

Inhibiting proteasome activity causes overreplication of DNA and blocks entry into mitosis in sea urchin embryos

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SUMMARY

The proteasome has been shown to be involved in exit from mitosis by bringing about destruction of mitotic cyclins. Here, we present evidence that the proteasome is also required for proper completion of S phase and for entry into mitosis in the sea urchin embryonic cleavage cycle. A series of structurally related peptide-aldehydes prevent nuclear envelope breakdown in their order of inhibitory efficacies against the proteasome. Their efficacies in blocking exit from S phase and exit from mitosis correlate well, indicating that the proteasome is involved at both these steps. Mitotic histone H1 kinase activation and tyrosine dephosphorylation of p34^{cdc2} kinase are blocked by inhibition of the proteasome, indicating that the

proteasome plays an important role in the pathway that leads to embryonic p34^{cdc2} kinase activation. Arrested embryos continued to incorporate [³H]thymidine and characteristically developed large nuclei. Pre-mitotic arrest can be overcome by treatment with caffeine, a manoeuvre that is known to override the DNA replication checkpoint. These data demonstrate that the proteasome is involved in the control of termination of S phase and consequently in the initiation of M phase of the first embryonic cell cycle.

Key words: Proteasome, Mitosis, p34^{cdc2} kinase, DNA synthesis, Sea urchin embryo

INTRODUCTION

It is widely believed that the activation of the mitotic kinase complex (MPF) is controlled by an intricate series of phosphorylation and dephosphorylation reactions, involving p34^{cdc2} in association with the mitotic cyclins. Dephosphorylation of tyrosine 15 and threonine 14 and the phosphorylation of threonine 161 of p34^{cdc2} kinase are considered to be critical steps governing the initiation of mitosis entry (Gould and Nurse, 1989; Norbury et al., 1991; Poon et al., 1993; Solomon et al., 1993; Fesquet et al., 1993; Patel et al., 1999). Gradual accumulation of cyclin B during the cell cycle pre-activates p34^{cdc2}, causing it to accumulate in the nucleus (Pines and Hunter, 1991; Ookata et al., 1992; Hagting et al., 1998), though full activation of p34^{cdc2} kinase occurs abruptly at the time of mitosis onset. Cyclin synthesis is absolutely required for entry into mitosis in sea urchin embryos; they accumulate suprathreshold cyclin levels long before the time of mitosis entry (Wagenaar, 1983; Dorée and Dubé, 1988; Hunt et al., 1992; Geneviere-Garrigues et al., 1995).

Proteolysis also plays an important part in regulation of mitosis. For example, cyclin B and the anaphase inhibitors Pds1p/cut2 and their homologues are destroyed at the metaphase/anaphase transition by the ubiquitin-proteasome

pathway (Glotzer et al., 1991; Holloway et al., 1993; Deveraux et al., 1995; Cohen-Fix et al., 1996; Funabiki et al., 1996). It has recently been shown that the degradation of Pds1p protein is essential for separation of sister chromatids at the metaphase/anaphase transition (Ciosk et al., 1998). All this suggests that the proteasome must be one of the key regulatory complexes involved in regulating mitosis exit. The endogenous G₁/S cyclin-dependent kinase (CDK) inhibitors such as p27 and p40^{sic1} are also destroyed by the ubiquitin-proteasome dependent pathway in the G₁ phase of the yeast and mammalian somatic cell cycle (Schwob et al., 1994; Peter and Herskowitz, 1994; Pagano et al., 1995).

The 26S proteasome, a high-molecular mass ATP-dependent proteinase complex, is thought to be a crucial component in the ubiquitin-dependent proteolytic system (Goldberg, 1992; Rechsteiner et al., 1993; Ciechanover, 1994; Hiltz and Wolf, 1995; Coux et al., 1996). The 26S proteasome consists of the 20S proteasome (Tanaka and Tanahashi, 1997) and regulatory complexes, which contain multiple ATPases that can activate the latent 20S proteasome (Orino et al., 1991; DeMartino et al., 1994; Hoffman and Rechsteiner, 1994; Kawahara and Tanaka, 1997). We and others have found that the intracellular distribution of the proteasome changes during the cell division cycle, particularly at M phase (Kawahara and Yokosawa, 1992,

Amsterdam et al., 1993; Rivett and Knecht, 1993; Palmer et al., 1994; Wilkinson et al., 1998). Moreover, proteasome activity is regulated during the mitotic cell cycle in the ascidian embryo: its activity increases both before NEB and at the metaphase/anaphase transition (Kawahara et al., 1992). There is evidence from *Xenopus* embryos that the cdk1 inhibitory kinase wee1 is degraded via the proteasome (Michael and Newport, 1998). To verify whether the proteasome has a crucial role in the embryonic cell cycle at this point, we undertook to inhibit the proteasome specifically in the sea urchin embryonic cleavage cycle (Lee and Goldberg, 1998). Rock et al. (1994) developed a series of peptide-aldehydes, which strongly inhibit the proteasome's chymotrypsin-like, peptidyl glutamyl hydrolase-like, caseinolytic and poly-ubiquitinated protein degrading activity.

Using a series of proteasome inhibitors, we present evidence that the proteasome-mediated pathway is involved not only at the mitosis exit step but also at the S/M boundary (entry into mitosis) where proteasome involvement had not adequately been characterised. Inhibition of the proteasome correlates with continuation of DNA synthesis and inhibition of mitotic p34^{cdc2} kinase activation and is rescued by treatment with caffeine, a substance known to override the DNA replication checkpoint at S/M (Boynton et al., 1974; Lau and Pardee, 1982; Schlegel and Pardee, 1987; Patel et al., 1997; Kumagai et al., 1998; Wang et al., 1999). We propose that the proteolysis of some crucial protein(s) is essential for the termination of S phase and the onset of mitotic p34^{cdc2} kinase activation during the first sea urchin embryonic cell cycle.

MATERIALS AND METHODS

Sea urchins

Lytechinus pictus eggs were obtained and fertilised as described before (Patel et al., 1997). To ensure good cell cycle synchrony, we have used eggs obtained from individual females unless otherwise described. In every experiment, we confirmed that more than 95% of control embryos underwent normal fertilisation to ensure the quality of eggs. All experiments were carried out at 14–15°C.

Treatment with inhibitors

The peptide-aldehyde inhibitors were usually added at 10 minutes after fertilisation unless otherwise stated. Briefly, synchronously fertilised eggs were immediately divided into portions in multiwell plates, allowed to settle and the supernatant of each culture replaced by artificial sea water (ASW) containing various peptide-aldehyde inhibitors at the final concentration described for each experiment. Then the embryos were gently resuspended every 10 minutes. All stock solutions of peptide-aldehyde inhibitors were prepared in DMSO and the final concentration of DMSO in ASW was 0.5%, including control experiments unless otherwise stated. At concentrations of DMSO up to 1%, the embryos develop normally to swimming mesenchyme blastula-stage larvae.

Caffeine (Sigma) was used at final concentration of 5 mM and dissolved in ASW as previously described (Patel et al., 1997).

Measurement of proteasome and histone H1 kinase activity

HMW protein fractions were prepared from fertilised sea urchin eggs as described previously (Kawahara and Yokosawa, 1994). Equal protein amounts of the HMW fractions were used for enzymatic assay as described previously (Kawahara and Yokosawa, 1994).

Histone H1 kinase assays were performed as in Meijer et al. (1989)

with modification. Harvested eggs were mixed with ice-cooled homogenisation buffer (60 mM β -glycerophosphate, 25 mM Mops, 15 mM EGTA, 15 mM MgCl₂, 1 mM DTT, 15 mM p-nitrophenylphosphate, 0.1 mM Na₃VO₄, 0.1 mM NaF, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 10 μ g/ml soybean trypsin inhibitor, 100 μ M benzamidine, pH 7.2) and crushed by sonication. Homogenates were centrifuged and diluted supernatants were added to an assay mixture that contained 1 mg/ml histone H1 (Sigma, type III-S) as described. Kinase reactions were carried out for 10 minutes at 30°C and these samples were subjected to 10% SDS-PAGE. Incorporation of ³²P into histone H1 was visualised by autoradiography using X-ray film or quantified phosphorylated signals of histone H1 using the FujiBAS1500 phosphorimager.

Measurement of protein synthesis

At 10 minutes after fertilization, eggs were mixed with ASW containing 45.5 μ Ci/ml L-[³⁵S]methionine (Amersham). At the times indicated, egg suspension was added to ice-cold 25% TCA and stored on ice. Samples were centrifuged, and air-dried pellets were dissolved in SDS-sample buffer. The protein concentrations of the samples were determined using a BCA protein assay kit (Pierce) and equal amounts of protein samples were subjected to SDS-PAGE. After fixation in 5% TCA, gels were stained using Amido black to confirm that each lane contained the same amounts of protein.

The incorporation of [³⁵S]methionine was visualised by autoradiography and quantified using a phosphorimager.

Immunoblotting

The extracts prepared for histone H1 kinase assay were also used for western blotting. Extracts were mixed with suspension of p13^{suc1} beads (Meijer et al., 1989), incubated for 60 minutes at 4°C and washed with beads buffer. The beads were resuspended in SDS-sample buffer, and then subjected to SDS-PAGE and immunoblot analysis.

For analysis of tyrosine phosphorylation of p34^{cdc2}, anti-phosphotyrosine monoclonal antibody (Sigma immunochem., 1:500 dilution) was used. Anti-PSTAIRE antibody (Santa-Cruz, 1:500 dilution) and anti-cyclinB antibody (1:4000 dilution) were used to determine the amounts of p34^{cdc2} and cyclinB, respectively.

Measurement of [³H]thymidine incorporation

Dejellied unfertilised eggs were preincubated in 1 μ Ci/ml [³H]thymidine (Amersham) in ASW for 1.5 hours at 16°C, fertilised and cultured in the presence of 1 μ Ci/ml [³H]thymidine at 14°C. The cells were harvested at the indicated times and the incorporation of [³H]thymidine (Whitaker and Steinhardt, 1981) was quantified using a phosphorimager with BAS-TR2040s plate.

Reagents

Ac-Leu-Leu-nLeu-al (MG-101), and Ac-Leu-Leu-nMet-al (LLM) were purchased from Calbiochem (San Diego, CA, USA). Z-Leu-Leu-nVal-al (MG-115) and Z-Leu-Leu-Leu-al (MG-132) were purchased from the Peptide Institute (Osaka, Japan) and from AFFINITY Research Products Ltd, Mamhead, UK.

Ac-Leu-Leu-Arg-al (LLR, Leupeptin), E-64d, emetine, Z-norvaline and aphidicolin were purchased from Sigma (St Louis, MO, USA) and olomoucine from Promega, inc. (Madison, WI, USA). All other chemicals were of analytical grade purchased from BDH (Poole, UK) or Sigma unless otherwise noted.

RESULTS

Proteasome inhibitors specifically delay the onset of nuclear envelope breakdown in sea urchin embryonic cell cycle

We have previously found in ascidian oocytes (Kawahara et al.,

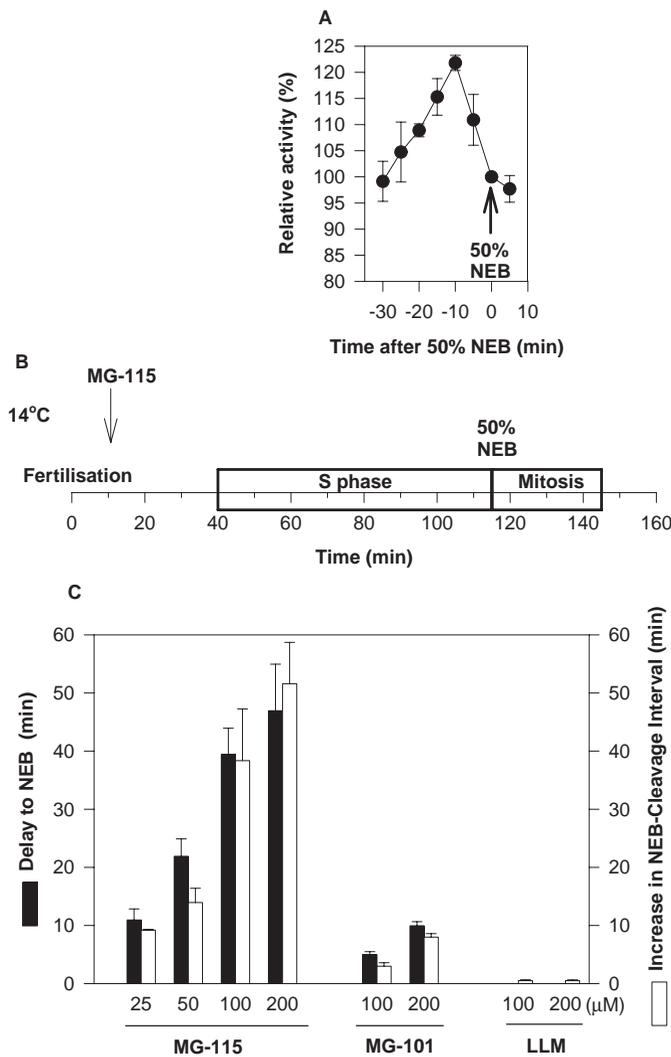


Fig. 1. (A) Changes in the activity of proteasomes before and after 50% NEB (nuclear envelope breakdown) during the first mitotic cell cycle of sea urchin. The protein fractions were prepared and their activity toward Succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide (0.02 mM) was assayed in the presence of 0.5 mM ATP. The activity at the time of 50% NEB was defined as 100%. (B) Cell cycle timing at 14°C for comparison, showing time of addition of MG-115. (C) Inhibition of NEB and cell cleavage by different peptide-aldehydes are well correlated in the first cell cycle of the sea urchin embryo. Peptide-aldehydes at different concentrations were added to the embryos, and the delay of NEB and the time elapsed between NEB and cell cleavage were determined. As a control 0.5% DMSO-treated embryos were used. Solid bar, delay of 50% NEB. Open bar, delay to the 50% NEB – 50% cell cleavage interval. Mean values \pm s.e.m. of three independent experiments are shown. Peptide-aldehydes were added to the embryo cultures within 10 minutes of fertilisation at the indicated concentrations. The proteasome inhibitor Z-Leu-Leu-nVal-al (MG-115) delayed the onset of NEB and also prolonged the time between 50% NEB to 50% cell cleavage in a dose-dependent manner, while the less potent proteasome inhibitor Ac-Leu-Leu-nLeu-al (MG-101) had only a small effect on both NEB and cell cleavage. Ac-Leu-Leu-Met-al (LLM), which is not effective against the proteasome, had no effect on the cell cycle progression.

1992) that the ATP-dependent activity of the 26S proteasome is increased at two points in the cell cycle: before NEB

(prophase) and during chromosome segregation (metaphase/anaphase). We confirmed the pre-NEB increase of proteasome activity in the sea urchin embryonic cell cycle (Fig. 1A). To determine whether the proteasome plays a critical regulatory role at these two points, we used a potent peptide-aldehyde proteasome inhibitor, Z-Leu-Leu-nVal-al (MG-115; Rock et al., 1994) to inhibit proteasome activity during the interphase of the sea urchin embryonic cell cycle.

We found that treatment with MG-115 caused a delay of first cleavage at concentrations above 25 μ M. 100 μ M MG-115 caused on average an 80 minute delay to first cleavage, compared to the control 0.5% DMSO-treated embryos at 15°C. Since mitotic cyclins have been shown to be degraded by the ubiquitin- and proteasome-dependent pathway (Glotzer et al., 1991; Gordon et al., 1993; Ghislain et al., 1993; Deveraux et al., 1995; Gordon et al., 1996) and the proteasome is an essential component of the ubiquitin-dependent proteolytic system (for a review, see Coux et al., 1996), it is natural to imagine that the delay to cleavage is caused by inhibition of mitotic cyclin degradation. Indeed, the interval from NEB to chromosome segregation is prolonged in MG-115-treated embryos compared to controls (Fig. 1C). However, our new finding is that MG-115 significantly delays the onset of nuclear envelope breakdown (NEB; Fig. 1C). 100 μ M MG-115 delays the onset of NEB by about 40 minutes compared to DMSO-treated control embryos. At a concentration of 100 μ M, the extent of delay to 50% NEB ranges from 20–135 minutes (42.4 ± 5.4 minutes, mean \pm s.e.m.) in 21 independent experiments at 15°C. The extent of delay to both NEB and cleavage is dose-dependent and both delays show very similar sensitivity to the inhibitor (Fig. 1C). These results suggest that MG-115 inhibits both transitions by the same mechanism.

The structurally related but less potent peptide-aldehyde, Ac-Leu-Leu-nLeu-al (MG-101), had a smaller effect; a significant delay of NEB was observed only above a concentration of 200 μ M. Furthermore, Ac-Leu-Leu-Met-al (LLM) and Ac-Leu-Leu-Arg-al (LLR) were without effect on the timing of NEB at concentrations up to 400 μ M (Fig. 1B, LLR not shown). This order of efficacies correlates well with their inhibitory potencies against the proteasome (Rock et al., 1994). Embryos treated with LLM or LLR developed to swimming mesenchyme blastula stage, indicating that there were no toxic effects of these peptide aldehydes. Neither L-norvaline, the characteristic constituent of MG-115, nor Z-norvaline (Z-nVal) affect the sea urchin cell cycle and development at a concentration of 200 μ M (not shown). These results strongly suggest that the effect of MG-115 is highly specific.

We found that MG-115 had to be added to the eggs within 15 minutes of fertilisation (see Fig. 1B) (this is about 70 minutes before NEB) in order to delay the first NEB significantly. The reasons why the inhibitor needs to be added so early relative to NEB may be that the peptide-aldehyde's inhibitory mechanisms are slow (Vinitsky et al., 1992) and because of the relatively poor cell membrane permeability of sea urchin embryos to this class of drug. There is a good evidence for involvement of the ubiquitin-proteasome pathway at mitosis exit (Glotzer et al; 1991; Ghislain et al., 1993; Gordon et al., 1993, 1996; Deveraux et al., 1995), so we have used the degree of delay of chromosome segregation in the same embryos as an internal control for blockage of the

intracellular proteasome pathway. We found that MG-115 (100 μM) had to be added 65 minutes before chromosome segregation in order to cause a significant delay to the first mitosis. These results indicate that inhibition of mitosis requires a preincubation comparable to that required for inhibition of NEB, leading us to conclude that the block to NEB represents a block at S/M, rather than G₁ or early S phase.

These observations indicate that proteasome-mediated protein destruction is required not only at mitosis exit but also prior to NEB in the embryonic cell cycle.

The proteasome inhibitor MG-115 prevents the activation of histone H1 kinase in early embryos

To examine how proteasome inhibition might block NEB, we determined whether treatment with the proteasome inhibitor would affect MPF activation during the first embryonic cell cycle. The results are shown in Fig. 2. In control embryos, histone H1 kinase activity began to increase from 40 minutes after fertilisation, and increased rapidly just prior to 50% NEB (at 80 minutes), reaching maximal levels at 90 minutes after fertilisation. The rate of histone H1 kinase activation was markedly slower in 100 μM MG-115-treated eggs and reached a reduced maximum level at around 130 minutes after fertilisation (Fig. 2); this delay of 40 minutes compared to controls corresponds to the delay of NEB observed in the same experiment, suggesting that the delay in NEB is due to a delay in MPF activation. Control peptide-aldehyde inhibitors had little effect on histone H1 kinase activation (Fig. 2). These data indicate that protein destruction mediated by the proteasome is required prior to the activation of histone H1 kinase.

Protein synthesis is one of the important factors that determine the onset of mitosis (Minshull et al., 1989; Solomon et al., 1990). To determine the effect of MG-115 on protein synthesis in sea urchin embryos, we quantified the newly synthesised protein using L-[³⁵S]methionine labelling. We confirmed that there are only small changes in the extent of protein synthesis in MG-115-treated embryos compared to LLM-treated embryos (Fig. 3A,B). Protein synthesis rates in DMSO- or LLM-treated embryos were essentially the same as in untreated controls (not shown). An anti-cyclin B western blot also indicated that both MG-115-treated and control embryos contain comparable amounts of cyclin B in egg extracts (not shown). Furthermore, emetine treatment experiments indicate that no further protein synthesis is required after 48 minutes post fertilisation for NEB to occur (not shown), as described previously (Wagenaar, 1983). When we compared the extent of NEB and the amounts of newly synthesised protein in embryos treated with emetine or MG-115, we found that MG-115-treated eggs contained significantly more newly synthesised proteins including cyclin B; nevertheless, emetine-treated eggs underwent NEB earlier (Fig. 3C). These observations suggest that suppression of mitosis entry in proteasome-inhibited embryos is mediated by a defect in the regulatory mechanism of p34^{cdc2} kinase activation, rather than by any effect on cyclin synthesis.

The [³⁵S]methionine labelling experiments also show the appearance of the phosphorylated form of cyclin (Fig. 3A) (Meijer et al., 1989; Patel et al., 1990; Whitaker and Patel, 1990). In control peptide-treated embryos, phosphorylated cyclin B is prominent at 90 minutes after fertilisation, and becomes essentially destroyed at 120 minutes (Fig. 3A, lanes

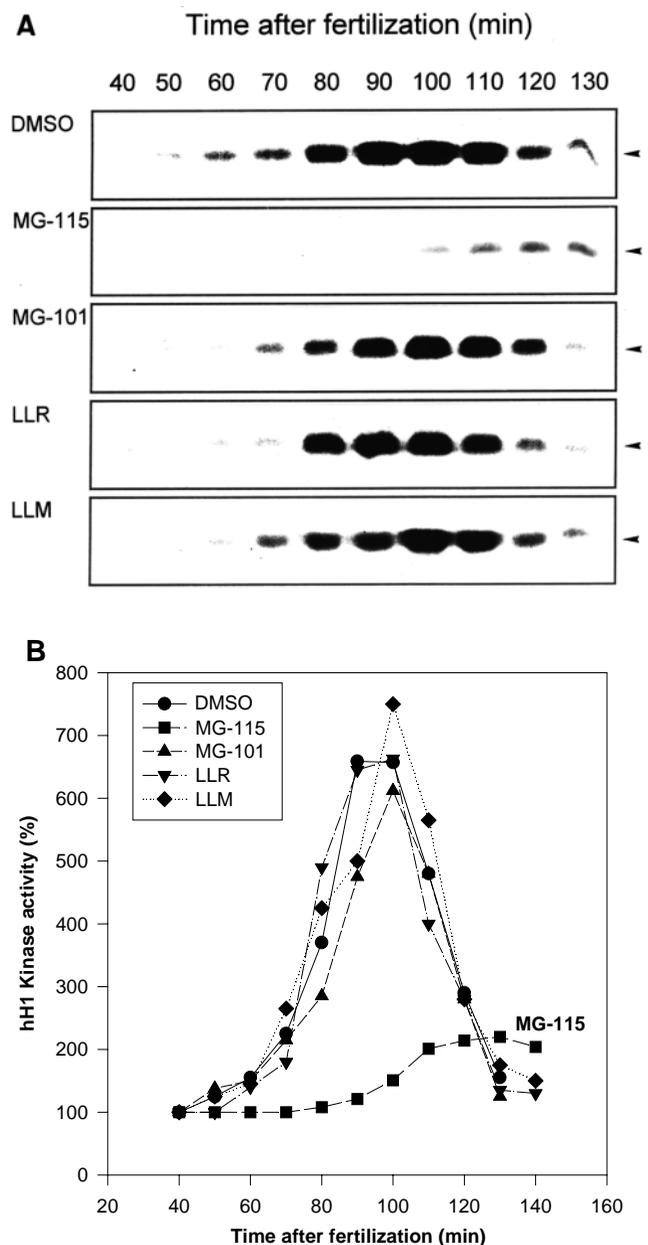


Fig. 2. MG-115 specifically blocks histone H1 kinase activation during the sea urchin cell cycle. (A) Synchronously fertilised embryos were divided into five portions just after fertilisation and different inhibitors were added to each culture within 10 minutes of fertilisation. 0.5% DMSO was used as a control. The embryos were harvested at the indicated times and the histone H1 kinase activity was measured as described in Material and Methods. 50% NEB was observed at 80 minutes (DMSO control, LLM, LLR), 85 minutes (MG-101) and at 120 minutes (MG-115), respectively. All peptide-aldehydes were dissolved in ASW, 0.5% DMSO to final concentration of 100 μM . The position of histone H1 (hH1) is shown by the arrowhead. Temperature 15°C. (B) Quantitation of the data in A (in arbitrary units) by phosphorimager analysis.

1-4). In contrast, phosphorylated cyclin B can be detected only after 120 minutes in MG-115-treated embryos (Fig. 3A, lanes 5-8). As phosphorylated cyclin B is thought to be generated by active p34^{cdc2} kinase itself (Solomon et al., 1990; reviewed in

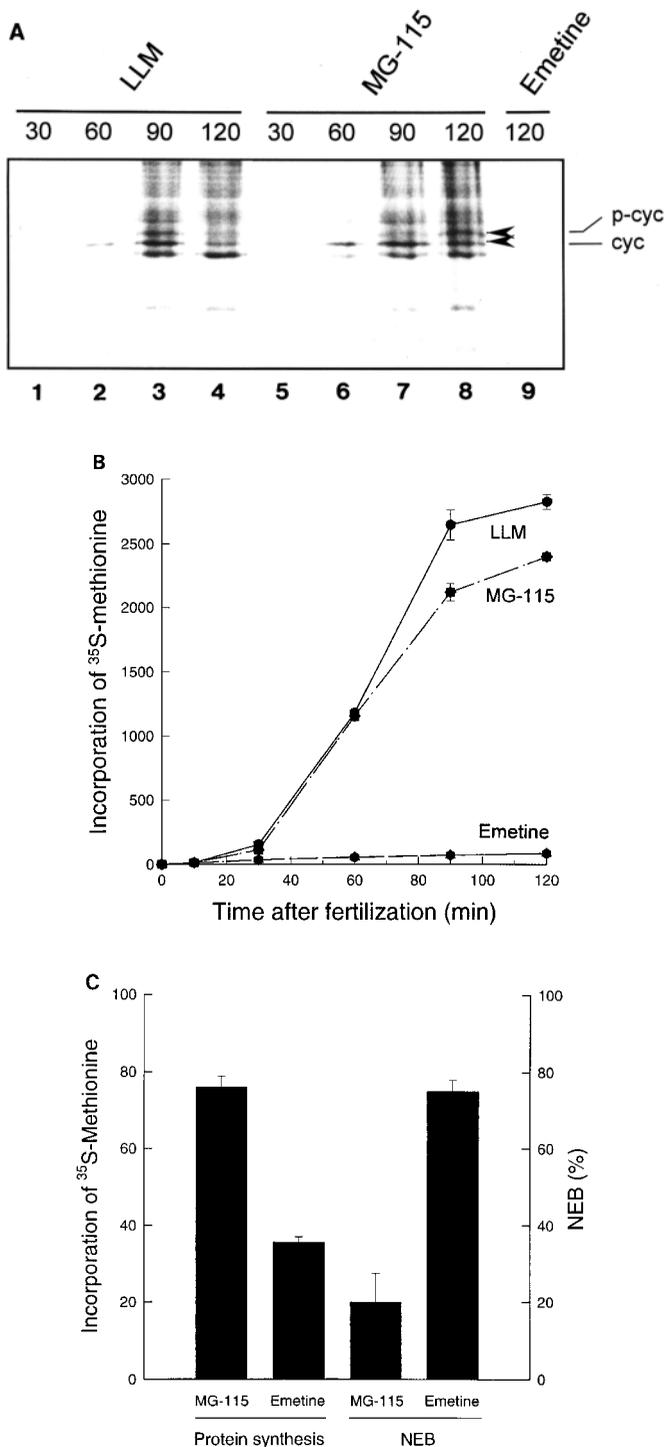


Fig. 3. Blockage of mitosis entry is not caused by the decrease of protein synthesis in MG-115-treated embryos. (A) LLM (100 μ M), MG-115 (100 μ M) and emetine (100 μ M) were added to the egg cultures within 10 minutes of fertilisation, together with 45.5 mCi/ml L-[35 S]methionine. Embryos were harvested at the indicated times and mixed immediately with cold 25% TCA. The samples were prepared as described in Materials and Methods, and subjected to SDS-PAGE, each lane having the same protein loading. The gels were stained with Amido black to verify that the amount of protein in each lane was constant and the radioactivity was measured and analysed by phosphorimager. Lanes 1-4, LLM; lanes 5-8, MG-115; lane 9, emetine. In the experiment represented here, 50% NEB was observed at 85 minutes in LLM-treated embryos, 120 minutes MG-115-treated embryos and never observed in emetine-treated embryos within 180 minutes. cyc, cyclin; p-cyc, phosphorylated cyclin. (B) Quantitation of the newly synthesised proteins (shown in A) in LLM, MG-115 and emetine-treated cells. (C) Comparison of protein synthesis and NEB ratio in MG-115- or emetine-treated embryos. Embryos were treated with 100 μ M MG-115 (added at 10 minutes after fertilisation) or 100 μ M emetine (added at 51 minutes after fertilisation) in the presence of [35 S]methionine. At 100 minutes after fertilisation, the % NEB and protein synthesis were determined as described in A.

observation further supports the existence of an intracellular pathway leading to p34^{cdc2}/cyclin B kinase activation that is controlled by proteasome-dependent proteolysis.

The rapid dephosphorylation of p34^{cdc2} phosphotyrosine is blocked by proteasome inhibition

It is well known that the activation of p34^{cdc2} is accompanied by the dephosphorylation of its Tyr15 and Thr14 residues, mediated by cdc25 phosphatase (Kumagai and Dunphy, 1991). Dephosphorylation of these residues is believed to activate the p34^{cdc2} kinase (Morla et al., 1989). Once p34^{cdc2} is partially activated, cdc25 is activated by its substrate cdc2, leading to an autocatalytic amplification of these activities and to a dramatic increase of MPF activity at the time of mitosis onset (Hoffman et al., 1993; Patel et al., 1999).

To examine how the suppression of histone H1 kinase activation was achieved by proteasome inhibition, we then investigated the rates of tyrosine dephosphorylation of p13^{suc1}-purified p34^{cdc2}. As shown in Fig. 4A, phosphorylated tyrosine in p34^{cdc2} disappeared rapidly at 80 minutes after fertilisation in 0.5% DMSO-treated control embryos; this was accompanied by the full activation of histone H1 kinase activity (Fig. 2). On the other hand, MG-115 clearly prevents the tyrosine dephosphorylation of p34^{cdc2} (Fig. 4A). The control peptide aldehyde MG-101 delays this dephosphorylation only slightly, and LLM does not significantly affect the rate of tyrosine dephosphorylation.

We confirmed that both MG-115-treated embryos and control embryos contained constant amounts of p34^{cdc2} throughout the course of the cell cycle (Fig. 4B), despite the temporal changes in the tyrosine phosphorylation signal level. Moreover, the slowly migrating form of p34^{cdc2} in SDS-gel electrophoresis (which indicates phosphorylation of Tyr14 and Thr15 on p34^{cdc2}; Solomon et al., 1992), does not disappear in MG-115-treated embryos, whereas in controls it disappears at the time of mitosis (Fig. 4B). Thus, it is clear that the delay of histone H1 kinase activation in MG-115-treated eggs

Nurse, 1990; Whitaker and Patel, 1990), this observation further supports our observations above that the proteasome inhibitor blocked the activation of mitotic p34^{cdc2}/cyclin B kinase, even though cyclin B is accumulated in comparable amounts to those in M phase control embryos.

Finally, in an in vitro kinase assay, MG-115 at concentrations up to 200 μ M does not affect the activity of histone H1 kinase at all (not shown), indicating that the suppression of histone H1 kinase activation in vivo is not caused by a direct effect of MG-115 on MPF itself. This

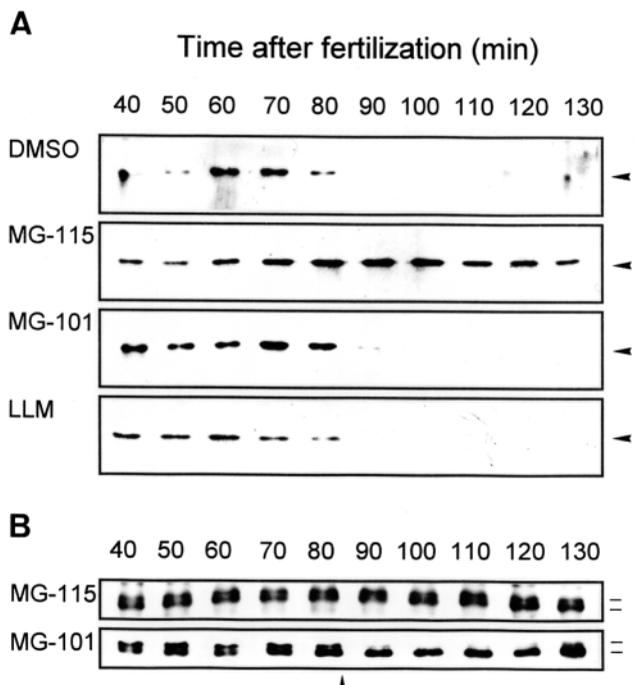


Fig. 4. Suppression of histone H1 kinase activation by MG-115 accompanied by inhibition of tyrosine dephosphorylation of p34^{cdc2}. (A) p34^{cdc2} does not undergo tyrosine dephosphorylation in MG-115 arrested cells. Western blotting of p34^{cdc2}, using anti-phosphotyrosine antibody. p34 was purified on p13^{suc1} beads from control, MG-101-, LLM- and MG-115-treated sea urchin embryos during the first mitotic cell cycle. (B) Western blotting of p34^{cdc2} using anti-PSTAIRES antibody. p34^{cdc2} was isolated as in A from MG-101- and MG-115-treated sea urchin embryos. The arrowhead represents 50% NEB in the control cells. Similar membranes as in A were reacted with anti-PSTAIRES antibody to verify the amounts and mobility of p34^{cdc2} during sea urchin cell cycle. p34^{cdc2} does not change its mobility on SDS-PAGE in MG-115 arrested cells. The upper band (which corresponds to the tyrosine phosphorylated form) disappears rapidly in control MG-101 (100 μ M)-treated embryos at 90 minutes after fertilisation in accordance with the onset of mitosis, while it persists for more than 130 minutes in embryos treated with the same concentration of MG-115. Arrowhead represents 50% NEB in control embryos.

is accompanied by a delay of the p34^{cdc2} tyrosine dephosphorylation.

These observations provide evidence of a requirement for proteasome-mediated control for dephosphorylation and subsequent activation of p34^{cdc2} during the cell cycle.

The proteasome inhibitor induces a 'Large nuclei' phenotype

In MG-115 arrested embryos, we observed a characteristic morphology which we have called 'Large nuclei' (Fig. 5). In control eggs the usual diameter of the nucleus at 70 minutes after fertilisation, just before NEB, is about 19 μ m. In contrast, the nuclei of 100 μ M MG-115-treated embryos gradually expand to reach a diameter of 28 μ m at 200 minutes after fertilisation (see Fig. 7Ca-c). When treated with 25 μ M MG-115, embryos showed a cell cycle arrest at 26 hours after fertilization, at which time these embryos contained several large nuclei (Fig. 5, MG-115). Embryos treated with the control peptide inhibitors had developed quite normally to swimming mesenchyme blastula stage at this time (Fig. 5, Control). These observations demonstrate that the volume of the nucleus increases more than threefold in MG-115 arrested cells.

The formation of the 'Large nuclei' phenotype depends on the continuation of DNA synthesis, because cell cycle arrest induced by the DNA synthesis inhibitor aphidicolin in the presence of MG-115 does not induce the 'Large nuclei' phenotype even after long incubations (not shown), so it appears that proteasome inhibition blocks the cells at a point after S phase initiation, probably during S phase and before entry into M phase.

Inhibition of the proteasome leads to excess incorporation of [³H]thymidine

To examine whether S phase is proceeding normally in MG-115-treated embryos, we measured the incorporation of [³H]thymidine in proteasome-inhibited eggs during the first cell division cycle. In control embryos, the incorporation of [³H]thymidine into DNA was observed to increase at 40 minutes after fertilisation and to reach a plateau between 90 and 120 minutes (Fig. 6A). In MG-115-treated embryos, the incorporation of [³H]thymidine began at a similar time to incorporation in the control embryos, indicating that S phase had started normally. To our surprise, the MG-115-treated embryos continued to incorporate [³H]thymidine to a level well above that of the control embryos by the end of S phase (Fig. 6A). As described above, NEB was delayed (Fig. 6A). By 120 minutes after fertilisation, the control embryos had completed the first S phase and MG-115-inhibited embryos had incorporated about double the control amount of [³H]thymidine. In three experiments, the relative rate of thymidine incorporation in MG-115 embryos compared to controls was 2.08 \pm 0.07 (mean \pm s.e.m.); this is a significant increase at the 0.5% level (Student's paired *t*-test). The incorporation of [³H]thymidine by MG-115-treated embryos is

Fig. 5. Embryos arrest at 26 hours after fertilization when treated immediately after fertilization with 25 μ M MG-115. They contain several very large (20-30 μ m) nuclei (left). A control blastomere at 5 hours that contains a similar number of nuclei is shown for comparison (right).

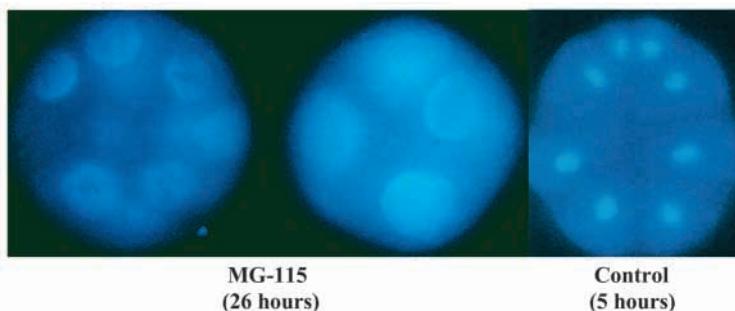
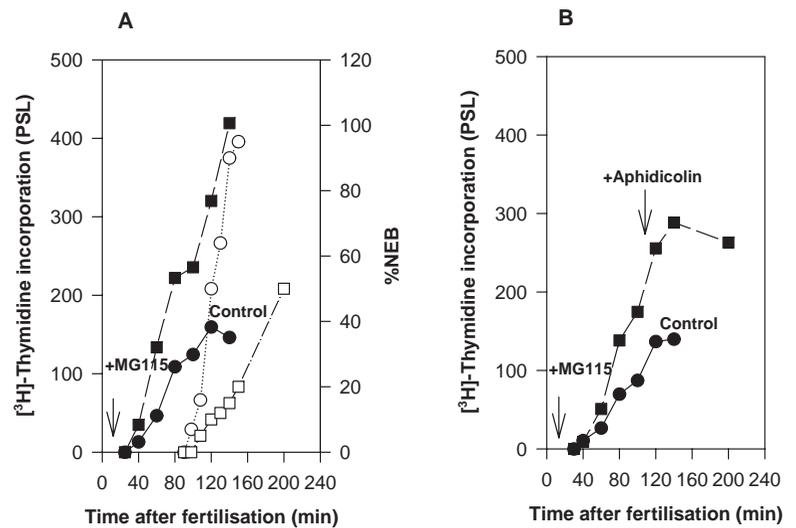


Fig. 6. DNA synthesis in MG-115-treated embryos. (A) Time-dependent incorporation of [^3H]thymidine during the cell cycle at 13.5°C. in 100 μM MG-115 (■) and in control 0.5% DMSO-treated (●) eggs. The % NEB in MG-115 (□) and control (○) cells is also shown. No MG-115-treated cells exit from the first mitosis at 200 minutes after fertilisation, while all control embryos are in the 2-cell stage. (B). Aphidicolin-sensitive incorporation of [^3H]thymidine in 100 μM MG-115 (■) and 0.5% DMSO-treated (control) (●) embryos. The intensity of radioactivity is measured by the phosphorimager in PSL (photostimulated luminescence) units.



aphidicolin-sensitive (Fig. 6B), indicating that it is DNA polymerase I-dependent and so presumably due to semireplicative DNA synthesis.

These data imply that proteasome inhibition leads to overreplication of DNA, causing overincorporation of [^3H]thymidine, and thus that the proteasome is involved in the mechanisms of termination of DNA synthesis, which in the sea urchin early embryonic cell cycle is closely linked to the initiation of mitosis.

Caffeine overrides the S-phase block caused by the proteasome inhibitor MG-115

It is known that caffeine treatment allows certain mammalian cells with damaged or improperly replicated DNA to override the DNA synthesis cell cycle checkpoint and enter M phase (Boynton et al., 1974; Lau and Pardee, 1982; Schlegel and Pardee, 1987). It has also been shown (Patel et al., 1997) that sea urchin embryos blocked at the late S-phase checkpoint by the DNA polymerase I inhibitor aphidicolin can be induced to enter mitosis by treatment with caffeine. To see whether the MG-115-inhibited embryos (which seem unable to terminate their DNA replication) could be induced to enter mitosis, we treated them at 165 minutes after fertilisation with 5 mM caffeine added to 100 μM MG-115 in ASW. The caffeine-treated embryos progressed through the cell cycle reaching 100% NEB at 270 minutes, while the MG-115-treated embryos remained blocked at 40–50% NEB, at least until 300 minutes after fertilisation (Fig. 7A; see also Fig. 7C). Caffeine treatment led to NEB and chromatin condensation (Fig. 7Cd), but the embryos did not segregate their chromatin into daughter cells normally. The condensed chromatin remained in one or other of the daughter cells or was found between them (Fig. 7Ce,f) and nuclear envelope did not reform. Furthermore, some of the cells formed not two but three daughter cells, the exact percentage varying from one experiment to another. The untreated control embryos had all undergone NEB by 120 minutes after fertilisation (Fig. 7A) and underwent a normal mitosis.

We also performed an experiment in which embryos were treated with both 100 μM MG-115 and 5 mM caffeine in ASW at 10 minutes after fertilisation. These embryos did not arrest

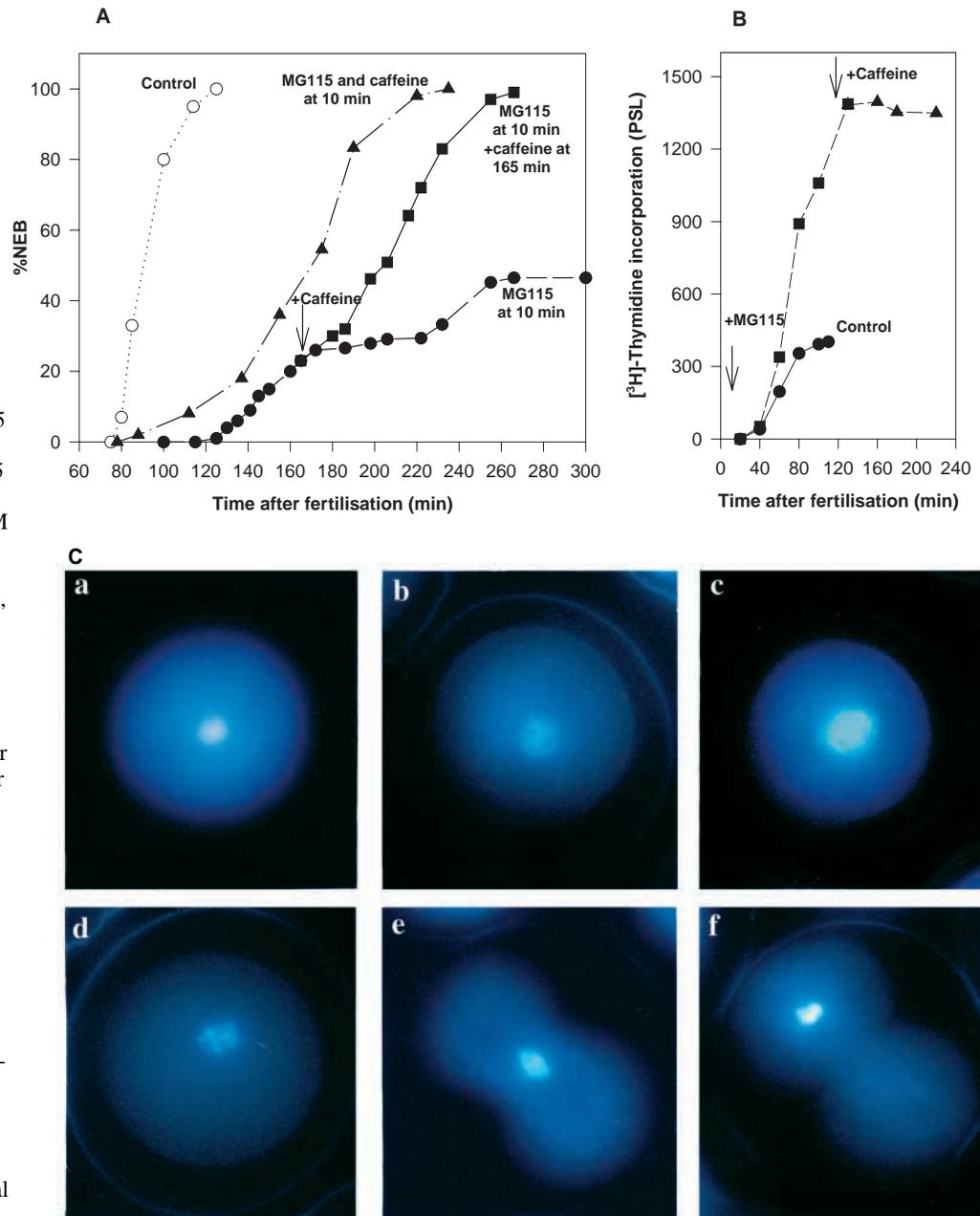
before mitosis (see Fig. 7A): they underwent delayed NEB (95% at 220 minutes) and divided, eventually arresting in mitosis with the same phenotype as in the experiments described above. The control MG-115-treated cells in this experiment underwent a partial and delayed NEB (not shown), identical to the MG-115-treated cells shown in Fig. 7A: at 275 minutes after fertilisation they achieved 45% NEB; the rest remained blocked in interphase.

These data suggest that MG-115 treatment invokes the DNA synthesis checkpoint, since the effects of caffeine rescue are identical to those described in aphidicolin-treated embryos (Patel et al., 1997).

Does caffeine suppress the overreplication of DNA? To answer this question, we carried out an experiment in which cells blocked by MG-115 were treated at 120 minutes with 5 mM caffeine and the incorporation of [^3H]thymidine was measured. Fig. 7B shows that caffeine prevents further overreplication of DNA, indicating that caffeine treatment does not merely override the DNA synthesis checkpoint downstream of overreplication, but can shut off DNA replication itself.

DISCUSSION

Earlier work identified two peaks of proteasome activity during the cell cycle in ascidian embryos (Kawahara et al., 1992). The late peak was associated with the metaphase/anaphase transition in mitosis: it is known that proteasome activity is required for metaphase/anaphase progression (Gordon et al., 1993, 1996; Ghislain et al., 1993; Deveraux et al., 1995). The earlier peak occurred before nuclear envelope breakdown and its significance was unclear. Here we show that a similar peak of activity occurs during the first cell cycle of sea urchin embryos and demonstrate that proteasome activation is essential for entry into mitosis using specific proteasome inhibitors. Lack of proteasome-mediated proteolysis leads to overreplication of DNA and the invocation of the DNA synthesis checkpoint, as the presence of inactive tyrosine-phosphorylated p34^{cdc2} and our caffeine rescue experiments demonstrate.



Efficacy and specificity of inhibitors

The panel of peptide-aldehydes used in this study are reported to inhibit not only proteasome activity but also certain cysteine proteases. However, the relative inhibitory potencies against cysteine proteases do not correlate with their efficacies in blocking NEB. In fact, LLR and LLM are rather more potent inhibitors of cysteine proteases than MG-115 (Rock et al., 1994). The reported inhibitory potencies against the proteasome's chymotrypsin-like activity *in vitro* are; MG-115, K_i 21 nM; MG-101, K_i 140 nM; LLM, K_i 1 000 nM; LLR, IC_{50} 8900 nM (Rock et al., 1994). Thus, the inhibitory potency for inhibition of proteasome activity correlates perfectly with the rank order of efficacy in blocking cell cycle progression. Furthermore, the concentration dependence of MG-115 for inhibition of NEB closely mirrors that of mitosis exit. We also report in passing that MG-132, an analogous peptide more

potent against the proteasome even than MG-115 (Jensen et al., 1995), has a twofold higher efficacy than MG-115 in blocking the sea urchin cell cycle. These observations further strongly suggest that the intracellular target of MG-115 is the proteasome, and indicate that inhibition of the proteasome causes arrest at both the entry and exit points of sea urchin embryonic mitosis.

Failure of mitotic CDK activity after proteasome inhibition

MG-115-treated embryos are blocked in interphase or markedly delayed before NEB in the first cell cycle. Delayed embryos are then blocked in first mitosis or are delayed in first mitosis and blocked completely either in interphase or in mid-mitosis of subsequent cell cycles. The delay in entry to mitosis is accompanied by a delay in the partial activation of histone

H1 kinase. In these embryos, cyclin accumulates normally and CDK1 is tyrosine phosphorylated. The absence of tyrosine dephosphorylation correlates with the low histone H1 kinase activity (Figs 2, 4). We conclude that CDK1 is present in a post-translationally modified state compatible with a blocked or delayed S/M transition.

Overreplication of DNA and the DNA replication/S phase completion checkpoint

Embryos treated with MG-115 and blocked or delayed before NEB continued to incorporate thymidine into their DNA in an aphidicolin-sensitive fashion. Longer treatments with MG-115 result in embryos with very large nuclei. These observations suggest that inhibition of the proteasome results in a failure to terminate DNA synthesis. The simplest hypothesis would be that the failure to complete S phase leads to the activation of the DNA synthesis checkpoint (Elledge, 1996; Wang, 1998). The current model suggests that Chk1-activated 14.3.3 protein binds to the cdc25 phosphatase to prevent activation, leaving cdk1 threonine/tyrosine phosphorylated and inactive and blocking mitosis entry (Wang, 1998). It has recently been shown that 14-3-3 protein inhibits nuclear import of cdc25 in *S. pombe* and *Xenopus* oocytes (Lopez-Girona et al., 1999; Yang et al., 1999). In human cells Cdc25 phosphatase is directly inhibited by the checkpoint kinase Cds1 (Blasina et al., 1998). This model is consistent with our observations of the post-translational modification state of cdk1 after MG-115 treatment. It is also consistent with our observation that caffeine can override the MG-115 arrest, when added subsequent to the MG-115 block and also when added together with MG-115. Caffeine is known to override the cdc25 inactivated DNA damage checkpoint in cells in culture and in sea urchin embryos (Boynton et al., 1974; Lau and Pardee, 1982; Schlegel and Pardee, 1987; Patel et al., 1997). There is evidence that caffeine acts by inhibiting ATM kinase activity; ATM kinase activity maintains active Chk1 (Blasina et al., 1999; Fig. 8).

Candidate proteins involved in rereplication suppressing mechanisms

Our data demonstrate that the rate of incorporation of thymidine into DNA is twofold greater in MG-115-treated embryos than in controls, indicating that rereplication begins early in S phase. Sea urchin and other embryos have short early mitotic cell cycles, lacking G₁ and G₂ phases and consisting only of S and M phases. S phase in early *Xenopus* embryos is 30 times shorter than in adult cells and S phase lasts only 4 minutes in early *Drosophila* embryos: the acceleration of the rate of DNA replication in these cells is achieved by increased number of origins rather than increased rate of fork progression along DNA (Laskey, 1985). Our experiments show an increased rate of [³H]thymidine incorporation in the sea urchin embryos during the whole interval of DNA synthesis from the very beginning of S phase. This most likely implies that early firing replication origins overreplicate in MG-115-treated cells, consistent with the idea of an undegraded and persistently present initiator of DNA replication.

The broad model of the control of replication is based on the idea of a licensing factor that binds to chromatin during mitosis and is destroyed within the nucleus once replication is initiated. Fresh licensing factor can attach to chromatin only once the nuclear envelope breaks down at the next mitosis (reviewed in Blow, 1996). In broad terms, then, it is likely that inhibition of the proteasome (which has a nuclear localization during S phase; Kawahara and Yokosawa, 1992) prevents destruction of a licensing factor, allowing reinitiation to occur at replication origins.

Mutation in the RPN11/MPR1 gene that encodes a regulatory proteasomal subunit in *S. cerevisiae* causes interphase cell cycle arrest accompanied by overreplication of both nuclear and mitochondrial DNA (Rinaldi et al., 1998).

The MCM proteins and the cdc18⁺ protein have also been suggested to behave as if they were licensing factors (Stillman, 1996). In fission yeast, cdc18p is critical for initiation of DNA replication; p65^{cdc18} levels oscillate, peaking at G₁/S;

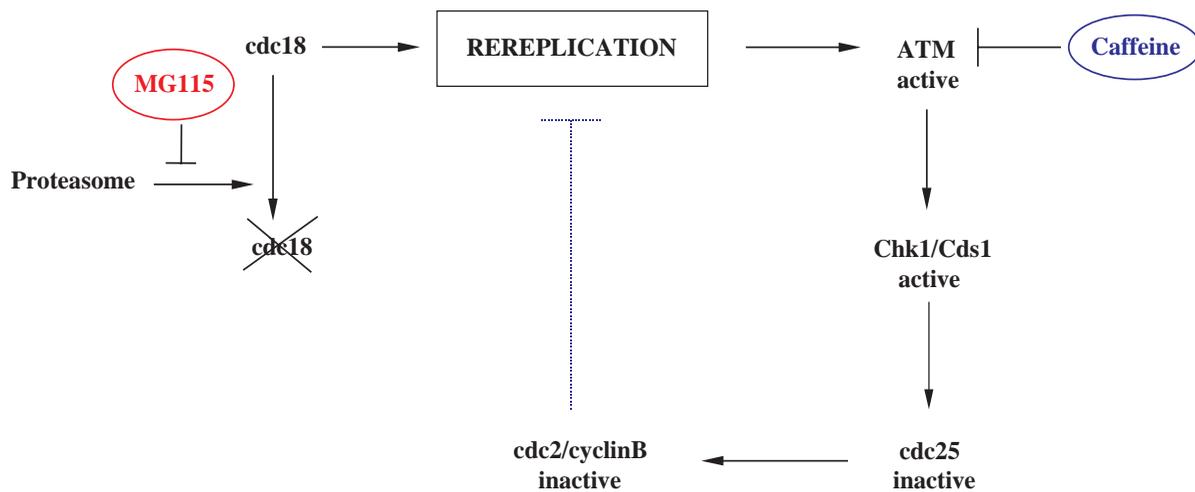


Fig. 8. Schematic diagram of a hypothesis to explain proteasome-dependent control of S-phase termination and mitosis entry. We postulate that MG-115 blocks proteasome-based degradation of cdc18 (or another putative initiator of DNA replication), leading to reinitiation of DNA synthesis at the replication origin. Mitosis is prevented because rereplication activates ATM kinase, which in turn suppresses cdc2/cyclinB activity via Chk1/Cds1 and cdc25. Caffeine induces chromatin condensation by blocking ATM kinase.

overexpression of p65 induces rereplication of DNA, without mitosis (Nishitani and Nurse, 1995). Expression of this protein is switched on in mitosis but it is continually targeted for proteolysis by cdc2 protein kinase-mediated phosphorylation until the inactivation of this kinase at anaphase, when the cdc18p stabilises and accumulates rapidly during G₁; according to the proposed model, after the START B-type cyclins reaccumulate, cdc2p/cyclin B can initiate S phase, at the same time destabilising cdc18p and so preventing rereplication, since expression of the protein is switched off during S phase (Baum et al., 1998). Thus, by control of cdc18p availability, the cell creates, once per cell cycle, a very short time window for initiation of DNA replication to occur. In fact, it has been suggested that, just as replication cannot occur during mitosis, so mitosis can be prevented by the persistence of replication factors (Stillman, 1996): the DNA synthesis completion checkpoint. Clearly, the effects of the proteasome inhibition in sea urchin embryos can be explained by the lack of destruction of putative sea urchin cdc18 homologue. Broadly, the work in yeast also suggests that MG-115 inhibits entry into mitosis by preventing completion of S phase, which then prevents mitosis, probably via inhibition of cdc25 activity (Fig. 8).

Other cell cycle targets of the proteasome

An effect of proteasome inhibition on the inhibitory protein kinase, wee1, is also conceivable. MPF activation is controlled by the balance between the activities of its activator, cdc25, and its inhibitors, Wee1 and Myt1. It has recently been shown in *Xenopus* egg extracts (Michael and Newport, 1998) that there is a link between the DNA replication checkpoint, degradation of Wee1 protein and entry into mitosis. The authors show that Wee1 protein degradation is necessary for the normal timing of mitosis entry. The DNA replication checkpoint specifically inhibits the proteolysis of Wee1 kinase, so shifting the balance toward MPF inactivation and preventing entry into mitosis (see also Baber-Furnari et al., 2000). The budding yeast homologue of Wee1, Swe1, is also destroyed at G₂/M by ubiquitin-mediated proteolysis (Kaiser et al., 1998); however, the first manifestation of proteasome inhibition in the sea urchin embryo is early rereplication during S phase, so while the proteasome may also be essential for degradation of Wee1 in late G₂, we have no evidence of this.

There is also evidence from yeast that the proteasome controls S phase initiation and the S/M transition, as well as functioning at anaphase. While the Sug1, Cim5 and mts2 26S proteasome mutants arrest cells in mitosis (Ghislain et al., 1993; Gordon et al., 1993), the Nin1 mutant blocks in G₁/S and S/M (Kominami et al., 1995, 1997). Destruction of the G₁-CDK inhibitor Sic1 is essential for S-phase initiation (Schwob et al., 1994), as is ubiquitin conjugating enzyme, Cdc34 (Dutcher and Hartwell, 1982); however, the Sic1/Cdc34 double mutant arrests not in G₁/S, but in S/M (Schwob et al., 1994). We cannot rule out a role for the proteasome in S phase initiation in sea urchin embryos, as our experiments suggest that the peptide inhibitors are relatively impermeant and take 30 minutes or more to reach inhibitory levels within the cell, by which time S phase of the first cell cycle has begun.

Links between mitotic kinase activity and rereplication

In yeast it seems clear that a major function of CDK activity

at the S/M boundary is to prevent rereplication (Hayles et al., 1994; Correa-Bordes and Nurse, 1995; Fisher and Nurse, 1996; Stillman, 1996; Dahmann et al., 1995; Tanaka et al., 1997; see also King et al., 1996). It is clearly possible that rereplication in sea urchin embryos is due simply to the absence of cdk1 activity after proteasome inhibition; however, it has not been reported that inhibition of cdk1 activity causes rereplication in sea urchin embryos. We report that inactivation of the DNA synthesis checkpoint pathway by caffeine brings an immediate end to rereplication. These findings certainly at first sight imply a link between suppression of rereplication and cdk1 activity in sea urchin embryos. We are, however, cautious about this sort of interpretation. The chromatin condensation induced by caffeine treatment is very abnormal in appearance. Caffeine-rescued embryos lack well formed chromosomes and do not undergo chromosome segregation at anaphase. It seems likely that the unobvious, unphysiological and ill-regulated chromatin condensation caused by caffeine is preventing rereplication simply because the physical state of condensed chromatin does not support DNA synthesis (Fig. 8).

Conclusion

Our data indicate that chymotrypsin-like proteasome activity is essential to prevent early rereplication during S phase in sea urchin embryos. The block to mitosis entry caused by inhibition of the proteasome is due to the DNA synthesis checkpoint, as it is overridden by caffeine treatment, which is known to inhibit the ATM/Chk1 pathway. A likely target of the proteasome is a sea urchin cdc18p homologue that licences DNA replication.

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