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cDNA Cloning of a Novel B Subunit of *Xenopus* Protein Phosphatase 2A and Its Biological Activity in Oocytes[1]

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We have cloned a cDNA encoding a novel B regulatory subunit of protein phosphatase 2A (PP2A) from a *Xenopus* oocyte cDNA library. The novel B subunit, termed Bβ', shows the strongest overall sequence similarity to, but a distinct N-terminal sequence from, the β isoform of the human/rat B subunit. When expressed ectopically in *Xenopus* oocytes, the Bβ' isoform can augment the endogenous PP2A activity and inhibit oocyte maturation induced by progesterone. These results suggest that the Bβ' isoform can form a complex with other PP2A subunits to make a trimeric PP2A holoenzyme in *Xenopus* oocytes and may negatively control the initiation of oocyte maturation. © 1997 Academic Press

Protein phosphatase 2A (PP2A), one of the major four classes of serine/threonine phosphatases in eukaryotes, is involved in the regulation of many cellular processes, such as the cell cycle, signal transduction and metabolism (1). PP2A is thought to exist and function in a heterotrimeric form *in vivo*, which consists of a common 'core' dimer (a catalytic C subunit and a regulatory A subunit) and either of the heterologous B, B′ and B″ regulatory subunits (1, 2). The B regulatory subunit, also called BR55 or PR55, is thought to determine the substrate specificity of the PP2A holoenzyme (2, 3) and may also determine its subcellular localization (4). At least three isoforms (α, β and γ) of the mammalian B subunit have been identified and shown to be expressed in a tissue-specific manner (2, 3, 5, 6).

Okadaic acid, a potent inhibitor of PP2A (2), can induce meiotic maturation of prophase-I-arrested immature oocytes from various species, suggesting that PP2A may be involved in prophase I arrest in oocytes (7, 8). In the amphibian *Xenopus*, only one isoform (or α) of the B regulatory subunit has been identified (9, 10), and a cytoplasmic activity (called INH) that is present in immature oocytes and can inhibit meiotic maturation in recipient oocytes has been shown to be a trimeric form of PP2A containing the Bα isoform (11, 12). And, in cell-free *Xenopus* egg extracts, addition of INH/PP2A can prevent entry into mitosis by inhibiting activation of M phase-promoting factor (MPF) (13, 14), a universal G_2/M regulator in eukaryotic cells (15). Thus, in oocytes, the ABαC trimeric form of PP2A is thought to mediate prophase I arrest by inhibiting activation of MPF (11, 14). In this study, we cloned a cDNA encoding a novel isoform of the PP2A B subunit (termed Bβ') from a *Xenopus* oocyte cDNA library, and show that the ectopically expressed Bβ' isoform can augment the endogenous PP2A activity in oocytes and potently inhibits oocyte maturation.

MATERIALS AND METHODS

cDNA cloning and sequence analysis. A *Xenopus* oocyte cDNA library (16) was screened for clones encoding the B regulatory subunit of *Xenopus* PP2A, by using a ^{32}P-labelled 1.1 kb *Alw* NI-*Bsp* HI fragment of the rat Bα subunit cDNA (6, 17) as a probe. Low-stringent hybridization was performed for 16 hr at 50°C in a buffer containing 5 × SSC, 50 mM Na_2HPO_4, 50 mM NaH_2PO_4, 0.5% sarcosyl, 0.1% skimmilk, 0.05% sodium pyrophosphate, 1 mM EDTA and 100 μg/ml salmon sperm DNA. Hybridized filters were washed three times, each for 45 min at 50°C, in a buffer containing 1 × SSC and 0.1% SDS. Positive clones were isolated, subcloned into a pUC18 plasmid vector and sequenced by the use of a dideoxy chain termination kit (Amersham).

Construction of recombinant plasmids and in vitro transcription. An *Eco* RI Bβ' cDNA fragment containing the whole Bβ'-encoding sequence was blunt-ended with a Klenow fragment and subcloned into a pSP64T plasmid vector (18), which had been cut with *Bgl* II and then blunt-ended. A mutant cDNA encoding the N-terminal two-thirds (positions 1-293) of the Bβ' subunit was prepared by PCR of the full-length Bβ' cDNA; the 5′ primer used (5′-CGCTCTAGAACC-ATGCCTTGTTCTGTAGAAGACAA-3′) contained an artificial *Xba* I

[1] The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number AB000406.

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Abbreviations: GVBD, germinal vesicle breakdown; kb, kilobases; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; MPF, M-phase-promoting factor; PP2A, protein phosphatase 2A.

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site (TCTAGA) and an initiator ATG codon, while the 3′ primer (5′-GCGCTCGAGTTAAGGCTCTTCAAACAATTTGGA-3′) contained an artificial *Xho* I site (CTCGAG) and a terminator TAA codon. The amplified fragments were cut with both *Xba* I and *Xho* I, blunt-ended and then inserted into the *Bgl* II-cut/blunt-ended pSP64T vector, as described above. pSP64T recombinant plasmids thus obtained were cut singly with *Eco* RI and then *in vitro* transcribed into 5′-capped mRNAs by using SP6 RNA polymerase, as described previously (19).

Measurement of PP2A activity. Oocytes were homogenized on ice in 250 μl (per oocyte) of a buffer containing 50 mM Tris (pH 7.5), 0.1 M NaCl, 5 mM EDTA, 50 mM NaF, 200 μM sodium orthovanadate, 20 mM sodium pyrophosphate, 100 μM leupeptin and 1% Triton X-100, and centrifuged briefly at 2°C. Two μl of the supernatant was added to 20 μl of a solution containing phosphorylated myelin basic protein (MBP; 0.5 μg/μl) (see below) with or without 40 nM okadaic acid; 40 nM okadaic acid can inhibit specifically PP2A activity in diluted oocyte extracts (13, 20). [MBP (Sigma) was phosphorylated *in vitro* by MAP kinase (UBI) in the presence of [^{32}P]ATP and separated from free [^{32}P]ATP on a NAP5 column (Pharmacia) by using a phosphorylation reaction buffer containing 25 mM Hepes (pH 7.4), 10 mM $MgCl_2$ and 1 mM DTT, exactly as described previously (21).] After 10 min at 23°C, the dephosphorylation reaction was stopped by the addition of an equal volume of 2 × Laemmli's sample buffer and then boiled for 5 min. Five μl of the boiled sample was mixed with 15 μl of a solution containing 10 mg/ml bovine serum albumin (as carrier) and 20 μl of 20% trichloroacetic acid. After incubation for 20 min on ice, the mixture was centrifuged briefly and 8 μl of the supernatant containing released ^{32}P was spotted on a filter paper. Quantitation of the released ^{32}P was performed by the use of an imaging analyzer BAS1000 (Fuji). PP2A activity was defined as the ^{32}P-releasing activity that was sensitive to 40 nM okadaic acid.

Preparation and microinjection of oocytes. Preparation and culture of oocytes, induction of oocyte maturation by progesterone, and microinjection of mRNA were all described previously (22).

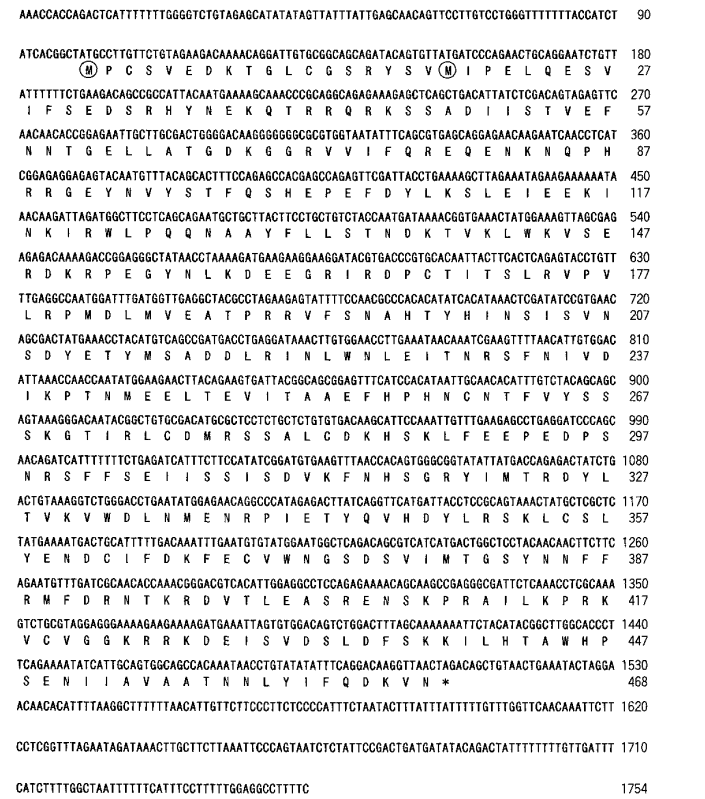

FIG. 1. Nucleotide sequence of the *Xenopus* Bβ' isoform cDNA and its deduced amino acid sequence. Potential initiator methionine residues are circled.

RESULTS

Cloning and sequencing of Xenopus PP2A B subunit cDNAs. We screened about 3×10^5 phages from a *Xenopus* oocyte cDNA library, using a cDNA encoding a rat PP2A Bα subunit as a probe. We obtained 22 positive clones and subcloned them each into a pUC18 plasmid vector. Sequence analysis revealed that 14 out of the 22 clones encoded an α isoform of the *Xenopus* PP2A B subunit, which had previously been cloned and sequenced (10); however, the deduced N-terminal amino acid sequence of our longest clone was different from that of the previously reported one, presumably due to the alternative splicing of the Bα transcripts (data not shown). The remaining eight clones apparently encoded a different isoform of the B subunit, which shared overall 81% identity with the Bα isoform. The longest cDNA of the eight clones had an open reading frame capable of encoding 468 amino acid residues (Fig. 1). However, it had a second methionine codon, which lied 54 bases downstream of the first methionine codon and had a better Kozak consensus motif (23). At present, we do not know which methionine codon is utilized as an initiator codon in cells.

We compared the deduced amino acid sequence of the newly isolated *Xenopus* B isoform cDNA with those of the three isoforms (α, β, and γ) of the rat B subunit (17, 24). It shared overall 90%, 83% and 81% identities with the β, α, and γ isoforms of the rat B subunit, respectively (Fig. 2). Interestingly, however, its N-terminal sequence (positions 1-48) did not show any similarity to those of the three rat isoforms (and also to those of human isoforms; data not shown but see ref. 25). Thus, despite its strongest overall sequence similarity to the β isoform, the *Xenopus* B subunit might not be a β isoform but may be a novel isoform. For this reason, we tentatively designate it Bβ'.

Increase in the endogenous PP2A activity by the ectopically expressed Bβ' isoform. Initially, we attempted to detect the Bβ' protein in *Xenopus* oocytes, but the results obtained were not clear, presumably because of the quality of the antibody we used and/or the low abundance of the Bβ' protein in oocytes. We therefore intended to examine the biochemical and biological activities of the Bβ' isoform by ectopically expressing it in *Xenopus* oocytes. First, we tested whether the ectopically expressed Bβ' isoform can influence the endogenous PP2A activity in oocytes. To do this, we injected oocytes with 18 ng of mRNA encoding the Bβ' isoform, and measured PP2A activity in the oocyte extracts using MAP kinase-phosphorylated MBP as a substrate (see MATERIALS AND METHODS for details). PP2A

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Xl Bβ'   MPCSVEDKTGLCGSRYSVMIPELQESVIFSEDSRHYNEKQTRRQRKSSADIISTVEFNNTGELLATGDKGGRVVI  75
Rat Bβ                          MEEDIDTRKIN:SFLRDHSYATE:::::::::H:::::::::::::::::  50
Rat Bα                       MAGAGGGNDIQWCFSQV:GAVDDDVAE:::::::::HS:::::::::::::::  54
Rat Bγ                          MGEDTDTRKINHSFLRDHSYVTE::V:::::::H:::::::::::::::  50

Xl Bβ'   FQREQENKNQPHRRGEYNVYSTFQSHEPEFDYLKSLEIEEKINKIRWLPQQNAAYFLLSTNDKTVKLWKVSERDK 150
Rat Bβ   ::::::S:::V:::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::: 125
Rat Bα   ::Q:::::I:S:S:::::::::::::::::::::::::::::::::::K::Q:::::::::I:::I::::: 129
Rat Bγ   ::::P:S::A::SQ:::D:::::::::::::::::::::::::::::K::::::::HS::::::::I::::IT:::: 125

Xl Bβ'   RPEGYNLKDEEGRIRDPCTITSLRVPVLRPMDLMVEATPRRVFSNAHTYHINSISVNSDYETYMSADDLRINLWN 225
Rat Bβ   ::::::::::::::L:::A:::T:::::::::::::::::::A:::::::::::::::::::::::::::::: 200
Rat Bα   ::::::::E:D::Y:::T:V:T:::::F::::::::S:::I:A:::::::::::I:::::::L:::::::::H 204
Rat Bγ   ::::::::::::KLK:LS:V:::Q:::K:::::::VS:::I:A:G:::::::::::::C:::::::::::::H 200

Xl Bβ'   LEITNRSFNIVDIKPTNMEELTEVITAAEFHPHNCNTFVYSSSKGTIRLCDMRSSALCDKHSKLFEEPEDPSNRS 300
Rat Bβ   F:::Q:::::::::A:::::::::::::::::H:::::::::::::::::::A:::::R:T:F:::::::::: 275
Rat Bα   ::::D:::::::::::A:::::::::::::::::NS:::::::::::::::::::::A:::::R:::::::::::: 279
Rat Bγ   :A::D:::::::::::A:::D:::::::S:::::H::L:::::::::SL::::::AA:::::::::::::::::: 275

Xl Bβ'   FFSEIISSISDVKFNHSGRYIMTRDYLTVKVWDLNMENRPIETYQVHDYLRSKLCSLYENDCIFDKFECVWNGSD 375
Rat Bβ   ::::::::::::::S:::::::::::::A:::::::::V:::::::::::::::::::::::::::::::::: 350
Rat Bα   ::::::::::::::S:::::M::::::S:::::::::::V::::::E:::::::::::::::::::::C:::: 354
Rat Bγ   ::::::::V::::::S:::::ML::::::::::::::::A:::::::::::::::::::::::S:::::::::A:::: 350

Xl Bβ'   SVIMTGSYNNFFRMFDRNTKRDVTLEASRENSKPRAILKPRKVCVGGKRRKDEISVDSLDFSKKILHTAWHPSEN 450
Rat Bβ   ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::: 425
Rat Bα   ::V:::::::::::::::::::I::::::::N:::TV:::::::AS:::K:::::::::::N:::::::::K:: 429
Rat Bγ   ::::::A:::::::::::::::::::::::::S:::::V::::R::::::::R:D::::::::T:::::::::A:: 425

Xl Bβ'   IIAVAATNNLYIFQDKVN*          468
Rat Bβ   ::::::::::::::::::*          443
Rat Bα   :::::T::::::::::::*          447
Rat Bγ   :::I::::::::::::::SDMH*      447
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FIG. 2. Comparison of the predicted amino acid sequence of the *Xenopus* (Xl) Bβ' isoform with those of the rat Bα (24), Bβ (24), and Bγ (17) isoforms. Amino acid residues identical to those of the *Xenopus* Bβ' isoform are indicated by two dots (:).

activity in these oocyte extracts was about 2-fold higher than that in uninjected-oocyte extracts (Fig. 3). In a control experiment, we injected oocytes with 18 ng of mRNA encoding a C-terminal third-truncated Bβ' isoform (B$\beta'\Delta$C), as such a C-terminus-lacking B subunit has been shown to be unable to form a complex with the AC core dimer (to make a trimeric PP2A holoenzyme) (26). These oocyte extracts showed no increase in PP2A activity, as compared with uninjected-oocyte

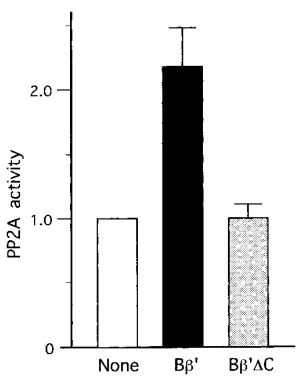

FIG. 3. Effect of ectopic Bβ' isoform expression on the phosphatase activity of PP2A in *Xenopus* oocytes. 18 ng of mRNA encoding either the full-length Bβ' isoform (Bβ') or the C-terminal third-truncated Bβ' mutant (B$\beta'\Delta$C) was injected into oocytes. Uninjected oocytes (None) and those injected with mRNA were cultured for 12 hr at 21°C and then subjected to *in vitro* PP2A phosphatase assay, as described in MATERIALS AND METHODS. The PP2A activities, shown in an arbitary unit, represent the averages from three independent determinations, the activity in uninjected control oocytes being standardized as 1.0.

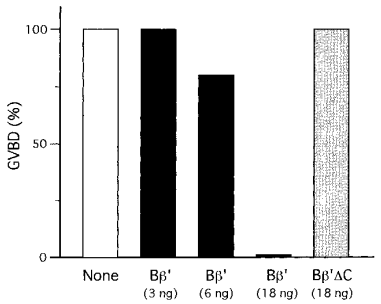

FIG. 4. Effect of ectopic Bβ' isoform expression on the initiation of oocyte maturation induced by progesterone. mRNA encoding either the full-length Bβ' isoform (Bβ') or the C-terminal third-truncated Bβ' mutant (B$\beta'\Delta$C) was injected into 20 oocytes at the doses indicated (per oocyte). Uninjected oocytes (None) and those injected with mRNA were cultured for 12 hr at 21°C and then treated with progesterone. These oocytes were scored for % GVBD (22), until 15 hr after the progesterone treatment. Essentially similar results to those presented in the figure were obtained with three other independent experiments.

extracts. These results show that the ectopic Bβ' isoform can increase the endogenous PP2A activity (towards MAP kinase-phosphorylated MBP) in oocytes, most probably by forming a heterotrimeric (ABβ'C) PP2A holoenzyme in the injected oocytes.

Inhibition of oocyte maturation by the ectopically expressed Bβ' isoform. We next examined whether the ectopically expressed Bβ' isoform had any effect on the initiation of oocyte maturation induced by progesterone. For this, we injected oocytes with mRNA encoding either the full-length Bβ' isoform or the non-functional B$\beta'\Delta$C mutant, and treated them with progesterone. Uninjected control oocytes and those injected with the B$\beta'\Delta$C-encoding mRNA (18 ng/oocyte) both matured normally [100% of the oocytes undergoing germinal vesicle breakdown (GVBD, a hallmark of oocyte maturation; ref. 22) within about 5 hr after the progesterone treatment] (Fig. 4). By contrast, oocytes injected with the full-length Bβ'-encoding mRNA did not mature or matured only slowly, depending on the dose of the injected mRNA: within 15 hr after the progesterone treatment, 80% of the oocytes injected with 6 ng of mRNA and none of the oocytes injected with 18 ng of mRNA underwent GVBD. (In some batches of oocytes, 100% of the injected oocytes underwent GVBD, albeit slowly, even at the highest dose or 18 ng of mRNA; concerning this, see Discussion.) These results, together with those described above, show that the ectopic Bβ' isoform can negatively control the initiation of oocyte maturation, presumably by increasing the endogenous PP2A activity towards some relevant substrates in oocytes.

DISCUSSION

Previous studies, using either a human Bα isoform cDNA or an antibody specific for the N-terminus of a

human Bα or Bβ isoform, suggested that *Xenopus* oocytes contain only Bα (or its transcripts) as an isoform of the B subunit of PP2A (9, 10). Using a rat Bα cDNA as a probe, however, we were able to isolate, from a *Xenopus* oocyte cDNA library, a cDNA encoding a B subunit that has the strongest overall sequence similarity to, but a distinct N-terminal sequence from, the human/rat Bβ isoform. This *Xenopus* B isoform may thus be either a homolog of the human/rat Bβ isoform or more likely a novel isoform (if we consider its unique N-terminal sequence); we tentatively designate it Bβ′.

The B regulatory subunit is thought to determine the substrate specificity and subcellular localization of the PP2A holoenzyme (2-4). Earlier, this subunit was thought to have a general inhibitory effect on the phosphatase activity of the AC dimeric PP2A holoenzyme (1). However, a growing body of evidence suggests that it may, in fact, activate the phosphatase activity of the PP2A holoenzyme towards some specific substrates but inhibit the activity towards other substrates (2, 3). Thus, for instance, proteins phosphorylated by proline-directed protein kinases, such as cdc2 kinase and MAP kinase, have been shown to be much better substrates for the ABC trimeric holoenzyme than for the AC dimeric holoenzyme (27-29). In this study, we found that when ectopically expressed in *Xenopus* oocytes, the Bβ′ isoform can augment the endogenous PP2A activity towards MAP kinase-phosphorylated MBP, while its Bβ′ΔC mutant (which is most probably unable to associate with the AC core dimer; confer ref. 26) cannot. These results suggest that the ectopically expressed Bβ′ proteins can associate with (free) AC core dimers, which are known to exist in *Xenopus* oocytes (9, 27, 30), and that the newly formed ABβ′C trimeric holoenzymes also have a substrate specificity for proteins phosphorylated by proline-directed protein kinases.

The ectopically expressed Bβ′ isoform could inhibit oocyte maturation induced by progesterone, in a dose-dependent manner. Formally, this inhibitory effect could be due either to interaction of the ectopic Bβ′ isoform with some unknown factor(s) or due to depletion itself (by the Bβ′ isoform) of free AC core dimers, which might themselves have some important function. As previously shown, however, INH-an ABαC trimeric form of PP2A purified from oocytes—has the same maturation-inhibitory effect as does the ectopic Bβ′ isoform (11, 14); moreover, triple expression of the A, Bβ′ (but not Bβ′ΔC) and C subunits in oocytes also showed the same inhibitory effect (our unpublished data). Thus, it is very likely that an inhibition of oocyte maturation by the ectopically expressed Bβ′ isoform is ascribed to its association with the endogenous AC core dimers and hence to the increased total phosphatase activity of the endogenous PP2A. (In some batches of oocytes, the maturation-inhibitory effect of the ectopic Bβ′ isoform was very small and accompanied only by a small increase in the endogenous PP2A activity, presumably because of the existence of a very limited amount of available free AC core dimers in those oocytes; our unpublished data.) At present, we do not know whether the Bβ′ protein is present endogenously in immature oocytes. If it is present in oocytes, however, then the trimeric PP2A holoenzyme containing it could naturally function to mediate prophase I arrest of immature oocytes, as appears to be the case with INH (11, 12).

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