Histone H3 phosphorylation during *Xenopus* oocyte maturation: regulation by the MAP kinase/p90Rsk pathway and uncoupling from DNA condensation

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Abstract Here we show that during the meiotic maturation of *Xenopus* oocytes, histone H3 becomes phosphorylated on serine-10 at about the time of maturation promoting factor activation and meiosis I entry. However, overexpression of cAMP-dependent protein kinase that blocks entry into M phase, also leads to massive serine-10 phosphorylation of histone H3 in intact *Xenopus* oocytes but does not cause chromosome condensation. We also show that the phosphorylation of histone H3 during oocyte maturation requires the activation of the mitogen-activated protein kinase/p90Rsk pathway. Our results indicate that in G2-arrested oocytes, which are about to enter M phase, histone H3 phosphorylation is not sufficient for chromosome condensation. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: cAMP-dependent protein kinase; Chromosome condensation; Histone H3 phosphorylation; Mitogen-activated protein kinase; Oocyte maturation

1. Introduction

Eukaryotic DNA is packaged into repeating units, known as nucleosomes (reviewed in [1]). Histone H3 is one of the core histones bound to DNA in the nucleosomes. Phosphorylation of histone H3 on Ser-10 has been observed in two different processes, the activation of transcription and chromosome condensation during mitosis and meiosis (reviewed in [2,3]). Chromosome condensation is an essential step for the accurate segregation of chromosomes during the cell cycle (reviewed in [4]). In *Tetrahymena*, Ser-10 phosphorylation of histone H3 is required for normal chromosome segregation during mitosis and meiosis [5]. Moreover, inhibition of histone H3 phosphorylation prevents the initiation of chromosome condensation and mitosis entry in mammalian cells [6] whereas the inhibition of histone H3 dephosphorylation induces abnormally condensed chromosomes and affects mitosis exit [7]. In *Xenopus* egg extracts, the phosphorylation of histone H3 at Ser-10 has been shown to correlate with the assembly of mitotic chromosomes [8]. Thus, both phosphorylation and dephosphorylation of histone H3 appear to be important features of the normal chromosome condensation/decondensation cycle. In contrast to these observations, in the plant *Zea mays*, histone H3 phosphorylation does not appear to play a role in the early steps of chromosome condensation [9].

Various histone H3 kinases have been identified (reviewed in [3]), although most of the available evidence points to the implication of members of the Aurora protein kinase family in mitotic histone H3 phosphorylation [10–13]. The cAMP-dependent protein kinase (PKA) has been shown to phosphorylate Ser-10 of histone H3 in vitro in the context of long chromatin or in the nucleosome core and, to a lesser extent, DNA-free histone H3 [14,15]. Recently, the follicle-stimulating hormone (FSH) has been shown to induce histone H3 phosphorylation in rat follicular granulosa cells, possibly mediated by PKA [16,17]. However, only a small percentage of histone H3 appears to be phosphorylated upon stimulation with FSH in contrast to the well-documented hyperphosphorylation of histone H3 during mitosis in a variety of cells [16]. In addition, the phosphorylation of histone H3 by PKA in this system seems to correlate with transcription initiation rather than mitosis entry [17].

The phosphorylation of histone H3 has not been investigated during the meiotic maturation of *Xenopus* oocytes, although antibodies raised against Ser-10-phosphorylated histone H3 can label the condensed DNA aligned on the metaphase spindle in mature *Xenopus* oocytes [18]. Here we report that histone H3 is phosphorylated on Ser-10 when oocytes enter meiosis I and this phosphorylation depends on the activation of the mitogen-activated protein kinase (MAPK)/p90Rsk pathway. However, inhibition of progesterone-induced oocyte maturation by overexpression of active PKA also correlates with the efficient Ser-10 phosphorylation of histone H3, uncoupling this phosphorylation event from chromosome condensation and M phase entry.

2. Materials and methods

2.1. Isolation of Xenopus oocytes and induction of meiotic maturation

The isolation of *Xenopus laevis* oocytes and the induction of meiotic maturation with progesterone (5 μg/ml, Sigma) or by injection of 50 nl of purified recombinant proteins has been previously described [19–21]. In some experiments, oocytes were pre-incubated with 50 μg/ml cycloheximide (CHX, Sigma) or 50 μM U0126 (Promega) for 30–60 min before injection. Maturation was scored by the appearance of a

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Abbreviations: CHX, cycloheximide; GVBD, germinal vesicle breakdown; H1K, histone H1 kinase; MAPK, mitogen-activated protein kinase; MPF, maturation promoting factor; PKA, cAMP-dependent protein kinase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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white spot on the animal pole of the oocytes. Germinal vesicle breakdown (GVBD) was confirmed by dissection of oocytes that had been fixed in 3% TCA for 10 min.

Capped mRNAs encoding myc-tagged *Xenopus* PKAα, either wild type or the K72R mutant, were obtained from FTX5 constructs using the MEGAscript™ in vitro transcription kit (Ambion) according to the manufacturer's instructions [21]. Oocytes were injected with 50 nl of mRNA solutions and left for 8–16 h at 18°C to allow protein expression.

2.2. Preparation of oocyte lysates, nuclei and membranes

Lysates were prepared by crushing oocytes in histone H1 kinase (H1K) buffer as described [19]. Nuclei were manually removed from oocytes fixed in 3% TCA for 10 min and washed with PBS before sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; 10 nuclei per lane). Membranes were prepared as described in [21]. The membrane pellets were resuspended in 1 μl H1K buffer per oocyte supplemented with 20 mM NaF, 2 μM microcystin and 0.1 mM vanadate and analysed by SDS–PAGE (equivalent to 10 oocytes per lane).

2.3. Immunoblotting and H1K assays

Immunoblotting was performed as previously described [19,22]. using the following antibodies: PKACγ, p90Rsk2, myc (Santa Cruz Biotechnology), Cdc2 (3E1 monoclonal provided by J. Gannon and T. Hunt), XMpk1-Ct [22], phospho-p44/42 ERK MAP kinase (Cell Signalling Technology), phospho-histone H3 (Upstate Biotechnology), α-tubulin (Sigma) and nucleoplasmin (provided by R.A. Laskey).

Oocyte lysates were prepared in ice-cold H1K buffer (10 μl per oocyte) and 4 μl lysate was incubated in a total volume of 12 μl H1K buffer containing 50 μM cold ATP, 2 μCi [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) and 4 μg histone H1 (Sigma) for 30 min at 22°C. The kinase reactions were analysed by SDS–PAGE and autoradiography.

2.4. Immunofluorescence confocal microscopy

Oocytes were fixed in methanol for 1 h at −80°C and rehydrated in PBS at room temperature. The nuclei of the fixed G2-arrested oocytes were manually isolated and incubated with 1.6 μg/ml phospho-histone H3 antibody in 5% milk in PBS for 1 h at room temperature. Alexa 488 labelled secondary antibodies were purchased from Molecular Probes. The nuclei were mounted in PBS supplemented with 2.5 μM of the DNA dye DRAQ5 (Biostatus Limited). The same results were obtained using 2.5 μM Hoechst 33342 (Molecular Probes). For metaphase II oocytes, the animal half was cut and processed in the same way as for the isolated nuclei. Half oocytes were then mounted and the chromosomes were imaged through the white spot. Images were acquired on a Zeiss LSM510 confocal microscope using the 488 and 633 nm laser lines for excitation with BP 520–550 and LP650 emission filters for Alexa 488 and DRAQ5, respectively. Images were assembled using Adobe Photoshop.

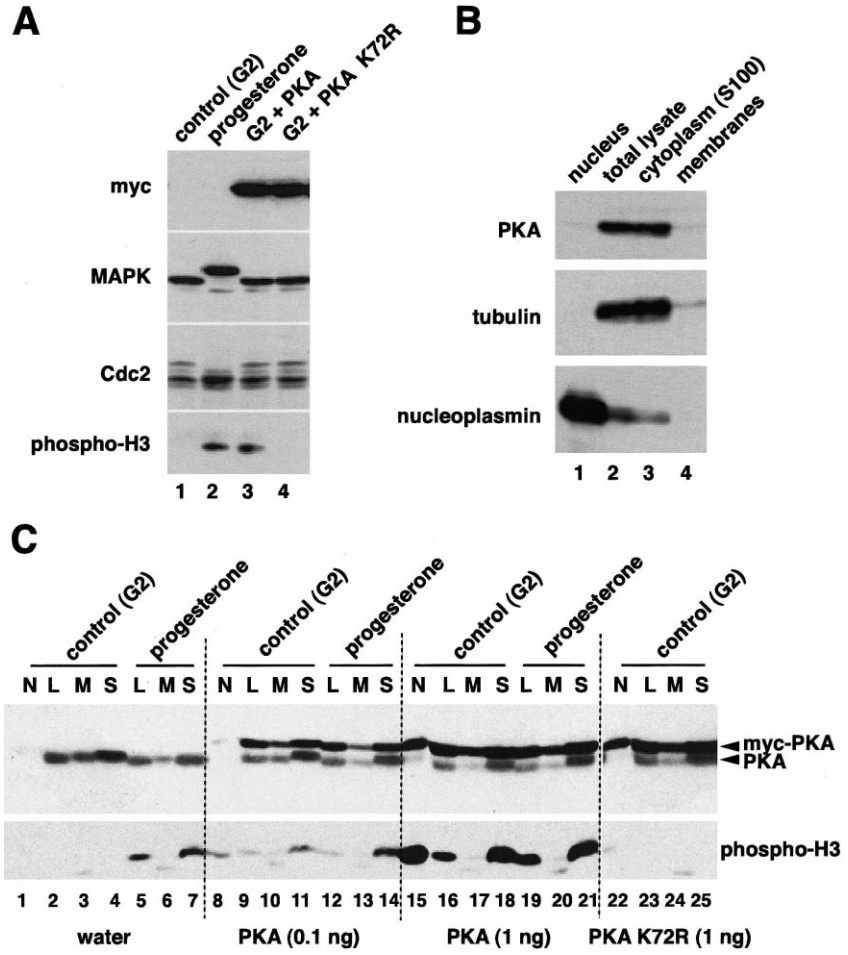

Fig. 1. Histone H3 phosphorylation by overexpressed PKA in *Xenopus* oocytes. A: Oocytes were injected with mRNAs (1 ng) encoding myc-tagged active PKA or the K72R mutant and incubated for 16 h. Total lysates prepared from the PKA-injected oocytes and from oocytes untreated or treated with progesterone were analysed by immunoblotting. B: Nucleus (20 oocytes), total lysate (1 oocyte), the supernatant after 100 000 × *g* ultracentrifugation of total lysates (cytoplasm, 1 oocyte) and the corresponding membrane fraction (membranes, 10 oocytes) were analysed by immunoblotting. C: Oocytes were injected with water, 0.1 or 1 ng of mRNA encoding myc-tagged active PKA or the K72R mutant and then were either left untreated or treated with progesterone 4 h after injection. Oocytes were collected 18 h after injection and nuclei (N, 10 oocytes), total lysates (L, 1 oocyte), membranes (M, 10 oocytes) and the 100 000 × *g* supernatant (S, 1 oocyte) were analysed by immunoblotting.

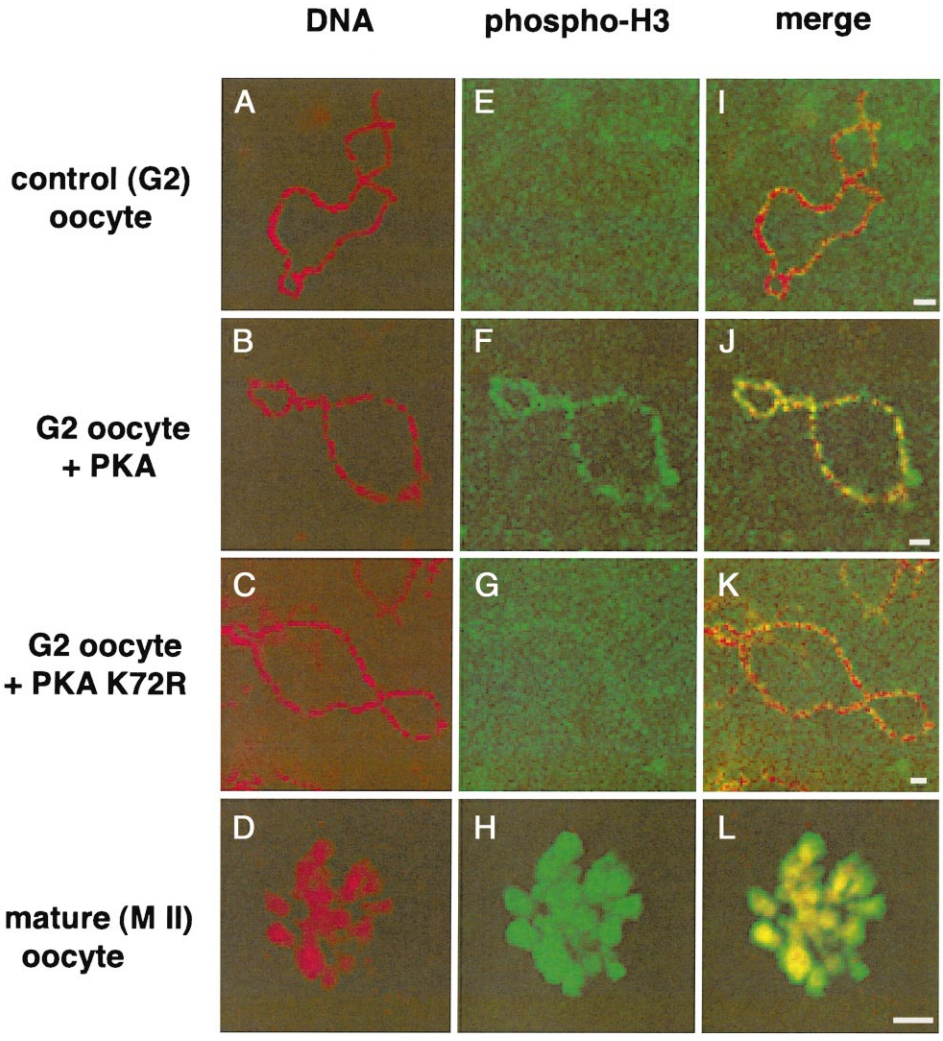

Fig. 2. PKA-induced phosphorylation of histone H3 does not correlate with DNA condensation. A–C: Visualisation of lampbrush chromosomes using DRAQ5 dye in isolated nuclei from G2-arrested oocytes. D: Metaphase II chromosomes from mature oocytes imaged through the white spot on the animal pole of the oocyte. E–H: Immunostaining with phospho-histone H3 antibody. I–L: Merge of both channels. Bars are 5 μm.

3. Results

3.1. *Overexpression of PKA in Xenopus oocytes leads to Ser-10 phosphorylation of histone H3*

We used an antibody raised against Ser-10-phosphorylated histone H3 to investigate this phosphorylation during the meiotic G2/M progression of *Xenopus* oocytes (Fig. 1A). The phospho-histone H3 antibody recognised a strong band with an apparent molecular weight of around 16 kDa in extracts prepared from progesterone-matured (Metaphase II-arrested) oocytes but not in extracts from G2-arrested oocytes (Fig. 1A, lanes 1 and 2). During the course of our studies on the inhibitory role of PKA in oocyte maturation, we also observed that overexpression in oocytes of the PKA catalytic subunit led to the same level of Ser-10 phosphorylation of histone H3 as in progesterone-matured oocytes (Fig. 1A, lanes 2 and 3, lower panel). However, the PKA-injected oocytes did not enter meiosis I and were still blocked in the G2 phase of the cell cycle, as indicated by the absence of GVBD and the lack of activation of MAPK and Cdc2/cyclin B (Fig. 1A, compare lanes 1 and 3) [21]. Overexpression of a kinase-inactive version of PKA (PKA K72R) did not cause phosphorylation of histone H3 (Fig. 1A, lane 4), indicating that the G2 phosphorylation required PKA catalytic activity. We also found that the endogenous PKA was normally localised in the cytoplasm of the oocyte and was undetectable in the nucleus (Fig. 1B). Therefore, it was of interest to know whether overexpressed PKA triggered histone H3 phosphorylation without entering into the oocyte nucleus. We examined the subcellular localisation of myc-tagged versions of active and K72R PKA overexpressed in oocytes (Fig. 1C). When low concentrations of PKA mRNA (0.1 ng) were injected, overexpressed PKA was mainly localised in the cytoplasm, like endogenous PKA (Fig. 1C, upper panel, lanes 8–14). Small amounts of overexpressed PKA were also detected in the nucleus (lane 8), whereas no endogenous PKA was found in the nuclei preparations (Fig. 1C, upper panel, lanes 1, 8, 15 and 22). In contrast, injection of higher concentrations of PKA mRNA (1 ng) resulted in PKA protein levels 5–10 times higher than the endogenous PKA levels in oocytes and led to the localisation of significant amounts of PKA in the nucleus (Fig. 1C, lanes 15 and 21). The presence of active PKA in the nucleus correlated with

increased levels of histone H3 phosphorylation (Fig. 1C, lower panel, compare lanes 1, 8 and 15). Histone H3 phosphorylation in total lysates from oocytes overexpressing PKA (lanes 16 and 19) exceeded the normal H3 phosphorylation levels observed in total lysates from progesterone-matured oocytes (lane 5). Overexpression of a kinase-inactive PKA mutant did not cause histone H3 phosphorylation although the protein was also present in the nucleus at high levels (Fig. 1C, upper panel, lane 22). These results indicate that overexpression of active PKA can trigger high levels of histone H3 phosphorylation which correlate with the localisation of PKA to the oocyte nucleus.

3.2. PKA-dependent phosphorylation of histone H3 does not correlate with chromosome condensation

As mentioned above, the oocytes overexpressing PKA were apparently arrested in G2; the germinal vesicle was intact and this correlated with lack of activation of the biochemical markers that accompany entry into meiosis I, such as MAPK and Cdc2/cyclin B (Fig. 1A). However, since PKA localised to the nucleus and phosphorylation of histone H3 has been correlated with chromosome condensation, we investigated the state of the DNA in these oocytes. Staining of the nuclei isolated from PKA-injected oocytes with two different DNA chromophores (Hoechst and DRAQ5) showed the presence of DNA fibres (characteristic of the prophase stage) but no sign of DNA condensation (Fig. 2B). These DNA fibres were indistinguishable in number and appearance from those observed in water-injected oocytes (Fig. 2A–C). In contrast, DNA was clearly condensed in metaphase II oocytes (Fig. 2D). As expected, the DNA in oocytes injected either with water or with a kinase-inactive PKA mutant was not stained with the phospho-histone H3 antibody (Fig. 2E,G). However, the DNA in oocytes injected with active PKA was efficiently stained by the phospho-histone H3 antibody (Fig. 2F), as was the condensed DNA in metaphase II-arrested oocytes (Fig. 2H). Nevertheless, the DNA in oocytes injected with active PKA remained decondensed. These results demonstrate that PKA can trigger the efficient phosphorylation of DNA-bound histone H3 on Ser-10, but that this phosphorylation is not sufficient for chromosome condensation.

3.3. PKA activity is not necessary for histone H3 phosphorylation during meiotic maturation

Inhibition of endogenous PKA activity in the oocytes by injection of the specific PKA inhibitor PKI is sufficient to induce meiotic maturation of *Xenopus* oocytes [23]. We investigated whether inhibition of PKA by PKI would reduce histone H3 phosphorylation in mature (metaphase II-arrested) oocytes. As shown in Fig. 3, phosphorylation of histone H3 was not affected in oocytes that matured upon injection of recombinant GST-PKI protein (Fig. 3, lane 4) compared to progesterone-matured oocytes (Fig. 3, lane 2). This indicates that PKA is not necessary for histone H3 phosphorylation during meiotic maturation of *Xenopus* oocytes.

3.4. Histone H3 phosphorylation during oocyte maturation requires activation of the MAPK cascade

Our results indicated that PKA was not involved in the phosphorylation of histone H3 observed in mature (metaphase II-arrested) oocytes. To investigate whether this phosphorylation was an early or late event during meiotic maturation, oocytes were collected at different time points after progesterone addition and analysed by immunoblotting (Fig. 4A). Histone H3 phosphorylation was essentially undetectable at the early stages of maturation, with only a faint band starting to be visible just before GVBD (Fig. 4A, lane 10). The phospho-histone H3 antibody only detected a strong band concomitant with the activation of MAPK and Cdc2/cyclin B (pre-maturation promoting factor (pre-MPF)), both of which occur at the time of meiosis I entry (Fig. 4A, lane 11). Thus, histone H3 phosphorylation occurred at the time of GVBD during progesterone-induced meiotic maturation of *Xenopus* oocytes.

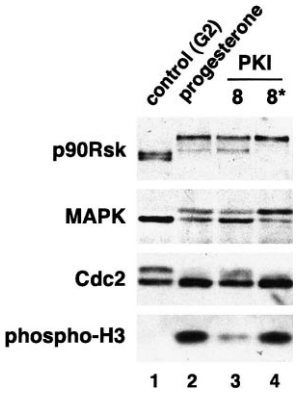

Fig. 3. Inhibition of PKA activity by PKI does not prevent histone H3 phosphorylation. G2-arrested oocytes (lane 1), progesterone-matured oocytes (lane 2) and oocytes injected with 50 ng of recombinant GST-PKI (lanes 3 and 4) were incubated for 8 h and analysed by immunoblotting. PKI-injected oocytes that reached GVBD are indicated with an asterisk (lane 4).

To investigate whether MPF activation was required for the phosphorylation of histone H3 induced by progesterone, we injected oocytes with recombinant Mos protein kinase, an activator of the MAPK cascade, in the presence or absence of the protein synthesis inhibitor CHX. Mos led to activation of MAPK and p90Rsk as early as 1 h after injection, which correlated also with the early phosphorylation of histone H3 to the same level as in progesterone-matured oocytes (Fig. 4B, lane 2, 3 and 14). These oocytes underwent GVBD about 5 h after Mos injection, which correlated with the activation of pre-MPF (presence of H1K activity and disappearance of the slow-migrating Cdc2 band in immunoblots) but did not lead to any further increase in histone H3 phosphorylation (Fig. 4B, compare lanes 3–6). As previously reported, the injection of Mos in CHX-treated oocytes-activated MAPK and p90Rsk with normal kinetics but GVBD and the activation of pre-MPF was delayed [19,24,25]. Interestingly, the phosphorylation of histone H3 was not diminished in these oocytes, even in the absence of detectable pre-MPF activation (Fig. 4B, lane 8–12). These results indicate a direct correlation in oocytes between activation of the MAPK/p90Rsk pathway and histone H3 phosphorylation, which apparently requires neither MPF activity nor protein synthesis.

Meiotic maturation can also be induced by injection of recombinant Cdc25 phosphatase [26]. Cdc25 directly dephosphorylates and thereby activates the Cdc2 present in the pre-MPF (Cdc2/cyclin B) complex [27]. In the presence of CHX, recombinant Cdc25 activates pre-MPF but not the MAPK cascade, probably due to the absence of Mos, which is normally synthesised during meiotic maturation [28,29]. We could

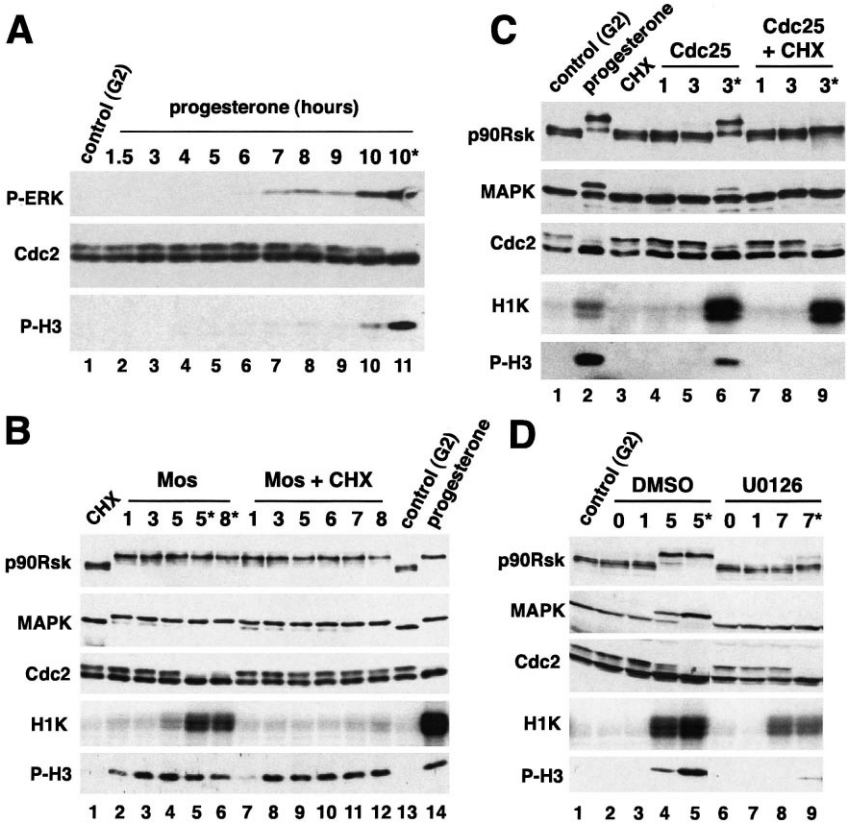

Fig. 4. Phosphorylation of histone H3 during *Xenopus* oocyte meiotic maturation. Oocyte lysates were analysed by immunoblotting using the indicated antibodies and Cdc2 activity was determined by H1K assays. Where indicated control G2-arrested oocytes and progesterone-matured oocytes were processed in parallel. The asterisks indicate oocytes that have undergone GVBD. A: Oocytes were untreated (lane 1) or incubated with progesterone for the times indicated in hours (lanes 2–11). B: Oocytes either untreated (lanes 2–6) or pre-incubated with CHX for 1 h (lanes 7–12) were injected with 25 ng recombinant MalE-Mos protein and samples of 8 oocytes were collected at the indicated times (hours) after injection. C: Oocytes either untreated (lanes 4–6) or pre-incubated with CHX for 1 h (lanes 7–9) were injected with 10 ng recombinant GST-Cdc25 protein and samples of 8 oocytes were collected at 1 and 3 h (when 50% of the oocytes underwent GVBD) after injection. D: Oocytes were pre-treated either with the dissolvent DMSO (lanes 2–5) or 50 μM of the MEK inhibitor U0126 (lanes 6–9) for 45 min prior to progesterone addition. Samples of 10 oocytes were collected at the indicated time points (hours) after progesterone addition.

detect phosphorylation of histone H3 in oocytes that underwent GVBD after injection of Cdc25 (Fig. 4C, lane 6) and this correlated with the activation of both MAPK and p90Rsk, as indicated by their shifts in immunoblots. In contrast, no histone H3 phosphorylation was detected in Cdc25-injected oocytes pre-incubated with CHX, even though these oocytes also underwent GVBD (Fig. 4C, lane 9). Immunoblot and H1K analysis demonstrated that in these oocytes, pre-MPF was activated whereas the protein kinases MAPK and p90Rsk were not active (Fig. 4C, compare lanes 6 and 9). These results show that even in the presence of high levels of Cdc2 activity, histone H3 did not become phosphorylated when MAPK and p90Rsk were not active in the oocytes.

To confirm that phosphorylation of histone H3 during oocyte maturation was related to the activation of MAPK, oocytes were incubated with the specific MAPK kinase inhibitor U0126, which blocks MAPK but not pre-MPF activation in response to progesterone [30]. In oocytes pre-treated with U0126 prior to stimulation with progesterone, phosphorylation of histone H3 was greatly reduced at the time of GVBD compared to oocytes incubated with DMSO only (Fig. 4D, compare lanes 5 and 9).

Taken together, these results demonstrated that the histone H3 kinase(s) activated during oocyte meiotic maturation did not require MPF activation but rather depended on activation of the MAPK/p90Rsk pathway.

4. Discussion

Phosphorylation of histone H3 at Ser-10 is considered to be a hallmark of chromosome condensation during both mitosis and meiosis in many different organisms (reviewed in [2]). We have investigated the phosphorylation of histone H3 during the meiotic cell cycle of *Xenopus* oocytes and its relationship with chromosome condensation.

We found that phosphorylation of histone H3 normally occurs at the time of meiosis I entry (GVBD) during progesterone-induced oocyte maturation, in agreement with the presence of condensed DNA. However, overexpression and nuclear localisation of PKA causes high levels of Ser-10 phosphorylation of histone H3 in oocytes that do not enter meiosis but remain arrested in G2 phase of the cell cycle. Our findings show that extensive Ser-10 phosphorylation of histone H3 does not induce chromosome condensation in G2 oocytes, demonstrating that this phosphorylation may be necessary but is not sufficient to compact the DNA. Our results suggest that PKA might be able to directly phosphorylate histone H3 in *Xenopus* oocytes. This is consistent with the

ability of PKA to phosphorylate Ser-10 of histone H3 in vitro [14,15] and with recent papers proposing histone H3 phosphorylation mediated by PKA in rodent follicular cells [16,17]. It should be noted, however, that these cells contain very low levels of histone H3 phosphorylation, which seem to correlate with transcription initiation [17], whereas we show that PKA is able to induce extensive histone H3 phosphorylation all over the chromosomes and in cells that are about to enter M phase, but still is not followed by chromosome condensation.

The uncoupling between histone H3 phosphorylation and chromosome condensation in intact *Xenopus* oocytes is in agreement with recent observations in *Xenopus* egg extracts [12,31]. Thus, the occurrence of chromosome condensation in egg extracts incubated with sperm nuclei requires the amino terminus of histone H2B, but not that of histone H3, which includes Ser-10 [31]. Moreover, the phosphatase inhibitor microcystin can induce Ser-10 histone H3 phosphorylation in interphase extracts but does not lead to chromosome condensation, indicating that the two processes can occur independently [12].

PKA is thought to be active in G2-arrested *Xenopus* oocytes and downregulated during meiosis entry (reviewed in [32]). Here we show that in oocytes that mature upon injection of PKI, histone H3 is normally phosphorylated at the time of GVBD as in progesterone-matured oocytes although PKA is completely inhibited. These results indicate that PKA is unlikely to be the physiological kinase phosphorylating histone H3 during oocyte maturation.

We found that phosphorylation of histone H3 is dependent on the activation of the MAPK/p90Rsk pathway but does not require MPF activation. p90Rsk has been shown to induce histone H3 phosphorylation [33,34] but this phosphorylation has been associated with transcriptional initiation rather than chromosome condensation. A candidate histone H3 kinase in *Xenopus* oocytes is Aurora B, which has been shown to be necessary for histone H3 phosphorylation in egg extracts [12]. It will be interesting to find out whether Aurora B might be directly regulated by the MAPK/p90Rsk cascade.

In conclusion, we found that phosphorylation of histone H3 on Ser-10 during *Xenopus* oocyte maturation correlates with DNA condensation at the time of metaphase I entry, but this phosphorylation cannot be used as an M phase marker, since it is not sufficient for chromosome condensation. Our results also indicate an important role for the MAPK/p90Rsk pathway in the regulation of histone H3 phosphorylation during the meiotic cell cycle of oocytes, whereas the activation of MPF is apparently not required.

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