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Authors: Yoshida, Noriyuki, Tanaka, Toyomi, and Yamashita, Masakane

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Changes in Phosphorylation Activities during Goldfish and *Xenopus* Oocyte Maturation

NORIYUKI YOSHIDA, TOYOMI TANAKA and MASAKANE YAMASHITA[1]

Laboratory of Molecular and Cellular Interactions, Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo 060, Japan

ABSTRACT—Oocyte maturation is promoted by the sequential actions of several kinases, of which MPF (a histone H1 kinase) and MAP kinase (a myelin basic protein (MBP) kinase) are known to play pivotal roles. However, other kinases responsible for inducing oocyte maturation have yet to be characterized. To identify these kinases, we examined phosphorylation activities toward 44 exogenous substrate proteins during oocyte maturation in goldfish and *Xenopus*. We found that 4 substrates in goldfish and 6 in *Xenopus* were phosphorylated and their phosphorylation states changed during oocyte maturation. Among them, only 3 substrates (histone H1, MBP and pepsin) were common to both species. Precipitation of cdc2 showed that, like histone H1, pepsin was also phosphorylated by cdc2 (MPF). These results suggest that different kinase cascades are involved in goldfish and *Xenopus* oocyte maturation, although MPF and MAP kinases are common to both species. We also found novel phosphorylation activities that precede the activation of MPF and MAP kinases using deoxyribonuclease I, casein, pepsin and protamine as exogenous substrates.

INTRODUCTION

Oocyte maturation is a process in which full-grown immature oocytes acquire the ability to undergo embryonic development after fertilization. This involves the reinitiation of meiosis arrested at the first meiotic prophase and is accompanied by such morphological changes as germinal vesicle break down (GVBD), chromosome condensation and spindle formation. Oocyte maturation is controlled by three major factors: gonadotropin (GTH), maturation-inducing hormone (MIH) and maturation-promoting factor (MPF) [24]. Oocyte maturation is initiated by GTH secreted from pituitary gland. GTH induces follicle cells around the oocytes to synthesize and secrete MIH. MIH is identified as 1-methyladenine in starfish [11], progesterone in amphibians [35], and 17α, 20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) in salmonid fishes [23]. The signal of MIH is received directly on the surface of oocytes and transduced into oocyte cytoplasm probably via the function of GTP binding proteins [2, 3, 42]. Finally, MPF is activated, bringing about all the changes accompanying oocyte maturation [12]. MPF consists of two components, cdc2 (a catalytic subunit) and cyclin B (a regulatory subunit), and its activity is controlled by phosphorylation of cdc2 after it binds to cyclin B [40].

Upon oocyte maturation, protein phosphorylation is greatly enhanced in many animal species [18]. Thus it is likely that kinases play pivotal roles in initiating and promoting oocyte maturation. Following the reception of MIH signals on the oocyte surface, a decrease in the activity of cyclic AMP-dependent protein kinase (A-kinase) is considered to play a part in the initial step of the signal transduction toward the activation of MPF [18, 35], although there are several species in which an increase in A-kinase activity seems to induce oocyte maturation [20, 37, 43]. Besides A-kinase, several kinases are also reported to be involved in oocyte maturation (c-*mos*, 31, 32, 41; S6 kinase, 22, 28, 29; MAP kinase, 19, 30, 34). However, the precise biochemical cascade which leads the MIH-stimulated oocyte to maturation through the activation of MPF is still unknown. We believe that many other unknown kinases play important roles in oocyte maturation. As a first step to identify kinases responsible for inducing and promoting oocyte maturation, we examined phosphorylation activities toward 44 exogenous substrate proteins during oocyte maturation in goldfish and *Xenopus*. Here we report novel phosphorylation activities which exhibit different patterns depending on the species, in addition to MPF and MAP kinase activities common to both species.

MATERIALS AND METHODS

Experimental animals and the preparation of oocyte extracts

Goldfish (*Carassius auratus*) and African clawed frogs (*Xenopus laevis*) were used in this study. Goldfish were obtained from a local fish farm (Yatomi, Aichi, Japan) and raised at 15°C until use. *Xenopus* were reared in our laboratory.

Full-grown immature goldfish oocytes were manually isolated from ovaries using a pipette and forceps, and induced to mature *in vitro* by incubating at room temperature in Ringer's solution [9] containing 1 μg/ml of 17α, 20β-DP. Oocyte extracts were obtained at 1 hr intervals following 17α, 20β-DP stimulation as described previously [8]. Briefly, 50 oocytes were homogenized with pestle (Pellet Pestle; Kontes) in 50 ml of ice-cold extraction buffer (100 mM β-glycerophosphate, 20 mM HEPES, 15 mM $MgCl_2$, 5 mM EGTA, 100 μM *p*-amidinophenylmethanesulfonyl fluoride, 3 μg/ml of

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[1] To whom correspondence should be addressed.

leupeptin, pH 7.5). The homogenate was centrifuged at 15,000xg for 10 min at 4°C, and the supernatant was frozen in liquid nitrogen and kept at −80°C until use. Oocytes were also extracted with an extraction buffer lacking β-glycerophosphate and EGTA. Maturational processes were assessed by immersing the oocytes in a clearing solution (5% formalin and 4% acetic acid in Ringer) [14], which facilitates microscopic examination of the occurrence of GVBD.

Full-grown immature *Xenopus* oocytes (Dumont stage VI) were isolated from ovaries by treating them with 2 mg/ml of collagenase in OR-2 (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 5 mM HEPES, pH 7.8) for 1 hr at room temperature with gentle agitation. They were then washed extensively with DB (111 mM NaCl, 1.3 mM KCl, 1.3 mM $CaCl_2$) several times, and induced to mature *in vitro* by incubating at room temperature in DB containing 10 μg/ml of progesterone. Maturational processes were evaluated by the appearance of a white spot on the animal pole, which indicates the occurrence of GVBD.

Phosphorylation assay

The phosphorylation assay was performed as follows. Oocyte extracts (2.5 μl) were incubated with 2 μg/ml of each substrate protein for 20 min at room temperature in 10 μl of a reaction buffer (15 mM $MgCl_2$, 1 mM DTT, 20 mM HEPES, 0.1 mM ATP) containing 3,000 Ci/mmol of [γ-^{32}P]ATP (HAS). The reaction was stopped by adding Laemmli's SDS sample buffer [13]. Proteins were separated by electrophoresis on a 12.5% or 15% polyacrylamide gel, and the radioactivities on the substrate bands were quantified using an imaging analyzer (FUJIX BAS2000), and expressed as photostimulated luminescence (PSL) values.

Suc1-precipitation

Extracts (25 μl) from mature oocytes of goldfish and *Xenopus* were incubated with 2.5 μl of suc1-conjugated beads overnight at 4°C [39]. The beads were washed three times with 1 ml of TTBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) and used for the phosphorylation assay.

RESULTS

Phosphorylation activities during goldfish oocyte maturation

We examined phosphorylation activities against 44 exogenous substrate proteins during goldfish and *Xenopus* oocyte maturation. The proteins examined in this study were listed in Table 1. We used two types of buffers for extracting kinases from oocyte cytoplasm: One was a HEPES buffer containing $MgCl_2$ and protease inhibitors, and the other contained β-glycerophosphate (a phosphatase inhibitor) and EGTA (Ca^{2+} chelater) in addition to the components of the former buffer. The latter buffer was used for extracting MPF. In spite of the presence of β-glycerophosphate and EGTA, however, no difference was observed between the two buffers (data not shown), suggesting that the phosphorylation activities described in this study are independent of phosphatases and Ca^{2+}.

Of the 44 proteins examined, 18 were phosphorylated in goldfish oocyte extracts, and the phosphorylation states of 4 proteins changed during oocyte maturation (Table 1). These proteins include pepsin, deoxyribonuclease I (DNase I), histone H1, and myelin basic protein (MBP). Figure 1 shows the changes in the phosphorylation states of the 4 proteins after 17α, 20β-DP stimulation. The changes in phosphorylation activity toward the 4 protein substrates can be classified into two different patterns. The phosphorylation activity to histone H1 and MBP increased gradually after 17α, 20β-DP stimulation, followed by an abrupt increase concurrent with the onset of GVBD (Fig. 1A and B). On the other hand, the changes in phosphorylation activity toward DNase I and pepsin exhibited two peaks before and after GVBD (Fig. 1C and D). The activities toward DNase I and pepsin differed in the height of the two peaks; the former showed a higher peak after GVBD, while the latter showed a higher peak before GVBD (Fig. 1C and D).

Phosphorylation activities during Xenopus *oocyte maturation*

Of the 44 proteins, 18 were phosphorylated in *Xenopus* oocyte extracts, and the phosphorylation states of 6 proteins changed during oocyte maturation (Table 1). These proteins were histone H1, MBP, pepsin, carbonic anhydrase, casein, and protamine (Fig. 2). The phosphorylation activities toward histone H1, MBP and pepsin showed similar changes during oocyte maturation. After a gradual increase following progesterone stimulation, a rapid increase occurred when oocytes underwent GVBD (Fig. 2A-C). Since histone H1 and pepsin are phosphorylated by cdc2 (MPF), and MBP is phosphorylated by MAP kinase, as will be indicated later, this confirms previous reports that progesterone-induced activation of cdc2 and MAP kinase occurs almost simultaneously in *Xenopus* oocytes [5, 19]. The change in the phosphorylation activity against carbonic anhydrase resembled those for histone H1, MBP and pepsin, but it was associated with a rapid decrease in the final step of oocyte maturation (Fig. 2D). The activities toward casein and protamine exhibited an apparently different pattern from those for other 4 proteins, with three peaks at 0.5, 2–2.5, and 3.5–4 hr after the progesterone treatment (Fig. 2E and F).

Pepsin phosphorylation by MPF

Table 1 shows that, of 44 exogenous protein substrates, only three (histone H1, MBP and pepsin) were phosphorylated regularly during goldfish and *Xenopus* oocyte maturation. Histone H1 and MBP are well known substrates for cdc2 (MPF) [38] and MAP kinase [25], respectively. However, no kinase is known to be responsible for pepsin phosphorylation. It is plausible that, like histone H1, pepsin is phosphorylated by cdc2. To confirm this, we precipitated cdc2 from mature oocyte extracts with suc1, a fission yeast *suc1*$^+$ gene product that binds specifically to cdc2 [39]. The suc1-precipitates and suc1-supernatants from mature goldfish and *Xenopus* oocyte extracts were used for the phosphorylation assay. In both goldfish and *Xenopus*, histone H1 was phosphorylated in the suc1 precipitate, but not in the supernatant (Fig. 3A and B), indicating that almost all active cdc2 was precipitated by suc1. In *Xenopus*, the phosphorylation activity against pepsin was removed from the oocyte extracts

TABLE 1. Exogenous substrate proteins

Proteins	Phosphorylation[1)]		Notes
	goldfish	*Xenopus*	
acid phosphatase	−	−	Boehringer Mannheim 108 219
aldolase	−	−	Pharmacia 17-0441-01
alkaline phosphatase	−	−	Sigma P 0780
apotransferrin	−	−	Sigma T 5761
α-amylase	−	−	Sigma A 6380
aprotinin	−	−	Boehringer Mannheim 236 624
bovine serum albumin	−	−	Pharmacia 17-0442-01
carbonic anhydrase	+	+O	Sigma C 3934
casein	+	+O	Sigma C 4032
catalase	−	−	Pharmacia 17-0441-01
cathepsin B	+	+	Sigma C 6286
chymotrypsinogen A	+	−	Pharmacia 11-A-239-02
creatine phosphokinase	+	−	Sigma C 3755
collagenase SI	−	+	Nitta Gelatin
cytochrome C	−	+	Sigma C 2506
deoxyribonuclease I	+O	−	Sigma D 5025
dispase	−	−	Boehringer Mannheim 165 859
elastase	+	−	Boehringer Mannheim 1027 891
enolase	−	−	Sigma E 6126
fetuin	−	−	Sigma F 2379
γ-globulin	−	−	Sigma G 9894
GF cdc2	+	+	goldfish cdc2 [10]
hemocyanin	−	−	Calbiochem 374805
hexokinase	+	+	Sigma H 4502
histone H1	+O	+O	Boehringer Mannheim 223 549
insulin	−	−	Sigma I 5500
GF KIN-7	+	+	goldfish MAP kinase-like protein [7]
β-lactoglobin	−	+	Sigma L 2506
lipase	−	−	Sigma L 3126
lysozyme	−	+	Sigma L 6876
GF cdk7 (MO15)	+	+	goldfish cdk7 (MO15) [26]
myelin basic protein (MBP)	+O	+O	Sigma M 1891
myoglobin	−	−	Sigma M 0630
ovalbumin	−	−	Pharmacia 11-A-240-02
pepsin	+O	+O	Sigma P 7012
phosphoglucose isomerase	+	+	Sigma P 5381
phospholipase D	−	+	Sigma P 8023
phosphorylase A	+	−	Sigma P 1261
protamine	−	+O	Sigma P 4255
proteinase K	−	−	Merck 24568
ribonuclease A	−	−	Pharmacia 11-A-238-02
thrombin	+	−	Boehringer Mannheim 602 418
trypsin inhibitor	+	+	Sigma T 9767
xanthine oxidase	−	−	Sigma X 1875

1) −, not phosphorylated; +, phosphorylated but no changes; +O, phosphorylated and changes in its phosphorylation state during oocyte maturation.

by suc1, indicating that the pepsin phosphorylation activity in *Xenopus* oocyte extracts is entirely derived from cdc2 (Fig. 3B). Similarly, the phosphorylation activity toward pepsin in goldfish oocyte extracts was also precipitated by suc1, although the precipitation was not complete and considerable activity remained in the supernatant (Fig. 3A). These results clearly show that, although a part of the pepsin phosphorylation activity was due to kinase(s) other than cdc2,

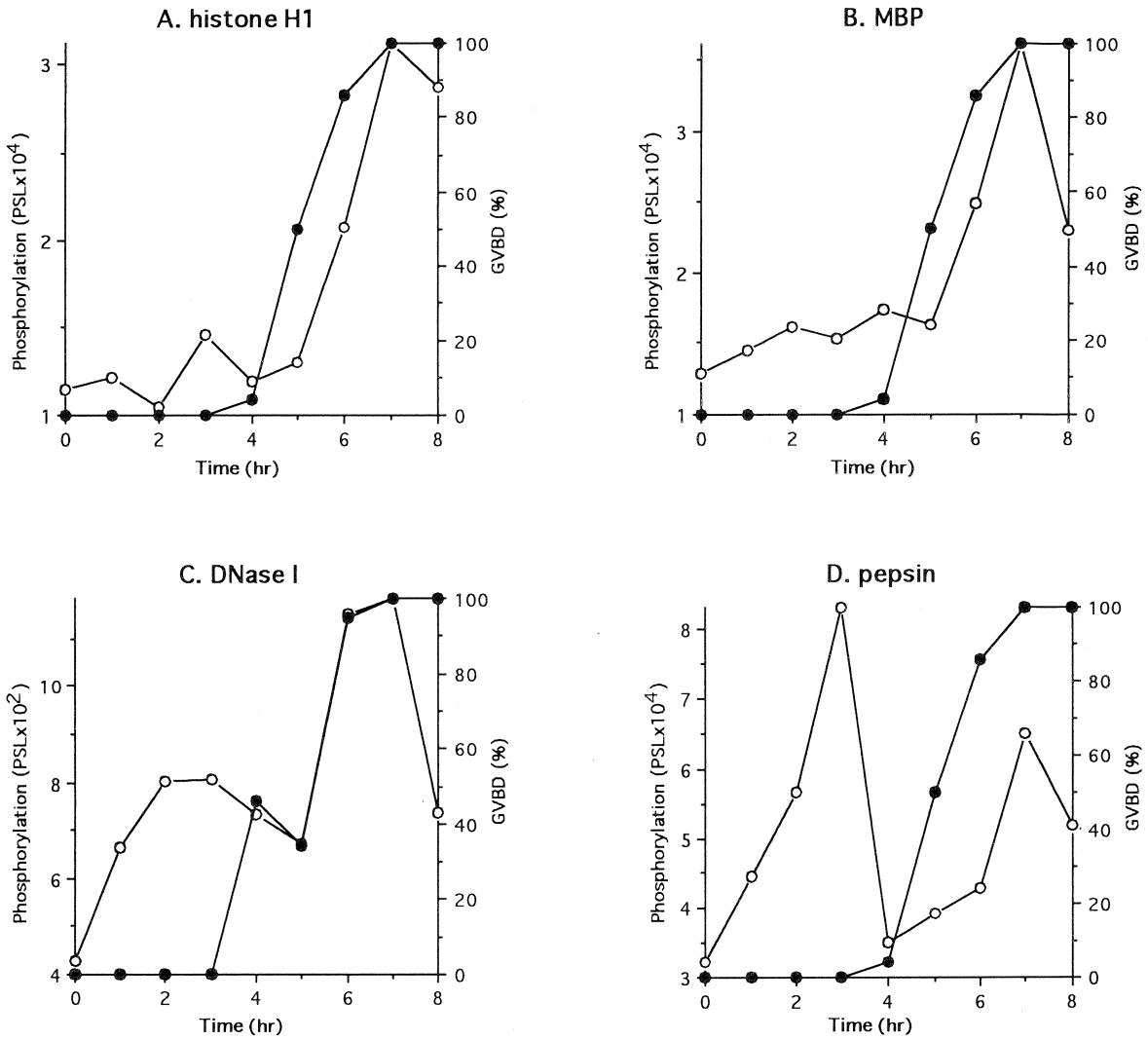

FIG. 1. Changes in phosphorylation activities during goldfish oocyte maturation induced by 17α, 20β-DP. Percentage of GVBD and phosphorylation activity are indicated by closed and open circles, respectively.

cdc2 is also responsible for pepsin phosphorylation in mature goldfish oocyte extracts.

DISCUSSION

Oocyte maturation induced by MIH is associated with the enhanced phosphorylation of many proteins in maturing oocytes of amphibians [1, 15–17, 36], starfish [4, 6, 33], *Urechis* [21] and annelids [27]. The enhanced protein phosphorylation is thought to be a compulsory process of oocyte maturation throughout the animal kingdom, but the only protein kinases well known to play crucial roles in oocyte maturation are cdc2 and MAP kinase. As a first step to characterize kinases which play crucial roles in oocyte maturation, we examined phosphorylation activities against 44 exogenous substrate proteins in maturing goldfish and *Xenopus* oocytes.

Of the 44 proteins examined, four were phosphorylated and their phosphorylation states changed during oocyte maturation in goldfish, whereas the number was six in *Xenopus*. These proteins included histone H1, MBP, pepsin, DNase I, carbonic anhydrase, casein, and protamine, among which the former three were common in the two species. Histone H1 and MBP are well known to be phosphorylated by cdc2 [38] and MAP kinase [25], respectively. However, phosphorylation activity toward pepsin has not been reported during oocyte maturation. By suc1 precipitation experiments, we showed that pepsin phosphorylation is also responsible for cdc2 in both goldfish and *Xenopus*, although a part of its activity is derived from kinase(s) other than cdc2 in goldfish. It is therefore concluded that only cdc2 and MAP kinase activity are common in both goldfish and *Xenopus* oocyte maturation.

Molecular mechanisms of the formation and activation of MPF differ completely between goldfish and *Xenopus*, in spite of the identity of the final molecular structure of active MPF [40]. In immature goldfish oocytes, all cdc2 is monomeric and cyclin B is absent. Cyclin B is *de novo*

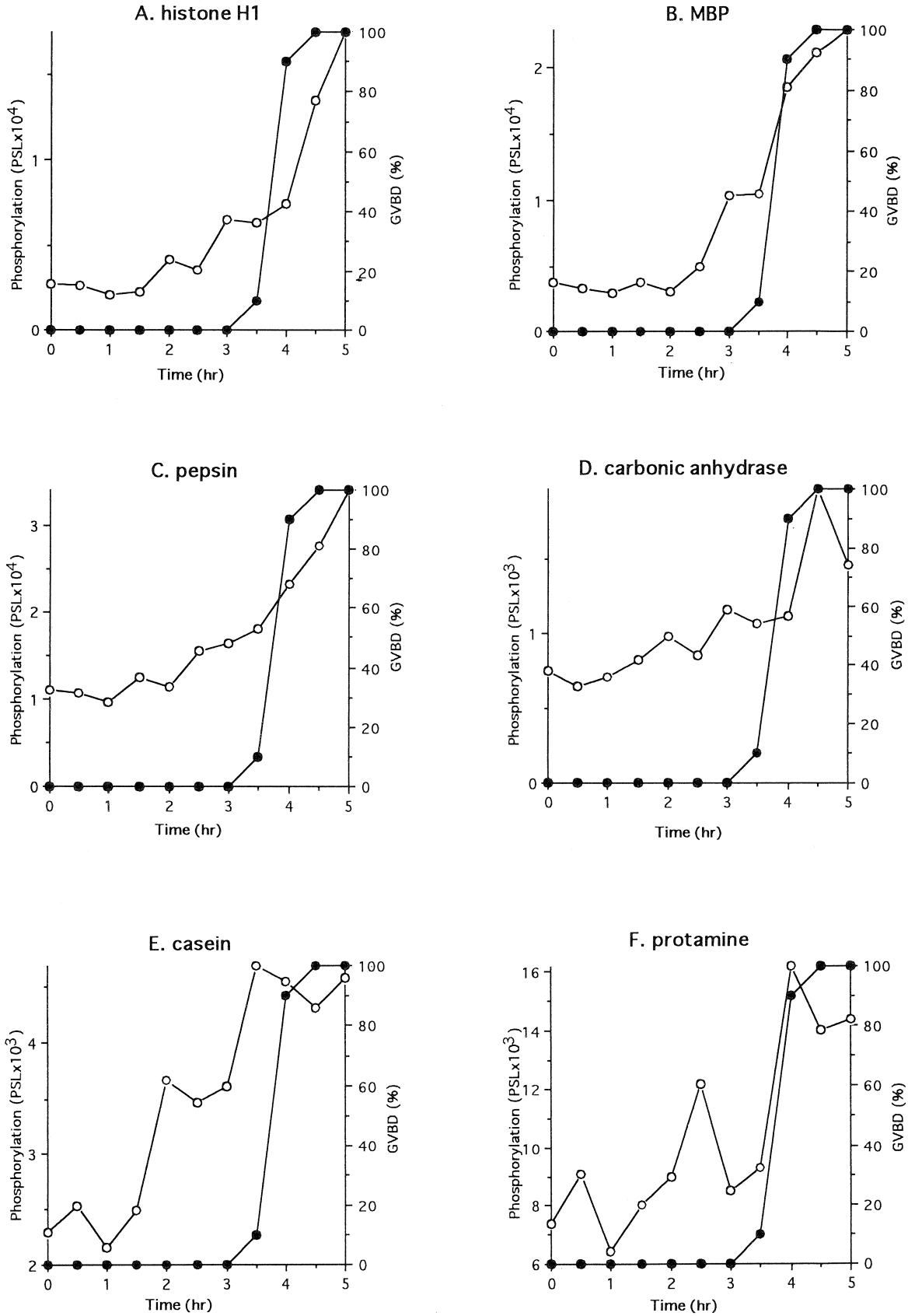

FIG. 2. Changes in phosphorylation activities during *Xenopus* oocyte maturation induced by progesterone. GVBD, closed circles; phosphorylation activity, open circles.

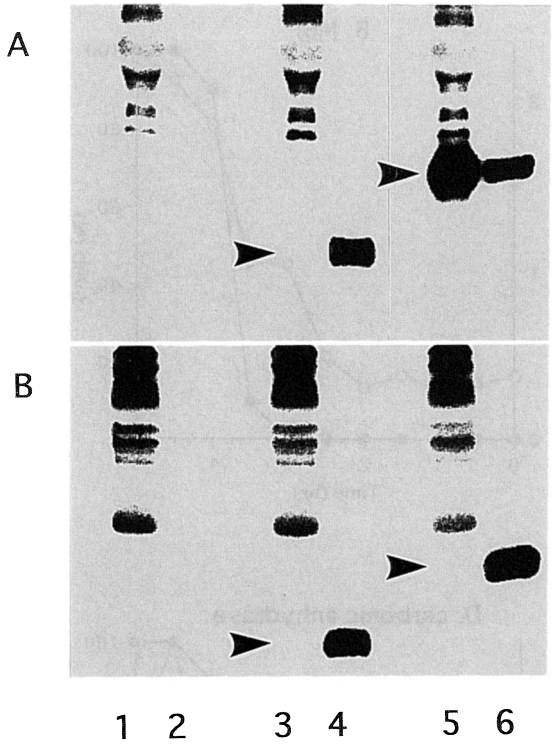

FIG. 3. Phosphorylation of histone H1 and pepsin in the suc1-supernatant (1, 3, 5) and the suc1-precipitate (2, 4, 6) from mature goldfish (A) and *Xenopus* (B) oocyte extracts. Samples were incubated in the absence (1, 2) of an exogenous substrate or in the presence of either histone H1 (3, 4) or pepsin (5, 6). The uptake of ^{32}P into histone H1 and pepsin (arrowheads) were analyzed by autoradiography following SDS-PAGE.

synthesized during oocyte maturation and bound to preexisting cdc2. After binding to cyclin B, cdc2 is phosphorylated on Thr161 and activated, yielding active MPF. In goldfish, therefore, MPF is formed and activated during oocyte maturation in accordance with the synthesis of cyclin B. On the other hand, cdc2 is already bound to cyclin B and phosphorylated on Thr161 in immature *Xenopus* oocytes. But, it is also phosphorylated on Thr14/Tyr15, thereby rendering it inactive. During oocyte maturation, cdc2 is dephosphorylated on Thr14/Tyr15 and thus acquires kinase activity. In the case of *Xenopus*, therefore, MPF is already present in immature oocytes as inactive form (pre-MPF), and its activation is dependent on Thr14/Tyr15 dephosphorylation and not on cyclin B. The present finding that, except for cdc2 and MAP kinase, phosphorylation activities revealed by exogenous substrates are quite different between goldfish and *Xenopus* also confirms the difference in the mechanisms of the promotion of oocyte maturation according to species.

Besides cdc2 and MAP kinase, we found novel phosphorylation activities that precede the activation of cdc2 and MAP kinase, when DNase I and pepsin (in the case of goldfish, Fig. 1) and casein and protamine (in the case of *Xenopus*, Fig. 2) were used as exogenous substrates. Although kinases responsible for the phosphorylation of these substrates have not yet been characterized, it is highly likely that these activities are derived from different kinases, since their phosphorylation patterns during oocyte maturation are apparently different from each other. Characterization of the kinases responsible for the phosphorylation activities that precede the activation of MPF and MAP kinase are of great importance, since we have little knowledge about the kinase cascade that links the reception of MIH signal on the oocyte surface and the activation of MPF in oocyte cytoplasm.

When a certain protein (DNase I or pepsin for goldfish and casein or protamine for *Xenopus*) was used, plural peaks of phosphorylation activities were observed during oocyte maturation. This can be explained in two ways. One explanation is that the activities of some kinases fluctuate with several peaks during oocyte maturation. In this case, these kinases may function at several points during oocyte maturation. Another explanation is that the plural peaks are the summation of plural kinase activities. In other words, certain proteins are phosphorylated by plural kinases simultaneously. For example, in *Xenopus*, all pepsin phosphorylation activity was due to cdc2 and exhibited only one peak (Figs. 2 and 3). In goldfish, however, pepsin phosphorylation activity exhibited two peaks before and after GVBD (Fig. 1D), and cdc2 only partially contributed to the activity found in the latter phase (in mature oocytes) (Fig. 3). This finding shows that pepsin indicates the activities of at least two different kinases in goldfish oocyte extracts. Like pepsin, it is plausible that plural peaks detected by other substrates reflect plural kinase activities. The identification of the kinases responsible for the phosphorylation activities found in this study is a prerequisite for understanding the precise role of each phosphorylation activity during oocyte maturation.

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