Protein phosphorylation during meiotic maturation of *Xenopus* oocytes: cdc2 protein kinase targets

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ABSTRACT M-Phase specific protein kinase or cdc2 protein kinase is a component of MPF (M-Phase promoting factor). During meiotic maturation of *Xenopus* oocytes, cdc2 protein kinase is activated in correlation with MPF activity. A protein phosphorylation cascade takes place involving several protein kinases, among which casein kinase II, and different changes associated with meiosis occur such as germinal vesicle breakdown, chromosome condensation, cytoskeletal reorganization and increase in protein synthesis. Our results provide a biochemical link between cdc2 protein kinase and protein synthesis since they show that the kinase phosphorylates *in vitro* a p47 protein identified as elongation factor EF1 (γ subunit) and that the *in vitro* site of p47 corresponds to the site phosphorylated *in vivo*. Immunofluorescence showed that the elongation factor (EF1-ßγ) is localized in the oocyte cortex. Furthermore, they show that cdc2 kinase phosphorylates and activates casein kinase II *in vitro*, strongly supporting the view that casein kinase II is involved in the phosphorylation cascade originated by cdc2 kinase.

KEY WORDS: *cdc2 protein kinase, H1-kinase, casein kinase II, elongation factor, protein synthesis*

Introduction

Prophase to metaphase transition of meiotic cell division as well as G2/M transition of mitotic cell division is controlled by MPF (M-Phase promoting factor) in all eucaryotic cells (Kishimoto *et al.*, 1982). During these last two years, considerable progress was made in the biochemical analysis of MPF when it was discovered that it corresponds to the association of the products of two genes involved in cell division control (see reviews in Dunphy and Newport, 1988; Hunt,1989; Lohka, 1989; Norbury and Nurse,1989). Cell division control genes (cdc) were found by genetic analysis in fission yeast (recent reviews in Lee and Nurse, 1988; Norbury and Nurse, 1989). The first protein immunologically and biochemically related to MPF (Arion *et al.*, 1988; Gautier *et al.*, 1988; Labbé *et al.*, 1988) is an analogue of cdc2 gene product, $p34^{cdc2}$. The second protein is an analogue of cdc13 product (Hagan *et al.*, 1988; Draetta *et al.*, 1989; Labbé *et al.*, 1989; Meijer *et al.*, 1989) which corresponds to proteins termed cyclins for their oscillating behavior during cell cycle; cyclin is high at metaphase and falls abruptly shortly before the onset of anaphase. MPF contains a protein kinase activity which phosphorylates histone H1 *in vitro* (Arion *et al.*, 1988; Labbé *et al.*, 1988) and whose catalytic activity is supported by $p34^{cdc2}$ protein. One main challenge in understanding how cdc2 protein kinase and cyclin regulate the cell cycle is to find the physiological substrates of the kinase and explain how they are involved in the numerous changes associated with G2/M transition (Fig. 1). For this purpose, we have used full-grown *Xenopus* oocytes and we have searched for cdc2 protein kinase substrates during meiotic maturation. Amphibian oocytes offer a good opportunity to investigate the role of cdc2 kinase since the cells are physiologically arrested both in prophase (G2 phase), when just isolated from the ovary, and in metaphase after progesterone administration (reviews in Masui and Clarke, 1979; Maller and Krebs, 1980; Ozon *et al.*, 1987).

Purification of p47, phosphorylated *in vivo* at M-phase

During meiotic maturation of oocytes, a burst in protein phosphorylation takes place when MPF appears in the cytoplasm (Maller *et al.*, 1977; Boyer *et al.*, 1983; Dorée *et al.*, 1983; Karsenti *et al.*, 1987). Since the increase in protein phosphorylation involves mostly threonine residue phosphorylation over serine (Boyer *et al.*, 1983; Spivack *et al.*, 1984; Capony *et al.*, 1986), we decided to purify from oocytes a phospho-protein phosphorylated on threonine as a molecular marker of MPF. Such a protein could be detected

Abbreviations used in this paper: cdc, cell division control; MF, M-phase promoting factor; EF 1, elongation factor 1; FPLC, fast protein liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; AMP-PCP, adenylyl (ßγ)-methylene diphosphonate; CK II, casein kinase type II.

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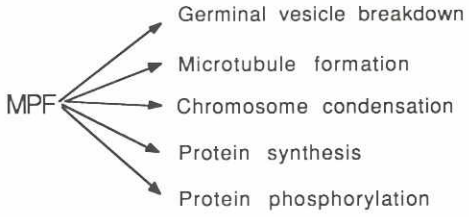

Fig. 1. Events in MPF action

after electrophoresis and autoradiography of oocyte extracts when the phospho-proteins were hydrolyzed *in situ* by alkali (Mulner-Lorillon *et al.*, 1989a) prior to autoradiography (Asselin *et al.*, 1984; Ozon *et al.*, 1987). Using this procedure, we were able to detect a p47 protein that was found to increase in phosphorylation in maturing oocytes, as well as in enucleated maturing oocytes; the increase was found very early after MPF transfer into oocytes (Ozon *et al.*, 1987). After several steps including ammonium sulfate precipitation, FPLC and affinity chromatography, the phospho-p47 protein was purified to near homogeneity (Mulner-Lorillon *et al.*, 1989).

The p47 purified phosphoprotein is in a high molecular weight complex

The purified p47 protein applied on a gel filtration column eluted at a high molecular weight (> 350 kDa). This result indicates that the protein is associated in its native form in a complex. As shown after SDS-PAGE, the complex resolves on the gels in at least three subunits of 47, 36 and 30 kDa, respectively (Bellé *et al.*, 1989; Mulner-Lorillon *et al.*, 1989b). An identical complex is also isolated when the purification procedure is applied to extracts prepared from prophase oocytes (Fig. 2). The p47 protein from matured oocytes is phosphorylated *in vivo* whereas the p47 protein from prophase oocytes is not (Mulner-Lorillon *et al.*, 1989b). In both cases, the amount of complex recovered after purification from 10,000 prophase or matured oocytes was 420µg indicating a large quantity of complex per oocyte; the p47 protein represented 2.6% of the total cytosolic proteins (Mulner-Lorillon *et al.*, 1989b).

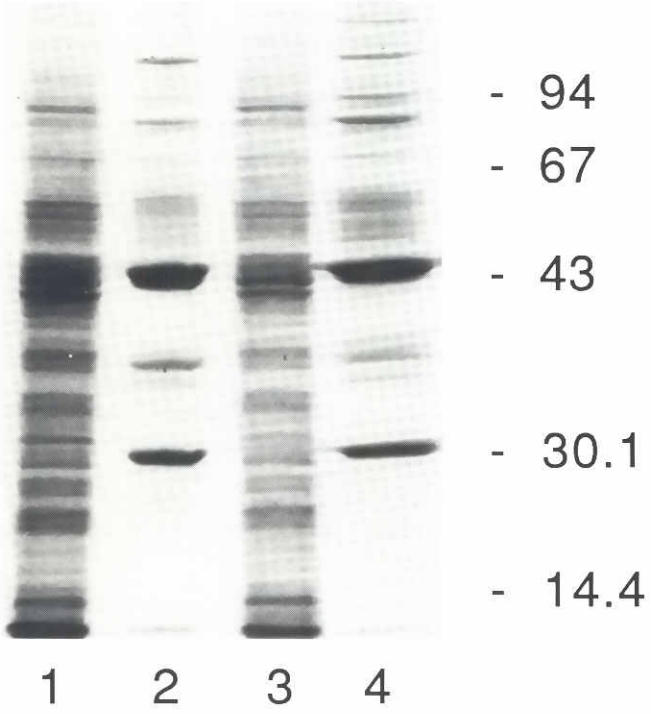

Fig. 2. Coomassie blue staining *after SDS-PAGE of initial (1,3: cytosols; 30 µg proteins) and final (2,4: 20 µg proteins) purification steps of p47 fractions purified from prophase (1,2) and metaphase (3,4)* Xenopus *oocytes.*

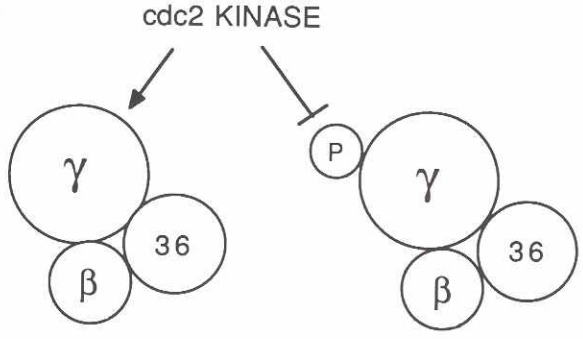

Fig. 3. Schematic representation of p47 fractions from prophase and metaphase oocytes. *ß and γ correspond to elongation EF-1ß and EF-1γ. cdc2 protein kinase can phosphorylate the prophase fraction which is purified in its non-phosphorylated form.*

cdc2 protein kinase phosphorylates p47 protein

While our work was in progress, cdc2 protein kinase was found to correspond to MPF (see introduction). We therefore studied phosphorylation of p47 protein by purified cdc2 protein kinase in collaboration with M. Dorée and J-C Labbé (CRBM Montpellier). The results clearly indicate that cdc2 protein kinase phosphorylates the p47 protein isolated from prophase oocytes and not the p47 isolated from matured oocytes (Mulner-Lorillon *et al.*, 1989b). To ascertain that the site phosphorylated *in vivo* was identical to the site phosphorylated *in vitro* by cdc2 protein kinase, proteolytic digestion of both p47 proteins was performed and the resulting peptides compared after electrophoresis. Results show identical phospho-peptide mapping and furthermore, in both cases, threonine is the phospho-aminoacid (Mulner-Lorillon *et al.*, 1989b). The stochiometry of the phosphorylation *in vivo* is found close to one, thus indicating that p47 has only one phosphorylation site that is fully phosphorylated in maturing oocytes since no more phosphate could be incorporated (Mulner-Lorillon *et al.*, 1989b).

Identification of the p47 and p30 proteins

The three polypeptides present in the purified complex (p47, p36 and p30) were partially sequenced in collaboration with J. Derancourt and J-P Capony (CRBM Montpellier). Since the NH_2 terminal of three proteins is blocked, sequencing was performed on peptides obtained by proteolytic digestion and separated by HPLC (Bellé *et al.*, 1989). When compared in the data banks, the p30 protein

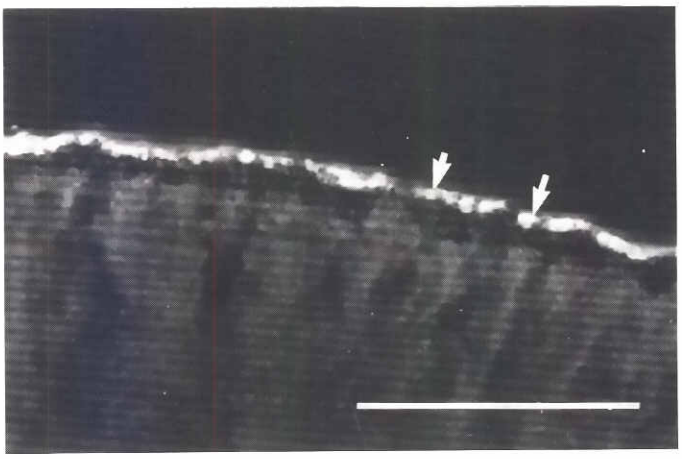

Fig. 4. Localization of the p47 complex in *Xenopus* oocytes. *Immunofluorescence staining of prophase arrested oocytes: cortical region of the animal hemisphere. Oocyte cryostat sections were incubated for 90 min at 37° C with the p47 antibody (dilution 1:1000) and revealed by a guinea pig anti-IgG (dilution 1:700). Arrowhead: subcortical spots of strong fluorescence. Bar represents 50 μm.*

polypeptides showed strong homology with elongation factor EF-1 (ß subunit) from *Artemia salina*. The p47 protein corresponds clearly to the γ subunit of *Artemia* elongation factor EF1. The p36 polypeptides do not correspond so far to any known protein. We conclude that the p47 protein phosphorylated by cdc2 protein kinase is the γ subunit of EF1; Fig. 3 shows a scheme of the purified complex from *Xenopus* and its cdc2 protein kinase phosphorylated site.

Localization of the p47 complex *in vivo*

A guinea pig polyclonal antibody was raised against the purified fraction containing p47, p36 and p30 proteins. The guinea pig serum was tested by immunoblotting of either purified fraction or crude cytosols. In both cases, the serums specifically recognized the p47 fraction at a dilution from 1:2000 to 1:5000 (data not shown). Immunofluorescence staining was then performed as described (Huchon *et al.*, 1985) using cryostat sections of full-grown *Xenopus* oocytes. Localization of the first antiboby was visualized by fluoresceine-conjugated antibody directed against guinea pig IgG (BL 2105 from Biosys). The fluorescence was found strongly concentrated at the level of the oocyte cortex suggesting that the p47 complex is located in the cortical region (Fig. 4); no vegetal or animal hemisphere polarity could be observed.

cdc2 protein kinase phosphorylates casein kinase II

Casein kinase II is a widely distributed protein kinase which phosphorylates a broad spectrum of endogenous substrates (Pinna *et al.*, 1980; Hataway and Traugh, 1982; Cochet and Chambaz, 1983; Edelman *et al.*, 1987). Its enzymatic activity was found to increase during meiotic cell division of Amphibian oocytes (Cicirelli *et al.*, 1988; Kandror *et al.*, 1989). Casein kinase II was highly purified from *Xenopus* ovary (Mulner-Lorillon *et al.*, 1988). Purified casein kinase II phosphorylates the p36 and p30 proteins of the purified p47 complex (Bellé *et al.*, 1989). We showed that cdc2 kinase phosphorylates casein kinase II *in vitro* on its ß subunit and that phosphorylation involved threonine residues different from the autophosphorylation sites of the enzyme (Mulner-Lorillon *et al.*, 1990). Interestingly, the consensus site of phosphorylation of cdc2 protein kinase (K(T,S)PXK) is found in the sequence of bovine casein kinase II ß-subunit (Takio *et al.*, 1987).

cdc2 protein kinase activates casein kinase II by phosphorylation

The activity of casein kinase II assayed after phosphorylation by cdc2 protein kinase increases 2.5-fold (Mulner-Lorillon *et al.*, submitted). Activation did not occur if ATP was replaced by a non-hydrolyzable analogue AMP-PCP and was reversed by alkaline phosphatase, thus demonstrating that the activation necessitated phosphorylation. These results demonstrate that cdc2 kinase can activate casein kinase II *in vitro*, by a phosphorylation reaction possibly involving the ß subunit of the enzyme. Since casein kinase II increases in activity during meiotic maturation when MPF is present (Cicirelli *et al.*, 1988), these results also suggest that cdc2 could activate casein kinase II *in vivo* and that casein kinase could be a part of the cdc2 protein kinase cascade. The finding that microinjection of purified casein kinase II, as well as spermine (its *in vitro* activator) into oocytes accelerates MPF-induced entry into M-

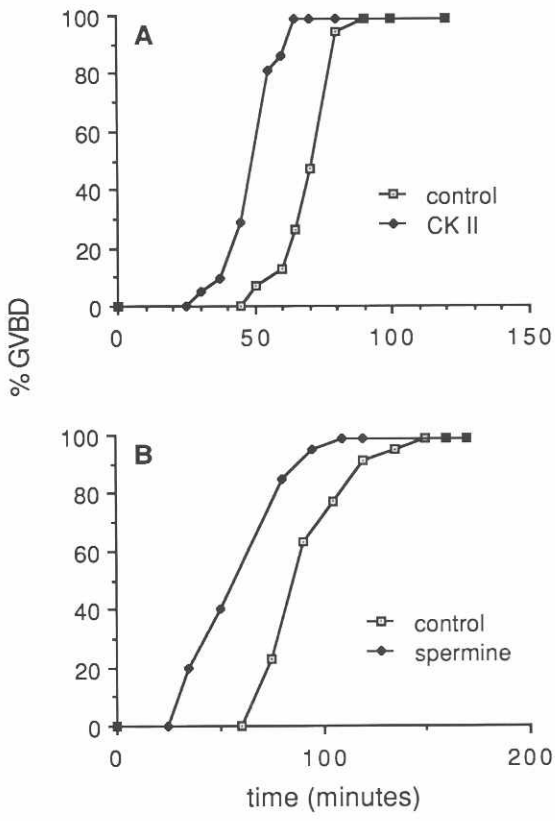

Fig. 5. Kinetic of germinal vesicle breakdown (GVBD) after MPF transfer into *Xenopus* oocytes. (A) *Oocytes microinjected with purified casein kinase II (5 ng; 80 mU per oocyte).* **(B)** *Oocytes microinjected with spermine, an in vitro activator of casein kinase II (2mM intracellular). Batches of 20 oocytes were incubated for one hour before MPF induction of maturation.*

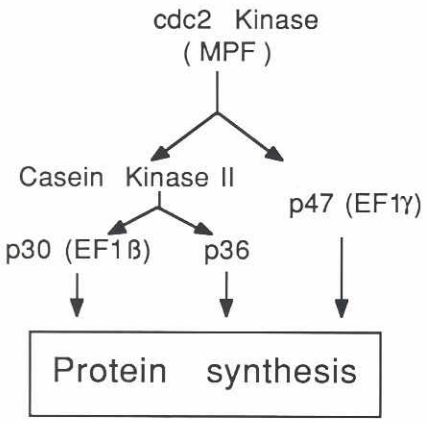

Fig. 6. Schematic representation of cdc2 protein kinase and casein kinase II involvement in regulation of protein synthesis by phosphorylation of the p47 complex.

phase provides strong experimental support to this view (Mulner-Lorillon *et al.*, 1988 and Fig. 5).

Concluding remarks

The findings briefly reviewed here show two targets for MPF during meiotic cell division. The first is a p47 protein which is a component of a high molecular weight complex containing elongation factor EF1-ßγ; therefore the protein is a biochemical link between MPF activity and protein synthesis. The second is a protein kinase, namely casein kinase II, which could be a component of the cdc2 protein kinase cascade leading to the pleiotropic effects of MPF. Fig. 6 shows how both cdc2 protein kinase and casein kinase II could be involved in the regulation of protein synthesis. It would now be of great interest to investigate regulation of protein synthesis *in vitro* and *in vivo* during meiotic maturation and also during early development in mitotic cell divisions.

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