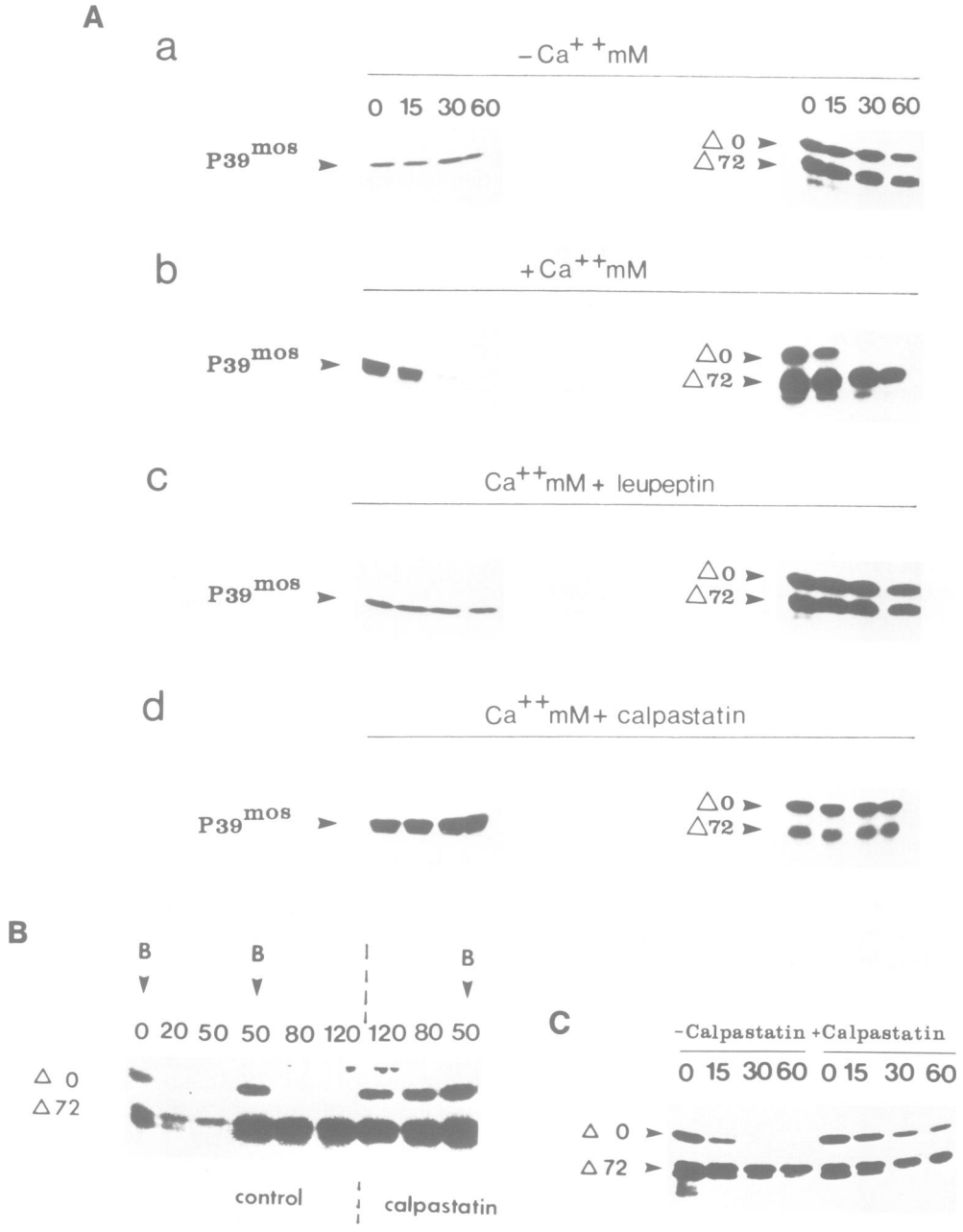

Fig. 1. Millimolar free Ca^{2+} induces degradation of full-length cyclin B and p39^{mos} in *Xenopus* extracts. (**A**) Autoradiograms of 10% polyacrylamide gels showing samples (4 μl) taken at the indicated times (min) after the addition of reticulocyte lysate (1.2 μl) containing [^{35}S]methionine-labelled *Xenopus* p39^{mos} (panels a–d, left), or full-length (Δ0) and truncated (Δ72) starfish cyclin B (panels a–d, right) to extracts prepared from metaphase II-arrested eggs (30 μl). $CaCl_2$ (6 mM, total amount) was added (b,c,d) or not (a) simultaneously with the labelled p39^{mos} or cyclin. Incubation was performed in the absence (a,b) or presence of either 10 μg/ml leupeptin (c) or 20 μM calpastatin peptide (d). (**B**) Millimolar free Ca^{2+} induces several rounds of cyclin degradation in extracts prepared from metaphase II-arrested eggs. At 50 min after Ca^{2+} addition (second and third arrows) (i.e. after inactivation of endogenous cdc2 kinase during the first round of cyclin degradation), the extract was divided into two parts. One received only *in vitro* translated cyclin B (control), the other received both cyclin B and 20 μM calpastatin peptide. Samples were taken at the indicated times (min) and processed as described in Materials and methods to monitor cyclin degradation. (**C**) Millimolar free Ca^{2+} induces degradation of full-length cyclin B via calpain in extracts prepared from immature *Xenopus* oocytes, which lack p39^{mos}. Calpastatin (20 μM) was added, or not, to the extracts containing *in vitro* translated cyclin. Degradation was triggered by adding 6 mM $CaCl_2$ (total amount).

left) and full-length cyclin B (Figure 1A, panel b, right). A truncated cyclin B lacking 72 amino acids from the N-terminus (Δ72) has been shown to be produced in the reticulocyte system by internal initiation on methionine 73 of starfish cyclin B (Lorca *et al.*, 1991). In contrast to full-length cyclin, this truncated cyclin B did not undergo Ca^{2+}-dependent proteolysis (Figure 1A, panel b, right). Degradation of both $p39^{mos}$ and full-length cyclin could be suppressed by adding either 10 μg/ml leupeptin (Figure 1A, panel c) or 20 μM of fragment (162−188) of calpastatin (Figure 1A, panel d), a synthetic peptide corresponding to the most effective domain of calpastatin. This peptide strongly inhibits calpain I and calpain II, but does not inhibit either papain (a cysteine protease) or trypsin (a serine protease) (Maki *et al.*, 1989).

Since $p39^{mos}$ is believed to protect cyclin from proteolysis, a possible interpretation for the above experiments was that calpain first destroys $p39^{mos}$, thereby releasing a Ca^{2+}-independent cyclin degradation pathway from its inhibited state. In apparent agreement with this view, the extracts were found to support several rounds of cyclin proteolysis after $p39^{mos}$ had been destroyed (Figure 1B). However, any round of cyclin proteolysis, not only the first one, could be suppressed by adding either the calpastatin peptide (Figure 1B), EGTA, leupeptin or E64 (data not shown). Moreover, millimolar amounts of Ca^{2+} also induced calpastatin-inhibited cyclin degradation in extracts prepared from immature oocytes (Figure 1C), which lack $p39^{mos}$ (Sagata *et al.*, 1988). Therefore calpain is able to proteolyse both $p39^{mos}$ and full-length cyclin, not only $p39^{mos}$, at least *in vitro*.

Calpain is not necessary for Ca^{2+}-induced cyclin degradation

In physiological conditions, the window for cyclin degradation remains open only for a very short time at each cycle (Standart *et al.*, 1987; Westendorf *et al.*, 1989). This was not the case in the above experiments, indicating that the conditions of assay were not those prevailing in the living cell. Free Ca^{2+} concentration was measured with a Ca^{2+}-specific electrode and found to be >500 μM following addition of $CaCl_2$ to 6 mM in the EGTA-containing extracts (data not shown). This contrasted with the transient increase of free Ca^{2+} at fertilization, which does not exceed 1.5 μM. In the next experiments, we investigated whether cyclin degradation could still be observed when the reaction was started by adding $CaCl_2$ to only 0.6 mM (total amount). This was found to increase free Ca^{2+} concentration from <0.1 μM to 0.5−1.5 μM (depending on the extracts), as monitored by a Ca^{2+} specific electrode. As shown in Figure 2A (panel a, left) this small increase of Ca^{2+} was sufficient to trigger cyclin degradation. In contrast with the previous conditions, neither calpastatin (Figure 2A, panel a, right) nor leupeptin (data not shown) was able to inhibit the cyclin degradation pathway. Moreover this pathway was turned off (Figure 2A, panel b) soon after the first round of cyclin degradation. Micromolar Ca^{2+} did not only trigger degradation of trace amounts of *in vitro* produced ^{35}S-labelled cyclin, but also that of endogenous cyclin B_2 or that of large amounts of cdc2-associated starfish cyclin B (data not shown). When free Ca^{2+} concentration was clamped at values intermediate between the micromolar and the millimolar range, neither the calpastatin-sensitive nor the calpastatin-insensitive cyclin degradation were detected (data not shown). This indicates, first that the calpastatin-insensitive pathway is inhibited when free Ca^{2+} is higher than the micromolar range, second that the calpastatin-sensitive pathway requires free calcium in the millimolar range. As shown in Figure 2B, cdc2 kinase activity is inhibited at high Ca^{2+} concentration, even in the

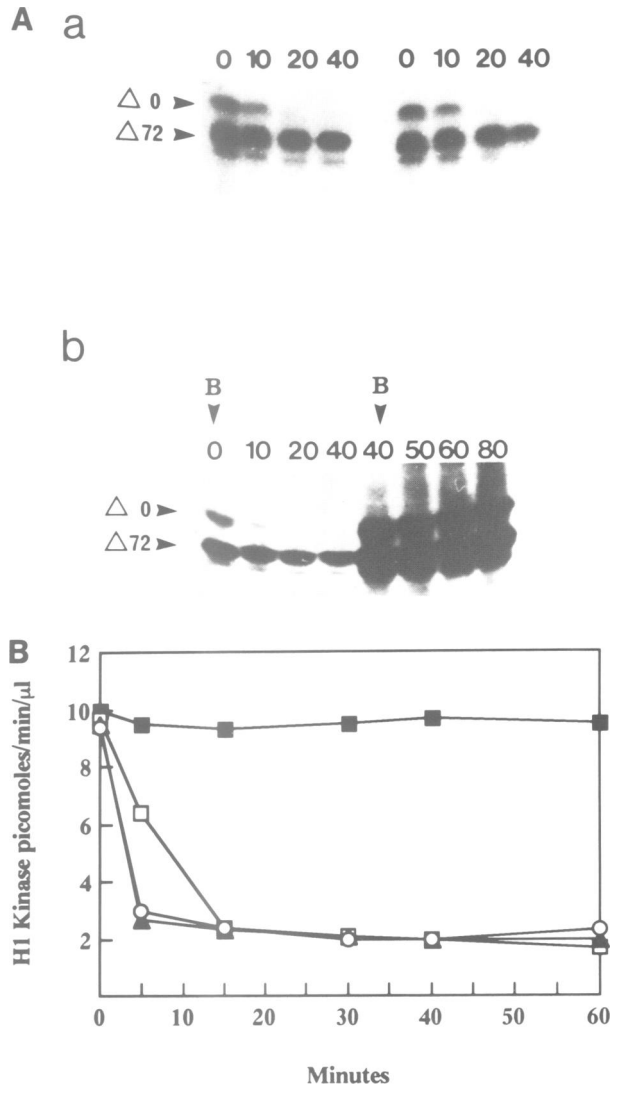

Fig. 2. Micromolar free Ca^{2+} activates a calpastatin-insensitive cyclin degradation pathway. (**A**) [^{35}S]methionine-labelled *in vitro* translated cyclin B was added to an extract prepared from metaphase II-arrested eggs. Degradation was started by raising free Ca^{2+} concentration to 1−1.5 μM at time 0. Panel a: incubation in the presence (right) or absence (left) of 20 μM calpastatin peptide. Panel b: at 40 min (second arrow), (i.e. after endogenous cdc2 kinase had been inactivated during the first round of cyclin degradation) *in vitro* translated cyclin B was added for the second time. Samples were taken at the indicated times and processed (see legend to Figure 1) to monitor cyclin degradation. (**B**) Time course of H1 histone kinase activity in extracts prepared from metaphase II-arrested eggs after increasing free Ca^{2+} concentration to 1−1.15 μM (□) or 0.5−1 mM) (○, ▲). Samples were taken at the indicated times after Ca^{2+} addition and processed as indicated in Materials and methods for determination of H1 histone kinase activities. In the absence of added Ca^{2+}, H1 histone kinase remained stable for at least 60 min at room temperature (■). At millimolar free Ca^{2+}, it decreased both in the absence (○) or presence (▲) of 20 μM calpastatin. In the last case (▲), ^{35}S-labelled cyclin B was not destroyed when added as a marker to monitor cyclin degradation (not shown). In all other cases degradation occurred following Ca^{2+} addition.

presence of calpastatin where no cyclin degradation occurs. Since cdc2 kinase has been shown to control cyclin degradation in the normal cell cycle (Felix *et al.*, 1990; Lorca *et al.*, 1991), this suggests that inhibition of the calpastatin-insensitive pathway at high Ca^{2+} concentration is due to inhibition of cdc2 kinase activity.

The above results suggested that the *in vitro* assay performed at micromolar free Ca^{2+} in egg extracts reflected the physiological process of cyclin degradation which occurs at fertilization, when eggs exit from meiotic arrest. During the regular cell cycle, cyclins are degraded by the ubiquitin pathway (Glotzer *et al.*, 1991). Degradation is impaired in mutants altered in the destruction box, a limited region in the N-terminus of cyclins. In starfish this corresponds to R31 GALENISN. To investigate whether the ubiquitin pathway was also involved in the Ca^{2+}-dependent cyclin degradation at fertilization, we converted the invariant arginine at position 31 to an alanine to create the derivative cycB Ala31. Figure 3 shows that this mutant could not be degraded efficiently when Ca^{2+} concentration was made micromolar in metaphase extracts. In contrast it was readily degraded by calpain when free Ca^{2+} concentration was raised to the millimolar range. These results support the view that cyclin is degraded by the ubiquitin pathway following fertilization of metaphase II-arrested eggs, as it is in the subsequent cell cycles.

Micromolar free Ca^{2+} does not induce p39^{mos} degradation in vitro

Since p39^{mos} is believed to protect cyclin from proteolytic degradation in metaphase II-arrested oocytes, we asked if the proto-oncogene product underwent degradation when free Ca^{2+} was raised to the micromolar range, although this was not sufficient to activate calpain in our *in vitro* conditions. In preliminary experiments, we observed that *in vitro* translated p39^{mos} remained stable at micromolar level of free Ca^{2+} (Figure 4A). We were concerned that p39^{mos} might escape degradation due to improper folding of the *in vitro* translated protein. To eliminate this possibility, we investigated whether the *in vivo* synthesized p39^{mos} was also resistant to degradation in our *in vitro* assay. Extracts were prepared from progesterone-stimulated oocytes which had been incubated in the presence of [^{35}S]methionine during the process of meiotic maturation. They were then tested by immunoprecipitation and autoradiography for the presence of p39^{mos}, either before or 30 min after free Ca^{2+} concentration was raised to the micromolar level. In these experiments we used a polyclonal antiserum raised against bacterially produced *Xenopus* p39^{mos}. As shown in Figure 4B, p39^{mos} was present in similar amounts before and after

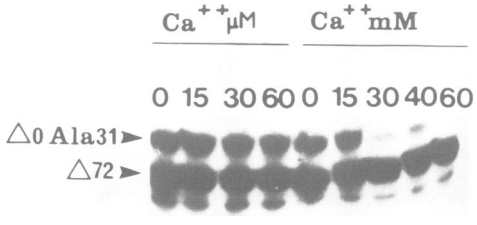

Fig. 3. An intact destruction box is required for cyclin degradation at micromolar but not millimolar free Ca^{2+}. The mutant cyclin B Ala31 (where an alanine residue was substituted to arginine at position 31 of starfish cyclin B) was translated *in vitro*, producing both the full-length and a truncated protein lacking 72 amino acids from the N-terminus, as for the wild type protein. It was added to extracts prepared from metaphase II-arrested eggs, then $CaCl_2$ was added to raise free Ca^{2+} concentration to either the micromolar (left) or the millimolar range (right). Degradation was monitored as a function of time after $CaCl_2$ addition, as described in legend to Figure 1.

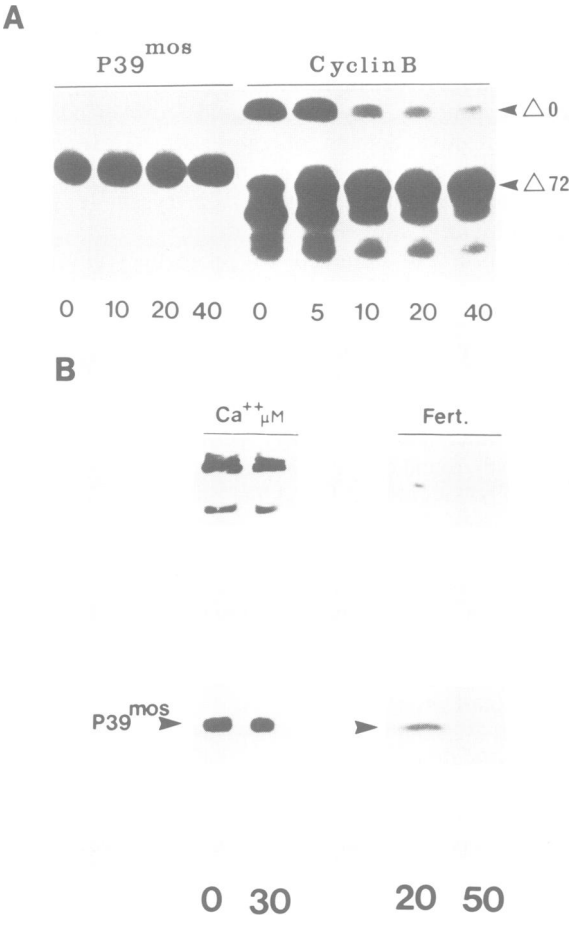

Fig. 4. Stability of p39^{mos} in extracts prepared from metaphase II-arrested eggs after raising free Ca^{2+} concentration to the micromolar range. (**A**) An extract prepared from metaphase II-arrested eggs was divided into two parts. One received [^{35}S]methionine-labelled *Xenopus* P39^{mos}, the other [^{35}S]methionine-labelled starfish cyclin B, both translated *in vitro* from their corresponding mRNAs, then free Ca^{2+} concentration was increased to the micromolar level. Samples were collected at the indicated times and processed as described in the legend to Figure 1 to monitor degradation of either p39^{mos} or cyclin B. (**B**) Left: stage VI oocytes were stimulated with progesterone in the presence of [^{35}S]methionine. An extract prepared from these oocytes after they had arrested at metaphase II was divided in two equal parts. One received $CaCl_2$ to raise free Ca^{2+} to the micromolar range, the other remained unchanged. Thirty minutes after $CaCl_2$ addition, a polyclonal antiserum against bacterially produced *Xenopus* p39^{mos} was added to both samples. Immunoprecipitation, analysis of the immunoprecipitated material by gel electrophoresis and fluorography were performed as described in Materials and methods. 0: sample without added Ca^{2+}. 30: sample immunoprecipitated 30 min after Ca^{2+} addition. It was checked in the same experiment that starfish cyclin B readily underwent degradation earlier than 15 min after Ca^{2+} addition to this extract (data not shown). Right: eggs were homogenized at various times after fertilization in XB buffer containing 5 mM EGTA, 10 µg/ml cytochalasin and 4 µg/ml nocodazole. After 15 min centrifugation at 10^5 *g*, the cytoplasmic layer was collected and analysed for the presence of p39^{mos} by SDS–PAGE followed by immunoblotting. Identical amounts of proteins were loaded for each sample, 20 and 50: samples prepared 20 and 50 min after fertilization, respectively (no change in the amount of p39^{mos} was detected earlier than 20 min).

Ca^{2+} treatment, while cyclin B was degraded. In the same way, no $p39^{mos}$ degradation was detected as late as 20 min after fertilization, although cyclin degradation had already occurred at that time (Watanabe *et al.*, 1989; T.Lorca, unpublished results).

A Ca^{2+}-calmodulin-dependent event triggers cyclin degradation in metaphase II-arrested eggs

The above results demonstrate that a Ca^{2+}-dependent event triggers cyclin proteolysis, which involves neither calpain nor $p39^{mos}$ degradation. To investigate more precisely how Ca^{2+} initiates cyclin degradation, free calcium was raised to the micromolar level, then returned to its initial value by adding EGTA after various times. As shown in Figure 5, cyclin degradation was irreversibly triggered when free Ca^{2+} was kept at a micromolar level for only 30 s, and occurred 10 min later.

Physiological effects of transient increases of Ca^{2+} are often mediated by Ca^{2+}−calmodulin dependent enzymes, which are almost inactive at basal level of free intracellular Ca^{2+} and become activated upon various cell stimulations that transiently elevate Ca^{2+} to 1 μM or higher (for review, see Manalan and Klee, 1984). To investigate whether the Ca^{2+}−calmodulin complex was involved in initiating the process of cyclin degradation, we used MLCK(488−511), a peptide of chicken gizzard myosin light chain kinase that binds Ca^{2+}−calmodulin with high affinity (K_a = 1 nM) and thereby inhibits the Ca^{2+}−calmodulin dependent protein kinase. This peptide was expected to compete for Ca^{2+}−calmodulin with any Ca^{2+}−calmodulin enzyme which could possibly be involved in triggering cyclin degradation. When added at a final concentration of ≥ 100 μM before raising free Ca^{2+} for 30 s to the micromolar range, the peptide readily suppressed both cyclin degradation (Figure 6A) and MPF inactivation (data not shown). The relatively high concentration of peptide required for inhibition is not surprising because calmodulin has been shown to be present at a minimal concentration of 34 μM in the cytosol of *Xenopus* oocytes (Cartaud *et al.*, 1980). In contrast MLCK(488−511) had no effect when added either simultaneously with EGTA to terminate the transient rise in Ca^{2+}, 30 s after Ca^{2+} addition (data not shown) or together with 15 μM calmodulin, before Ca^{2+} (Figure 6B). As expected, MLCK(488−511) had no effect on calpain-dependent cyclin degradation (data not shown). Besides binding to Ca^{2+}−calmodulin, the synthetic peptide MLCK(480−511), whose sequence overlaps that of the peptide used in the present work, has been shown to inhibit to some extent protein kinase C (Smith *et al.*, 1990). We found, however, that PKC(19−36), a synthetic peptide corresponding to the auto-inhibitory domain of protein kinase C, and which strongly inhibits this kinase (Smith *et al.*, 1990), had no effect on cyclin degradation (Figure 6C), although it did not undergo significant degradation in egg extracts before the end of the Ca^{2+}-dependent period (data not shown). This eliminated the possibility that inhibition of cyclin degradation by MLCK(488−511) might be due to non-specific inhibition of protein kinase C.

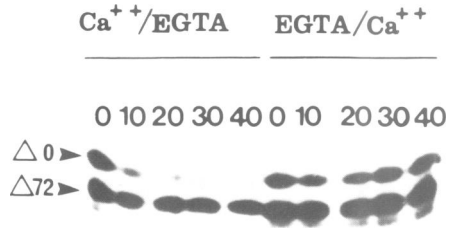

Fig. 5. A 30 s period of elevated free Ca^{2+} is sufficient to trigger cyclin degradation. [^{35}S]methionine-labelled cyclin B (1.2 μl) was added to a metaphase extract (30 μl containing 5 mM EGTA), which was divided in two equal parts. One (left) received 1 μl of 20 mM $CaCl_2$ (this raised free Ca^{2+} concentration to 1−1.5 μM) and 30 s later 4 μl of 200 mM EGTA (this clamped free Ca^{2+} concentration to 0.1 μM or less). The second part (right) first received 4 μl of 200 mM EGTA, then 1 μl of 20 mM $CaCl_2$ (free Ca^{2+} concentration did not increase above 0.1 μM in this case). Samples were taken at the indicated times after either $CaCl_2$ (left) or EGTA addition (right) and processed for determination of cyclin degradation.

Discussion

It has been proposed that the *c-mos* proto-oncogene product undergoes specific proteolysis by calpain on fertilization of *Xenopus* eggs (Watanabe *et al.*, 1989). This was based on

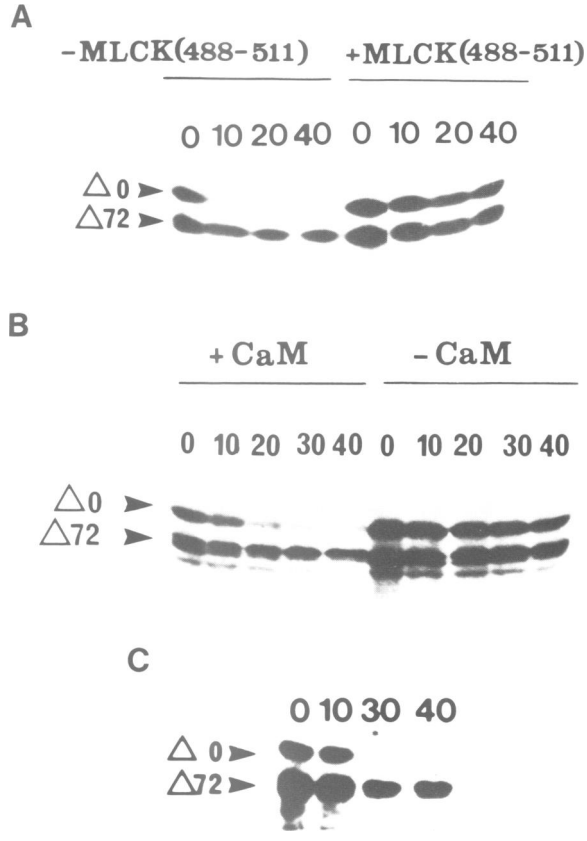

Fig. 6. Effect of synthetic peptides MLCK(488−511) and PKC(19−36) on proteolysis of ^{35}S-labelled starfish cyclin B, induced by raising free Ca^{2+} to the micromolar range in metaphase extracts. (**A**) incubation was in the presence (right) or absence (left) of 80 μM MLCK(488−511). (**B**) Calmodulin reverses inhibition of cyclin proteolysis by MLCK(488−511). ^{35}S-labelled starfish cyclin B was added to metaphase extracts in the presence or absence of 17 μM ram testis calmodulin. MLCK(488−511) was added to 80 μM in both samples, then free Ca^{2+} was raised to the micromolar level by adding $CaCl_2$ to 0.6 mM. Samples were taken at the indicated times after $CaCl_2$ addition and processed as described in the legend to Figure 1 to monitor cyclin degradation. (**C**) as in (A), but 100 μM PKC(19−36) was used in place of MLCK(488−511). Samples were taken at the indicated times and processed to monitor cyclin degradation.

the following immunoprecipitation experiments. *In vivo*, it was shown that [^{35}S]methionine-labelled p39^{mos} can be immunoprecipitated with specific antibodies against the *Xenopus* proto-oncogene from progesterone-stimulated oocytes arrested at second meiotic metaphase, but not from the same oocytes after they had been activated either by pricking with a needle or with the calcium ionophore A23187. In addition, p39^{mos} could be detected in 150 000 *g* supernatants prepared from crushed unfertilized, but not fertilized eggs. *In vitro* it was shown that addition of 5 mM $CaCl_2$ to cytosols prepared from unfertilized eggs destroys p39^{mos}, and that calpastatin inhibits this degradation.

In the present work, we confirmed that calpain is able to degrade p39^{mos} in extracts prepared from unfertilized *Xenopus* eggs. However, it is not specific for p39^{mos} since it also degrades cyclin. Moreover calpain-dependent degradation of either p39^{mos} or cyclin was observed only when free Ca^{2+} concentration was raised to the millimolar range. No calpastatin-sensitive degradation of either p39^{mos} or cyclin was detected at micromolar free Ca^{2+}, indicating either that only calpain II activity, not I, is present in eggs extracts or that calpain I degrades neither p39^{mos} nor cyclin. This argued against the possibility that calpain might be involved in the degradation of either p39^{mos} or cyclin at fertilization, where free Ca^{2+} is elevated only to the micromolar range. There is no major difficulty in explaining the results of Watanabe *et al.* Indeed, unphysiological activation by pricking or ionophore treatment is expected to elevate cytosolic Ca^{2+} at least transiently to its millimolar value in the external medium, and this could have artefactually activated calpain. Moreover, failure to detect p39^{mos} after fertilization in high speed supernatants could be due to its translocation from cytosol to a pelletable material. This would not have been surprising because extensive reorganization of the cytoskeleton occurs when cells exit from M phase (Karsenti *et al.*, 1984; Verde *et al.*, 1990; Chou *et al.*, 1990; Yamashiro *et al.*, 1991). Nonetheless we were able to confirm, by direct immunoblotting of homogenates treated with both cytochalasin and nocodazole, that p39^{mos} disappears after fertilization although it does so only after cyclins have undergone proteolysis.

Although it does not primarily trigger p39^{mos} destruction, the burst of free Ca^{2+} associated with fertilization releases the cyclin degradation pathway from its CSF-inhibited state, resulting in MPF inactivation (Masui and Markert, 1971; Gerhart *et al.*, 1984; Dorée *et al.*, 1990). In the intact egg, free Ca^{2+} rises from a resting value of 0.4 μM to a peak of ~1.2 μM over the course of 2 min after fertilization (Busa and Nucitelli, 1985). The calcium transient propagates with a velocity of ~8 μm/s as a 45 s 'wide' ring of elevated free Ca^{2+} from the sperm entry point to its antipodes (Kubota *et al.*, 1987). The 45 s width of the Ca^{2+} wave fits well our finding that increasing free Ca^{2+} to the micromolar level for only 30 s is sufficient to release the cyclin degradation pathway from its inhibited state in extracts prepared from metaphase-arrested eggs. In such conditions, the degradation machinery was activated for a single round and inactivated soon after cyclin proteolysis, as it is during the normal cell cycle. This was not the case when calpain was activated at millimolar Ca^{2+} concentration. Moreover substitution of Ala31 for R31 in the destruction box suppressed cyclin B degradation at micromolar Ca^{2+}, suggesting that cyclin is degraded by the ubiquitin pathway following fertilization of oocytes arrested at second meiotic metaphase, as it is in the regular mitotic cell cycle. These results suggest that the fine regulation of the cyclin degradation machinery is conserved in our *in vitro* conditions and validate our main result that degradation of the proto-oncogene p39^{mos} is not necessary for cyclin proteolysis and exit from meiotic metaphase.

What may be the nature of the Ca^{2+}-dependent event required to free the cyclin degradation pathway? It was recently proposed that protein kinase C acts downstream of calcium to induce cytoplasmic events of the first mitotic cell cycle (Bement and Capco, 1990). To support this view, it was shown that inhibitors of protein kinase C blocked cortical granule exocytosis and cortical contraction, when eggs were challenged for activation by treatment with either the calcium ionophore A23187 or phorbol 12-myristate-13 acetate (PMA), an activator of protein kinase C. In the present work we showed that PKC(19–36), a strong inhibitor of protein kinase C, failed to inhibit Ca^{2+}-dependent activation of cyclin degradation. This indicates that protein kinase C is probably not involved in the basic mechanisms that control exit from M phase, although it may play a role downstream of the calcium wave in morphological changes associated with egg activation, such as membrane fusion events. Protein kinase C may also be involved in the Ca^{2+} wave propagation, by decreasing Ca^{2+} concentration through the activation of the Ca^{2+} transport ATPase and the Na^+/Ca^{2+} exchanger, both of which remove Ca^{2+} from the cytosol (Ogita *et al.*, 1990). In contrast, we found that a synthetic peptide corresponding to the auto-inhibitory domain of myosin light chain kinase, which binds Ca^{2+}–calmodulin with high affinity, prevented micromolar free Ca^{2+} to trigger cyclin degradation. The peptide had no effect when added either simultaneously with EGTA to terminate the transient rise in Ca^{2+}, or together with calmodulin. This provided strong evidence that formation of a Ca^{2+}–calmodulin complex is required at fertilization or parthenogenetic activation as an early event (the first 30 s) following elevation of intracellular free Ca^{2+}, to release the cyclin degradation pathway from its inhibited state, resulting in exit from M phase. The target for Ca^{2+}–calmodulin remains to be identified. Possible candidates include a Ca^{2+}–calmodulin-dependent protein kinase and calcineurin.

Materials and methods

Egg extracts

Extracts from unfertilized eggs were prepared according to Murray *et al.* (1989). Eggs were dejellied in 2% cysteine (pH 7.8), washed four times in MMR/2 (50 mM NaCl, 1 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.05 mM Na–EGTA, 2.5 mM NaHEPES, pH 7.7) and two times in XB (100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM K–HEPES, 50 mM sucrose, pH 7.7). Eggs were then transferred to centrifuge tubes containing XB plus 5 mM EGTA, 10 μg/ml cytochalasin B and 10 μg/ml cycloheximide at 4°C. Excess buffer was removed prior to centrifugation for 10 min at 15 000 *g*. The cytoplasmic layer was collected from the crushed eggs and an ATP-regenerating system consisting of 10 mM creatine phosphate, 80 μg/ml creatine kinase, 1 mM ATP (final concentrations), was added. After spinning for 15 min at 15 000 *g*, the supernatant was collected and kept at −70°C.

[^{35}S]methionine-labelled extracts were prepared in a similar way from progesterone-stimulated oocytes incubated in the presence of 1 mCi/ml [^{35}S]methionine during progesterone-induced meiotic maturation, except that the cysteine treatment was omitted.

In vitro production of [^{35}S]methionine-labelled proteins and purification of calmodulin

Starfish cyclin B and *Xenopus* p39^{mos} mRNA were transcribed from the corresponding full-length clones (pTZ-cycB and pRF145) (Labbé *et al.*, 1989; Freeman *et al.*, 1989) with T7 and SP6 RNA polymerases, respectively. Translation was made in the presence of [^{35}S]methionine in a rabbit reticulocyte lysate. The mutant cycB Ala31 was generated according to an oligonucleotide-directed *in vitro* mutagenesis system from Amersham, UK.

Calmodulin was purified to apparent homogeneity from ram testis, as described previously (Autric *et al.*, 1980).

Assays for cyclin B and p39^{mos} degradation and H1 histone kinase in extracts

The translation mix containing ^{35}S-labelled proteins was added to the metaphase extracts in a proportion of 1 vol, per 30 vol of extract. After incubation at room temperature, 4 μl aliquots were transferred into 20 μl of SDS gel sample buffer. Samples were run on 10% polyacrylamide gels. Gels were treated with an enhancer for low energy radiations (Dupont), dried, and kept at −70°C for fluorography. H1 histone kinase assays were run as described in Felix *et al.* (1989).

Peptide synthesis

Solid phase synthesis of the MLCK(488−511) peptide was performed on Milligen 9050 peptide synthesizer using a fluorenylmethoxycarbonyl (FMOC) group as temporary amino protection. An analogue of the MLCK(488−511) peptide with replacement of W497 and F497 was also synthesized using the same method, with identical results in the inhibition assay of cyclin degradation.

For synthesis of PKC(19−30), *t*-butoxycarbonyl aspartic cyclohexyl ester (BOC) was used as temporary amino protection, and trifluoroacetic acid (TFA) to remove BOC protection. All peptides were purified by HPLC and assessed by sequencing before use.

Antibodies and immunochemical procedures

Polyclonal antibodies were produced in rabbit against bacterially produced starfish cyclin B (amino acids 26−362) and *Xenopus* p39^{mos} (full length). The proteins to be injected were eluted from gels run in the presence of SDS. For blotting experiments, antiserum were further purified by affinity on a matrix consisting of the corresponding protein covalently bound to Sepharose.

For immunoprecipitation experiments, extracts (40 μl) were diluted 25-fold in a RIPA buffer consisting of 10 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 80 mM β-glycerophosphate, 50 mM sodium fluoride and 2 mM phenylmethylsulphonyl fluoride (PMSF) at pH 7.6, then the anti-*Xenopus* p39^{mos} antiserum (20−30 μl) was added. After a 1 h incubation at 4°C immune complexes were pelleted by centrifugation after the addition of protein A−Sepharose. Pellets were washed three times in RIPA buffer and once in 50 mM Tris pH 7.6, then the immune complexes were analysed by gel electrophoresis followed by fluorography.

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References

Autric,F., Ferraz,C., Kilhoffer,M.C., Cavadore,J.C. and Demaille,J. (1980) *Biochim. Biophys. Acta*, **631**, 139−147.

Bement,W.M. and Capco,D.G. (1990) *Cell Regul.*, **1**, 315−326.

Busa,W.B. and Nucitelli,R. (1985) *J. Cell. Biol.*, **100**, 1325−1329.

Capony,J.P., Picard,A., Peaucellier,G., Labbé,J.C. and Dorée,M. (1986) *Dev. Biol.*, **117**, 1−12.

Cartaud,A., Ozon,R., Walsh,M.P., Haiech,J. and Demaille,J. (1980) *J. Biol. Chem.*, **255**, 9404−9408.

Chou,Y.H., Bischoff,J.R., Beach,D. and Goldman,R.D. (1990) *Cell*, **62**, 1063−1071.

Cross,N.L. (1981) *Dev. Biol.*, **85**, 380−384.

Dorée,M., Cavadore,J.C. and Picard,A. (1990) *J. Reprod. Fertil. Suppl.*, **42**, 135−140.

Felix,M.A., Labbé,J.C., Dorée,M., Hunt,T. and Karsenti,E. (1990) *Nature*, **346**, 379−382.

Freeman,R.S., Pickham,K.M., Kanki,J.P., Lee,B.A., Pena,S.V. and Donoghue,D.J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 5805−5809.

Gautier,J., Minshull,J., Lohka,M.J., Glotzer,M., Hunt,T. and Maller,J.L. (1990) *Cell*, **60**, 487−494.

Gerhart,J., Wu,M. and Kirschner,M. (1984) *J. Cell. Biol.*, **98**, 1247−1255.

Glotzer,M., Murray,A.W. and Kirschner,M.W. (1991) *Nature*, **349**, 132−138.

Jaffe,L.F. (1983) *Dev. Biol.*, **95**, 265−276.

Karsenti,E., Newport,J., Hubble,R. and Kirschner,M. (1984) *J. Cell Biol.*, **98**, 1730−1745.

Kemp,B.E., Pearson,R.B., Guerriero,V., Bagchi,I. and Means,A.R. (1987) *J. Biol. Chem.*, **262**, 2542−2548.

Kubota,H.Y., Yoshimoto,Y., Yoneda,M. and Hiramoto,Y. (1987) *Dev. Biol.*, **119**, 129−136.

Labbé,J.C., Picard,A., Karesenti,E. and Dorée,M. (1988) *Dev. Biol.*, **127**, 157−169.

Labbé,J.C., Capony,J.P., Caput,D., Cavadore,J.C., Derancourt,J., Kaghad,M., Lelias,J.M., Picard,A. and Dorée,M. (1989) *EMBO J.*, **8**, 3053−3058.

Lorca,T., Fesquet,D., Zindy,F., le Bouffant,F., Cerruti,M., Brechot,C., Devauchelle,G. and Dorée,M. (1991) *Mol. Cell Biol.*, **11**, 1171−1175.

Maki,M., Bagci,H., Hamaguchi,T., Vedo,M., Murachi,T. and Hatanaka,M. (1989) *J. Biol. Chem.*, **264**, 18866−18869.

Manalan,A.S. and Klee,C.B. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res.*, **18**, 226−278.

Masui,Y. and Markert,C.L. (1971) *J. Exp. Zool.*, **177**, 129−146.

Melloni,E. and Pontremoli,S. (1989) *Trends Neurosci.*, **12**, 438−443.

Meyerhof,P.G. and Masui,Y. (1977) *Dev. Biol.*, **61**, 214−229.

Murray,A.W., Solomon,M.J. and Kirschner,M.W. (1989) *Nature*, **339**, 280−286.

Ogita,K., Koide,H., Kikhawa,V., Kishimoto,A. and Nishizuka,Y. (1990) *Adv. Second Messenger Phosphoprotein Res.*, **24**, 218−224.

Sagata,N., Oskarson,M., Copeland,T., Brumbaugh,J. and Vande Woude,G.F. (1988) *Nature*, **335**, 515−525.

Sagata,N., Watanabe,N., Vande Woude,G.F. and Ikawa,Y. (1989) *Nature*, **342**, 512−518.

Shibuya,E.K. and Masui,Y. (1988) *Dev. Biol.*, **129**, 253−264.

Smith,M.K., Colbran,R.J. and Soderling,T.R. (1990) *J. Biol. Chem.*, **265**, 1837−1840.

Standart,N., Minshull,J., Pines,J. and Hunt,T. (1987) *Dev. Biol.*, **124**, 248−258.

Verde,F., Labbe,J.C. Dorée,M. and Karsenti,E. (1990) *Nature*, **343**, 233−238.

Watanabe,N., Vande Woude,G.F., Ikawa,Y. and Sagata,N. (1989) *Nature*, **342**, 505−511.

Westendorf,J.M., Swenson,K.I. and Ruderman,J.V. (1989) *J. Cell Biol.*, **108**, 1431−1444.

Yamashiro,S.Y., Yamakita,Y., Hosoya,H. and Matsurama,F. (1991) *Nature*, **349**, 169−172.

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