

## Purification of a p47 phosphoprotein from *Xenopus laevis* oocytes and identification as an in vivo and in vitro p34<sup>cdc2</sup> substrate

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This paper describes the purification of a 47 kDa protein from *Xenopus laevis* oocytes that becomes phosphorylated when the oocytes undergo meiotic maturation. This protein (p47) is part of a high molecular mass complex containing at least two other proteins of molecular mass 30 and 36 kDa. This complex can be isolated from stage VI oocytes before maturation. We obtained a pattern for phosphopeptides in p47 phosphorylated in vivo very similar to that of the purified protein phosphorylated in vitro by p34<sup>cdc2</sup> (a H1 kinase which is a component of the M-phase promoting factor) and [ $\gamma$ -<sup>32</sup>P]ATP. Therefore, the purified p47, already described as a marker of MPF activity, is the first reported in vivo substrate for the cell division control kinase.

Protamine kinase; Meiosis; Promoting factor, M-phase

### 1. INTRODUCTION

Full-grown oocytes from *Xenopus laevis* are naturally arrested at the very end of G<sub>2</sub> phase. Upon hormonal stimulation they enter M phase and mature into metaphase-arrested fertilizable eggs. A cytoplasmic factor called MPF (maturation promoting factor) has been demonstrated by transfer experiments to be responsible for the maturation of *Xenopus* oocytes: cytoplasm taken from maturing oocytes induces resumption of meiosis when microinjected into unstimulated prophase-arrested oocytes [1]. MPF has been detected in meiotic and mitotic cells from a number of species ranging from yeast to man, being high in M phase and undetectable in inter-

phase. MPF is therefore considered as a fundamental regulator of M phase in eukaryotes [2,3].

MPF has recently been published using unfertilized oocytes from *Xenopus*. It is composed of two major proteins, p34 possessing H1-kinase activity and identified as the product of the homolog of the yeast *cdc2* gene, and p45, in vitro, a substrate of the p34 kinase [4–8].

At the time of MPF appearance, a burst in cAMP-independent phosphorylation is observed [9,10]. Among the numerous phosphorylated proteins detected at that time, we previously characterized a protein, p47, which had been phosphorylated on alkali-resistant threonine residues [11]. The appearance of this phosphoprotein is highly correlated to that of MPF [12]. Our intention therefore was to purify p47 and to investigate its relation with MPF.

Here, we describe the purification of p47 and demonstrate that it is a substrate for p34<sup>cdc2</sup> mitotic kinase in vitro and in vivo.

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## 2. MATERIALS AND METHODS

### 2.1. Materials

*X. laevis* adult females were obtained from Serea-CNRS (France) and maintained under laboratory conditions. [ $^{32}$ P]Orthophosphate (carrier-free, 10 mCi/ml) and adenosine 5'-[ $\gamma$ - $^{32}$ P]triphosphate (3000 Ci/mmol) were from Amersham (France); hydroxyapatite gel from Bio-Rad; other gels for chromatography from Pharmacia (France); histone H1 from Boehringer Mannheim; mixed casein, catalytic subunit of cAMP-dependent protein kinase (A-kinase) and *Staphylococcus aureus* (strain V8) protease from Sigma; pancreatic  $\alpha$ -chymotrypsin from Calbiochem. Casein kinase II was purified from *X. laevis* ovary as described [13] and p34<sup>cdc2</sup> (P11 phosphocellulose fraction) was prepared from starfish eggs [7].

### 2.2. Preparation and labelling of oocytes

Defolliculated full-grown oocytes were isolated as in [14]. Labelling was performed by incubating oocytes (usually 1000 oocytes/ml) for one night at room temperature with gentle shaking in medium A containing [ $^{32}$ P]orthophosphate (0.5 mCi/ml). Oocytes were then washed four times with medium A. To obtain maturing labelled oocytes, 1  $\mu$ M progesterone was added to the incubation medium 2 h after the radioactivity. Maturation was judged according to the presence of the white maturation spot on the animal pole of the oocyte.

### 2.3. p47 purification

Oocytes were transferred to 1 vol. ice-cold homogenization medium B (75 mM KCl, 50 mM NaF, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM EDTA, 10 mM ATP, 100  $\mu$ M orthovanadate, 5 mM 4-nitrophenyl phosphate, 1.14 mM PhMeSO<sub>2</sub>F, 0.3 mM L-1-tosylphenylalanylchloromethane (TosPheCH<sub>2</sub>Cl), 0.3 mM  $\alpha$ -N-benzoyl-L-arginine methyl ester (BzArgOMe), 5  $\mu$ M soybean trypsin inhibitor, 1 mM benzamidin, 0.04 IU/ml aprotinin, 50 mM Tris; pH 7.4). They were homogenized with five manual strokes of a Potter-Elvehjem homogenizer. Vitellin platelets and melanosomes were discarded by centrifugation for 5 min at 800  $\times$  g followed by 15 min at 2000  $\times$  g at 4°C. The cytosol fraction was then obtained by centrifugation (16 min, 130000  $\times$  g) in a Beckman TL100 ultracentrifuge. When labelled oocytes were processed, the bulk of free and lipid-bound radioactivity was discarded by rapid filtration of the cytosol fraction on a Sephadex G-25 column equilibrated with medium C (1 mM DL-dithiothreitol, 2 mM EDTA, 50 mM Tris; pH 7.4).

A 40–50% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fraction was then prepared at 4°C as follows: the cytosol fraction was adjusted to 40% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> by adding 243 mg/ml solid ammonium sulfate, incubated for 60 min and then centrifuged for 15 min at 2000  $\times$  g. The pellet was discarded and the supernatant brought to 50% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> by addition of 63 mg/ml solid ammonium sulfate. After 60 min incubation the fraction was centrifuged (2000  $\times$  g, 15 min) and the supernatant discarded. The pellet, corresponding to the 40–50% fraction, was dissolved in 10 ml medium C.

All chromatography steps were performed in medium C. The 40–50% fraction was applied to a hydroxyapatite column (1.5  $\times$  5.0 cm). After washing, elution was performed with 40 ml medium C containing 0.5 M potassium phosphate. The p47-containing fraction was dialysed overnight vs medium C and loaded onto a MonoQ HR 5/5 column, a continuous gradient (40 ml) to 700 mM NaCl in medium C being applied to

elute proteins. Further purification of p47 was achieved by applying the MonoQ fraction (diluted 3 times with medium C) to a heparin-Sepharose column (1.0  $\times$  2.5 cm), with elution by a continuous gradient (10 ml) to 1 M NaCl in medium C.

The protein content of fractions was determined by the method of Bradford [15].

### 2.4. In vitro phosphorylation of p47

Incubations were performed at room temperature with gentle shaking in 50  $\mu$ l containing 5  $\mu$ g p47, 50 mM Hepes (pH 7.4), 5 mM pNPP, 5 mM MgCl<sub>2</sub>, 10  $\mu$ M [ $\gamma$ - $^{32}$ P]ATP (spec. act.  $\sim$ 2  $\mu$ Ci/nmol) and 50 IU catalytic subunit of A-kinase (Sigma). 5 U CKII [13] or 1.25  $\mu$ l p34<sup>cdc2</sup> (standard activity: 300 pmol/min per  $\mu$ l [7]). After 20 min, 25  $\mu$ l electrophoresis buffer were added to the incubation mixture which was then immediately boiled and analysed on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described below.

### 2.5. H1-kinase activity of p34<sup>cdc2</sup>

To assay H1-kinase activity, 25 nl p34<sup>cdc2</sup> was incubated in 50  $\mu$ l medium containing 10  $\mu$ g histone H1, 5 mM MgCl<sub>2</sub>, 10  $\mu$ M [ $\gamma$ - $^{32}$ P]ATP (spec. act.  $\sim$ 4  $\mu$ Ci/nmol), 5 mM pNPP and 50 mM Hepes (pH 7.4) in the presence or absence of 0.5  $\mu$ g p47 heparin fraction from prophase or progesterone-matured oocytes. After 20 min, 25  $\mu$ l of each incubation mixture were spotted onto pieces of P81 phosphocellulose paper, which were washed as in [16], and the radioactivity was determined by Cerenkov counting in 10 ml water.

### 2.6. Electrophoresis, phosphoamino acid analysis and peptide mapping

Proteins were analysed by SDS-PAGE on slabs according to [17], using 12% polyacrylamide separation gel. Alkali treatment of gels and phosphoamino acid analysis were performed as described [13]. The procedure of Cleveland et al. [18] for partial hydrolysis of proteins in gel slices was used for peptide mapping, in the presence of *Staphylococcus aureus* V8 protease or  $\alpha$ -chymotrypsin using 4  $\mu$ g enzyme per line.

Radioactive proteins and peptides were detected by exposure of gels to Kodak X-AR film at 20°C (untreated gel) or at  $-80^\circ$ C (alkali-treated gel) in the presence of an intensifying screen. Quantitative analysis was performed by densitometric scanning of the autoradiographs with a Shimadzu scanner.

## 3. RESULTS

### 3.1. Purification of p47

The distinguishing feature of p47 is the alkali resistance of its phosphorylation which occurs when MPF is present in oocytes. We therefore studied the purification of p47 from progesterone-matured oocytes whose phosphoproteins were labelled. 1000 oocytes were incubated in the presence of  $^{32}$ P, induced to mature by 1  $\mu$ M progesterone and homogenized at the time of metaphase II, in a phosphoprotein-conserving medium. Autoradiography of the electrophoretic

pattern of the different purification steps is shown in fig.1A. The cytosol fraction was prepared by centrifugation. p47 was detected in the cytosol after SDS-PAGE, alkali treatment of the gel and autoradiography as the major alkali-resistant phosphoprotein. After rapid gel filtration on Sephadex G-25 to eliminate labelled nucleotides, lipids and free  $^{32}\text{P}$ , the cytosol was fractionated by ammonium sulfate precipitation. Alkali-resistant p47 was recovered in the 40–50% saturated ammonium sulfate fraction. Three chromatographic steps were then performed: the p47 eluted at 0.5 M potassium phosphate from hydroxyapatite, at 0.45 M NaCl from MonoQ and at 0.8 M NaCl from heparin-Sepharose. The overall purification yield was calculated from the total amount (estimated by densitometric scanning for NaOH-treated gel autoradiography) of alkali-resistant p47 in the most purified fraction, that of heparin-Sepharose, vs that in the initial cytosoluble fraction as shown in fig.2C; this yield was 5% (SD = 1.9;  $n = 4$ ). Radioactivity in protein p47 was 29.5% (SD = 12.8;  $n = 7$ ) of the total radioactivity in proteins of the heparin fraction.

When labelled prophase arrested oocytes were processed by using the same purification protocol, no phosphorylated (alkali-resistant or -susceptible) protein with the electrophoretic mobility of p47 could be detected (fig.1B, upper and lower panels).

A Coomassie blue-stained band of 47 kDa was enriched in the same fractions as those determined for alkali-resistant labelled p47 from progesterone-matured oocytes. Furthermore, the elution profile of the blue band on heparin-Sepharose coincided with that of the labelled protein (not shown), strongly suggesting that they were the same protein. The most highly purified fraction (heparin-Sepharose) contained three major stained bands (fig.2A): p47 [accounting for 37% (SD = 9,  $n = 9$ ) of proteins in the fraction], and proteins of 30 kDa [corresponding to 24% (SD = 6,  $n = 9$ ) and 36 kDa [accounting for 11% (SD = 3,  $n = 9$ )]. The latter two are also phosphorylated although less intensely than p47 (fig.2B). The relative ratio between the three bands (p47, p36, p30) remained constant during the last three purification steps. When analysed by gel filtration on Superose 12 (Pharmacia), all proteins contained in the heparin-Sepharose fraction were recovered in a fraction eluting before lipovitellin (350 kDa). These results suggest that

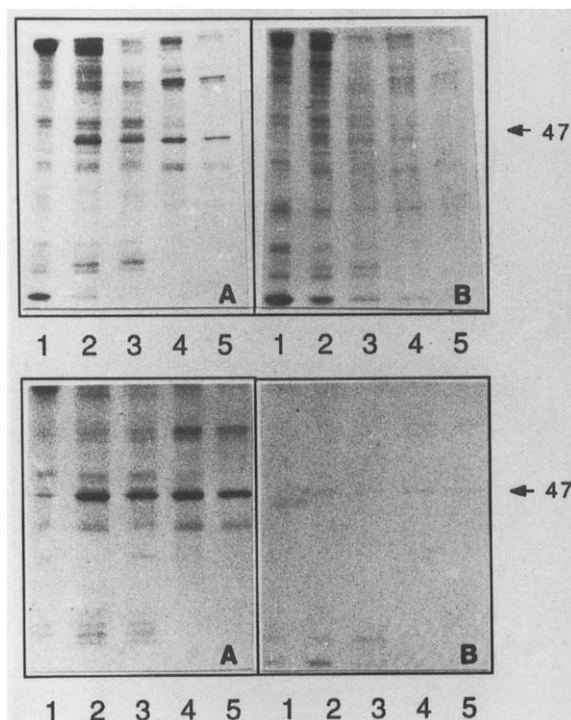


Fig.1. Autoradiography of 12% SDS-PAGE of the different fractions from p47 purification. Fractions (lane): (1) cytosol, (2) 40–50% ammonium sulfate, (3) hydroxyapatite, (4) MonoQ, (5) heparin-Sepharose. (1–3) 10  $\mu\text{g}$  proteins, (4,5) 5  $\mu\text{g}$  proteins. Preparation from (A) progesterone-matured and (B) prophase oocytes. (Upper) Untreated and (lower) alkali-treated gels.

native p47 exists as a high molecular mass complex of greater than 350 kDa.

Table 1 shows the total protein concentration of each fraction prepared from 10000 progesterone-matured oocytes from a typical experiment, which is representative of the 30 preparations which have been made. Based on the amount of the p47 Coomassie-stained band in the heparin fraction, we have calculated that one oocyte contains 0.43  $\mu\text{g}$  p47, corresponding to 2.6% of the proteins in the cytosol fraction. On estimation of the specific activity of the intracellular ATP pool after  $^{32}\text{P}$  labelling of oocytes based on the data reported in [19], we calculated the specific activity of p47 in progesterone-matured oocytes to be 1.0 mol phosphate/mol protein.

When unlabelled prophase arrested oocytes were processed according to the purification procedure

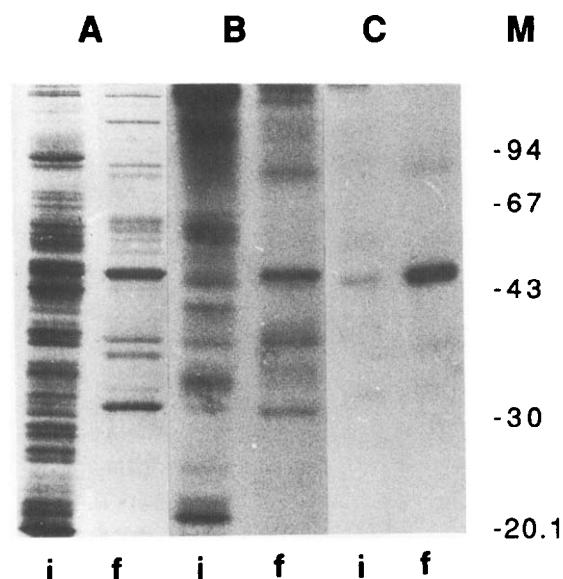


Fig.2. Electrophoretic pattern of initial (i: cytosol, 30  $\mu$ g proteins) and final (f: heparin fraction, 10  $\mu$ g proteins) purification steps of protein p47 from 2000 progesterone-matured oocytes. (A) Coomassie blue-stained gel; (B) autoradiography of (A); (C) autoradiography after alkali treatment of (A).

for p47, the heparin fractions from progesterone-matured and prophase oocytes were very similar with the same associated proteins (p47, p30, p36) present in both cases (not shown).

### 3.2. Phosphorylation of p47

Incubation of the most highly purified fraction of p47 (heparin fraction) in the presence of

Table 1

Protein content (in  $\mu$ g) of the different fractions from purification of p47

Fraction	Protein content ( $\mu$ g)
Cytosol	163 000
C 50	28 000
HAP	7320
Mono Q	1760
Heparin	578
Protein p47	216
Protein p36	63
Protein p30	139

Proteins were determined as in [15]. The amounts of p47 and p30 in the heparin fraction were determined by densitometric integration of the scanned Coomassie-stained gel

[ $\gamma$ - $^{32}$ P]ATP demonstrated the existence of an endogenous kinase activity in the fraction. The amount of kinase activity was variable among different preparations, indicating that copurification of p47 and the kinase was partial. This kinase activity was inhibited by low doses of heparin (3  $\mu$ g/ml), suggesting that it could be a casein kinase II type enzyme. This idea was supported by the finding that it phosphorylated mixed casein as exogenous substrate. The endogenous proteins phosphorylated by this kinase were p30 and p36. Purified casein kinase II from *Xenopus* oocytes was also found to be capable of phosphorylating p30 and p36. On the other hand, neither the endogenous kinase nor purified casein kinase II could phosphorylate p47 irrespective of whether it had been obtained from prophase-arrested or progesterone-matured oocytes.

We next tested the activity of other purified protein kinases for the ability to phosphorylate p47 in vitro. Both p34<sup>cdc2</sup> purified from maturing starfish oocytes and the catalytic subunit of cAMP-dependent protein kinase (A-kinase) were capable of phosphorylating p47 purified from stage VI oocytes although A-kinase was much less effective. Fig.3A shows that p47 prepared from progesterone-matured oocytes was only weakly phosphorylated by p34<sup>cdc2</sup>, the level of labelling representing only 10% of that on p47 purified from stage VI oocytes. By contrast, phosphorylation of p47 from progesterone-matured oocytes by A-kinase represented 35% of that for p47 from immature oocytes. The same result was obtained in three independent experiments. We verified that the p47 fraction from progesterone-matured oocytes contained no inhibitor of p34<sup>cdc2</sup> activity. Table 2 shows that the H1-kinase activity of p34<sup>cdc2</sup> was unaffected by the presence of p47 in the reaction mixture. This experiment was repeated twice. We interpret these results to suggest that the low degree of phosphorylation of p47 from progesterone-matured oocytes by p34<sup>cdc2</sup> was due to the site on p47 having already been phosphorylated by p34<sup>cdc2</sup> in vivo.

In order to confirm that the same site was indeed phosphorylated in intact cells as that in vitro, we performed alkaline hydrolysis on a gel of the protein phosphorylated by p34<sup>cdc2</sup> in vitro. As shown in fig.3B, the labelled protein was resistant to treatment with alkali. By contrast, in vitro

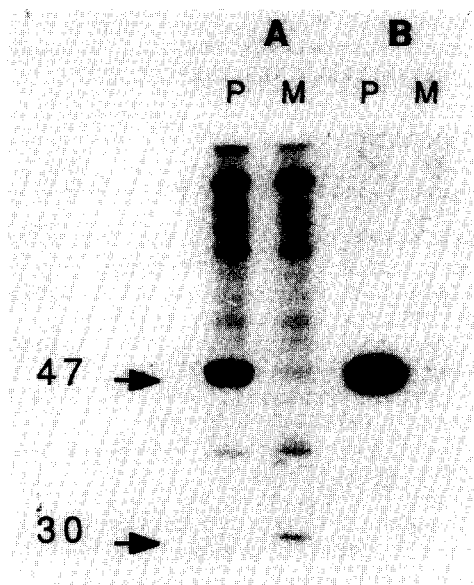


Fig.3. Autoradiography of 12% SDS-PAGE analysis of p47 phosphorylation by p34<sup>cdc2</sup>. Heparin fractions from prophase (P) or progesterone-matured (M) oocytes were phosphorylated as indicated in the text. (A) Untreated and (B) alkali-treated gels.

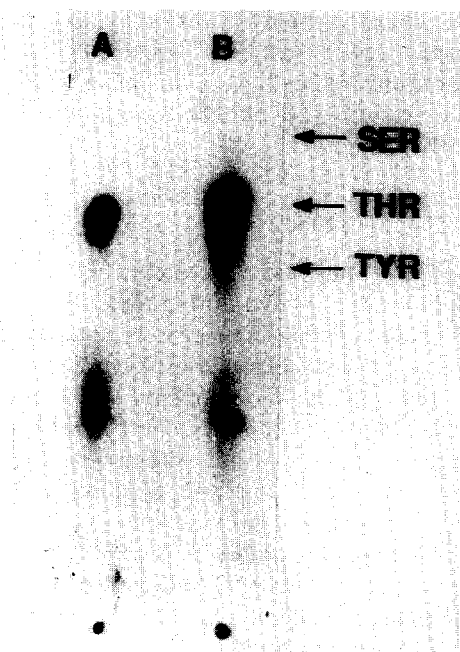


Fig.4. Autoradiography of phosphoamino acid analysis of p47 protein after (A) in vivo and (B) in vitro phosphorylation by p34<sup>cdc2</sup>. Arrows: authentic phosphoamino acids.

phosphorylation by A-kinase was not alkali-resistant (not shown), indicating that the in vivo phosphorylation could not be due to A-kinase activity. The alkali resistance of p47 phosphorylated in vivo is known to be due to threonine residues [11]. Fig.4 confirms that phosphorylation of p47 by p34<sup>cdc2</sup> occurred exclusively on threonine residues.

We finally compared peptide maps of p47 phosphorylated in vivo and in vitro by p34<sup>cdc2</sup>.

Table 2

Effect of p47 fraction on in vitro histone kinase activity of p34<sup>cdc2</sup>

	Activity (pmol/min per 25 nl enzyme)
p34 <sup>cdc2</sup>	0.096
p34 <sup>cdc2</sup> + prophase p47	0.096
p34 <sup>cdc2</sup> + matured p47	0.101

The kinase activity of p34<sup>cdc2</sup> was determined in the presence of purified p47 fractions prepared from prophase and progesterone-matured oocytes as described in section 2. Each point corresponds to duplicate determinations

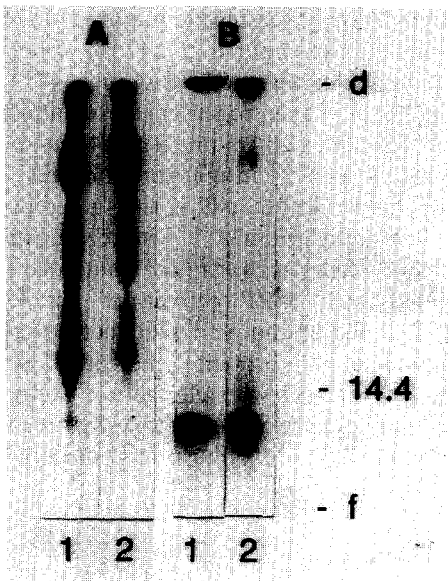


Fig.5. Autoradiography of 15% SDS-PAGE of p47 phosphopeptides after proteolytic digestion by (A)  $\alpha$ -chymotrypsin and (B) V8 protease. Peptide patterns from p47 phosphorylated: (lane 1) in vitro by p34<sup>cdc2</sup> and (lane 2) in vivo.

After partial proteolysis by  $\alpha$ -chymotrypsin, the labelled peptide patterns obtained from both preparations of p47 were identical (fig.5A). A similar result was obtained with V8 protease (fig.5B).

#### 4. CONCLUSION

We have purified a protein, p47, which is phosphorylated *in vivo* during meiotic maturation of *Xenopus* oocytes. The protein appears to be a part of a high molecular mass complex that contains three major components of 30, 36 and 47 kDa. The same complex can be purified from immature oocytes or progesterone-matured oocytes; however, the p47 component is only phosphorylated during maturation.

Multiple protein kinases are activated at the time of appearance of MPF [20–22] and any one of these activities could account for p47 phosphorylation. Our results suggest that p47 is a direct substrate for phosphorylation by the p34<sup>cdc2</sup> mitotic protein kinase. This is indicated by the equal degree of alkali resistance of phosphorylated residue(s), the identical peptide pattern obtained by partial proteolysis by two different proteases and the inability of p34<sup>cdc2</sup> to phosphorylate p47 prepared from progesterone-matured oocytes. In addition, the time course of p47 phosphorylation *in vivo* coincides with activation of p34<sup>cdc2</sup> during meiosis [7,12]. Taken together, these data strongly support the idea that protein p47 is a direct substrate for p34<sup>cdc2</sup> in maturing oocytes.

As far as we know, p47 is the first reported *in vivo* substrate of the p34<sup>cdc2</sup> kinase. Phosphorylation of p47 occurs at the time of numerous changes associated with MPF activation. Interestingly, analysis of the amino acid sequences of p47 and p30 associated protein indicates sequence homologies of p30 with elongation factors [23]. Accordingly, we are currently investigating the role of this complex and its phosphorylation in protein synthesis.

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