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Protein phosphatase 2_A from *Xenopus* oocytes

Characterization during meiotic cell division

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A polyclonal antibody was raised against bacterially produced catalytic α subunit of protein phosphatase 2_A (PP2AC) cloned from *Xenopus* ovarian library. The amount of PP2AC in *Xenopus* oocytes determined by Western blot analysis was 1 ng/μg of cytosolic protein. The antibody depleted PP2AC from oocyte extracts in association with 6 components (40, 62, 65, 80, 85 and 90 kDa). Prophase- and metaphase-arrested oocytes contained identical amounts of PP2AC. Metaphase oocytes showed one specific change in the 62 kDa protein associated with PP2AC.

Protein phosphatase 2_A; Associated protein; Metaphase-specific form; *Xenopus* meiotic cell division

1. INTRODUCTION

Protein phosphatases play an essential role in the regulation of many cellular processes including cell cycle events (reviewed in [1]). Among the protein phosphatases, protein phosphatase 2_A (PP2A) was proposed to play a role in tumor suppression since it is a target of a tumor promoter, okadaic acid, which inhibits its activity in the nanomolar concentration range [2,3]. Genetic anaysis also demonstrated that PP2A plays an essential role in cell cycle control in fission yeast [4]. Moreover, the M-phase promoting factor (MPF), a factor universally regulating the G2-to-M transition of the cell cycle [5] is negatively regulated by PP2A [6–13]. An important question that remains unanswered however, is whether PP2A is regulated during cell division? Only one report suggests changes in PP2A activity during the course of MPF activation in *Xenopus* meiotic division, shortly and transiently after progesterone exposure of the oocytes [14].

We have cloned a cDNA encoding the catalytic subunit of PP2A from *Xenopus* oocytes. We have raised a polyclonal antibody against the bacterially produced protein which allowed us to characterize the oocyte enzyme and to show the presence of a metaphase-specific form during meiotic cell division.

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2. MATERIALS AND METHODS

A λgt10 *Xenopus* ovarian cDNA library [15] was used for isolation of a clone encoding the catalytic subunit of protein phosphatase 2A (PP2AC). Both strands of the cDNA insert were completely sequenced by the dideoxy chan termination method [16] after subcloning of the appropriate restriction fragments (see Fig. 1A) in the Bluescript KS phagemid [17]. Northern analysis of RNA isolated from oocytes were performed as previously described [18].

The *Pvu*II–*Pst*I fragment of the *Xenopus* PP2AC cDNA was cloned between the *Eco*RI and the *Pst*I sites of the pT7-7 procaryote expression vector (a gift from Dr. Tabor, Department of Biological Chemistry, Harvard Medical School, Boston). Expression of the mRNA from the T7 promoter was induced in K38/pGP1-2 cells. The majority of the bacterially expressed recombinant protein was recovered as insoluble material. The protein was solubilised in 2% SDS and extensively dialysed against 100 mM NaCl, 50 mM Tris-HCl, pH 7.4.

Guinea pig (Dunkin-Hartley, IFFA-CREDO, France) antiserum was obtained by injection of the recombinant protein. Antibody was purified on a column of protein A-Sepharose CL4B (Sigma) [19] and coupled to immobilized protein A using either Pierce kit or as described in [20].

Prophase- and metaphase-arrested stage VI [21] oocytes were obtained as already described [22]. Oocytes were homogenised in 2 mM dithiothreitol, 10 mM $MgCl_2$, 10 mM EGTA and 50 mM Tris-HCl, pH 7.4, and the cytosoluble fraction prepared [23]. Labelling was achieved by incubating oocytes overnight in 250 μCi/ml [^{35}S]methionine (1000 Ci/mmol, Amersham). Oocytes were washed in order to remove external radioactivity and further incubated in the absence or presence of progesterone. The cytosoluble fraction was then prepared after homogenisation of the oocytes in an antiphosphatase buffer [23]. The protein content of the fractions was determined by Bradford assay [24].

Protein phosphatase 2A (low-M_r polycation-stimulated, PCS_L protein phosphatase) was purified from *Xenopus* ovary [25].

Protein phosphatase activities were measured on diluted extracts using casein kinase II-phosphorylated β-casein as a substrate. β-Casein was phosphorylated by casein kinase II [26] in a 0.3:1 ratio using

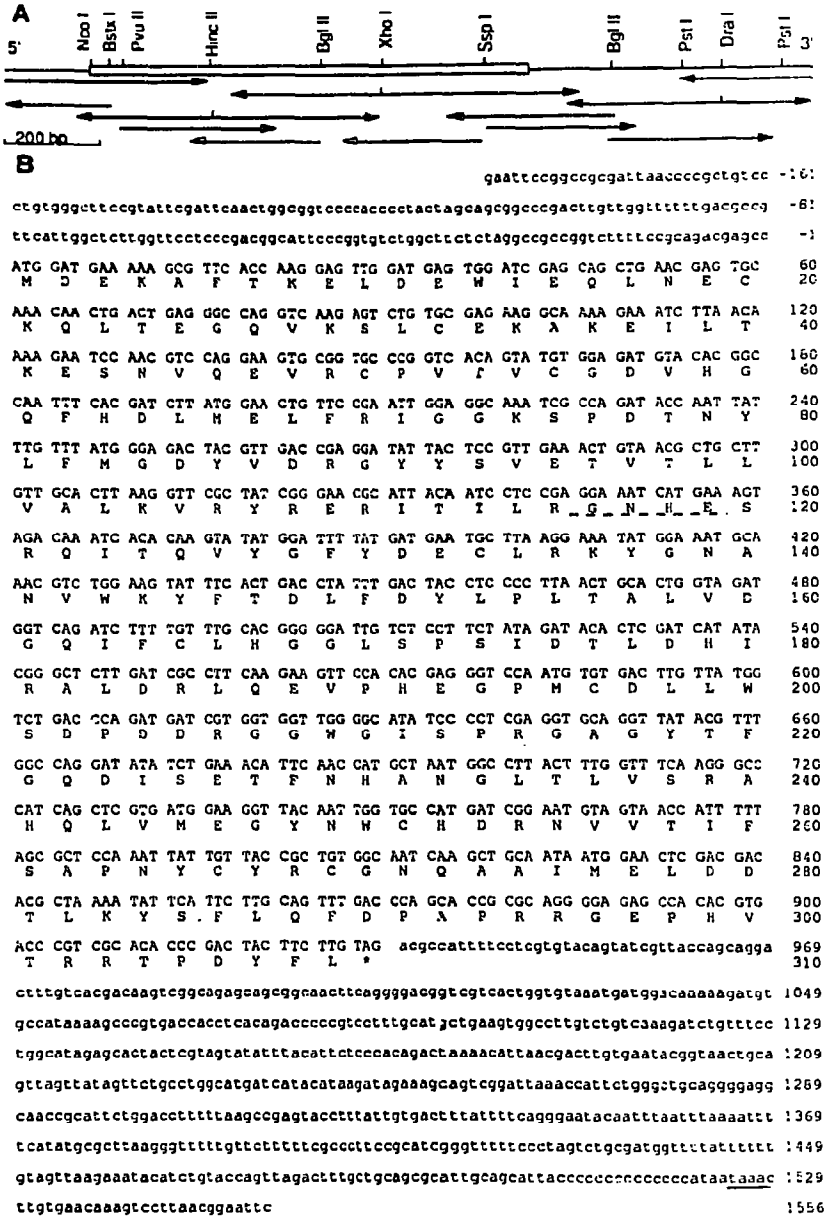

Fig. 1. cDNA sequence and deduced amino acid sequence of the catalytic subunit of *Xenopus* protein phosphatase 2_A. (A) Restriction map and sequencing strategy. (B) Nucleotide and deduced amino acid sequences. The first 8 bases at the 5′ end and the last 8 bases at the 3′ end correspond to the *Eco*RI linker sequence. A polyadenylation signal (AATAAA) is underlined. A feature characteristic of all known serine/threonine protein phosphatases (RGNHE) is indicated (dashed line).

the procedure of Cormier et al. [14] except that 500 μM [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) was used. Protein phosphatase activity was assayed at 30°C in buffer containing 1 mM dithiothreitol, 10 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4, and phosphorylated β-casein 80–400 pmol/100 μl. After addition of 1 vol. of 20% trichloracetic acid, ^{32}P released was determined [14]. The reaction was linear with time from 0–30 min when the extract concentration was 2.5–10 μg/ml. Okadaic acid was a gift of Pr. Daisuke Uemura (Shizuoka University, Japan).

Depletion of protein phosphatase activity from extracts was performed with immobilized antibody (4 mg IgG per ml bead). Immobilized antibody was saturated with 1% BSA in 20 mM Tris-HCl, pH 7.4. Beads (25 or 50 μl) were incubated with 500 μl extract containing 30 μg protein for 60 min at 4°C. Supernatants were decanted by centrifugation and the beads washed twice in 20 mM Tris-HCl, pH 7.4. Protein phosphatase activity of 50 μl aliquot of each fraction was assayed as described above.

Analysis of the proteins associated with PP2AC was performed by incubating [^{35}S]-labelled cytosoluble extracts with immobilized antibody. 50 μl beads saturated with 1% BSA in 500 mM NaCl, 50 mM Tris-HCl, pH 7.6, were incubated in 1.5 ml containing 100–200 μg cytosoluble proteins in the same buffer with the addition of 1% NP40. After 2 h incubation at 4°C, the supernatant was decanted by centrifugation and the beads were washed 3-times with the incubation buffer and twice with the buffer without BSA and NP40. The antigen was

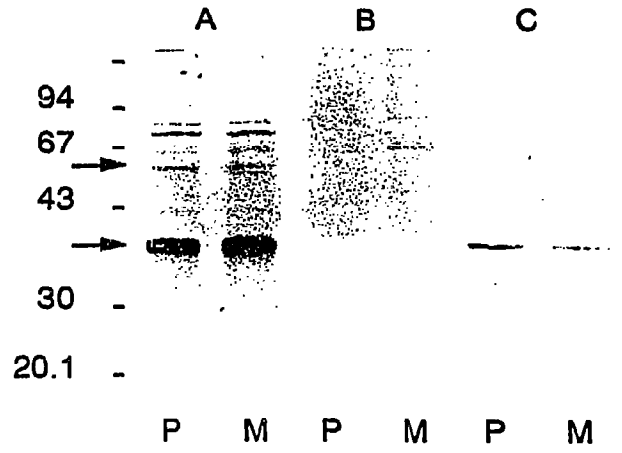

Fig. 2. Immunoaffinity purification of protein phosphatase 2_A from prophase-arrested oocytes (P) and metaphase-arrested oocytes (M). (A) Autoradiography of [^{35}S]-labelled proteins resolved on SDS-PAGE after adsorption of 200 μg cytosoluble extract on (50 μl) PP2AC-antibody coupled to Sepharose beads. (B) As for (A) with control Sepharose beads. (C) Western blot analysis of the total cytosoluble proteins run on a foster gel (80 μg per slot), the blot is revealed with affinity-purified PP2AC antibody (1:1000).

then released from the coupled antibody by solubilization in 2% SDS, 62.5 mM Tris-HCl, pH 6.8, for 2 min at 100°C. After centrifugation, the supernatant was diluted in Laemmli buffer [27] and electrophoresed.

SDS-PAGE was carried out according to Laemmli [27]. Western blot analysis was made after electrophoretic transfer of proteins to nitrocellulose using the guinea pig antibody at a dilution of 1:1000. The antigen-antibody complex was revealed by the chloronaphthol-peroxidase reaction using peroxidase-coupled secondary antibody (Dakopatts, Denmark) at a dilution of 1:500.

3. RESULTS AND DISCUSSION

3.1. *Characterization of PP2A in the* Xenopus *full-grown oocyte*

Fig. 1A shows the partial restriction analysis and the sequencing strategy of the insert encoding the *Xenopus* PP2A catalytic α subunit. The complete nucleotide sequence and the deduced protein sequence of 309 amino acids are presented in Fig. 1B. The sequence conservation between the *Xenopus* PP2AC and the corresponding proteins from other species was more than 98% with mammalian proteins.

The antibody, raised against bacterially produced PP2AC, recognized biochemically purified *Xenopus* PP2A at 35 kDa on Western blot (data not shown). When a crude cytosol prepared from *Xenopus* oocytes was analysed, the antibody showed the presence of one single band at 35 kDa (Fig. 2C). This result indicates that the antibody presents no cross-reaction with other cytosoluble proteins. In particular, no reaction was detected at 61 or 37 kDa, corresponding to the expected position of the catalytic subunits of protein phosphatase 2_B and protein phosphatase-1, respectively, although these proteins share sequence similarities (between 40 and 50%) with PP2AC [1]. The amount of PP2AC present in cytosoluble proteins, calculated from densitometric scanning of immunoblots, was 1 ng of PP2AC per 1 μg of cytosoluble proteins.

PP2A activity in oocyte cytosoluble extracts was 0.38 pmol/min/μg protein (SD = 0.20, n = 19). This activity was inhibited by okadaic acid with an ED_{50} of 6.3 nM, demonstrating the presence of PP2A. The immobilized antibody depleted the cytosoluble phosphatase activity by 76% (SD = 12, n = 5). Protein phosphatase activity remained totally unadsorbed using a non-related antibody which was coupled to beads and used in parallel with the PP2AC antibody.

An analysis of proteins associated with PP2AC was then performed by immunoadsorption of in vivo labelled [^{35}S]methionine oocytes. Four independent experiments were performed. Electrophoretic analysis of the adsorbed proteins is illustrated in Fig. 2A. The major adsorbed protein migrated at 35 kDa, corresponding to the position of PP2AC. In addition, 6 other proteins (40, 62, 65, 80, 85 and 90 kDa) of lesser intensity were detected. The relative ratios of these proteins varied between experiments. Bands at 65 and 85 kDa were detected in association with control beads free of antibody (Fig. 2B) or with control beads coupled to unrelated antibody (not shown). The others, at 40, 62, 80 and 90 kDa, are specific PP2AC-associated proteins.

3.2. *Change in PP2AC-associated proteins during meiotic cell division*

Changes in PP2AC and the associated proteins were investigated in prophase- and metaphase-arrested oocytes. The amount of PP2AC, analysed by Western blotting, was identical in both oocyte extracts (Fig. 2C). PP2AC-associated proteins were analysed by immunoadsorption of [^{35}S]-labelled proteins of prophase and metaphase extracts. As illustrated in Fig. 2A, PP2AC-associated proteins were very comparable. However, a quantitatively minor change was consistently observed at the level of the 62 kDa protein which was resolved as a doublet in metaphase compared to prophase extracts (arrowed in Fig. 2A). This change was observed in all 4 experiments. Although this difference appears to involve less than 10% of PP2A, it suggests the existence of a metaphase-specific form of PP2A. This result is the first evidence that PP2A can be under cell cycle regulation. When protein phosphatase activities of prophase and metaphase extracts were investigated, no significant change was found using our experimental conditions. Considering the amount of the metaphase-specific form, this lack of difference in the total PP2A activity is not surprising.

It will now be important to purify the metaphase-specific form of PP2A or to search for its putative specific substrate(s), for instance to analyse the phase-spe-

cific 62 kDa PP2AC-associated protein with respect to INH, a negative regulator of MPF, recently reported to be a peculiar form of protein phosphatase 2_A [13]. Another attractive hypothesis would be that this form of PP2A could play a role in the metaphase II arrest of the oocyte. Exit from M-phase requires cyclin degradation and it was shown that okadaic acid added to metaphasic extracts is sufficient to induce cyclin degradation [28]. This suggests that PP2A negatively controls the cyclin degradation pathway. The metaphase specific form of PP2A could be the one responsible for this control.

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