Analysis of Subunit Isoforms in Protein Phosphatase 2A Holoenzymes from Rabbit and Xenopus*

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A dimeric and two trimeric forms of protein phosphatase 2A (PP2A) were purified from rabbit and Xenopus tissues and analyzed using antisera specific for the catalytic and regulatory subunits. The dimeric holoenzyme consists of a complex of a 36-kDa catalytic subunit associated with a ~65-kDa regulatory subunit. The two trimeric holoenzymes consist of the catalytic subunit complexed with 65- and 55-kDa subunits, or 65- and 72-kDa subunits. Antisera were raised against synthetic peptides specific for the α- and β-isofoms of the 65-kDa (PR65α/β) and 55-kDa (PR55α/β) subunits identified by molecular cloning. Anti-peptide antisera to the 36-kDa catalytic subunit complexed with 65- and 55-kDa subunits, or 65- and 72-kDa subunits, reacted with both α- and β-isoforms. Immunochemical analysis of all three mammalian holoenzymes showed that the catalytic, 55- and 65-kDa subunits are both predominantly of the α-isofom, which is consistent with the peptide sequence data. The 65-kDa subunit of PP2A holoenzymes isolated from Xenopus skeletal muscle reacted with both anti-α and anti-β PR65-specific antisera whereas the PP2A holoenzymes isolated from Xenopus oocytes reacted preferentially with the β-specific antisera, indicating developmental changes in the expression of the 65-kDa subunit isoform. Taken together, these results show that the "core" subunits of the PP2A holoenzymes consist of the catalytic complexed with the 65-kDa subunit and that the association of the third subunit does not appear to be influenced by the isoform of these two core subunits.

Recent data have implicated protein phosphatase 2A (PP2A) in processes such as viral DNA replication (Virshup et al., 1989; Weinberg et al., 1990), suppression of cell cycle progression (Goris et al., 1988; Felix et al., 1990; Kinoshita et al., 1990), and identified the dimeric holoenzyme of PP2A as one of the targets for the transforming activity of the middle T and small t antigens of polyoma virus and the small t antigen of SV40 (Pallas et al., 1990; Walter et al., 1990). In addition, several protein kinases appear to be regulated by dephosphorylation mediated by PP2A (Ramachandran et al., 1987; Agostonis et al., 1987a; Ballou et al., 1988; Sturgill et al., 1988; Anderson et al., 1990; Haccard et al., 1990; Gomez and Cohen, 1991). These observations have added new perspectives to the previously recognized central role of PP2A.

Several oligomeric forms of PP2A have been purified to homogeneity (reviewed in Ballou and Fischer, 1986; Cohen, 1989; Shenolikar and Nairn, 1991). The dimeric holoenzyme of PP2A consists of a 36-kDa catalytic subunit and a regulatory subunit of 65 kDa. The two trimeric forms of PP2A contain a 36-kDa catalytic subunit complexed with a 65-kDa polypeptide associated with an additional subunit of either 55 or 72 kDa, respectively. However, other holoenzymes have been isolated where the third subunit appears to be different with a molecular mass of about 54 (Tung et al., 1985) or 74 kDa (Usui et al., 1988).

PP2A can be activated in vitro by polycations (Waellkens et al., 1987a, 1987b), but the mechanism is unknown. The presence of different regulatory subunits has been shown to determine the substrate specificity of PP2A holoenzymes in vitro (Iamaoka et al., 1983; Agostonis et al., 1987b, 1990, 1992; Munby et al., 1987; Usui et al., 1988; Chen et al., 1989; Cayla et al., 1990; Kamibayashi et al., 1991; Yang et al., 1991; Scheidtmann et al., 1991). Additionally these enzymes can also function as phosphotyrosyl phosphatases in vitro (Cheroff et al., 1983; Hermann et al., 1988; Goris et al., 1988; Jessus et al., 1989; Cayla et al., 1990; Haystead et al., 1990).

Molecular cloning techniques revealed that the catalytic subunit is encoded by different genes (Stone et al., 1987; Khe-Goodall et al., 1991; reviewed in Shenolikar and Nairn, 1991). The amino acid sequence of the two isoforms is 98% identical, with the differences between the two sequences being confined to the amino-terminal region. In addition, two different genes were identified that encode the 65-kDa regulatory subunit (PR65α and PR65β) from the dimeric PP2A (Hemmings et al., 1990; Walter et al., 1990); the predicted protein sequences show 87% homology. Furthermore, the 55-kDa subunit is encoded by three genes (PR55α, β, and γ) that are expressed in a tissue-specific manner (Mayer et al., 1991a; Healy et al., 1991). Recent data from the characterization of the 72-kDa subunit reveals that there are two forms of this protein, apparently derived from alternatively spliced tran-
scripts, that have predicted molecular masses of 62-kDa and 130-kDa.

Since the different holoenzymes of PP2A have distinct enzymatic properties, we wanted to investigate whether the primary structure of the 65-kDa regulatory and catalytic subunits was a determining factor in the subunit composition and hence the catalytic properties of a particular holoenzyme.

For the 65-kDa regulatory subunit, two strategies were followed: analysis of these subunits using peptide specific antisera, and direct sequence analysis of tryptic peptides generated from the 65-kDa subunit obtained from three holoenzymes of PP2A. We found that the 65-kDa subunits in the different holoenzymes, isolated from rabbit skeletal muscle, was predominantly the α-isoform, whereas holoenzymes isolated from Xenopus oocytes and muscle cross-react with both PR65α and β peptide-specific antisera. Interestingly, the PP2A holoenzymes isolated from Xenopus oocytes apparently only contain the PR65α epitope. A similar immunological approach was used for the analysis of the catalytic and 55-kDa subunit of the different oligomeric holoenzymes from rabbit skeletal muscle.

MATERIALS AND METHODS

Purification of Protein Phosphatase 2A Holoenzymes—The dimeric PP2A and the trimeric holoenzyme, containing the 72-kDa subunit, were purified from rabbit skeletal muscle according to Waelkens et al. (1987b), employing an additional FPLC Superose 12 gel filtration step for the latter. The trimeric holoenzyme containing the 55-kDa subunit from rabbit skeletal muscle was purified as described in Mayer et al. (1991a). The dimeric and trimeric form of PP2A were isolated from Xenopus oocytes and muscle as described by Hermann et al. (1988) with minor modifications (see "Results").

Preparation of Peptide-specific Antisera—Peptides were obtained from Multiple Peptide System, Cambridge Research Biochemicals or synthesized on a MilliGene Pepsynthesizer 9050. PR65-65-peptides (see Fig. 1) were coupled to either Keyhole Limpet Hemocyanin (KLH) (Calbiochem) (α-, β-, and β'-peptides), or ovalbumin (Sigma) (α- and β-peptides), or bovine serum albumin (BSA) (Sigma) (β'-peptide) using glutaraldehyde. Approximately 4 mg of the protein was mixed with 5-7 µg of the peptide in 500 µl of 0.1 M phosphate buffer, pH 7.5, and 20-µl aliquots of glutaraldehyde (20 mM) were added three times at 5-min intervals. After incubation for 1 h at 25 °C, the reaction was quenched by addition of 250 µl of 1 M glycine and incubated for 1 h. The peptide-protein conjugates were dialyzed extensively against water or TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and stored at −20 °C.

A similar procedure was followed for coupling the three peptides derived from the catalytic subunit (see Fig. 1, α-specific, β-specific, and the carboxyl-terminal peptide common to both isoforms).

For preparation of PR55α- and β-specific antisera, the two aminoterminal peptide sequences (α, MAGAGGGNDIQGWFSQVKYG; and β, MEEDDTTRKINSFLRDHSX; in both cases the carboxyl-terminal tyrosine was added for coupling as described below) were chosen and coupled by the method of Bassiri et al. (1979) as described in Harlow and Lane (1988). Briefly, 9 mg of KLH in 0.1 M borate buffer, pH 9.0, was incubated with 4 mg of the appropriate peptide plus 50 µl of bis-diazobenzenide, and incubated at 4 °C for 2 h. Following coupling the peptide-KLH mixture was dialyzed into PBS (140 mM NaCl, 2.5 mM KCl, 1.5 mM NaH2PO4, 8 mM Na2HPO4).

Rabbits (New Zealand White or Chinchilla) were immunized intradermally with 500 µg of KLH-peptide conjugates in complete Freund’s adjuvant (Difco). Every 4-6 weeks, boosts were given with 100-200 µg of the conjugates using incomplete Freund’s adjuvant. Blood was taken weekly from the ear vein and sera stored at −20 °C in 0.02% (w/v) Na3 EDTA. Specificity of the antisera was assessed using recombinant proteins and ELISA.

A similar procedure was followed for the PR65-peptide conjugates (BSA or ovalbumin) were coupled to 4 ml of Affi-Gel 15 (Bio-Rad) according to the manufacturer’s protocol. IgG from 1 ml of serum was precipitated with 50% saturated (NH4)2SO4, resuspended in 3 ml of TBS, and loaded onto an equilibrated peptide-Affi-Gel 15 column followed by extensive washing with TBS. Elution of peptide-specific antisera was achieved in two steps using 0.1 M glycine, pH 2.5, followed by 0.1 M Tris, pH 11.5. Between these two steps, the columns were equilibrated with 10 mM Tris-HCl, pH 8.8. Eluted antisera were combined and dialyzed against TBS containing 1% NaN3, and stored at 4 °C with 0.02% (w/v) NaN3.

Preparation of Monoclonal Antibodies—Monoclonal antibodies to the catalytic subunit were generated by immunizing mice with the dimeric form of PP2A using standard procedures (Harlow and Lane, 1988). Using the monoclonal antibodies monoclonal antibodies to the catalytic subunit and none were found toward the PR65 subunit. Full characterization of this monoclonal antisera will be published elsewhere.

ELISA and Western Blot Analysis—ELISA plates (Maxi-Sorb, InterMed Corp.) were coated with 500 ng of coupling peptide conjugates (prepared as described above) per well, blocked with PBS containing 3% (w/v) BSA, and incubated with primary antisera in PBS containing 1% BSA. Swine anti-rabbit IgG coupled peroxidase (Dakopatts) (1:1000) was used as secondary antisera. Peroxidase activity was quantified using ortho-phenylenediamine (0.06 mg/ml) and H2O2 (0.012% v/v) in 100 µl of 100 mM citrate buffer, pH 5.0. The reaction was started with 150 µl of NaH2SO4, and absorbance was measured at 492 nm.

Western blots were carried out as follows. After electrophoresis and blotting onto nitrocellulose (NitroScreen-West, Dupont) or PVDF membranes (Immobilon, Millipore), blots were blocked with PBS containing 3% BSA or 5% skimmed milk and incubated with primary antisera in PBS containing 1% BSA or 5% skimmed milk for 1-2 h. Anti-rabbit IgG-coupled peroxidase or 125I-labeled donkey anti-rabbit IgG (Amersham) was used at 1:1000 dilution for 1 h to detect the primary antibody. Peroxidase activity was detected using diaminobenzidine and H2O2 with nickel enhancement (Harlow and Lane, 1988).

For Western blots using the monoclonal antibody to the catalytic subunit, the ascites fluid was diluted 1:10,000, and the second antibody was an anti-mouse IgG-coupled peroxidase (diluted 1:10,000) and enhanced chemiluminescence (ECL) reagents as recommended by the manufacturer (Amersham).

Generation of Tryptic Peptides of the 65-kDa Subunit and Amino Acid Sequencing—To obtain partial amino acid sequences of the 65-kDa subunit from the rabbit trimeric holoenzyme containing the 72-kDa subunit, the procedure was followed as described in Mayer et al. (1991a). The purified enzyme was resolved into its constituent subunits by SDS-PAGE and subsequently blotted onto PVDF membrane (Millipore). Amido Black-stained bands were excised from the membrane, and protein-binding sites were blocked by incubation in 30-kDa polyvinyl pyrolidone (Janssen Chimica). The protein was digested in situ with trypsin (2 µg in 100 µl) and the resulting peptides were purified by reverse-phase chromatography (Millipore) and a 13-column using a 3-h gradient from 0 to 42% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. Amino acid sequencing was performed using an Applied Biosystem model 470A or 473A gas-phase sequencer with on-line phenylthiohydantoin (PTH) analysis.

Generation of PCR Products of the α- and β-Isotypes of PP2A Subunits—The T7 promoter-driven bacterial expression vector pR1K72 (McLeod et al., 1987) was used to express the human PP2A α and β catalytic subunit cDNAs (Stone et al., 1988; Hemmings et al., 1988), the human PR55α and β subunit cDNAs (Mayer et al., 1991a), the human PR65α cDNA (HHPR65a-3; Hemmings et al., 1990), and the porcine PR65α cDNA (assembled from PP6R5-A1 and PP6R5-2; Hemmings et al., 1990). To pace the cDNAs at the correct distance from the Shine-Dalgarno sequence, an NdeI site (CATATG) was introduced at the initiator ATG of the cloned human cDNA. In case of the PR65α clone, which is lacking an initiator codon, an in-frame ATG was added at the 5'-end of the published sequence. The NdeI site was introduced into the cDNAs by polymerase chain reaction using mutant oligonucleotides (Ca, TGGGGG GGGTGCGATATGGAGGAAGG; Cb, CGCGCCCATATGAGGAAGG; Pr65α, TCCCTCATCTAGAAACG; Pr65ß, TCCCTCATCTAGAAGGA; Pr65'T, TTCTACATATGATTTCCGCGGCGG; Pr55α, CCAT ATGCAGGAGGCTTGG; PRR55ß, GCCGTCATAGGGAGGAG; A CAT TGT G) and oligonucleotides complementary to the vector sequences following the 3'-cloning site (reverse primer or T7 promoter primer).

Amplification products were cloned into a full-length 10-µl mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% (w/v) gelatin, 0.25 mM of each dNTP, 0.25-1 µM of each primer, 0.1-1 µg of cDNA template, and 1.25 units of Taq polymerase (Perkin-Elmer Cetus). After 10 min denaturation at 95 °C, amplification was followed by electrophoresis.
culation was performed in a Perkin-Elmer Cetus thermal cycler. The cycles were started with 1 min of denaturation at 94 °C, followed by 1 min of cooling to the annealing temperature, 1 min of annealing, and 2 min of extension at 72 °C. The annealing temperatures were 45 °C (2 cycles)/60 °C (30 cycles) for Ca, 35 °C (10 cycles)/45 °C (10 cycles) for Caβ, 64 °C (2 cycles)/65 °C (18 cycles) for PR65α, 50 °C (2 cycles)/66 °C (18 cycles) for PR65β, and 45 °C (25 cycles) for PR55α and PR55β. The reaction products were digested with NdeI and EcoRI (partial digests were used in the case of PR65β and PR55α due to internal EcoRI sites) and ligated into the corresponding cloning sites of pRK172. The correctness of the constructs was verified by DNA sequence analysis.

For bacterial expression, 1 liter of Luria-Bertani medium containing 200 µg/ml ampicillin was inoculated with an overnight culture of freshly transformed JM109/DE3 cells. Cultures were grown at 37 °C and induced at an absorbance of 0.5 at 600 nm with 0.5 mM isopropyl-β-D-thiogalactoside for 3 h (Ca/β and PR55α/β) and 5 h (PR65α/β). The bacteria were collected by centrifugation at 5,000 g for 20 min at 4 °C, the Ca, Cβ, PR65α, PR55α and PR55β proteins were found almost exclusively in the insoluble fraction (inclusion bodies) where they constituted 30–50% of the total protein. In contrast, the PR65α protein was exclusively soluble and constituted about 5% of the total soluble protein. It was purified to homogeneity using (NH₄)₂SO₄ precipitation, chromatography on DEAE-Sepharose and phenyl-Sepharose, and finally by FPLC on a Mono Q column (all Pharmacia). The detailed purification scheme will be published elsewhere.3

RESULTS

Preparation of Isotype-specific Antisera—Three peptides (α, β, and β') were used to generate specific antisera for the PR65 isoforms (Fig. 1). Antisera to the three peptides were affinity purified on the appropriate peptide-protein conjugates linked to Affi-Gel 15 (see “Materials and Methods”). The specificity of the α-, β-, and β'-antipeptide antisera was confirmed by ELISA using comparable amounts of the peptide conjugated to a carrier protein (data not shown), as well as by Western blot analysis using bacterially expressed recombinant PR65α and β proteins (see Fig. 2). This analysis showed that the antisera were specific for their target sequences at the appropriate dilutions.

Similarly, antisera for the catalytic subunit were raised using an α-specific peptide and one common to both isoforms (Fig. 1). The antisera were specific at high dilutions without further purification (see Fig. 6). Specific antisera for the PR55 subunit isoforms were raised against unique peptides derived from the amino-terminal regions of these proteins (Fig. 1, and see Fig. 5).

Three Holoenzyme Forms of Protein Phosphatases 2A from Rabbit Muscle Contain the PR65α Subunit—The dimeric and two trimeric forms of PP2A were purified from rabbit skeletal muscle in order to analyze the 65-kDa subunit in different holoenzymes. Equal amounts of the 65-kDa subunit from the different holoenzymes (based on densitometric scanning of Coomassie Blue-stained gels) were analyzed by Western blotting. Immunoreactions were carried out with the affinity purified PR65α, β-, and β'-specific antisera. As can be seen in Fig. 3, the 65-kDa subunits of the different oligomeric forms from rabbit skeletal muscle reacted to a similar extent with the antisera specific for the α-epitope. The reaction with the β- and β'-antisera was very low compared to that observed with the α-antisera. These data indicate that, in rabbit skeletal muscle, the 65-kDa subunit is largely of the PR65α isoform in the different oligomeric PP2A holoenzymes.

We also used an alternative approach to determine the

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Fig. 1. Location and sequences of the peptides from the 65- and 55-kDa regulatory and the catalytic subunits (PR65, PR55, and C) of protein phosphatase 2A used for antisera production. The numbers in brackets indicate the corresponding residues in the proteins. The corresponding regions from the related proteins that were not used in this study are indicated with (*). PP-X stands for protein phosphatase X (da Cruz e Silva et al., 1988; Cohen et al., 1990).

Fig. 2. Specificity of the affinity purified peptide-specific antisera to PR65α and PR65β determined by Western blot analysis using recombinant proteins. Purified bacterially expressed PR65α was loaded at the amounts indicated. Inclusion body preparations of bacterially expressed PR65β were loaded at equivalent amounts as determined by densitometric scanning. The upper panel shows the Coomassie Blue-stained gel of the 65-kDa proteins, and the lower panels are Western blots developed with affinity purified antisera either against the α peptide (PR65α Ab), the β peptide (PR65β Ab), or the β'-peptide (PR65β' Ab), all diluted 1:100. After incubation with an 125I-labeled anti-rabbit IgG antisera, the blots were exposed for 2 days (or for 3 days for the PR65β' Ab) at −70 °C with two intensifying screens. The center lane contains α and β recombinant protein in equal amounts (0.125 µg), and the total PR65 protein is shown above.
sequence and thus the isoform of the 65-kDa subunit in the different holoenzyme preparations. The 65-kDa subunit was isolated from the trimeric holoenzymes by SDS-PAGE, transferred to PVDF membrane, and digested in situ with trypsin. The resulting peptides were separated by reverse-phase high performance liquid chromatography and sequenced. In Fig. 4 the amino acid sequence data obtained from 14 different peptides of the 65-kDa subunit from three different holoenzyme preparations are compared to the corresponding sequences predicted from the human PR65a and β isoforms (Hemmings et al., 1990). All sequences correspond to the PR65a isoform. Only one difference was found, valine (β-specific) instead of a serine residue (α-specific) at position 122 in the 65-kDa subunit of the dimeric PP2A, however, the other 4 specific residues of this peptide correspond to the α-isoform. Previous sequence data of more than 300 amino acids obtained from the porcine kidney and muscle 65-kDa subunit from the dimeric PP2A corresponded also to the α-isoform (Hemmings et al., 1990).

These two independent methods indicate that the isoform of the 65-kDa subunit is the same in the different holoenzymes of PP2A. Therefore, it can be concluded that the isoform of the 65-kDa subunit does not apparently determine the association of the 55- or 72-kDa subunits in the trimeric forms of PP2A.

Analysis of the 55- and 72-kDa Subunit Isoforms in Trimeric PP2A Holoenzymes from Rabbit Skeletal Muscle—Sequence analysis of 140 residues of the 55-kDa subunit were previously found to correspond to the PR55α isoform (Mayer et al., 1991a). In order to confirm these observations, we prepared PR55α- and β-specific antisera by immunizing with NH2-terminal peptides (see “Material and Methods”). The specificity of the antisera was confirmed using recombinant protein expressed in bacteria (see “Material and Methods”). These data show that each antisera were specific for the appropriate isoform (Fig. 5). Immunoblot analysis of the trimeric holoenzyme with the isoform-specific antisera confirmed that only the PR55α isoform was present in this enzyme preparation (Fig. 5).

Sequence analysis of the 72-kDa subunit indicates that it
is not related to the 55-kDa subunit. The holoenzyme from rabbit skeletal muscle appears to contain only the lower molecular mass form of the 72-kDa subunit, since only peptides common to the two isoforms, or specific for this form were sequenced, and none were found for the unique region of the high molecular mass 130-kDa form (to be described in detail elsewhere).

**Rabbit Skeletal Muscle Protein Phosphatases 2A Holoenzymes Contain the α-Isform of the Catalytic Subunit**—Molecular cloning revealed two isoforms of the PP2A catalytic subunit in mammalian cells that differ only in eight amino acids, with seven of the differences located in the amino terminus of the protein. A related protein phosphatase catalytic subunit, termed protein phosphatase X (PP-X), cloned from a rabbit liver cDNA library shows 69% sequence identity to the catalytic subunit of PP2A (da Cruz e Silva et al., 1988; Cohen et al., 1990). It has been suggested that PP-X might be the catalytic subunit of the trimeric PP2A holoenzyme containing the 72-kDa subunit. We investigated this hypothesis using a peptide-specific antisera against the carboxy terminus of the PP2A catalytic subunit (PHVTTRTPDYFL). Preincubation of the crude antisera (1:2000) with 20 μM of the 12-amino-acid carboxyl-terminal peptide abolished completely the immunoreaction while preincubation with the same concentration of a peptide consisting of the four last amino acids (DYFL) had no detectable effect (Fig. 6A). This indicates that there are almost no antibodies unique for the last four amino acids of the peptide, which are in common between the two proteins (see Fig. 1), and that the antisera preferentially react with PP2A catalytic subunit (low reactivity toward PPX cannot be excluded but would appear to be unlikely). Furthermore, analysis with equal amounts of the catalytic subunit of the different holoenzymes by SDS-PAGE followed by Western blotting using the carboxyl-terminal-specific antisera showed that all three holoenzymes reacted with this antisera, as did the recombinant Ca and Cβ proteins (Fig. 6B).

To determine the isoform of the catalytic subunit in the different PP2A holoenzyme preparations, an identical blot as used above was incubated with antisera raised against a peptide of 10 amino acids unique for the Ca-isoform (amino acids 22–31, QLSESQVKSL). This sequence is located in the amino-terminal part of the catalytic subunit where the α- and the β-isoforms show four differences (see Fig. 1). The antisera were specific for the α-isoform as shown by Western blot analysis with recombinant proteins (Fig. 6B). Immunization with a peptide corresponding to the β-isoform of the catalytic subunit (amino acids 22–31, QLNEQVRRTL) produced low affinity antisera that cross-reacted with both α- and β-isoforms. (Further work, such as affinity purification, will be required to generate β specific antisera.) Analysis of the three purified holoenzymes using α-specific antisera revealed that all three preparations reacted to a similar extent indicating that they all contained predominantly the α-isoform. (The sequence of PP-X in this region differs from the Ca peptide, five differences in 10 amino acids.)

From our previous protein sequence results of rabbit skeletal muscle PP2A (Stone et al., 1987), we only obtained evidence for the α-isoform in the catalytic subunit purified by ethanol fractionation, which is likely to contain all different forms of PP2A. These results confirm that all holoenzymes from rabbit skeletal muscle contain the α-isoform of the PP2A catalytic subunit. Similar results have been obtained using a monoclonal antiserum raised against the catalytic subunit of the dimeric form of PP2A (data not shown).

**Purification of Protein Phosphatase 2A Holoenzymes from Xenopus Oocytes and Skeletal Muscle**—At least two different forms of PP2A are present in the cytosolic fraction of *Xenopus* oocytes (Cayla et al., 1990). In previous studies the dimeric PP2A was purified from oocytes (Hermann et al., 1988) using an assay procedure that selectively identified the dimeric phosphatase. However, trimeric PP2A holoenzyme containing the 55-kDa subunit can also be partially purified using this purification procedure if the polycation-stimulated dephosphorylation of phosphorylase is used to identify the phosphatase activity. The trimeric holoenzyme and the dimeric PP2A holoenzyme were partially purified from *Xenopus* skeletal muscle and oocytes and used for immunochemical analyses. Panel A of Fig. 7 shows a Coomassie Blue-stained gel of the PP2A holoenzyme preparations from *Xenopus* after Mono Q FPLC. The 100 and 40 kDa bands are contaminants and do not comigrate with phosphatase activity (see below).

**Immunochemical Analysis of Protein Phosphatase 2A Holoenzymes from Xenopus Muscle and Oocytes**—Purified preparations of PP2A were analyzed using the α-, β- and β'-PR65-specific antisera. These analyses indicated that protein phosphatase preparations isolated from *Xenopus* muscle reacted equally well with all three antisera preparations (Figs. 3 and 7). This suggests that in *Xenopus* skeletal muscle both the α- and β-PR65 isoforms are present in the trimeric as well as in the dimeric protein phosphatase. Alternatively, it could be argued that both holoenzymes isolated from *Xenopus* skeletal muscle contain a unique 65-kDa subunit recognized by all the specific antisera. (We also detected immunoreaction with a 100–110-kDa protein with the PR65α-specific antisera, but...
FIG. 7. Western blot analysis of protein phosphatase 2A holoenzymes purified from Xenopus oocytes (OC) and Xenopus skeletal muscle (MU). Each lane corresponds to 200 ng of the 65-kDa subunit loaded; T55 indicates the trimeric holoenzymes containing the 55-kDa subunit, and D the dimeric holoenzymes. Panel A shows a Coomassie Blue-stained gel. Panels B–E show blots incubated with PR65α (1:50), PR65β (1:100), PR65β′ (1:250), and a catalytic subunit monoclonal antiserum. Antiserum incubations and staining of immunoreactive proteins were performed as described under "Materials and Methods."

Recent results suggest that this is unrelated to PP2A.4) With PP2A preparations from Xenopus oocytes, the 65-kDa subunit appeared as a doublet (compared to a single band in muscle) in the trimeric form containing the 55-kDa subunit, as well as in the dimeric holoenzyme. Both 65-kDa proteins of the doublet were strongly immunoreactive with the PR65β as well as with the β′-antisera (Figs. 3 and 7). Surprisingly, with the PR65α-specific antisera only the upper band showed a very weak reaction while the lower band showed no immunoreactivity. Therefore, it is unlikely that this reaction with the PR65α-specific antisera was due to a nonspecific binding. It suggests that two different gene products were present in the purified enzyme preparation.

In summary, there appear to be three different forms of the 65-kDa subunit in Xenopus: 1) a muscle 66-kDa form equally reactive with the α- and β-specific antisera; 2) a 66-kDa form from oocytes reacting mainly with the β-specific antisera; and 3) a 64-kDa form from oocytes which only reacts with the β-specific antisera. Interestingly, through comparison of the data from Xenopus muscle and oocytes, it is clear that the PR65 isoforms are differentially expressed during embryogenesis or organogenesis.

Using a monoclonal antibody directed toward the rabbit PP2A catalytic subunit, we quantified the catalytic subunit in the different holoenzyme preparations. As stated above the amount of PP2A loaded was normalized using the 65-kDa subunit. Analysis of the catalytic subunit in each enzyme preparation was constant as shown in Fig. 7E. This result indicates that the ratio of catalytic to 65-kDa PR65 subunit in each preparation was apparently constant. In addition these data reveal that the proteins of 38–40 kDa in the muscle preparations are contaminants because they do not react with the monoclonal antibody.

In another series of experiments, we analyzed the trimeric forms of Xenopus PP2A purified from muscle and oocytes with the α- and β-specific PR55 antisera (Fig. 8). This revealed that the α-isofrom was present in the holoenzyme from

4 Immunostaining of the 100–110-kDa protein in Fig. 7 was only observed with the PR65α-specific antisera. Experiments using a second PR65α antibody, prepared by immunization with recombinant PR65α did not cross-react with this protein. This suggests that the 100–110-kDa protein shares an epitope with the immunizing peptide or was due to nonspecific binding.

FIG. 8. Western analysis of 55 kDa subunit in trimeric holoenzymes isolated from Xenopus muscle and oocytes. Purified trimeric PP2A holoenzymes containing the 55 kDa as described in Fig. 7 were separated by SDS-polyacrylamide electrophoresis. Each lane contains approximately 0.2 μg of 55 kDa subunit. The blots were treated with anti-α PR55 or anti-β PR55 and developed as described in Fig. 5.

Both sources. No immunoreactivity was observed with the β-specific antisera. This could reflect either (a) sequence divergence between the mammalian and frog proteins or (b) Xenopus does not produce a second PR55 isoform in muscle and oocytes.

Discussion

The different PP2A holoenzymes show specific catalytic properties, such as substrate specificity or response to effectors (Imaoka et al., 1983; Agostinis et al., 1987b, 1990; Mumbey et al., 1987; Waelkens et al., 1987b; Usui et al., 1988; Chen et al., 1989; Cayla et al., 1990, Kamibayashi et al., 1991; Yang et al., 1991; Scheidtmann et al., 1991). These differences could be caused by different subunit compositions, differences in primary structure of the subunits, or covalent modifications. Thus, it was of interest to know whether specific isoforms of the 65-kDa or the catalytic subunits are found in a particular holoenzyme. We have examined the structure of the dimeric and trimeric holoenzyme forms of PP2A using peptide-specific antisera for the 65-kDa and catalytic subunits. These two subunits are found in all holoenzymes isolated so far and therefore constitute the core subunits. In addition, the 55- and 72-kDa subunits found in the trimeric holoenzymes were examined using isoform-specific antisera for PR55α and PR55β and by direct sequence analysis of the 72-kDa (PR72) subunit.

Using isoform-specific antisera, we found that three holoenzymes isolated from rabbit skeletal muscle contained mainly the α-isofrom of the 65-kDa subunit. We also generated tryptic peptides of the 65-kDa subunits of the different oligomeric forms of PP2A. Amino acid sequencing of these peptides revealed only sequences that correspond to the predicted amino acid sequence derived from the PR65α cDNA (Hemmings et al., 1990; Walter et al., 1990; Mayer et al., 1991a; this study). Therefore, these two independent approaches revealed that the PR65α isoform is the major isofrom in the dimeric and the trimeric holoenzymes of PP2A, but the antisera data revealed a low level of the PR65β isoform in all three holoenzyme preparations. Previous peptide mapping of the 65-kDa protein present in different oligomeric forms of PP2A also showed similar patterns (Tung et al., 1985; Usui et al., 1988).

These immunochemical data are in agreement with previous observations. Northern blot analysis of a number of mammalian cell lines indicated high levels of the PR65α compared to the β mRNA (Hemmings et al., 1990). Analysis of the PR65 transcripts from porcine skeletal muscle also revealed a high α to β ratio (28:1) (Mayer et al., 1991b). Thus, the protein level appears to reflect the transcript levels in this tissue, and it is not surprising that only PR65α peptide sequences were obtained.

Analysis of the catalytic subunit present in the different holoenzymes of PP2A using specific antisera revealed the...
presence of the α-isoform in all the purified holoenzymes from rabbit skeletal muscle. The data make it unlikely that one of the holoenzymes contains a distinct, but closely related, catalytic subunit, such as, PP-X (da Cruz e Silva et al., 1988). This is in agreement with the fact that previously no enzymological differences could be detected with the catalytic subunits isolated from the different oligomeric forms of PP2A (Waelkens et al., 1987c). In addition, peptide mapping of the catalytic subunit of dimeric and trimeric PP2A holoenzymes revealed no differences (Tung et al., 1985; Usui et al., 1988). The detection of mainly the α-isoform is also consistent with the peptide sequence data obtained for the catalytic subunit from rabbit skeletal muscle and bovine heart (da Cruz e Silva et al., 1987; Green et al., 1987; Stone et al., 1987). In all cases the enzyme was purified using an ethanol precipitation step which recovers the catalytic subunit from all PP2A holoenzymes.

The protein data presented in this paper reflect the higher abundance of the Ca mRNA compared with the Cβ mRNA (Khw-Goodall and Hemmings, 1988). Recent work indicates that this difference can be explained by the promoter strength in the catalytic subunit genes, since the Ca promoter is about eight times stronger than the Cβ promoter (Khw-Goodall et al., 1991).

Since the core subunits of PP2A (catalytic and 65 kDa) are predominantly α-isoforms in the dimeric and both trimeric PP2A holoenzymes, it is apparent that the isoform of these proteins cannot determine the association of the third subunit. Association of the 55- or 72-kDa subunits seems to be mutually exclusive since no holoenzyme has been isolated with both subunits bound in a heterotetrameric holoenzyme. The mechanism(s) governing the relative amounts of the 55- or 72-kDa subunit bound to the core dimer, and the way they interact with the core proteins to form a trimeric PP2A holoenzyme are currently unknown. Further analysis is required to explain how they manifest their dramatic effect on the substrate specificity and regulation of PP2A.

From our analysis of skeletal muscle PP2A holoenzymes, the PR65/5 isoform appears to be present at extremely low levels. Further analysis using PP2A holoenzyme preparations from different tissues should help to delineate the abundance and function of the β'-isoforms of the catalytic and 65-kDa subunits. Northern analysis revealed that in other tissues such as the liver and brain the transcript levels of the PR65α isoform are particularly high (Mayer-Jaekel et al., 1993). In the future it will be important to investigate the structure and properties of PP2A holoenzymes isolated from other tissues where the β- (or γ-) isoforms are more abundant. In vitro reconstitution experiments with cloned proteins offers a direct approach to assessing the properties of the different isoforms of catalytic and regulatory subunits. Indirect evidence, however, favors the idea that the isoforms of the catalytic subunit do not perform specific functions in vivo. In fission yeast (Kinosita et al., 1990), as well as in budding yeast (Sneddon et al., 1990), the two genes encoding the catalytic subunit of PP2A are apparently functionally overlapping since only double disruptants are lethal. Furthermore, recent analysis of PP2A from Drosophila shows that both the catalytic, 55-, and 65-kDa subunits are each encoded by single genes (Mayer-Jaekel et al., 1992, 1993). In the case of the PR55 gene from Drosophila, two isoforms are produced by alternate splicing of NH2-terminal encoding exons. Further studies are required to elucidate how they determine the subunit levels in different tissues and modulate subunit composition of the different holoenzymes during development. The completion of the molecular characterization of the remaining subunits will be an important step for the unravelling of these mechanisms. This approach will eventually lead to an understanding of how PP2A can perform with precision the myriad of functions in vivo predicted from in vitro studies.

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