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 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 p34cdc2 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, p34cdc2 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 Peloch 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 p34cdc2 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 phosphorylation of p34cdc2 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). p34cdc2 can be phosphorylated on tyrosine, serine and threonine residues in accordance with the cell cycle (Krek and Nigg, 1991; Nigg et al., 1989).

Abbreviations used in this paper: BSA, bovine serum albumin; CSF, cytokostatic factor; 6-DMAP, 6-dimethylaminopurine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene bis-(oxosnylhenenitritiolo)) tetraacetic acid; MPF, M-phase promoting factor; NSW, natural sea water; TBS, Tris-buffered saline; TCA, trichloroacetic 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 60 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 philippinarum 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
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 (Dube and Dufresne, 1990), treatment of metaphase I

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.

Fig. 3. Stimulation of 3H-thymidine incorporation by 6-DMAP. 3H-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 3H-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 35S-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.

DNA synthesis

It was previously reported (Dubé and Dufresne, 1990) that emetine induces DNA synthesis in the interphase 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 3H-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 interphase 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

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.

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

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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\textsuperscript{sup1}\textsuperscript{-}-sepharose beads, known to bind complexes of p34\textsuperscript{cdc2}\_homologs and associated proteins, like cyclins, in several other cell systems. It is worth noting that all the samples used for p13\textsuperscript{sup1}\textsuperscript{-} 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 35S-methionine-labeled oocytes are sampled at desired times after activation, for analysis of p13\textsuperscript{sup1}-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\textsuperscript{sup1} precipitated proteins after 6-DMAP treatment, support the view that this treatment does not lead to cyclin destruction or dissociation of the p34\textsuperscript{cdc2}-cyclin complex, even though modifications of putative cyclins take place.

Figure 5 depicts a similar experiment where 32P-phosphate-labeled proteins are precipitated with p13\textsuperscript{sup1}-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\textsuperscript{cdc2} mussel homolog was detected with anti-p34\textsuperscript{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 32P-labeled samples precipitated with p13\textsuperscript{sup1} beads (Fig. 6). p34\textsuperscript{cdc2} modifications induced by 6-DMAP are totally...
Histone kinase assays

Histone H1 kinase activity was measured from whole homogenates and from p135c1-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 p34cdc2 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 p34cdc2 component and continuous 3H-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 p34cdc2 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 p34cdc2 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.
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 p13Suc1-precipitated proteins (Fig. 5A). In both cases we also observed the disappearance of 32P-labeled bands of 48 and 52 kDa in p13Suc1-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 35S-methionine-labeled proteins precipitated with p13Suc1 (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 p13Suc1-precipitated proteins (Figs. 4 and 5B). In addition, a washout of 6-DMAP (Fig. 5B) rapidly reestablishes both bands to their original location. Finally, since no 32P-labeled protein in the region of 52 kDa is seen in p13Suc1 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 32P-phosphate-labeled proteins present in metaphase-arrested oocytes, and detected after p13Suc1 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 p34cdc2 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 p34cdc2 for tyrosine phosphorylation in other species (Solo-

<table>
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<tr>
<th>Assay</th>
<th>CPM in histone excised band (mean±s.d.)</th>
<th>Decrease (%) compared to unfertilized oocytes</th>
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<tr>
<td>p13Suc1-bound material</td>
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<tr>
<td>Unfertilized</td>
<td>32,628±1,543</td>
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<tr>
<td>Fertilized 15 mn</td>
<td>4,346±153</td>
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<tr>
<td>Emetine 1 mM, 50 mn</td>
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</tr>
<tr>
<td>6-DMAP 800 µM, 60 mn</td>
<td>14,027±2,662</td>
<td>57.0%</td>
</tr>
<tr>
<td>Total homogenates</td>
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</tr>
<tr>
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<td>Fertilized, 15 mn</td>
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<td>Emetine 1 mM, 50 mn</td>
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</tr>
<tr>
<td>6-DMAP 800 µM, 60 mn</td>
<td>17,596±905.0</td>
<td>66.2%</td>
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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 Markeri, 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 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 interphase stage, is not accompanied by the destruction of putative cyclins even though they apparently undergo slight post-translational modifications.

**p34^cdcc homolog**

Precipitation of proteins by p1^32Pc1 reveals that a 32P-labeled p34^cdcc homolog is detectable in unfertilized metaphase I-arrested oocytes from Mytilus, but it is no longer seen after fertilization or emetine treatment (Fig. 6). Western blots performed with anti-phosphotyrosine further show that this 32P-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^cdcc 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^cdcc 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^cdcc homolog that is rapidly lost upon wash-out of the drug (Fig. 7) and might correspond to the additional band seen in 32P-labeled proteins precipitated by p1^32Pc1 beads (Fig. 6). This is in agreement with the fact that cyclins, which are required for targeting p34^cdcc 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^cdcc homolog subunit.

p34^cdcc 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^cdcc.

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^cdcc phosphorylation on tyrosine and these complexes are maintained in inactive form, characterized by low level of histone 1 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 Dutrèse, 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 3H-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^cdcc 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^cdcc 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 nuclear 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 cytoplasmic accumulation favors the tyrosine phosphorylation of p34cdc2, and therefore, decreases histone H1 kinase activity. It will be of interest to further determine which residues of the p34cdc2 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 (3-aminomethyl ether) (EGTA), Tris-HCl, B-glycerophosphate, sodium fluoride, 6-aminohippuric acid (6-DMAP), emetine, leupeptin, aprotonin, soybean trypsin inhibitor, benzamidine, phenylmethylsulfonyl fluoride (PMSF), and histone (type III-S), were from Sigma Chemical Co., St. Louis, Missouri, USA.

-[32P]ATP, [35S]methionine, [32P]orthophosphate and [3H]thymidine were purchased from Amersham. Anti-p34cdc2 antibodies (G8) were kindly provided by Dr. G. Draetta (Cold Spring Harbor, USA) and antiphosphotyrosine 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 x 10^6 spermatozoon/ml, a condition yielding optimal fertilization with little polyspermy (Dubreusse-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% OsO4 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% uranyl 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 the presence of 5 μCi/ml [3H]-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 precipitation with TCA overnight at 4°C was followed by redissolution in NaOH. 0.1 ml were 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 precharget for 2 to 4 h with [35S]methionine (100-200 μCi/ml, final concentration) or [32P]-orthophosphate (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).

p12351-Sepharose beads precipitation
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 8-glycerophosphate, 5 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate). 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 Ultrospec XL Laser densitometer (LKB-Pharmacia) coupled to a MIP's 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 units x mm) 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 MgCl2, 2 mM EGTA, 5 mM 8-glycerophosphate, adjusted to pH 7.4 with 1 N 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 μl[γ-32P]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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