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Original Article

The release from metaphase arrest in blue mussel oocytes

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ABSTRACT In *Mytilus edulis*, shed oocytes are arrested at metaphase I of meiosis until fertilization. We previously suggested (Dubé and Dufresne, *J. Exp. Zool. 256:* 323-332, 1990) that such a metaphase arrest depends upon a continuous synthesis of short-lived proteins, the destruction of which is sufficient to induce meiosis resumption. We further investigated the mechanism of metaphase release in blue mussel oocytes as triggered either by fertilization or by inhibition of protein synthesis (emetine) or phosphorylation (6-dimethylaminopurine, 6-DMAP). Treatment of unfertilized oocytes (UF) with emetine induces completion of the first meiotic cycle including extrusion of the polar body, followed by chromosome decondensation and by the formation of large membrane-bound nuclei, as visualized by Hoechst staining and transmission electron microscopy (TEM). Inhibition of protein phosphorylation with 6-DMAP induces directly chromosome decondensation and the formation of multiple nuclei surrounded by nuclear membrane. These interphasic nuclei exhibit continuous ^{3}H-thymidine incorporation. p13 precipitation of p34 and associated proteins reveals «putative» cyclins in UF, no longer detected after metaphase/anaphase transition due to fertilization or emetine treatment. In the presence of 6-DMAP, new migrating forms are observed. The phosphorylated p34^{cdc2} homolog becomes dephosphorylated after fertilization or emetine treatment, whereas 6-DMAP induces its phosphorylation on tyrosine. Histone H1 kinase activity is reduced after these treatments, compared to the UF sample. Our results suggest that the metaphase/anaphase transition triggered by fertilization in blue mussel oocytes is induced by the rapid destruction of a set of continuously synthesized proteins accompanied by decreased histone H1 kinase activity. These events can be mimicked by inhibiting protein synthesis. Inhibition of protein phosphorylation would drive the cell to interphase without commitment to meiosis I.

KEY WORDS: *metaphase, p34^{cdc2} homolog, cyclins, phosphorylation, DNA synthesis*

Introduction

Animal oocytes, before fertilization, are arrested at one of four specific stages of meiotic maturation. While several analyses investigated the mechanisms of arrest in prophase I or metaphase II (review in Pelech *et al.*, 1990; Masui, 1991), much less is known concerning the metaphase I arrest, as seen in several invertebrate species. Recent progress in the biology of the cell cycle has identified a universal M-phase promoting factor or MPF (Masui and Markert, 1971; Gerhart *et al.*, 1984, 1985; Lohka *et al.*, 1988) exhibiting maximal histone H1 kinase activity during metaphase (Draetta and Beach, 1988; Labbé *et al.*, 1988a,b; Meijer and Pondaven, 1988), and decreasing at the metaphase/anaphase transition. MPF is formed by the association of a catalytic subunit of p34^{cdc2} with specific regulatory proteins which are periodically synthesized and destroyed during the cell cycle: the cyclins (Evans *et al.*, 1983; Dunphy *et al.*, 1988; Draetta *et al.*, 1989; Labbé *et al.*, 1989; Meijer *et al.*, 1989). Loss of MPF activity is due to cyclin destruction (Luca and Ruderman, 1989; Murray *et al.*, 1989; Felix *et al.*, 1990) and rephosphorylation of p34^{cdc2} on tyrosine prevents precocious reactivation of MPF during the cell cycle (Gautier *et al.*, 1989; Gould and Nurse, 1989). Although cyclins can be phosphorylated in the M-phase stage (Pondaven *et al.*, 1990), this posttranslational modification does not appear to be a prerequisite for their destruction (Izumi and Maller, 1991). p34^{cdc2} can be phosphorylated on tyrosine, serine and threonine residues in accordance with the cell cycle (Krek and Nigg, 1991; Nigg *et al.*,

Abbreviations used in this paper: BSA, bovine serum albumin; CSF, cytostatic factor; 6-DMAP, 6-dimethylaminopurine; DTT, dithiothreitol; EDTA, ethylene diaminetetraacetic acid; EGTA, (ethylene bis-(oxyethylenenitrilono)) tetraacetic acid; MPF, M-phase promoting factor; NSW, natural sea water; TBS, Tris-buffered saline; TCA, tricholoroacetic acid; TEM, transmission electron microscopy.

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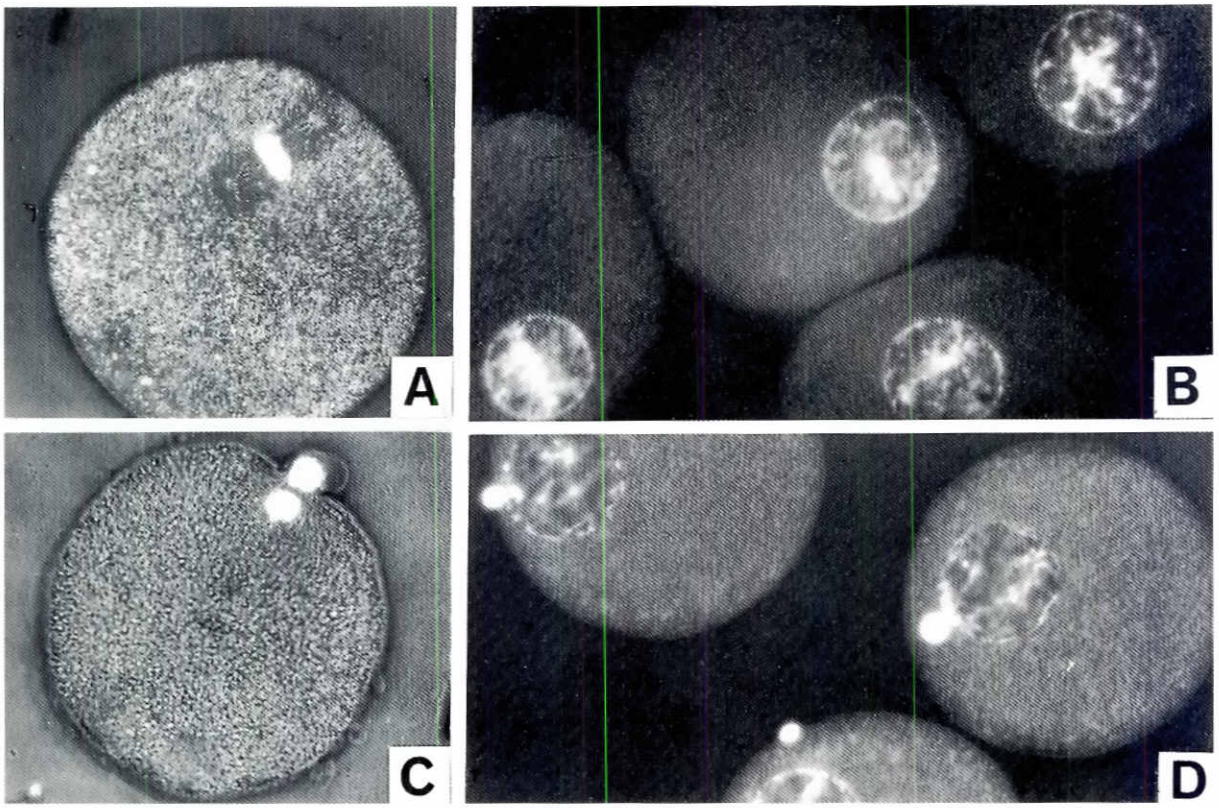

Fig. 1. The effects of 6-DMAP and emetine on metaphase I-arrested oocytes. *The oocytes were stained live with Hoechst 33342.* **(A)** *Untreated metaphase I-arrested oocyte;* **(B)** *oocytes treated with 150 μM 6-DMAP showing a large resting nucleus by 80 min after treatment;* **(C and D)** *oocytes treated with 150 μM emetine and having extruded the first polar body by 40 min after addition of the drug* **(C)** *followed, 80 min after treatment, by the formation of a large decondensed nucleus* **(D)**.

1991), and the regulation of its activity during the cell cycle is controlled by wee1 kinase and cdc25 phosphatase, in concert with associated cyclins (for review, see Feilotter *et al.*, 1992; Solomon, 1993).

Blue mussels *(Mytilus edulis)* are pelecypod molluscs shedding oocytes arrested at metaphase I of meiosis prior to fertilization. Since partial inhibition of protein synthesis with emetine induced completion of the first meiotic division in unfertilized oocytes, as evidenced by the extrusion of the first polar body and the subsequent formation of a large DNA-synthesizing nucleus (Dubé and Dufresne, 1990), we already suggested that this metaphase arrest required continuous protein synthesis. In fertilized oocytes, two cycling proteins, behaving like well-known cyclins, exhibited fluctuating levels of abundance in phase with specific stages of the meiotic or mitotic divisions. One of these two proteins was clearly seen to disappear early after fertilization or after treatment of the oocytes with emetine (Dubé and Dufresne, 1990), supporting the assumption that the early destruction of a continually synthesized protein, related to cyclins, is the main initial trigger of the metaphase/anaphase transition in this species.

In another molluscan species, the prosobranch gastropod *Patella vulgata,* naturally shed oocytes are similarly arrested at the metaphase I stage of meiotic maturation, prior to fertilization (Guerrier *et al.*, 1986, 1990). In this species, it was first reported that inhibition of protein synthesis by emetine induced the formation of numerous decondensed nuclei (Néant and Guerrier, 1988a; van Loon *et al.*, 1991). More recently, using Hoescht 33342 for chromatin staining, it has been shown that emetine treatment could induce a true metaphase/anaphase transition in *Patella* oocytes (Colas *et al.*, 1993), as also observed for oocytes of *Mytilus* (Dubé and Dufresne, 1990), the Japanese clam *Ruditapes philipinarum* and the ascidian *Phallusia mammillata* (Abdelmajid *et al.*, 1993).

On the other hand, 6-dimethylaminopurine (6-DMAP), a serine/threonine kinase inhibitor (Meijer and Pondaven, 1988; Néant and Guerrier, 1988b), was able to induce a reversible chromosome decondensation without initiating the metaphase/anaphase transition in unfertilized oocytes from *Patella* (Néant and Guerrier, 1988a) or mouse (Rime *et al.*, 1989; Szöllösi *et al.*, 1991), as well as in echinoderm dividing embryos (Néant *et al.*, 1989; Dufresne *et al.*, 1991). Unlike emetine, 6-DMAP does not alter protein synthesis (Rebhun *et al.*, 1973; Néant and Guerrier, 1988a) and does not inhibit cyclin synthesis (Néant *et al.*, 1989).

This prompted us to further study the process of emetine-dependent activation of blue mussel oocytes, as compared to the effects brought about by 6-DMAP. Also, we wanted to elucidate whether the processes, initiated during this critical period of the metaphase/anaphase transition, were related to those suggested to take place in other species, such as frog and mouse oocytes.

The present work shows that in blue mussel oocytes, the inhibition of protein synthesis (by emetine) and the inhibition of

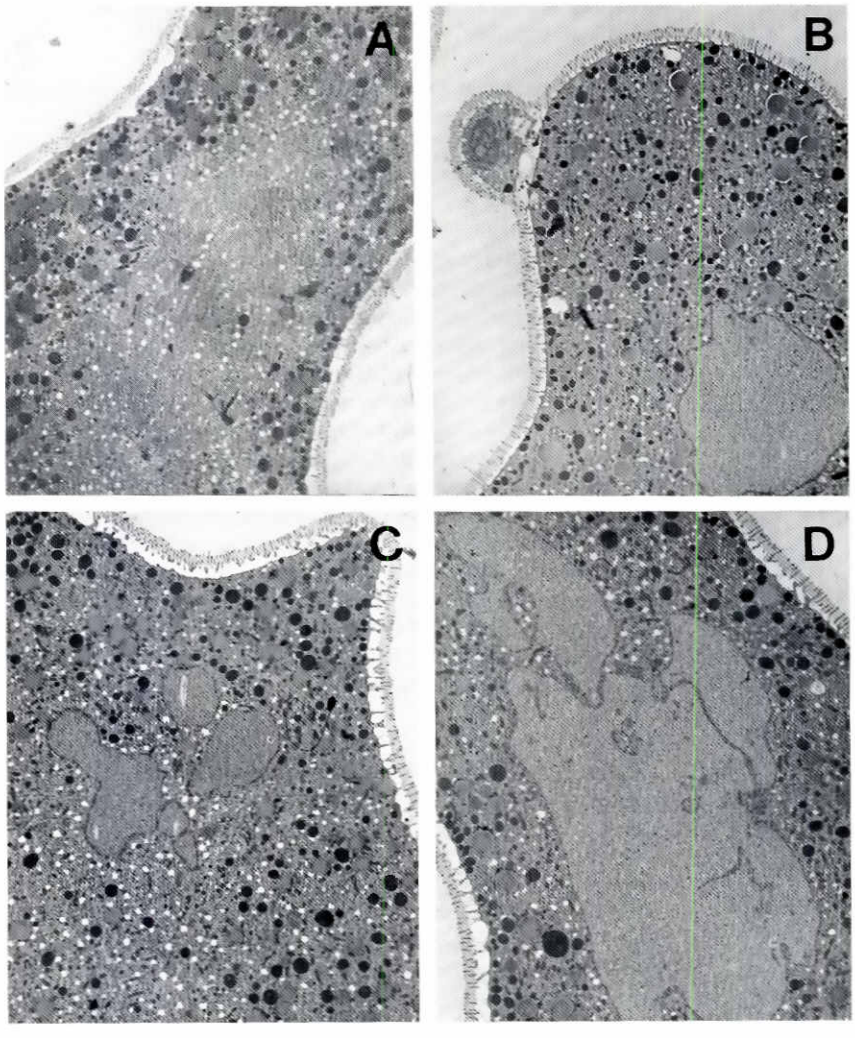

Fig. 2. The effects of 6-DMAP and emetine on metaphase I-arrested oocytes seen by transmission electron microscopy. (A) *Longitudinal section through the meiotic spindle in metaphase I-arrested unfertilized oocytes. Chromosomes are seen aligned at the equator of the spindle, which fibres point to each end.* **(B)** *Emetine-treated oocytes 90 min after addition of the drug. A first polar body is formed while remaining maternal chromatin develops into a large nucleus surrounded by a nuclear envelope.* **(C and D)** *6-DMAP-treated oocytes after 90 min are never seen with an extruded polar body but the maternal chromosomes decondense and develop into numerous resting nuclei eventually fusing together and also surrounded by a nuclear envelope. Magnification: A, x3200; B, x3000; C, x3200; D, x3800.*

protein phosphorylation (by 6-DMAP) have different morphological and biochemical effects. The inhibition of protein synthesis by emetine triggers a true metaphase/anaphase transition soon followed by a permanently decondensed nuclear state, whereas altering protein phosphorylation by 6-DMAP directly interferes with the normal control of the cell cycle, driving the cell precociously to interphase. In both conditions, DNA synthesis is initiated.

Results

Morphological effects of emetine and 6-DMAP

The cytological effects of these drugs were followed after staining live or fixed oocytes with Hoechst fluorochromes. In unfertilized oocytes exposed to 6-DMAP at concentrations greater than 75 μM, the metaphase chromosomes (Fig. 1A) decondense within 30 min and then merge to produce a large resting nucleus (Fig. 1B). This process is direct and the tetrads do not segregate nor initiate any metaphase/anaphase transition. Thus, no polar body is formed under these conditions. The effect of 6-DMAP is perfectly reversible; washed oocytes returned to normal seawater undergo chromosome recondensation (not shown). As previously described (Dubé and Dufresne, 1990), treatment of metaphase I

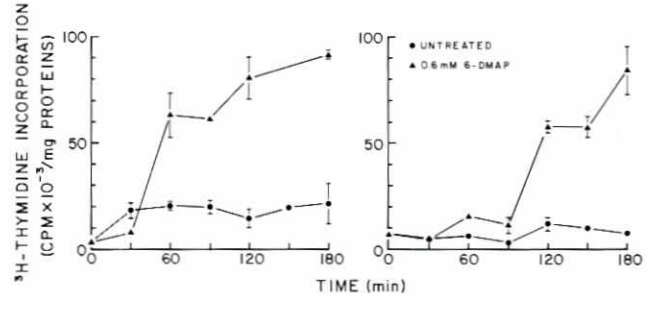

Fig. 3. Stimulation of ^{3}H-thymidine incorporation by 6-DMAP. *^{3}H-thymidine incorporation into the TCA-precipitable fraction was measured in the absence (control, ●) or in the presence of 0.6 mM 6-DMAP added at 0 min (6-DMAP, ▲). These two graphs present ^{3}H-thymidine incorporation of two batches of oocytes which exhibit different delay in the response, depending on their own sensitivity to the drug. Mean results of duplicate samples (±s.d.) per time.*

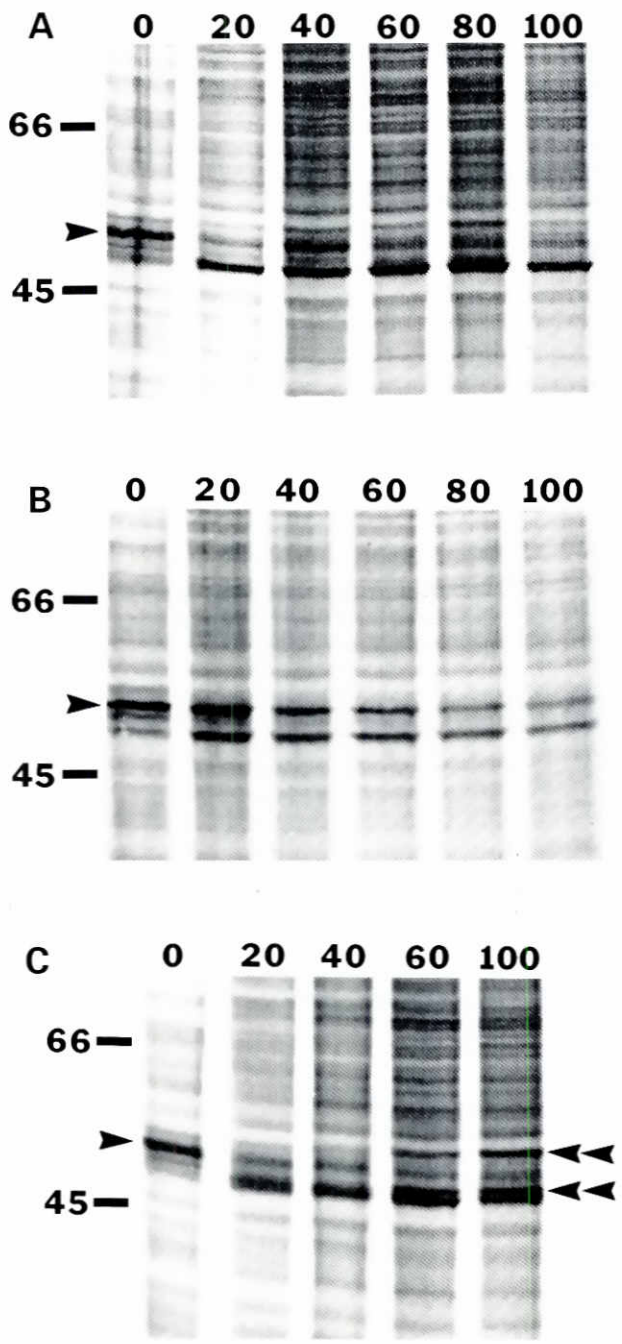

Fig. 4. Patterns of total synthesized proteins after fertilization or artificial activation. *Samples were taken (at the different times as indicated in min above each panel) from a ^{35}S-methionine-preloaded oocyte suspension stimulated by fertilization* **(A)**, *or by activation with 1 mM emetine* **(B)** *or 0.6 mM 6-DMAP* **(C)**. *Single arrow at 52 kDa, double arrow at 48 and 54 kDa. Molecular weight markers are in kDa.*

oocytes with emetine (0.1 to 1 mM) triggers entry into anaphase within 30 min (Fig. 1C), and leads to the extrusion of the first polar body and the formation of a large decondensed nucleus (Fig. 1D). Sometimes, the two sets of anaphase chromosomes are retained within the oocytes, producing two large pronuclei, which may further fuse together.

Transmission electron microscopy of 6-DMAP and emetine-treated oocytes

The effects after a 90 min treatment of emetine and 6-DMAP on nuclear events were examined by transmission electron microscopy. Figure 2A depicts the meiotic spindle area of normal untreated oocytes, characterized by the absence or paucity of particular organelles and the presence of condensed chromosomes aligned on the metaphase plate. In emetine-treated oocytes (Fig. 2B), first polar body extrusion is completed, and the intracytoplasmic chromatin, originating from the maternal chromosomes, has undergone decondensation and is included in a membrane-bound nucleus. Oocytes incubated in presence of 6-DMAP for the same period never show a polar body, but rather either numerous nuclei (Fig. 2C) or a single nucleus (Fig. 2D) issued by fusion of the former ones. They are also surrounded by a nuclear envelope. This TEM analysis further confirms the observations from optical microscopy, that the metaphase arrest is released upon treatments with emetine or 6-DMAP and that complete chromosome decondensation and nuclear envelope formation accompany this process. It should be noted however that emetine induces a true metaphase/anaphase transition, with completion of first meiotic division, which is not the case after treatment with 6-DMAP.

DNA synthesis

It was previously reported (Dubé and Dufresne, 1990) that emetine induces DNA synthesis in the interphasic stage taking place after completion of first meiotic division. Because 6-DMAP drives the metaphase I-arrested oocytes into interphase, the effect of this drug on DNA synthesis was thus investigated.

6-DMAP induces a large increase of ^{3}H-thymidine incorporation (Fig. 3). This burst begins between 30 and 90 min after addition of the drug, depending on the sensitivity of the different batches used (4 independent experiments). This delay could be related to the variable time needed for the cells to decondense their chromosomes and to revert to interphasic nuclei.

Biochemical analysis of the release from the metaphase arrest

We further investigated the biochemical processes underlying the observed biological effects of emetine or 6-DMAP, as compared to normal embryos. Previously, we reported that a protein at ≈52 kDa, synthesized in unfertilized oocytes undergoes a rapid disappearance upon fertilization to further oscillate along with the cell cycles (Dubé and Dufresne, 1990). This experiment is partly reproduced in Fig. 4, along with the patterns of synthesized proteins observed in emetine and 6-DMAP treated oocytes. Times at which oocytes were sampled after each of these treatments are indicated above each panel. Fertilization (Fig. 4A) induces cyclic variations in the intensity of the 52 kDa protein band (single arrow) which peaks at metaphase I (0 min), metaphase II (40 min) and metaphase of first cleavage (80 min), and decreases between these periods. During the same time, another major synthesized protein of 48 kDa increases progressively. In emetine-treated oocytes (Fig. 4B), the 52 kDa protein band progressively declines in intensity, while the 48 kDa band decreases more slowly. The ratio between these two bands (52 kDa/48 kDa), estimated by densitometric analysis, also decreases over time (not shown). Finally, in 6-DMAP treated oocytes (Fig. 4C), the 52 kDa protein

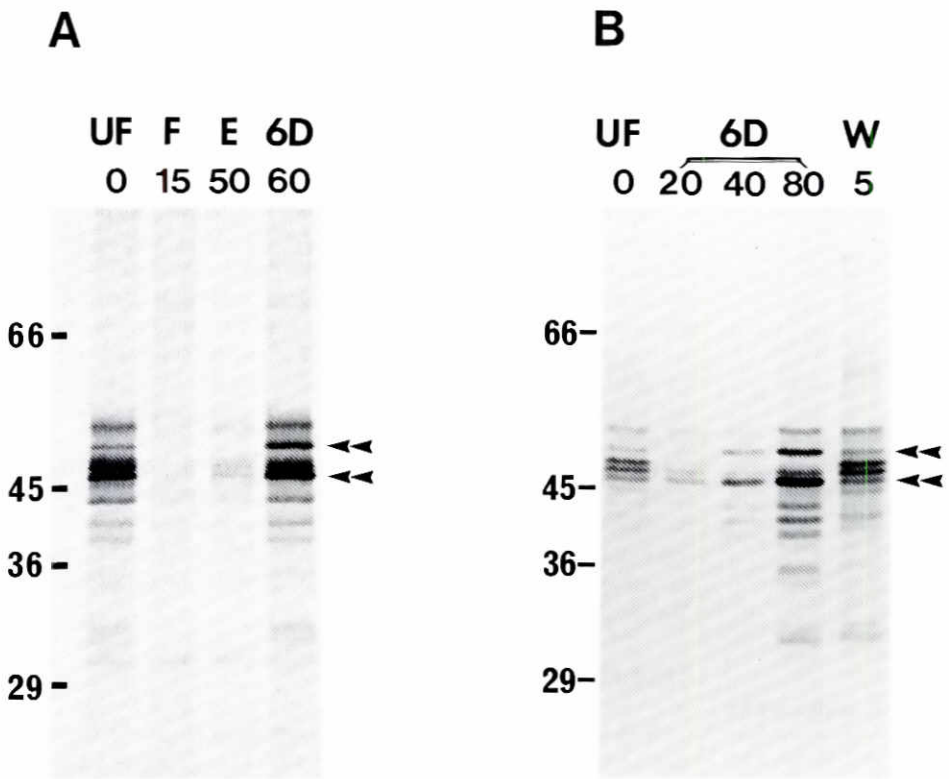

Fig. 5. Patterns of ^{35}S-methionine labeled proteins precipitated by p13^{suc1}-sepharose beads. (Panel A) *^{35}S-methionine-preloaded oocytes were sampled for p13^{suc1} precipitation before fertilization (UF), 15 min after fertilization (F), 50 min after incubation with 1 mM emetine (E) or after 60 min with 0.6 mM (6D). Fertilized and emetine-treated oocytes were in anaphase (72% and 97% respectively), while 100% of the oocytes had a decondensed nucleus after 6-DMAP treatment.* **(Panel B)** *^{35}S-methionine-preloaded oocytes were sampled at the indicated times (in minutes above each line), before activation (UF), during 6-DMAP treatment (6D) and after wash-out of the drug (W). 100% decondensed nuclei in 6-DMAP-treated oocytes were followed by chromatin recondensation after the drug was washed out. Molecular weight markers are in kDa.*

band, as observed after fertilization, declines in intensity after 20 min of treatment. Another striking observation is the appearance of two new protein bands (double arrows), one migrating slightly above 48 kDa, and the other at ≈54 kDa. Both bands start to accumulate around 60 min after treatment.

We then wanted to ascertain whether these proteins belonged to the cyclin family, as suggested by their cyclic appearance and disappearance after fertilization. To address this question, we have taken advantage of the properties of p13^{suc1}-sepharose beads, known to bind complexes of p34^{cdc2} homologs and associated proteins, like cyclins, in several other cell systems. It is worth noting that all the samples used for p13^{suc1} precipitations are taken at different times in order to compare the patterns after exit from metaphase arrest, which takes longer with 6-DMAP or emetine treatments as compared to fertilization. Aliquots of ^{35}S-methionine-labeled oocytes are sampled at desired times after activation, for analysis of p13^{suc1}-bound material (Fig. 5A). The first lane (UF), showing the bound material from unfertilized oocytes, reveals the presence of several protein bands, some of which migrate between 48 and 54 kDa. None of these bands are observed 15 min after fertilization (F), at the approximate time of metaphase/anaphase transition, while they are faintly apparent in the 50 min- emetine-treated oocytes (E), and totally absent after 80 min (not shown). In the 6-DMAP-treated oocytes (6D), sampled at 60 min, we observe a major band at 48 kDa, and also an accumulation of the 54 kDa form. The enrichment in both bands is further confirmed as a progressive event with time (B) that is reversible when 6-DMAP is washed out.

These observations suggest that meiosis resumption, triggered either by fertilization or emetine, involves the destruction of putative cyclins. The accumulation and modifications of p13^{suc1}-precipitated proteins after 6-DMAP treatment, support the view that this treatment does not lead to cyclin destruction or dissociation of the p34^{cdc2}-cyclin complex, even though modifications of putative cyclins take place.

Figure 6 depicts a similar experiment where ^{32}P-phosphate-labeled proteins are precipitated with p13^{suc1}-sepharose beads. In the area where putative cyclins are expected to migrate, one band around 48 kDa is detected in the metaphase I-arrested oocytes. This band is absent from fertilized and emetine treated samples, whereas it is barely detectable in the 6-DMAP treated oocytes. An also barely phosphorylated band is detectable in the UF sample at 52 kDa. It appears that fertilization, emetine and 6-DMAP all induce a decrease in the abundance of phosphorylated forms of putative cyclins. In the 34 kDa area, a single band at 32 kDa is detected in the unfertilized oocyte samples (UF), which is very faint in the fertilized ones (F), and absent in the emetine-treated oocytes (E). In the 6-DMAP samples, a higher band is also detected (6D).

This 34 kDa area was further analyzed and p34^{cdc2} mussel homolog was detected with anti-p34^{cdc2} antibodies in Western blots (Fig. 7). A high resolutive blot permitted to visualize two bands in unfertilized samples (UF), at 32 and 34 kDa. The 32 kDa band corresponds to the phosphorylated one revealed in Fig. 6, as verified in independent experiments (not shown). This faster-migrating band is gradually lost after addition of emetine (E). Upon 6-DMAP treatment (6D), there is also a gradual loss of the faster-migrating form but this is followed by the reappearance of both these bands plus an additional one of intermediate size (≈33 kDa), as previously seen in ^{32}P-labeled samples precipitated with p13^{suc1} beads (Fig. 6). p34^{cdc2} modifications induced by 6-DMAP are totally

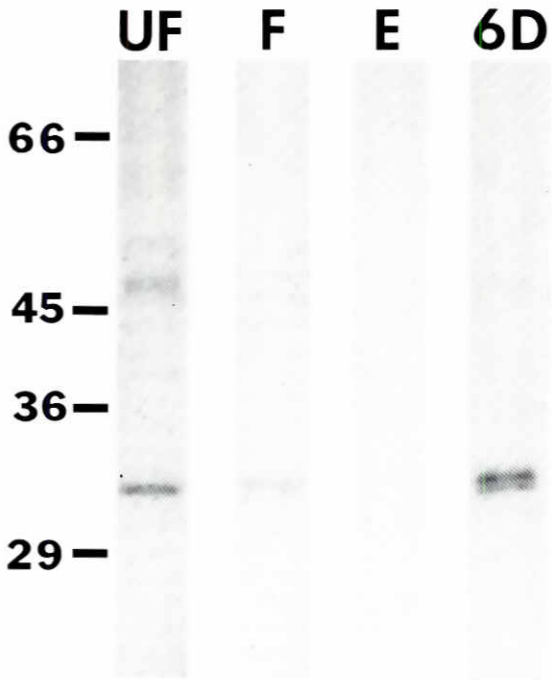

Fig. 6. Patterns of ^{32}P-phosphate labeled proteins precipitated by p13^{suc1}-sepharose beads. *^{32}P-phosphate-preloaded oocytes were sampled before fertilization (UF), 27 min after fertilization (F), 75 min after incubation with 1 mM emetine (E) or 55 min with 0.6 mM 6-DMAP (6D). 66% of the fertilized eggs were in anaphase, whereas 100% of the oocytes had decondensed chromatin after drug treatments.*

reversed within 5 min after a simple wash-out of the drug (W). For comparison, fertilized oocytes (Fig. 7, F) exhibit a faintly resolved doublet of p34 homolog.

Incubation with anti-phosphotyrosine antibodies (Fig. 8) reveals that the new intermediate p33 band appearing after 6-DMAP treatment (Fig. 7, 6D) is the only one phosphorylated on tyrosine, but it is no longer phosphorylated 5 min after wash-out of the drug (W). No signal is detected by this antibody in the unfertilized (UF) or emetine (E) samples. The residual signal detected in emetine treatment is due to incomplete dehybridization of the nitrocellulose membrane after anti-p34^{cdc2} antibody incubation.

Histone kinase assays

Histone H1 kinase activity was measured from whole homogenates and from p13^{suc1}-bound material (Table 1). In both cases, it appears that histone phosphorylation is respectively 80%, 90% and 60% lower after fertilization, emetine and 6-DMAP treatments, as compared to the values recorded for metaphase I-arrested unfertilized oocytes. Thus, all of these treatments dramatically reduce the histone kinase activity of the oocytes, whereas the total level of p34^{cdc2} remains stable during all the experiments (not shown).

In conclusion, our results show that normal completion of meiosis requires cyclin destruction. A decrease in histone H1 kinase activity is not sufficient for completion of M-phase but it appears enough for reverting to interphasic stage, accompanied by tyrosine phosphorylation of p34^{cdc2} component and continuous ^{3}H-thymidine incorporation.

Discussion

The main goal of the present work was to clarify the early processes leading to meiosis resumption in metaphase I-arrested *Mytilus* oocytes. Our results reveal that metaphase I is characterized by a high level of histone H1 kinase activity, the presence of MPF complex consisting of phosphorylated p34^{cdc2} and associated proteins. Release of the metaphase arrest by fertilization is accompanied by a rapid decrease of histone H1 kinase activity, a decrease in the level of the phosphorylated form of p34^{cdc2} homolog without any apparent decrease in its total concentration, and the loss of synthesized proteins. This scheme coincides with current knowledge on the cell cycle as determined in several other species.

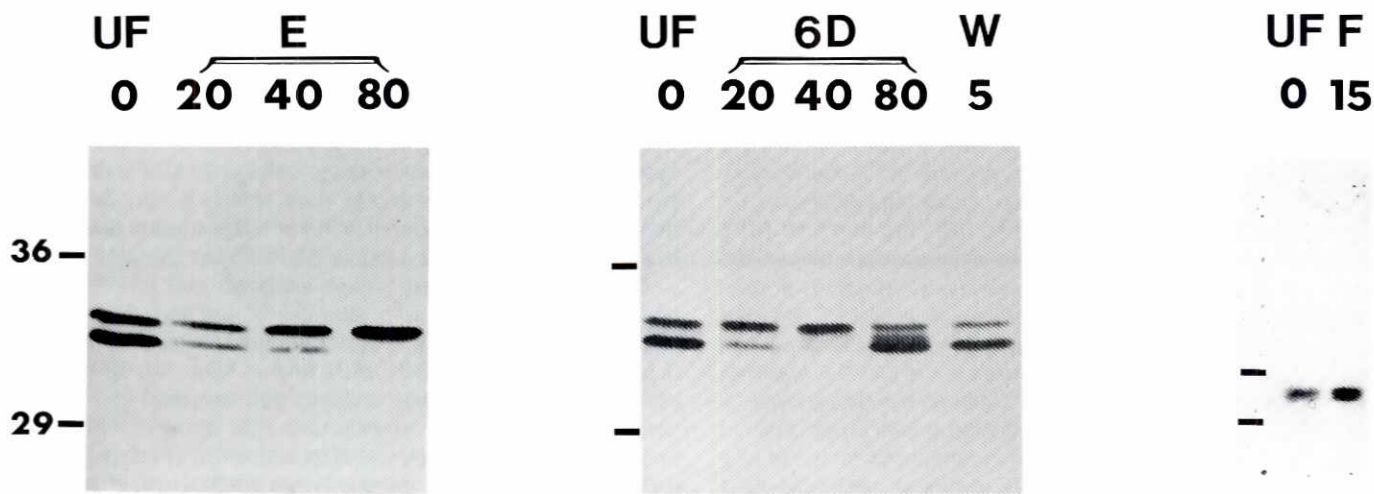

Fig. 7. Detection of p34^{cdc2} blue mussel homolog with anti-p34^{cdc2} antibody. *Samples from metaphase I-arrested oocytes (UF lanes), 1 mM emetine-treated oocytes (E), 0.6 mM 6-DMAP-treated oocytes (6D), and 6-DMAP-washed-out (W), precipitated at the indicated times by p13^{suc1}-beads, were blotted with anti-p34^{cdc2} antibody (G8), compared to 15 min after fertilization (F).*

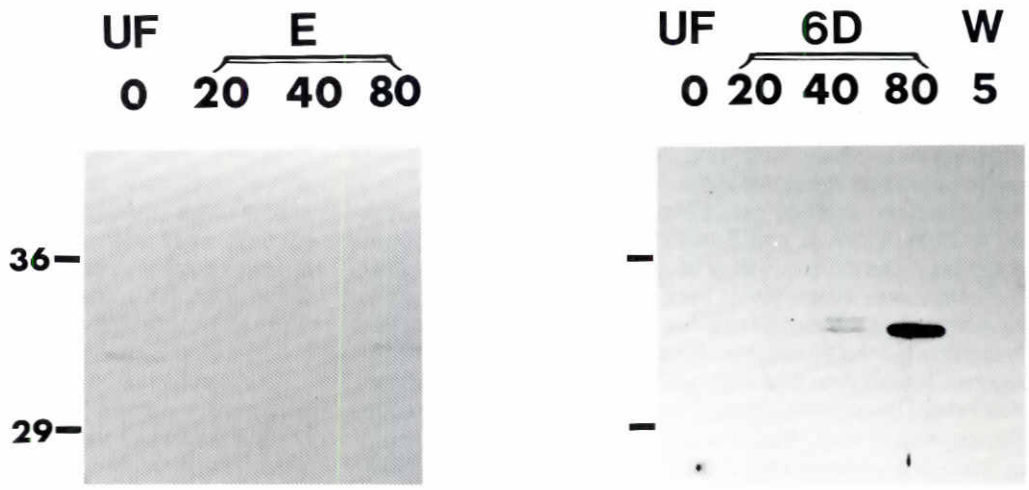

Fig. 8. Phosphorylated tyrosine form(s) at the 34 kDa level. *The membranes previously blotted in Figure 7 were incubated with anti-phosphotyrosine antibody to detect the tyrosine-phosphorylated forms of p34^{cdc2} from unfertilized (UF), emetine-treated (E), 6-DMAP-treated (6D) or 6-DMAP-washed-out (W) oocytes.*

Indeed, maximal histone H1 kinase activity develops at metaphase and is rapidly lost, simultaneously with the cyclin destruction step, which occurs at the metaphase/anaphase transition (reviewed by Pelech *et al.*, 1990; Feilotter *et al.*, 1992).

Cyclins

When comparing the results obtained under the various conditions tested, several characteristics either link or distinguish the mechanism of metaphase release. For the putative cyclins, emetine-treated and fertilized oocytes share common features in the early process: in the two conditions the loss of a major 52 kDa band is seen as the first step both *in vivo* with total proteins (Fig. 4) and with specific p13^{suc1}-precipitated proteins (Fig. 5A). In both cases we also observed the disappearance of ^{32}P-labeled bands of 48 and 52 kDa in p13^{suc1}-precipitated proteins (Fig. 6). The putative 52 kDa cyclin is later seen to reappear and cycle in normal embryos undergoing additional division cycles (Fig. 4A; Dubé and Dufresne, 1990). 6-DMAP-treated oocytes, in contrast, show no clear evidence of specific destruction of any given band *in vivo* (Fig. 4) and even more clearly in ^{35}S-methionine-labeled proteins precipitated with p13^{suc1} (Fig. 5A,B). Instead, we detect an additional 54 kDa band and an apparent shift of the 52 kDa one to a faster-migrating form of ≈48 kDa band. We suggest that the 54 kDa band might well be another cyclin appearing, or detectable, only at later times, as we already observed in a previous report (Dubé and Dufresne, 1990). The new appearing ≈48 kDa band, on the other hand, seems to arise from a conversion of the 52 kDa band as suggested by the parallel kinetics in the decrease of 52 kDa band and increase of ≈48 kDa band as seen *in vivo*, and again much more clearly in p13^{suc1}-precipitated proteins (Figs. 4 and 5B). In addition, a wash-out of 6-DMAP (Fig. 5B) rapidly reestablishes both bands to their original location. Finally, since no ^{32}P-labeled protein in the region of 52 kDa is seen in p13^{suc1} precipitates of 6-DMAP-treated oocytes (Fig. 6), this further suggests that dephosphorylation of the 52 kDa band seen in unfertilized oocytes might convert into this new ≈48 kDa band seen only in 6-DMAP-treated oocytes.

The 6-DMAP-induced modifications in the distribution of these putative cyclins may effectively be due to phosphorylation changes, since (1) 6-DMAP has been shown to inhibit protein phosphorylation (Meijer and Pondaven, 1988; Néant and Guerrier, 1988a,b; Néant *et al.*, 1989), (2) the ^{32}P-phosphate-labeled proteins present in metaphase-arrested oocytes, and detected after p13^{suc1} precipitation, are no longer seen after 6-DMAP treatment, and (3) a similar modification in the pattern of cyclins was observed after 6-DMAP treatment of metaphase oocytes from *Patella*, and this observed redistribution can be mimicked by *in vitro* incubation of the metaphasic extracts with alkaline phosphatase (van Loon *et al.*, 1991; Néant, unpublished).

Furthermore, Luca and Ruderman (1989) reported that 6-DMAP completely blocks cyclin destruction *in vitro*, supporting the hypothesis that cyclins stay present *in vivo*, and thus inhibit the metaphase/anaphase transition. Under these conditions, the accumulation of putative cyclins, at above 48 kDa and at 54 kDa, would allow tyrosine phosphorylation of p34^{cdc2} blue mussel homolog, a feature that would account for the observed decrease in the histone H1 kinase activity (Table 1), as discussed below. This coincides with the demonstration that cyclin B accumulation targets p34^{cdc2} for tyrosine phosphorylation in other species (Solo-

TABLE 1

HISTONE KINASE ACTIVITIES AFTER ACTIVATION OF UNFERTILIZED OOCYTES WITH SPERM, EMETINE OR 6-DMAP, MEASURED FROM TOTAL HOMOGENATES OR FROM p13^{suc1}-BOUND MATERIAL

Assay	CPM in histone excised band (mean±s.d.)	Decrease (%) compared to unfertilized oocytes
p13^{suc1}-bound material		
Unfertilized	32,628 ± 1,543	—
Fertilized 15 mn	4,346 ± 153	86.7%
Emetine 1 mM, 50 mn	2,036 ± 34	93.8%
6-DMAP 600 µM, 60 mn	14,027 ± 2,662	57.0%
Total homogenates		
Unfertilized	52,057 ± 951.0	—
Fertilized, 15 mn	14,152 ± 60.0	72.8%
Emetine 1mM, 50 mn	4,017 ± 178.9	92.2%
6-DMAP 600 µM, 60 mn	17,596 ± 905.0	66.2%

mon *et al.*, 1990, 1992; Meijer *et al.*, 1991; Parker *et al.*, 1991; Devault *et al.*, 1992).

While metaphase II arrest and MPF activity are stabilized by a cytostatic factor (CSF) in vertebrate species (Masui and Markert, 1971; Sagata *et al.*, 1989; Masui, 1991), there is until now no indication that a similar stabilizing factor also exists in invertebrate species arrested in metaphase I. Indeed, it has been shown in *Patella* oocytes, that anti-sense oligonucleotide-directed destruction of both cyclins A and B mRNA releases oocytes from the metaphase arrest (van Loon *et al.*, 1991). This strongly suggests that continuous synthesis of cyclins is absolutely required for maintenance of the metaphase I arrest. One possibility is that, in metaphase I-arrested oocytes, a fragile dynamic equilibrium exists between proteolysis and the synthesis of short-lived proteins, which might be either the cyclins themselves, as shown in mouse oocytes (Kubiac *et al.*, 1993), or some other regulatory proteins, as reported for *Patella* oocytes (Colas *et al.*, 1993). If this equilibrium is affected beyond a critical threshold, the whole process leading to release of the metaphase arrest would be triggered. In the absence of *de novo* synthesized proteins during emetine treatment, proteolysis involved in the mechanism of cyclin destruction (Picard *et al.*, 1985; Glotzer *et al.*, 1991; Holloway *et al.*, 1993), leads to cyclin disappearance. Anaphase is thus initiated, followed by the completion of cell division (i.e. polar body extrusion in this case).

Thus, taken altogether, our results indicate that the release from metaphase I arrest in blue mussel oocytes, by fertilization and by protein synthesis inhibition, is accompanied by putative cyclin disappearance, and the differential biological response of 6-DMAP, characterized by direct transition to an interphasic stage, is not accompanied by the destruction of putative cyclins even though they apparently undergo slight post-translational modifications.

p34^{cdc2} homolog

Precipitation of proteins by p13^{suc1} reveals that a ^{32}P-labeled p34^{cdc2} homolog is detectable in unfertilized metaphase I-arrested oocytes from *Mytilus*, but is no longer seen after fertilization or emetine treatment (Fig. 6). Western blots performed with anti-phosphotyrosine further show that this ^{32}P-labeled band seen in unfertilized oocytes is not phosphorylated on tyrosine (Fig. 8). Thus, this indicates that release from metaphase arrest after emetine treatment or fertilization is accompanied by some serine/threonine dephosphorylation of p34^{cdc2} homolog. These metaphase/anaphase transitions are accompanied by a drop in histone H1 kinase activity (Table 1). Even though the total amount apparently remains stable as determined by Western blots with anti-p34^{cdc2} antibodies (data not shown), the progressive disappearance, in the presence of emetine, of the lower band in the p34 doublet observed in metaphase-arrested oocytes (Fig. 7), is an unexpected result. Dephosphorylation is indeed generally accompanied by a shift to a faster-migrating form. We expect that further phosphoamino acid analysis will contribute to clarify this behavior.

6-DMAP treatment, unlike emetine activation, results in a dramatic appearance of tyrosine-phosphorylated form of p34^{cdc2} homolog that is rapidly lost upon wash-out of the drug (Fig. 7) and might correspond to the additional band seen in ^{32}P-labeled proteins precipitated by p13^{suc1} beads (Fig. 6). This is in agreement with the fact that cyclins, which are required for targeting p34^{cdc2} for tyrosine phosphorylation (Solomon *et al.*, 1990, 1992; Meijer *et al.*, 1991; Parker *et al.*, 1991), are already synthesized in the presence of 6-DMAP. In 6-DMAP-treated, but not in emetine-treated oocytes, maintenance of cyclin synthesis allows accumulation of inactive MPF complexes. Thus, release from the metaphase arrest by 6-DMAP is also accompanied by a drop of histone H1 kinase activity (Table 1), which is maintained at low level by tyrosine rephosphorylation of p34^{cdc2} homolog subunit.

p34^{cdc2} tyrosine dephosphorylation and subsequent activation of histone H1 kinase are normally due to the activation of cdc25 phosphatase (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Kumagai and Dunphy, 1991; Strausfeld *et al.*, 1991). cdc25 activity is itself regulated by phosphorylation (Kumagai and Dunphy, 1992; Clarke *et al.*, 1993; Mellgren *et al.*, 1993). We suggest that 6-DMAP might inhibit phosphorylation of cdc25 and maintain this phosphatase in its inactive form, thus preventing activation of p34^{cdc2}.

Our results show that metaphase release in blue mussel oocytes is accompanied by a drop in MPF activity, evidenced as a drop in histone H1 kinase activity, directly attributable to the disappearance of the putative cyclins after fertilization or emetine treatment. In the presence of 6-DMAP, newly synthesized cyclins target p34^{cdc2} phosphorylation on tyrosine and these complexes are maintained in inactive form, characterized by low level of histone H1 kinase activity.

DNA synthesis

The inhibition of protein synthesis by emetine mimics the early processes, associated with normal fertilization, which drive the cell cycle forward into the metaphase/anaphase transition and all the subsequent steps of meiosis I, including polar body extrusion. Afterwards, however, under the same conditions of protein synthesis inhibition, the chromosomes undergo decondensation, 2n equivalent DNA containing nuclei form and enter in a permanent DNA-synthesizing phase (Dubé and Dufresne, 1990). Moreover, the inhibition of protein phosphorylation by 6-DMAP in metaphase-arrested oocytes also triggers chromosome decondensation, but without allowing completion of the first meiotic cycle, and the 4n equivalent DNA-containing nuclei undergo continuous ^{3}H-thymidine incorporation (Fig. 3).

During meiosis, gametes undergo two cell divisions, allowing the transition from 4n to 1n equivalent stock of DNA through the extrusion of the polar bodies. No nuclear membrane reforms between these two sets of division and no DNA synthesis takes place. This indicates that the regulation cascade of DNA replication is inhibited during meiosis, until the cell enters mitosis, and this inhibition may be reverted in the presence of emetine or 6-DMAP.

Recent reports demonstrate that the wee1 kinase is inhibited after DNA replication (Dasso and Newport, 1990; Lehner and O'Farrell, 1990; Devault *et al.*, 1992; Pagano *et al.*, 1992) and that p34^{cdc2} stays in a tyrosine-phosphorylated inactive form, controlled by wee1 kinase, as long as DNA replication is not achieved (Heald *et al.*, 1993). Since 6-DMAP may be considered as a partly specific inhibitor of protein kinases, allowing activity of tyrosine kinases but not of serine/threonine kinases (Jessus *et al.*, 1991), one may hypothesize that wee1 protein kinase homolog is not affected by the drug, and its substrate, the p34^{cdc2} blue mussel homolog, might be phosphorylated on tyrosine in the presence of 6-DMAP. On the other hand, cdc25 phosphatase activity also requires completion of DNA synthesis (Edgar and O'Farrell, 1989; Enoch and Nurse, 1990; Kumagai and Dunphy, 1991). cdc25 might stay inactive in continuously DNA-synthesizing oocytes treated by 6-DMAP.

In conclusion, our results provide a possible explanation for the differing biological effects of emetine and 6-DMAP in the oocytes of *Mytilus edulis* and other invertebrates. Emetine, like fertilization,

results in the destruction of one (or more) synthesized protein(s), a process that would be sufficient to drive meiosis I to its completion. 6-DMAP, on the other hand, would not directly result in the specific destruction of any given set of proteins, while some of them might be dephosphorylated, and not destroyed, upon such a treatment. The result, as seen, is the loss of active MPF and histone H1 kinase activity, an event which would be sufficient to cause chromosome decondensation and nuclei reformation but not sufficient to drive meiosis into its normal subsequent steps. This leads us to propose the view that, unlike emetine which drives the cell cycle forward, 6-DMAP reverts it backward. In other words, 6-DMAP-treated oocytes are not triggered to complete meiosis I, but are reverted into an interphase stage where cyclin accumulation favors the tyrosine phosphorylation of $p34^{cdc2}$, and therefore, decreases histone H1 kinase activity. It will be of interest to further determine which residues of the $p34^{cdc2}$ homolog are involved during this process, and to analyze the cdc25 and wee1 homologs. Further work on these peculiar mechanisms of metaphase I arrest in invertebrate species is required to fully elucidate the basic mechanism of cell cycle regulation as well as the specific pathways adopted by different species along the evolution.

Materials and Methods

Chemicals

Sodium orthovanadate, ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis (ß-aminoethyl ether) (EGTA), Tris-HCl, ß-glycerophosphate, sodium fluoride, 6-dimethylaminopurine (6-DMAP), emetine, leupeptin, aprotinin, soybean trypsin inhibitor, benzamidine, phenylmethylsulfonide fluoride (PMSF), and histone (type III-S), were from Sigma Chemicals (St. Louis, Missouri, USA).

[γ-^{32}P]ATP, [^{35}S]methionine, [^{32}P]*ortho*phosphate and [^{3}H]thymidine were purchased from Amersham. Anti-$p34^{cdc2}$ antibodies (G8) were kindly provided by Dr. G. Draetta (Cold Spring Harbor, USA) and anti-phosphotyrosine by Dr J.Y.J. Wang (University of California at San Diego, USA).

Handling of gametes

Adult specimens of the blue mussel *Mytilus edulis* were collected at Métis (Québec, Canada) and kept in running sea water tanks. The animals were induced to spawn in individual finger bowls filled with seawater, after electrical shock treatments (30 V, 15 seconds, electrical shock applied between the closed valves). The oocytes were washed several times with natural filtered seawater (NSW) adjusted to pH 8.0 with Hepes 10 mM. Sperm was collected as concentrated as possible and kept at 4°C until use. Fertilization was achieved by adding sperm to oocyte suspensions at 0.5-2% v/v, for a final sperm density of approximately $3.0x10^6$ spermatozoa/ml, a condition yielding optimal fertilization with little polyspermy (Dufresne-Dubé *et al.*, 1983). All experiments were performed at a constant temperature of 15°C maintained with a temperature-controlled water bath. Oocyte suspensions were incubated in NSW containing penicillin G (50 U/ml) and streptomycin sulphate (0.05 mg/ml).

Cytological procedures

Optical microscopy

The morphological effects of emetine or 6-DMAP upon the state of chromosome condensation were studied on live oocytes stained for 10 min with 0.5 µg/ml of the DNA-specific fluorescent probe Hoechst 33342 or after fixation and staining with Hoechst 33258 as described earlier (Dubé and Dufresne, 1990).

Transmission electron microscopy

Oocytes were fixed for 1 h in 3% glutaraldehyde in NSW at room temperature. After 2 washes with seawater, they were post-fixed with 0.5% OsO_4 in sea water for 15 min. They were then dehydrated in a series of ethanol and embedded in Spurr (1969). Thin sections were stained with 4% uranium acetate and lead citrate dissolved in 50% methanol (Reynolds, 1963).

Biochemical analysis

Measurement of DNA synthesis

The protocol used was a modification of the method described in Dubé and Dufresne (1990): 1-2% oocyte suspensions were incubated for 2 h in presence of 5 µCi/ml ^{3}H-thymidine (final concentration). After 3 washes with NSW, the oocytes were divided into 2 batches, one receiving 6-DMAP to a final concentration of 0.6 mM. At different time intervals, duplicate samples of 0.2 ml were transferred to an equal volume of ice-cold 20% TCA containing 1 mg/ml cold thymidine. After 1 h at 4°C, the pellets were washed 3 times with 1 ml TCA 5%+0.1 mg/ml thymidine, and dissolved in 0.2 ml NaOH 0.5 N. A reprecipitation with TCA overnight at 4°C was followed by redissolution in NaOH. 0.1 ml were then mixed with 5 ml Scintiverse and counted in a Beckman LS5801 scintillation counter. The remaining 0.1 ml were used for protein quantification by the method of Bradford (1976).

Protein synthesis and phosphorylation

10% v/v oocyte suspensions were precharged for 2 to 4 h with ^{35}S-methionine (100-200 µCi/ml, final concentration) or ^{32}P-*ortho*phosphate (200 µCi/ml, final concentration). The oocytes were then washed three times with NSW and adjusted to 2% v/v. 0.2 ml samples were transferred at desired times to an equal volume of 20% TCA containing 1 mg/ml methionine and were left on ice for 30 min; the pellets were then washed twice with 1 ml ice-cold acetone and dissolved in 0.15 ml Laemmli sample buffer (1970).

$p13^{suc1}$-sepharose beads precipitations

The beads, prepared as described by Meijer *et al.* (1989), were obtained from M. Barrot (ENS, Lyon, France). 1 ml of labeled oocyte suspensions were sampled at desired times, centrifuged at 13000 g, washed with 1 ml of calcium free-sea water, dry pellets were immediately frozen in liquid nitrogen and stored at -80°C before use. Frozen aliquots were disrupted in 0.5 ml of Bead Buffer (complemented with 20 mM ß-glycerophosphate, 5 mM sodium pyrophosphate, 0.1 mM sodium *ortho*vanadate). After 30 sec centrifugation at 13000 g, the supernatants were removed, adjusted for equal CPM, and incubated at 4°C end over end for 1 h with 10 to 20 µl of p13-sepharose beads. After a brief centrifugation at 13000 g, the beads were washed four times with Bead Buffer and the precipitated material dissolved in Laemmli sample buffer.

Electrophoresis and Western blot analysis

SDS-polyacrylamide gels were prepared according to Laemmli (1970), fixed, dried and exposed on an X-ray film, or transferred onto nitrocellulose membranes (Towbin *et al.*, 1979). Blots were incubated for 2 h at room temperature in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.6) containing 0.1% Tween 20, 3% BSA, 2 h in the presence of antibodies (1/500 in TBS-Tween-BSA) and 1 h with horseradish peroxidase-conjugated secondary antibodies (1/3000). The antibodies were detected with enhanced chemiluminescence kit (ECL, Amersham).

Dehybridization of the nitrocellulose membranes for further analysis, such as incubation with another antibody or direct autoradiography, was performed according to the antibody purification method described by Jessus and Beach (1992): the remoistened membrane was incubated for 30 min in 0.1 M glycine pH 2.5, washed in 1 M Tris pH 8.0 and rinsed by TBS-Tween.

When necessary autoradiographs were scanned using an Ultroscan XL Laser densitometer (LKB-Pharmacia) coupled to a MIPS computer. Analysis of the scans were performed using the GSXL2 software (LKB-Pharmacia). After scanning of the lanes, peaks were integrated and their absolute areas (absorbance unitsxmm) compared. All descriptions presented in the text concerning the behavior of specific proteins were confirmed by these quantitative analysis.

Histone H1 kinase assays

The oocytes were suspended as a 2% v/v suspension, and treated as previously described. At desired times, aliquots were withdrawn, centrifuged, washed once with CaFSW, and frozen in liquid nitrogen. The pellets (0.1 ml packed oocytes) were homogenized with 1 ml of PK buffer (20 mM Tris, 1 mM DTT, 10 mM $MgCl_2$, 2 mM EGTA, 5 mM ß-glycerophosphate, adjusted to pH 7.4 with 1N HCl) at 4°C. The homogenates were centrifuged for 15 min at 13000 g in a cold chamber and the supernatants were frozen in liquid nitrogen. After determining the protein content according to Bradford (1976), 1.0 µg/µl of proteins were mixed with 0.1 mM cold ATP with or without 1 µg/µl added histone, in a final volume of 200 µl. The reaction, started by the addition of 0.7 µCi/µl γ-^{32}P-ATP, was carried out at 30°C for 15 min and stopped by adding 1 vol of 20% ice-cold TCA. Samples were then run on SDS-PAGE as previously described and silver stained (Bio-Rad kit) to visualize the histone bands. These were carefully excised from the gels, transferred to scintillation vials containing 5 ml of scintiverse-BD (Fisher Scientific) for CPM determinations. The results, expressed as the mean±s.d. of triplicates, are the differences between the CPM in samples with added histone minus those without histone.

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