MEIOTIC MATURATION OF AMPHIBIAN OOCYTES INCREASES DEPHOSPHORYLATION OF MICROINJECTED HISTONES

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SUMMARY. Histone H2b phosphorylated by a cAMP dependent protein kinase was injected into *Xenopus laevis* oocytes and the hydrolysis of phosphate groups measured. Two hours after injection 60% of the (^{32}P)histone radioactivity had been released. Direct microinjection of (^{32}P)histone into oocyte nuclei showed that this organelle also has phosphatase activity. (^{3}H)methylhistone and (^{125}I)histone are not degraded for at least 4 hours in the oocyte. Exposure of oocytes to progesterone for 6 hours to induce meiotic maturation results in a threefold increase in the rate of dephosphorylation. Papaverine and cycloheximide which block maturation prevent this increment in histone phosphatase activity.

INTRODUCTION. Protein phosphorylation and dephosphorylation are biochemical reactions that play important roles in many regulatory mechanisms. Several lines of evidence have pointed to the involvement of the reversible phosphorylation of cellular proteins in the induction of the maturation of amphibian oocytes (1). The most compelling evidence is the observation that microinjection of the catalytic subunit of cAMP dependent protein kinase blocks hormonal induction of oocyte maturation while injection of the regulatory subunit of the enzyme or its heat stable inhibitor results in the induction of maturation in the absence of hormone (2). A general increase in protein phosphorylation three to four hours after the induction of maturation by progesterone (3,4) and a decrease in the intracellular cAMP levels shortly after hormone treatment of oocytes (5,6) have been observed.

The present report presents evidence that the protein phosphatase activity increases significantly as a result of oocyte maturation. Microinjection of (^{32}P)-phosphorylated histones H2b and H1 has been used to assay the phosphatase activity present in living oocytes and oocyte nuclei. It is shown that the breakdown of the oocyte nucleus which occurs during meiotic maturation is accompanied by a threefold increase in the dephosphorylation of these proteins.

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MATERIALS AND METHODS. Mature female *Xenopus laevis* were obtained from South African Snake Farm, Fish Hoek, RSA. Ovaries were removed surgically and full grown oocytes hand dissected out and kept at 4° in amphibian saline solution containing: 88 mM NaCl, 2.4 mM $NaHCO_3$, 1 mM KCl, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 10 mM Tris-HCl pH 7.6, and 10 µg per ml each penicillin and streptomycin. Nuclei were removed from oocytes manually and microinjections performed essentially as described by Gurdon (7). Maturation induction and the assay for maturation were as described (6).

(^{32}P)Histone H2b and H1. 1 mg of histone H2b or H1 (Sigma) was incubated for 30 minutes at 37° with 200 µg of bovine heart cAMP dependent protein kinase (Sigma) in a 400 µl volume containing: 50 mM sodium phosphate buffer pH 7.1, 10 mM $MgCl_2$, 10 mM dithiothreitol, 2 µM cAMP and 100 µM (^{32}P)-γ-ATP. The reaction was stopped by the addition of cold 2% trichloroacetic acid which precipitates the protein kinase. The 1500 x g supernatant fraction was dialyzed against water to remove excess (^{32}P)ATP. The (^{32}P)phosphorylated histone was concentrated by lyophilization to contain 2.5×10^7 cpm and 0.5 mg protein/ml.

(^{3}H)methylhistone H2b. 1 mg of histone H2b was incubated with 100 µg of a partially purified preparation of S-adenosyl methionine-arginine protein methyltransferase from *Xenopus laevis* ovary (kindly donated by V. Hinrichsen) in a 0.5 ml final volume containing : 50 mM Tris-HCl pH 9.0, 4 mM dithiothreitol, 10 mM EDTA and 20 µM (^{3}H)methyl-S-adenosylmethionine (sp. act. 1000 mCi per mmol). After 40 minutes incubation at 37° the reaction was heated to 100° for 90 seconds and dialyzed. The (^{3}H)methylhistone contained 3×10^6 cpm per mg protein.

(^{125}I)Histone. Histone H2b was iodinated with ^{125}Iodine using the technique of Greenwood et al (8). The (^{125}I)histone was purified by Sephadex G-50 gel filtration and contained 5×10^8 cpm per mg protein.

The modified labelled histones were analyzed on Panyim and Chalkley (9) electrophoretic gels. More than 95% of the radioactivity recovered from the gels corresponded to the position of the H2b histone band of control gels.

RESULTS. The (^{32}P) phosphate groups incorporated into histone H2b by a cAMP dependent protein kinase are rapidly hydrolyzed when the modified histone is microinjected into *Xenopus laevis* oocytes. Figure 1A shows the time dependence of the loss of (^{32}P) radioactivity that is precipitable with 10% trichloracetic acid as the injected oocytes are incubated at 22°. Two hours after the injection less than 40% of the radioactivity remains bound to the protein. Similar results were obtained after the injection of (^{32}P)histone H1 (data not shown). Also in Figure 1A it is clear that the (^{3}H)methyl groups incorporated enzymatically into the arginine residues of histone H2b or the ^{125}I introduced by reaction with chloramine T remain bound to the protein several hours after injection into the oocytes. These results indicate that the loss of radioactivity in the case of the (^{32}P)histone is not due to proteolysis. A previous report has shown that histones microinjected into oocytes are not appreciably degraded (10).

Direct microinjection of (^{32}P) disodium orthophosphate into oocytes using amounts of radioactivity comparable to the (^{32}P)histone demonstrated

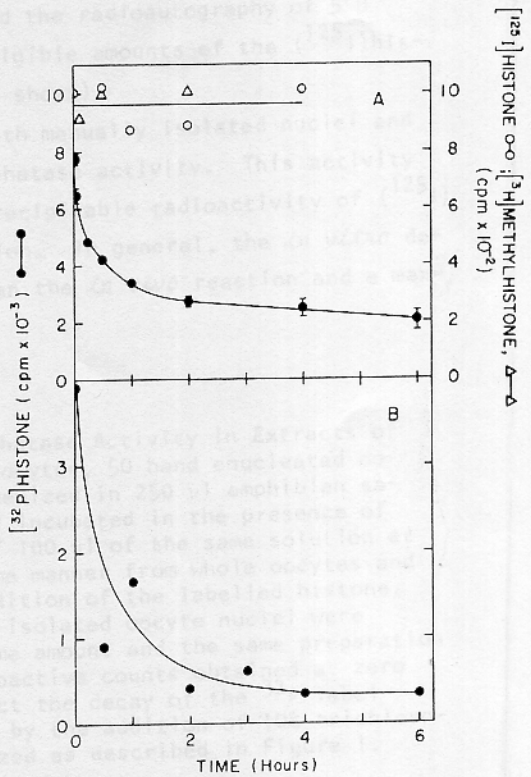

Figure 1. The Stability of Radioactive Modified Histones Microinjected into Cytoplasm and Nucleus of *Xenopus laevis* Oocytes.
A. Duplicate groups of 6 oocytes were microinjected into the cytoplasm with 40 nl each of either (^{32}P)histone (●), (^{125}I)histone (o), or (^{3}H)methylhistone (Δ) and incubated in 50 µl of amphibian saline solution at 21°. At the times given the oocytes were fixed by addition of 2 ml 10% trichloroacetic acid and the cells homogenized and heated to 90° for 10 minutes. The precipitated protein was collected by centrifugation at 1500 x g for 5 minutes and was redissolved in 0.1 ml of 1 N NaOH and reprecipitated with 10% trichloroacetic acid. The final precipitate was collected by filtration on glass fiber filters and the radioactivity measured in a liquid scintillation system.
B. Duplicate groups of 6 to 10 oocytes were microinjected into the nucleus with 20 nl of (^{32}P)histone H2b and incubated in 50 µl of amphibian saline. At the times given the oocytes were fixed in 10% trichloroacetic acid and the nuclei dissected out free of cytoplasmic material and were processed and counted as described in A.

that less than 3% of the radioactivity became precipitable with 10% trichloroacetic acid. Recycling of (^{32}P)inorganic phosphate, released from the histone, back into the oocyte macromolecules is therefore not a significant source of error in these studies.

Since histones microinjected into oocytes are known to migrate to the nucleus (10, 11) the hydrolysis of the phosphate groups of (^{32}P)histone H2b microinjected directly into these organelles was measured. Figure 1B shows that the dephosphorylation occurs efficiently in the oocyte nuclei. Parallel experiments in which (^{125}I)histone was injected into oocyte nuclei showed that this compound was stable in this organelle and the radioautography of 5 µ slices of fixed oocytes showed that only negligible amounts of the (^{125}I)histone were found outside the nucleus (data not shown).

Table 1 shows that extracts from both manually isolated nuclei and of enucleated oocytes contain a histone phosphatase activity. This activity was heat-labile and did not alter the acid precipitable radioactivity of (^{125}I) histone H2b after similar periods of incubation. In general, the *in vitro* dephosphorylation of histone H2b was slower than the *in vivo* reaction and a maximum of 50% hydrolysis was obtained.

TABLE 1. The Presence of (^{32}P)Histone Phosphatase Activity in Extracts of Enucleated Oocytes and Nuclei. 100 intact oocytes, 50 hand enucleated oocytes or 50 hand separated nuclei were homogenized in 250 µl amphibian saline solution and 15 µl aliquots removed and incubated in the presence of radioactive histone H2b in a final volume of 100 µl of the same solution at 21°. Heated extract was prepared in the same manner from whole oocytes and heated to 100° for 10 minutes before the addition of the labelled histone. The experiments with enucleated oocytes and isolated oocyte nuclei were carried out at different times using the same amount and the same preparation of 32P-labelled histone. The different radioactive counts obtained at zero time in these experiments, therefore, reflect the decay of the ^{32}P label of the substrate. The reaction was stopped by the addition of 10% trichloroacetic acid and the radioactivity was analyzed as described in Figure 1. Values are numerical averages of duplicate assays.

Histone H2b	Extract Source	Protein-Bound Radioactivity (10% Trichloroacetic acid precipitable) cpm		
		Zero time	6 Hours	% Initial value
(^{32}P)	Intact oocyte	10,353	6,798	66
"	Heated extract	10,353	10,858	104
"	Enucleated oocytes	5,762	3,302	57
"	Oocyte nuclei	2,798	1,354	48
(^{125}I)	Intact oocyte	15,397	14,819	96

Figure 2A shows that the meiotic maturation of the oocytes caused by progesterone treatment significantly increases the rate of dephosphorylation of the injected histone. The time required to hydrolyze 50% of the phosphate groups was reduced from 90 minutes in the control cells to 30 minutes in the oocytes that had matured after 6 hours with progesterone. However, treatment of oocytes for 3 hours with progesterone, which is not enough time to cause nuclear breakdown, did not affect the rate of hydrolysis of the histone phosphate groups. Papaverine and cycloheximide which block maturation, inhibit this increment of the histone phosphatase activity (Figure 2B). Indeed, the presence of 0.1 mM papaverine which is a potent inhibitor of oocyte cAMP phosphodiesterase, inhibits the histone phosphatase activity to levels below that of the control oocytes.

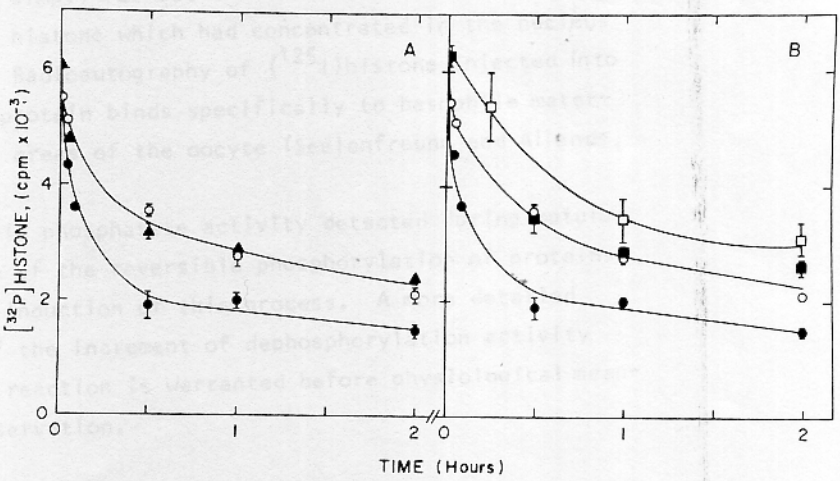

Figure 2. The Effect of Meiotic Maturation Induced by Progesterone on the Dephosphorylation of Microinjected (^{32}P)histone H2b into Oocytes of *Xenopus laevis*.
A. Prior to microinjection oocytes were incubated in amphibian saline for 6 hours (o), or with 1 µM progesterone for 3 hours (▲), or for 6 hours (●). Oocytes were injected into the cytoplasm with 40 nl of (^{32}P)histone H2b and the radioactivity remaining at the times given was measured as described in Figure 1.
B. The oocytes were treated in duplicate groups of 6 as follows: Prior to injection, control oocytes (o) were incubated 11 hours in amphibian saline. Two groups of oocytes were preincubated for 5 hours with 0.1 mM papaverine (□) or 0.1 mM cycloheximide (■) and then were incubated with 1 µM progesterone for 6 hours before microinjection. A fourth group (●) was preincubated for 5 hours with amphibian saline and then incubated with 1 µM progesterone for 6 hours before microinjection with labelled histone. All microinjections were into cytoplasm and the measurement of radioactivity was as described in Figure 1.

DISCUSSION. The results presented above establish the presence in the living oocyte of a protein phosphatase activity that efficiently hydrolyzes the phosphate groups of microinjected histones. This activity is present both in the oocyte nucleus and in the cytoplasm of these cells.

Since protein kinases that phosphorylate histones *in vitro* are known to be present in oocytes (12) it is likely that the loss of radioactive phosphate groups that is described above entails an exchange of these groups on the injected histones with endogenous non-radioactive phosphate introduced by protein kinases of the oocytes.

The abundant phosphate groups of lipovitellin and phosvitin, the major proteins in the oocyte yolk platelets, apparently turn over very slowly if at all (13). It would seem, therefore, that these groups are not available to the protein phosphatase that attacks phosphorylated histone. This discrimination could be due to substrate specificity or due to compartmentation of these proteins in the lipid-rich yolk platelets.

The increase in the dephosphorylation activity detected in the mature oocytes coincides with the breakdown of the cell nucleus, which is known to concentrate injected histones. It seems possible, therefore, that the increase in dephosphorylation may simply reflect the redistribution in the cell of the injected substrate. The histone which had concentrated in the nucleus is dispersed into the cytosol. Radioautography of (^{125}I)histone injected into mature oocytes shows that this protein binds specifically to basophile material which is present in several areas of the oocyte (Seelenfreund and Allende, unpublished results).

The increase in protein phosphatase activity detected during maturation supports the proposed role of the reversible phosphorylation of proteins in the control of the hormonal induction of this process. A more detailed study of the characteristics of the increment of dephosphorylation activity and of the specificity of this reaction is warranted before physiological meaning can be ascribed to this observation.

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