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Separation of PP2A Core Enzyme and Holoenzyme with Monoclonal Antibodies against the Regulatory A Subunit: Abundant Expression of Both Forms in Cells

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Protein phosphatase 2A (PP2A) holoenzyme is composed of a catalytic subunit, C, and two regulatory subunits, A and B. The A subunit is rod shaped and consists of 15 nonidentical repeats. According to our previous model, the B subunit binds to repeats 1 through 10 and the C subunit binds to repeats 11 through 15 of the A subunit. Another form of PP2A, core enzyme, is composed only of subunits A and C. It is generally believed that core enzyme does not exist in cells but is an artifact of enzyme purification. To study the structure and relative abundance of different forms of PP2A, we generated monoclonal antibodies against the native A subunit. Two antibodies, 5H4 and 1A12, recognized epitopes in repeat 1 near the N terminus and immunoprecipitated free A subunit and core enzyme but not holoenzyme. Another antibody, 6G3, recognized an epitope in repeat 15 at the C terminus and precipitated only the free A subunit. Monoclonal antibodies against a peptide corresponding to the N-terminal 11 amino acids of the Aα subunit (designated 6F9) precipitated free A subunit, core enzyme, and holoenzyme. 6F9, but not 5H4, recognized holoenzymes containing either B, B′, or B″ subunits. These results demonstrate that B subunits from three unrelated gene families all bind to repeat 1 of the A subunit, and the results confirm and extend our model of the holoenzyme. By sequential immunoprecipitations with 5H4 or 1A12 followed by 6F9, core enzyme and holoenzyme in cytoplasmic extracts from 10T1/2 cells were completely separated and they exhibited the expected specificities towards phosphorylase *a* and retinoblastoma peptide as substrates. Quantitative analysis showed that under conditions which minimized proteolysis and dissociation of holoenzyme, core enzyme represented at least one-third of the total PP2A. We conclude that core enzyme is an abundant form in cells rather than an artifact of isolation. The biological implications of this finding are discussed.

Protein phosphatase 2A (PP2A), the most abundant serine/threonine-specific phosphatase in mammals, plays a role in many fundamental cellular processes, including cell division (7, 30), signal transduction (36), gene expression (52), and *Drosophila* development (34). The PP2A holoenzyme consists of a 36-kDa catalytic C subunit and a 65-kDa regulatory A subunit, which together form the core enzyme to which one of several B subunits is bound (8, 38). The A and C subunits both exist as two isoforms (α and β) (19, 63), whereas the B subunits fall into three families called B, B′, and B″, which are unrelated to each other by protein sequence. The B family has three members, Bα, Bβ, and Bγ, each with a molecular mass of around 55 kDa (18, 33, 41); the B′ family consists of numerous isoforms and splice variants, whose molecular masses range from 54 to 68 kDa (10, 35, 57, 69); and the B″ family has two members, which have molecular masses of 72 and 130 kDa and are splice variants of the same gene (20). The combination of these subunits can give rise to a large number of PP2A variants, which differ in substrate specificity, subcellular localization, or tissue specificity and could form the molecular basis for the regulation of PP2A in many cellular events.

A fourth class of proteins able to associate with core enzyme are the tumor (T) antigens. The small T antigens of simian virus 40 (SV40), polyomavirus, and BK virus and middle T antigen, the transforming protein of polyomavirus, bind to the N terminus of the A subunit by replacing the B subunit (16, 17, 25, 46–49, 62, 64, 67). SV40 small T antigen inhibits the activity of the core enzyme (50, 66) and promotes cell proliferation by activating signal transduction through the mitogen-activated protein (MAP) kinase pathway (13, 55). It has been proposed that PP2A normally inhibits this pathway by dephosphorylating and inactivating MAP kinase and MAP kinase kinase. By binding to PP2A, SV40 small T antigen prevents this inhibition (55). Polyomavirus middle T antigen, in addition to binding PP2A, also associates with other proteins involved in growth control. They include $pp60^{c\text{-}src}$ (9) and the closely related c-yes (27) and fyn proteins (6, 21, 28), phosphatidylinositol 3-kinase (65), Shc (3, 11), Grb2 (3, 11), and 14-3-3 protein (40). The binding of middle T antigen to PP2A is a prerequisite for its association with $pp60^{c\text{-}src}$, phosphatidylinositol 3-kinase, and Shc protein as well as for its ability to transform cells (2, 15).

The structural basis for the interaction between the A, B, and C subunits in holoenzyme and core enzyme and between the core enzyme and T antigens has been studied by site-directed mutagenesis of the A subunit (43, 44) and of T antigens (2, 14, 15, 39, 42, 55) and by in vitro assembly of purified components (22, 24). The A subunit polypeptide consists of 15 nonidentical repeats of 38 to 43 amino acids, which are arranged in a linear fashion and form a rod-shaped molecule (5, 19, 63). Our studies indicated that the Bα subunit binds to repeats 1 through 10 and the C subunit binds to repeats 11 through 15 of the A subunit, whereas SV40 small T antigen binds to repeats 3 through 6 and polyomavirus small T antigen and polyomavirus middle T antigen bind to repeats 2 through 8 (43, 44) (see Fig. 4). Using the yeast two-hybrid system, McCright and Virshup demonstrated recently that, like Bα, B′

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and B″ bind to the N-terminal domain (35). Given that they share a binding site on the A subunit, it is remarkable that there is no sequence similarity between T antigens and B subunits as there is none between the three B subunit families, except for a short stretch of four amino acids common to T antigens and Bα (53).

An unresolved question is whether in vivo PP2A occurs only in the trimeric form or whether the core enzyme is also present and participates in regulating cellular events. The common view is that core enzyme is an artifact of enzyme purification that can be avoided (58, 60, 68). This question is important because holoenzyme and core enzyme differ markedly in their substrate specificity (1, 4, 12, 22, 54). The holoenzyme, particularly when bound to Bα (69), is more active than the core enzyme towards substrates phosphorylated by cyclin-dependent kinases (1, 12, 54), whereas the core enzyme is equally or more active towards most other substrates. In the present study we isolated and characterized monoclonal antibodies (MAbs) against the A subunit. They were used to quantitatively separate active core enzyme from active holoenzyme. Our data indicate that core enzyme is an abundant form in cells. These findings have important implications for how we view the regulation of PP2A. Furthermore, we demonstrated that members of all three families of B subunits bind to the N terminus of the A subunit, whereas the C subunit binds to the C terminus, confirming and extending a previously proposed model of PP2A.

MATERIALS AND METHODS

MAbs against the A subunit. Lou/C rats were immunized three times in intervals of 3 weeks with 70 μg of purified Aα subunit or with an 11-amino-acid peptide (MAAADGDDSLY), corresponding to the N terminus of the Aα subunit, coupled to bovine serum albumin (BSA) through the tyrosine residue with bis-diazobenzidine as previously described (61). Aα subunit was expressed in insect cells following infection with a recombinant baculovirus and purified by chromatography on aminohexyl-Sepharose and Mono Q as described previously (24). The first injection was done in complete Freund adjuvant, the second was done in incomplete Freund adjuvant, and the third was done without adjuvant. Fusion of the myeloma cell line P3X63 Ag 8.653 with rat immune spleen cells was performed according to the general procedure described by Köhler and Milstein (26). Hybridoma supernatants were tested in a solid-phase immunoassay with purified Aα subunit (3 μg/ml) bound to polystyrene microtiter plates. Following incubation with culture supernatants for 1 h, bound MAbs were detected by using peroxidase-labeled goat anti-rat immunoglobulin G (IgG) antibodies and *o*-phenylenediamine as the chromogen in the peroxidase reaction. Cells yielding supernatants that reacted positively in the enzyme-linked immunosorbent assay (ELISA) were cloned at least twice by limiting dilution. Solid-phase ELISA on microtiter plates coated with mouse anti-rat Ig antibodies was used to determine the Ig type. IgG MAbs were purified from culture supernatant by affinity chromatography on a column of protein G Sepharose 4 Fast Flow (Pharmacia). 5H4 and 1A12 antibodies belong to the IgM class, and 6G3 and 6F9 belong to the IgG2a class. Polyclonal antibodies against the N-terminal 11-amino-acid peptide were prepared in rabbits immunized with the coupled peptide as described previously (61).

In vitro protein synthesis. Plasmids encoding wild-type and mutant Aα subunits, the various B subunits, and the SV40 small T antigen were transcribed with T7 RNA polymerase and translated in the presence of L-[^{35}S]methionine (37 TBq/mmol) (Amersham) with 10 μl of reticulocyte lysate (Promega) and 50 to 100 ng of DNA, as described previously (43). The Bα, Bβ, and B′α1 transcription plasmids were provided by Craig Kamibayashi and Marc Mumby, the B″ plasmid was provided by Brian Hemmings, and the SV40 small T expression plasmid, pGEM 4t, was provided by Kathy Rundell.

Cell lines and labeling of cells. The 10T1/2 and HeLa cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were labeled for 3 h with L-[^{35}S]methionine (37 TBq/mmol) at 250 μCi/ml in methionine-free medium supplemented with 10% dialyzed fetal calf serum. Cells were lysed at 4°C in Triton X-100 lysis buffer (1 ml/10-cm-diameter plate) containing 1% Triton X-100, 3 mM $MgCl_2$, 150 mM NaCl, 50 mM Tris-HCl (pH 8), 1 mM dithiothreitol, 50 μM leupeptin (Sigma), 1 μg of aprotinin (Boehringer) per ml, 0.2 mg of soybean trypsin inhibitor (Calbiochem) per ml, and 1 mM Pefabloc (Boehringer). Lysis was carried out for either 90 s or 10 min as described in the figure legends.

Immunoprecipitation, immunoblots, and ELISA. Immunoprecipitation of in vitro-synthesized A subunit was carried out by adding either 6 μg of purified 6G3 IgG, 1.2 μg of 5H4 IgM from hybridoma supernatant, or 3 μg of 1A12 IgM from hybridoma supernatant to the in vitro-synthesized protein in 10 μl of reticulocyte lysate. This was followed by the addition of 5 μl of protein G Sepharose (GBP) (Pharmacia) containing 10.8 μg of bound antibodies to IgG and/or IgM and by incubation for 2 h on ice. Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (44). Immunoprecipitation of [^{35}S]methionine-labeled proteins was performed with GBP to which 6F9, 1A12, 5H4, 6G3, or KT3 antibodies had been covalently coupled with dimethylpimelimidate as previously described (51). One milliliter of GBP contained approximately 15 mg of 6F9, 5 mg of 6G3, or 15 mg of KT3. For coupling 1A12 and 5H4 antibodies, both of which are IgM, GBP (1 ml) was first incubated for 3 h at 4°C with goat anti-rat IgG/IgM (10 mg) and then with concentrated 1A12 or 5H4 hybridoma supernatants containing approximately 5 mg of IgM. Immunoblotting was carried out with the enhanced chemiluminescence system (Amersham) as previously described (44, 45). Polyomavirus middle T antigen immunoprecipitations were performed with extract from 293 cells (90 μg of protein) infected with adenovirus type 5, containing the cDNA for polyomavirus middle T antigen under control of the adenovirus late promoter, as previously described (44).

The antibodies used for immunoblotting PP2A subunits were MAb 6G3, an antipeptide serum against residues 18 through 29 of the 55-kDa Bα subunit, and a MAb against the catalytic subunit (37). Polyomavirus middle T antigen was detected with the Glu-Glu MAb (16).

To evaluate the relative strengths of the different rat MAbs, polystyrene microtiter plates (Maxisorb; Nunc) were coated with $\log_2$ dilutions of native Aα subunit in 50 mM carbonate-bicarbonate buffer, pH 9.5, starting with 220 μg of native Aα subunit per ml. Wells were blocked with 1% nonfat milk in phosphate-buffered saline, and equal amounts of rat MAb were added in excess. Bound rat MAbs were detected with peroxidase-labeled goat anti-rat antibodies. Endpoints were defined as three times the background levels. 6G3 had the highest affinity, being positive at a 1:2,048 dilution of the antigen, followed by 6F9 (1:512) and 1A12 and 5H4 (1:128).

Phosphatase assays. Phosphorylase α and retinoblastoma (Rb) peptide, ING SPRTPRRGQNR (1), corresponding to amino acids 246 through 259 of the Rb protein, were used to assay the 6F9 and 5H4 immunoprecipitates. Phosphorylase *a* was labeled with [^{33}P]ATP (1,000 cpm/pmol) by using phosphorylase kinase as previously described (32), and Rb peptide was phosphorylated by cdk1-cyclin B under the conditions previously described (1) with [^{33}P]ATP (1,000 cpm/pmol). The cdk1-cyclin B used in the reaction was expressed in Sf9 cells infected for 48 h with recombinant baculoviruses expressing human cyclin B and cdk1 at a multiplicity of infection of 5 (31). The kinase-cyclin B complex was partially purified by immunoprecipitation with antibodies raised in rabbits against the peptide YFNDLDNQIKKM, which corresponds to the C-terminal sequence of cdk1 (29). The peptide was phosphorylated with a stoichiometry of 0.37 mol/mol and was assayed at a final concentration of 5 μM. A total of 3.08×10^5 cpm of Rb peptide and 7.7×10^5 cpm of phosphorylase *a* were used per assay. The immunoprecipitates were prepared from 25 μl of 10T1/2 extract containing 25 μg of protein. For each precipitation, 75 μg of 6F9 coupled to 5 μl of GBP or 10 μg of 5H4 coupled to 10 μl of GBP was used. The precipitates were resuspended in 1 volume containing 0.1% BSA, 50 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, and 0.1% 2-mercaptoethanol and kept on ice until assayed for 30 min at 30°C as described previously (32). The samples were agitated every 2 min to keep the beads in suspension. The extraction of released phosphate was carried out as previously described (32).

RESULTS

Mapping of epitopes recognized by MAbs against the A subunit. The approximate location of the epitopes recognized by the anti-A subunit MAbs was determined by immunoprecipitation of in vitro-synthesized mutant A subunits. The construction of the mutants, which contained a series of N- and C-terminal deletions and internal deletions, has been described previously (43, 44), and they are shown schematically in Fig. 1. MAb 6G3 immunoprecipitated the wild-type A subunit but not the C-terminal-deletion mutants 553 through 237, as shown in Fig. 2A. Polyclonal antibodies against a peptide corresponding to amino acids 1 through 11 of the A subunit (anti-1–11) precipitated all subunits with C-terminal deletions (positive control). Conversely, 6G3 precipitated the subunits with N-terminal deletions 123 and 162 that were not recognized by the anti-1–11 antibodies (Fig. 2B). These results indicate that the epitope for 6G3 is located in repeat 15, which has been deleted in mutant 553 and all other C-terminal-deletion mutants (Fig. 2A). Since 6G3 recognized mutant 583, which is missing six amino acids from the C terminus, but not mutant 570, which is missing 19 amino acids, the 6G3 epitope

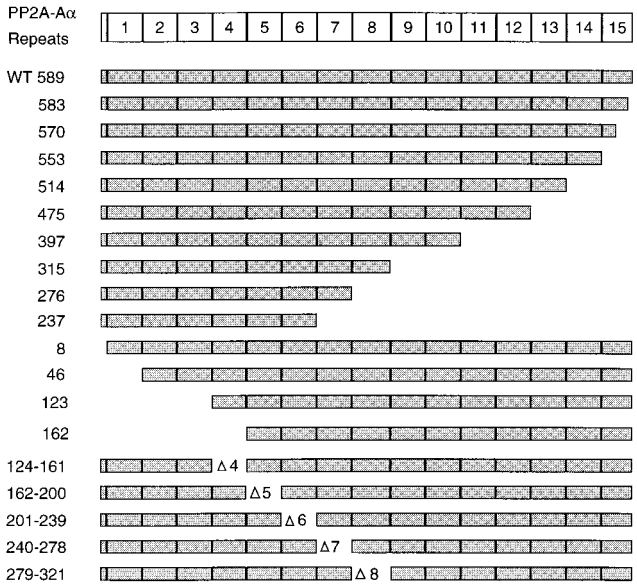

FIG. 1. Scheme of A subunit mutants used for localization of epitopes. The top bar symbolizes the linear arrangement of 15 repeats. The shaded bars represent the repeats present in wild-type protein (WT), N- and C-terminal-deletion mutants, or single-repeat-deletion mutants. The numbers on the left indicate the last amino acid for C-terminal deletions, the first amino acid after the initiating methionine for N-terminal deletions, and the stretch of deleted amino acids for single-repeat deletions.

includes some or all of the amino acids between 571 and 583 (Fig. 2C). The weak signal obtained with mutant 570 and anti-1–11 antibodies is due to a low level of in vitro synthesis of the mutant 570 protein in this particular experiment (Fig. 2C). If 6G3 had recognized the mutant 570 protein, the expected signal would have been stronger than that obtained with anti-1–11, as was the case with the mutant 583 and wild-type proteins (Fig. 2C).

A set of polypeptides smaller than the wild-type A subunit was observed in immunoprecipitates of wild-type A subunit (Fig. 2A and B). One or more of these bands were also seen in immunoprecipitates of the mutant proteins 397, 315, 276, and 237. They were only weakly detectable in total in vitro-synthesized proteins (Fig. 2A, right) because only 1/20 of the amount used for immunoprecipitation was analyzed on the gel (Fig. 2A, left). These polypeptides most likely originated by initiation at internal methionines located downstream of the termination codon inserted to generate each mutant (43, 44). This idea is supported by the fact that the number of extra proteins corresponded to the number of downstream methionines for each mutant. Furthermore, the apparent molecular weights of these proteins were approximately as expected from the positions of methionines. Since all internally initiated polypeptides shared a C terminus, they were precipitated by 6G3 but not by anti-1–11 antibodies. When this 6G3 epitope was removed, the extra proteins were not observed (data not shown).

Another MAb, 5H4, immunoprecipitated the C-terminal-deletion mutants 276 through 475, as well as mutant 8, which has a deletion of the N-terminal eight amino acids (Fig. 3A). On the other hand, 5H4 did not recognize mutant proteins 46, 123, and 162 with deletions of one, two, and four N-terminal repeats, respectively. 6G3 was used as a positive control for the N-terminal deletions, and anti-1–11 antibodies were used for the C-terminal deletions. 5H4 also recognized mutant proteins with internal deletions of repeats 4 through 8 (mutants Δ4 through Δ8) (Fig. 3B). These results demonstrate that the 5H4 epitope is located in the first repeat.

Results similar to those obtained with 5H4 were obtained with MAb 1A12, which precipitated mutant 8 but not the mutants with N-terminal deletions of repeats 1 (mutant 46), 1 through 3 (mutant 123), or 1 through 4 (mutant 162). The subunits with C-terminal deletions (mutants 276 and 315) and the internal-deletion mutants Δ4 through Δ8 were precipitated by 1A12 (data not shown). A summary of the data on epitope localization and a model of the PP2A trimer are presented in Fig. 4.

It might be argued that the failure to be recognized by 6G3, 5H4, or 1A12 when a particular repeat has been deleted does not necessarily imply deletion of the epitope but rather a conformational change induced in some other part of the A subunit which contains the conformation-sensitive epitope. However, the finding that all these antibodies recognized the SDS-denatured A subunit (data not shown) suggests that the epitopes are conformation insensitive and located on those repeats that were deleted.

Differential immunoprecipitation of dimeric and trimeric PP2A. As mentioned above, previous mutagenesis studies indicated that the Bα subunit binds to repeats 1 through 10 of the A subunit whereas the C subunit binds to repeats 11 through 15 (43, 44) (Fig. 4). Thus, it was of interest to find out whether the B subunit prevents 5H4, 1A12, or 6F9 from recognizing the A subunit in holoenzyme and whether the C subunit interferes with recognition of the A subunit by 6G3 in core enzyme and holoenzyme. To test these possibilities, [^{35}S]methionine-labeled extract of 10T1/2 cells, containing soluble and membrane-bound proteins and the bulk of cellular PP2A, was prepared by lysis in Triton X-100 buffer. Immunoprecipitations were carried out with 6F9, 1A12, 5H4, and 6G3.

6F9 precipitated the A, B, and C subunits (Fig. 5A, lane 2) and precipitation was inhibited by the addition of peptide (lane 3). Presumably, the C subunit band represents Cα, which is the predominant form in many cell types. This was confirmed by immunoblotting with antibodies against Cα (data not shown). Precipitation of the 55-kDa B subunit was somewhat obscured by background bands that were also seen with control antibody KT3 (Fig. 5A, lane 7), although the lower of the two bands was reduced relative to the upper one by peptide competition (compare lanes 2 and 3) and is likely to represent Bα. By immunoblotting with antipeptide antibodies the Bα subunit was clearly detectable (Fig. 5B, lane 2) and disappeared with the addition of peptide (Fig. 5B, lane 3). Thus, 6F9 can precipitate the holoenzyme and, as shown below, the core enzyme.

Next, we showed that 1A12 and 5H4 precipitated the A and C subunits (Fig. 5A, lanes 4 and 5) but not the 55-kDa Bα subunit (Fig. 5B, lanes 4 and 5). Occasionally, a small amount of the B subunit was seen when 1A12 immunoprecipitates were analyzed by Western blotting with anti-B antibodies. Since this was never observed with 5H4, it indicates that 1A12 and 5H4 recognize different epitopes. Another reason for assuming that 1A12 and 5H4 epitopes are different is the fact that 1A12 strongly cross-reacts with a cytoskeletal protein by immunofluorescence whereas 5H4 does not (data not shown). 6G3 antibodies precipitated only the A subunit and neither B nor C (Fig. 5, lanes 6).

Nonspecific proteins, as defined by immunoprecipitation with a control antibody, KT3, were detected in all 6G3, 5H4, 1A12, and 6F9 precipitates. Some bands were specific for individual MAbs. These have not been further analyzed except for one protein of approximately 42 kDa that was precipitated with 6F9 (Fig. 5A, lane 2) and strongly inhibited by peptide

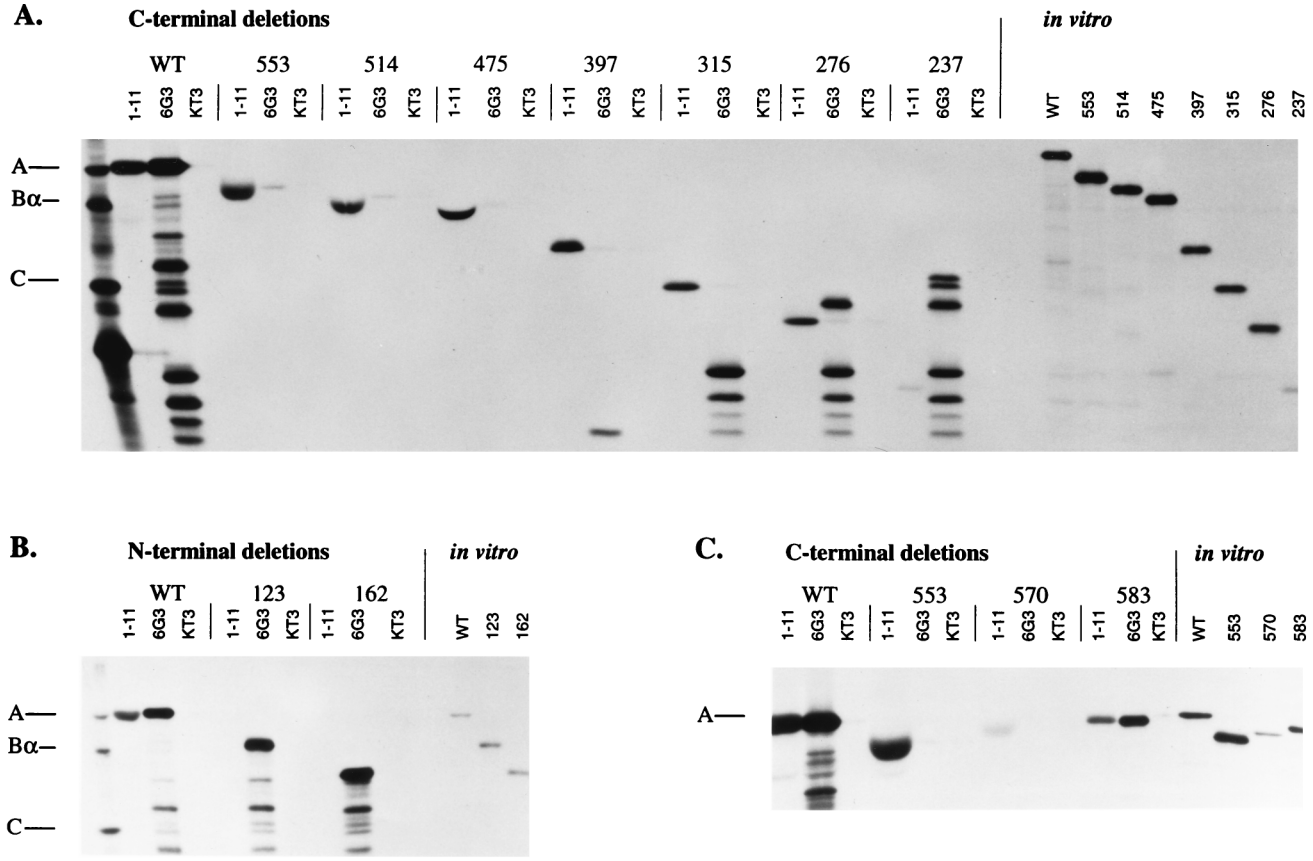

FIG. 2. Localization of the epitope recognized by 6G3. Wild-type (WT) and mutant A subunits, named at the top of each set of lanes by a number corresponding with an amino acid as described in the legend to Fig. 1, were synthesized in vitro in the presence of [^{35}S]methionine. The in vitro-synthesized proteins used for immunoprecipitation are shown on the right in each panel. Immunoprecipitations were carried out with 6G3, anti-1–11 antibodies (positive or negative control), or unrelated MAb KT3 (negative control). (A) C-terminal-deletion mutants 553 to 237 were precipitated by anti-1–11 antibodies but not by 6G3. (B) N-terminal-deletion mutants 123 and 162 were precipitated by 6G3 but not by anti-1–11 antibodies. (C) C-terminal-deletion mutant 583 was precipitated by 6G3 but deletion mutants 553 and 570 were not. A mixture of in vitro-synthesized A subunit (65 kDa), B subunit (55 kDa), and C subunit (36 kDa) was used for molecular weight markers (far left unmarked lanes in panels A and B).

competition (lane 3). We have identified this protein as actin (data not shown).

In summary, the Bα subunit prevents binding of 5H4 and of 1A12, but not of 6F9, to repeat 1, and the Cα subunit prevents binding of 6G3 to repeat 15. These data are consistent with the model of PP2A that was based on site-directed mutagenesis of the A subunit.

Separation of core enzyme and holoenzyme. The immunoprecipitation data indicate that 6F9, 5H4, and 1A12 might be used to differentiate between various PP2A subpopulations and that core enzyme is an abundant form that might exist inside cells. To further investigate these possibilities, sequential immunoprecipitations of [^{35}S]methionine-labeled cytoplasmic extracts with 6F9, 5H4, and 1A12 were carried out. When 1A12 antibodies were used three times, the bulk of the A and C subunits was removed in the first precipitation (Fig. 6A, lane 2), and little was precipitated in the second (lane 3) and third (lane 4) precipitations. A protein migrating slightly faster than the C subunit was precipitated only by 1A12 and is unrelated to the C subunit. When 6F9 was used instead of 1A12 for the third precipitation, a significant amount of A and C was precipitated. In addition, the B subunit was then detected (Fig. 6A, lane 5). In contrast, no additional A, B, or C was found when 5H4 was used in the third precipitation (Fig. 6A, lane 6). These results confirm that 1A12 recognizes the core enzyme but not the holoenzyme whereas 6F9 recognizes the holoenzyme. In the second set of experiments, 6F9 antibodies were used once (Fig. 6A, lane 7) or twice (Fig. 6A, lane 8). The second precipitation was followed by another with either 1A12 (Fig. 6A, lane 9), 5H4 (lane 11), or 6F9 for a third time (lane 10). 6F9 removed all A-C complexes and all A-B-C complexes in the first and second precipitations, and there was no further precipitation by 1A12 or 5H4. Thus, 6F9 quantitatively precipitated holoenzyme and core enzyme. In the third experiment, 5H4 was used twice (Fig. 6A, lanes 12 and 13), followed by 1A12 (lane 14), 6F9 (lane 15), or 5H4 for a third time (lane 16). The results were similar to the experiment with 1A12 in that 5H4 removed A and C but not B, and 1A12 precipitated nothing in addition. On the other hand, 6F9 was able to precipitate additional A, B, and C. The background levels in these experiments are high because the antibodies were used in excess in order to ensure immunodepletion and because the exposure of the fluorogram was long in order to visualize weak bands in the third rounds of immunoprecipitation.

The results from sequential immunoprecipitation were complemented by the results from immunoblotting of the extracts that remained after immunoprecipitation. Note that Fig. 6A and B are complementary in that strong signals shown in Fig. 6A correlate with weak signals shown in Fig. 6B. As shown in Fig. 6B, three precipitations with 1A12 removed approximately

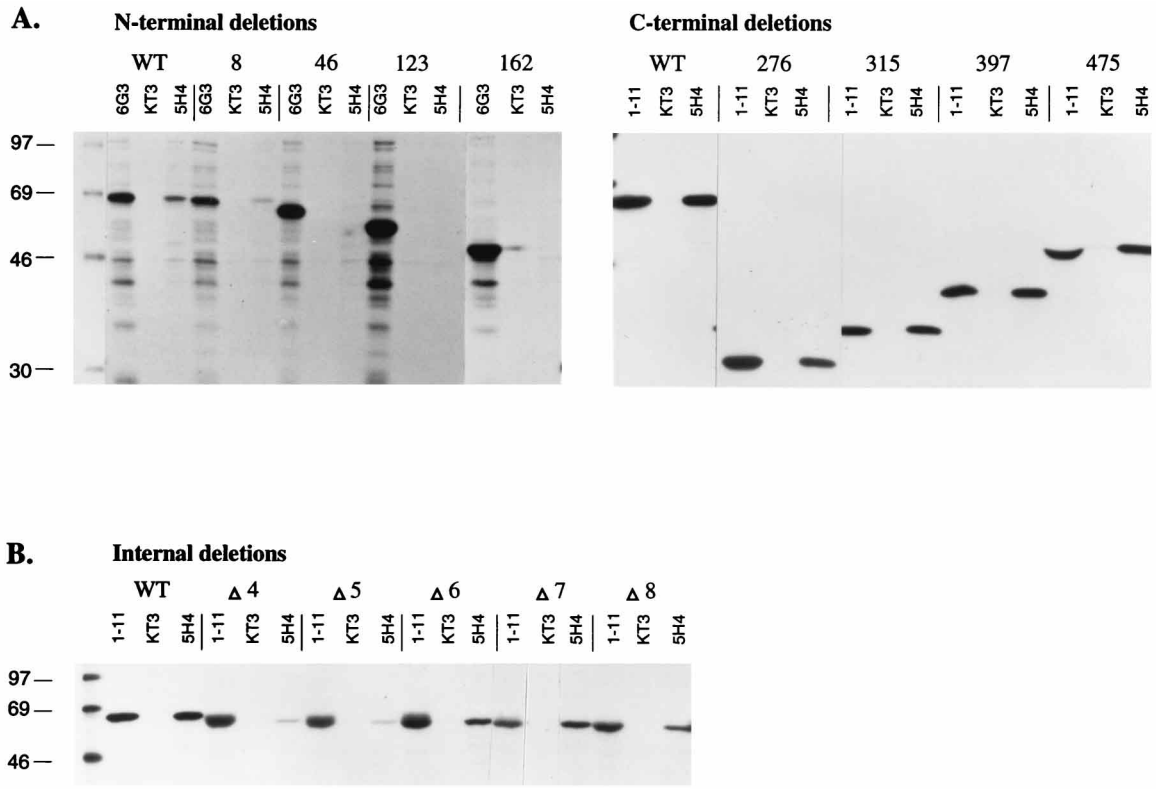

FIG. 3. Localization of epitope recognized by 5H4. (A) Only N-terminal-deletion mutant 8 was precipitated by 5H4; N-terminal-deletion mutants 46, 123, and 162 were not precipitated. All C-terminal-deletion mutants were precipitated by 5H4. (B) Single-repeat-deletion mutants Δ4 through Δ8 were precipitated by 5H4. 6G3, anti-1–11 antibodies, and KT3 were used as controls. The unmarked far left lanes in panels A and B show molecular weight markers phosphorylase *b* (97 kDa), BSA (69 kDa), ovalbumin (46 kDa), and carbonic anhydrase (30 kDa). WT, wild type.

one-half of the total amount of A and C from the extract (compare lanes 2 and 3). A similar amount remained after three precipitations with 5H4 (Fig. 6B, lane 11). On the other hand, three precipitations with 6F9 exhausted the extract of almost all of the A, B, and C subunits (Fig. 6B, lane 7). 6F9 also removed most of the A, B, and C subunits remaining after two precipitations with either 1A12 (Fig. 6B, lane 4) or 5H4 (lane 10). In contrast, 5H4 precipitated nothing in addition to what was precipitated by 1A12 (Fig. 6B, compare lanes 3 and 5) nor did 1A12 precipitate anything more than 5H4 (compare lanes 9 and 11).

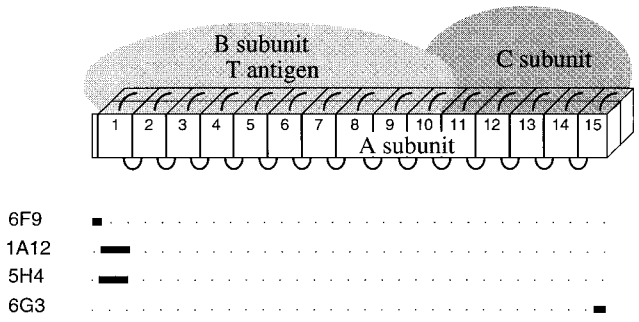

FIG. 4. Model of PP2A subunit interaction and approximate localization of epitopes on the A subunit. The B subunit and T antigens bind to the N terminus, and the C subunit binds to the C terminus of the A subunit. The regions where the epitopes of 6F9, 1A12, 5H4, and 6G3 antibodies are located are indicated by horizontal bars.

These experiments demonstrate that, under conditions of antibody excess, 5H4 or 1A12 can be used together with 6F9 for quantitation and separation of core enzyme and holoenzyme. When comparing the amount of core enzyme immunoprecipitated by 1A12 (Fig. 6A, lane 2) or 5H4 (lane 12) with the amount of holoenzyme immunoprecipitated by 6F9 after removal of core enzyme (lanes 5 and 15), we found approximately equal amounts of both forms. Because the experiments were carried out under conditions which minimized or prevented dissociation of holoenzyme and proteolytic degradation of the B subunit, we conclude that core enzyme is not an artifact but is present in cells at a concentration similar to that of holoenzyme (see Discussion).

To test whether the antibodies 5H4, 1A12, and 6G3 might cause dissociation of purified holoenzyme (A-Bα-C), they were incubated in a 10-fold excess with holoenzyme for 3 h at 4°C or at 37°C. The mixture was then analyzed on a nondenaturing gel, followed by immunoblotting with antibodies against the B subunit. No dissociation of the holoenzyme was detected (data not shown).

Phosphatase activities of immunoprecipitated core enzyme and holoenzyme. To determine whether the immunoprecipitated enzymes are active and have the expected substrate specificities, cytoplasmic extracts from 10T1/2 cells were sequentially precipitated with saturating amounts of 5H4 and 6F9. One half of each precipitate was analyzed by immunoblotting with antibodies against the A, B, and C subunits, and the other half was assayed for activity by using phosphorylase *a* and Rb peptide as substrates. The Bα subunit strongly stimulates the activity of the core enzyme towards substrates of cdc2-cyclin B,

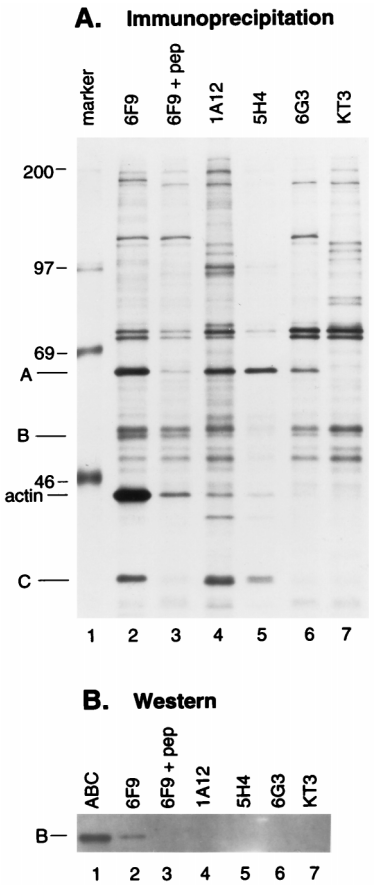

FIG. 5. Precipitation of PP2A with MAbs. Subconfluent 10T1/2 cells were labeled with [^{35}S]methionine and lysed in 1 ml of cold Triton X-100 lysis buffer per 10-cm-diameter plate (see Materials and Methods) for 90 s. The lysate was removed from the plate and spun for 10 min at 14,000 rpm in a cooled microcentrifuge (Eppendorf 5402). The supernatant was used for the immunoprecipitations shown in lanes 2 through 7. For immunoprecipitation, 60 μl of the lysate was incubated for 2 h with 10 μl of settled protein G Sepharose to which 6F9, 1A12, 5H4, 6G3, or KT3 antibodies were coupled (see Materials and Methods). Precipitates were washed three times in wash buffer containing 0.5% Triton X-100, 150 mM NaCl, 50 mM Tris (pH 8.0), 3 mM $MgCl_2$, 1 mM dithiothreitol, and 50 μM leupeptin. Precipitates were solubilized in 20 μl of SDS-PAGE sample buffer and boiled for 5 min. Ten microliters from each sample was loaded on two 10% polyacrylamide gels 12.5 cm long. (A) One gel was PPO (2,5-diphenyloxazole) treated and exposed for 2 days to X-ray film. (B) The other gel was used for Western blotting with polyclonal antibodies against the Bα subunit (IgG dilution, 1:1,000) (exposure time, 5 s). 6F9 antibodies were blocked with 10 μg of peptide 1–11 per 60 μl of cell lysate (lanes 3 and 9). Numbers on the left show positions of molecular weight markers (in thousands). Lane B1 contains purified holoenzyme (ABC) from bovine heart. The positions of A, B, and C were determined by comigration with in vitro-synthesized subunits and also by immunoblotting with anti-A, -B, and -C antibodies (data not shown).

such as Rb peptide (1, 12, 54). The enzymatic activities obtained were corrected for equal amounts of protein based on the immunoblots. As shown in Fig. 7A, 5H4 precipitated only core enzyme (lane 1), and 6F9 precipitated holoenzyme (lane 2). By scanning, it was determined that the molar ratio of holoenzyme to core enzyme was 2:1. The 5H4 precipitate (core enzyme) was 4.8 times more active in dephosphorylating phosphorylase *a* than the 6F9 precipitate (holoenzyme) (averages of 105,991 versus 21,988 counts released from substrates). On the other hand, the 6F9 precipitate was 6.5 times better than the 5H4 precipitate at dephosphorylating Rb peptide (averages of 5,233 versus 805 counts). These results demonstrate that the immunoprecipitated enzymes were active and had the expected substrate specificities.

To check whether other cell types also contain a large amount of core enzyme, we sequentially precipitated HeLa cell extracts with saturating amounts of 5H4 and 6F9. As shown in Fig. 7B, the extracts contained equal quantities of core enzyme and holoenzyme.

Immunoprecipitation of different holoenzymes by 6F9 but not 5H4; binding of B′ and B″ to the N terminus of the A subunit. Having shown that 6F9 but not 5H4 precipitates A-Bα-C, we asked whether it also precipitates holoenzymes containing a B′ or B″ family member. In addition, we tested whether 5H4 was unable to recognize B′- or B″-containing holoenzymes. We used Bα as a positive control and also included Bβ, which was expected to behave similarly to Bα. We used B′α1 as a representative of the B′ family (57). All B subunits were synthesized in vitro with a reticulocyte lysate and labeled with [^{35}S]methionine. As shown previously, in vitro-synthesized Bα associates with core enzyme that is present in the lysate (43). The lysates were precipitated with 6F9, 5H4, or KT3 (control antibody), and the precipitates were analyzed by PAGE. As shown in Fig. 8A, 6F9 precipitated B′α1- and B″-containing holoenzymes (lanes 5 and 11, respectively), whereas 5H4 did not (lanes 6 and 12, respectively). These results indicate that B′α1 and B″ bind to repeat 1 of the A subunit and cover up the epitope recognized by 5H4, as do Bα and Bβ.

Our model of PP2A predicts that both 6F9 and 5H4 should recognize complexes of core enzyme and T antigens, whose binding sites on the A subunit do not include repeat 1. As shown in Fig. 8A, lanes 14 and 15, in vitro-synthesized SV40 small T antigen was indeed precipitated by 6F9 and 5H4. To study middle-T-antigen binding with 6F9 and 5H4, a different approach was necessary because in vitro-synthesized middle T antigen does not form a complex with core enzyme (reference 2 and unpublished data). Therefore, as an alternative, extracts from 293 cells expressing a high concentration of middle T antigen were precipitated with 6F9 and 5H4, and middle T antigen was identified by immunoblotting. Both 6F9 and 5H4 precipitated the middle-T-antigen–core enzyme complex (Fig. 8B, lanes 1 and 3, respectively). As the control for 6F9, the immunoprecipitation was carried out in the presence of competing peptide (Fig. 8B, lane 2). As the control for 5H4, KT3 antibody was used (Fig. 8B, lane 4).

DISCUSSION

Evidence that core enzyme is abundant in cells. We have demonstrated that MAb 5H4 or 1A12 in combination with 6F9 can be used to separate core enzyme from holoenzyme, and quantitative analyses showed that 10T1/2 and HeLa cell extracts contain similar amounts of core enzyme and holoenzyme. We believe that intact cells also contain a large amount of core enzyme, in contrast to the common view that core enzyme is an artifact resulting from dissociation and proteolytic breakdown of B subunits during isolation. The reasons for our assumption are the following.

(i) Core enzyme was removed from extracts by three consecutive precipitations with 5H4 or 1A12. The bulk of core enzyme was already removed by the first immunoprecipitation. If core enzyme was generated by continuous dissociation of A-B-C during the immunoprecipitation procedure, each step involving 3 h of incubation at 4°C, one would expect to find large amounts of A-C in the second and third precipitations. It seems unlikely that decay of holoenzyme took place only during cell lysis and not during the subsequent immunoprecipitation.

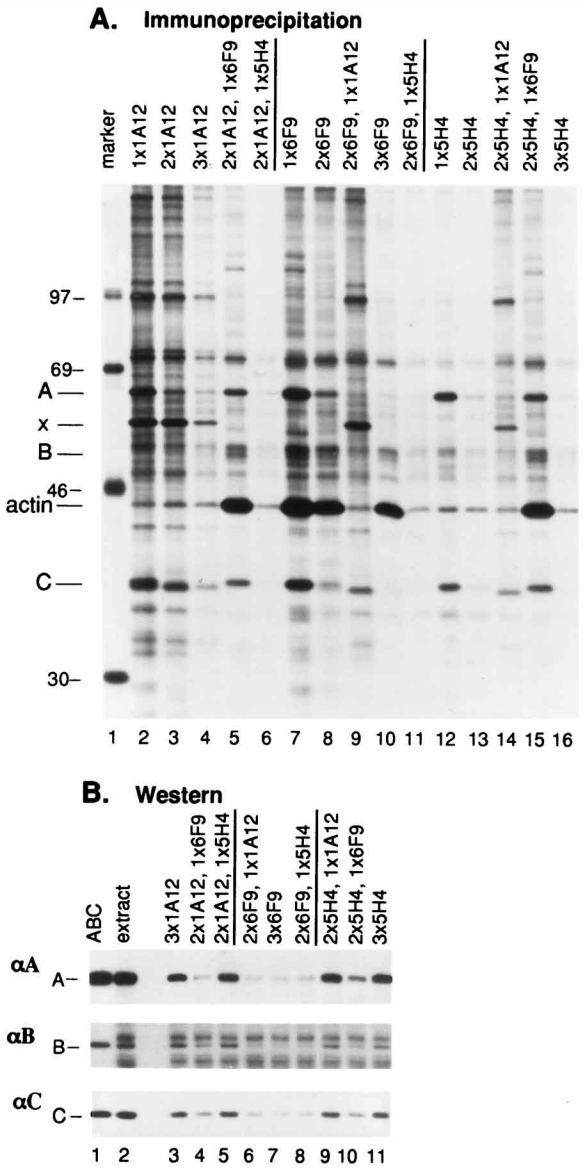

FIG. 6. Quantitative separation of core enzyme and holoenzyme by sequential immunoprecipitation. 10T1/2 cells were labeled with [^{35}S]methionine and lysed for 10 min in cold Triton X-100 lysis buffer. (A) Aliquots of 150 μl of lysate were precipitated with 15 μl of Sepharose-coupled 1A12, 6F9, or 5H4 antibody and incubated for 2 h on ice. The Sepharose was spun, washed, and boiled in 20 μl of SDS-PAGE sample buffer. A 10-μl aliquot was analyzed on a 10% polyacrylamide gel (lanes 2, 7, and 12). The supernatants were again incubated with 15 μl of the same antibody and the precipitates were processed as before (lanes 3, 8, and 13). The supernatants from the second precipitation were split into three parts. One aliquot was incubated a third time with the same antibody (lanes 4, 10, and 16), and the remaining aliquots were incubated with the other two antibodies as indicated at the top of lanes 5, 6, 9, 11, 14, and 15. Molecular weight markers are shown in lane 1 (numbers on the left are in thousands). x, unidentified protein. (B) Aliquots (20 μl) from the extracts remaining after three sequential precipitations were diluted with 2× SDS-PAGE sample buffer and analyzed on immunoblots. The sample designations at the top of the lanes are analogous to those in panel A. Lane 2 shows a blot of 20 μl of the initial extract. Lane 1 shows a blot with purified PP2A holoenzyme from bovine heart. Only the relevant sections of the blots are shown. The following antibodies were used for blotting: 6G3 (120 ng/ml) against the Aα subunit, MAbs against the Cα subunit (100 ng/ml) (29), and polyclonal antibodies (1:1,000 dilution of IgG) against the Bα subunit. Incubation with primary antibodies was for 3 h. The following horseradish peroxidase-conjugated secondary antibodies were used: goat anti-rat IgG/IgM (Jackson) at a 1:5,000 dilution for 6G3, donkey anti-rabbit antibodies (Amersham) (1:2,000) for anti-B subunit antibodies, and sheep anti-mouse antibodies (Amersham) (1:2,000) for anti-C subunit antibodies. The incubation time was 1 h.

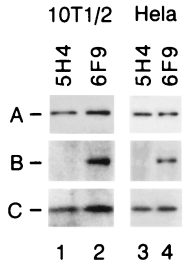

FIG. 7. Immunoprecipitation of core enzyme and holoenzyme by 5H4 and 6F9 for use in phosphatase assays. 10T1/2 cell extract was immunoprecipitated with 5H4 and 6F9 and analyzed by PAGE and immunoblotting with antibodies against the A, B, and C subunits, as described in Materials and Methods. The same amounts of core enzyme (lane 1) and holoenzyme (lane 2) were used for the phosphatase assays described in the text.

(ii) Previous investigators have described holoenzyme as unstable during purification, but the methods they used involved harsh conditions, such as extreme pH changes, high salt concentrations, long dialysis, and chromatography. In addition, the entire purification procedure took several days to complete. It is not surprising that under such conditions some of the B subunit, which is very sensitive to proteolytic enzymes, gets degraded and dissociates from the core (23). All of these harsh conditions were avoided during the immunoprecipitation protocol used here, which was carried out with all possible pre-

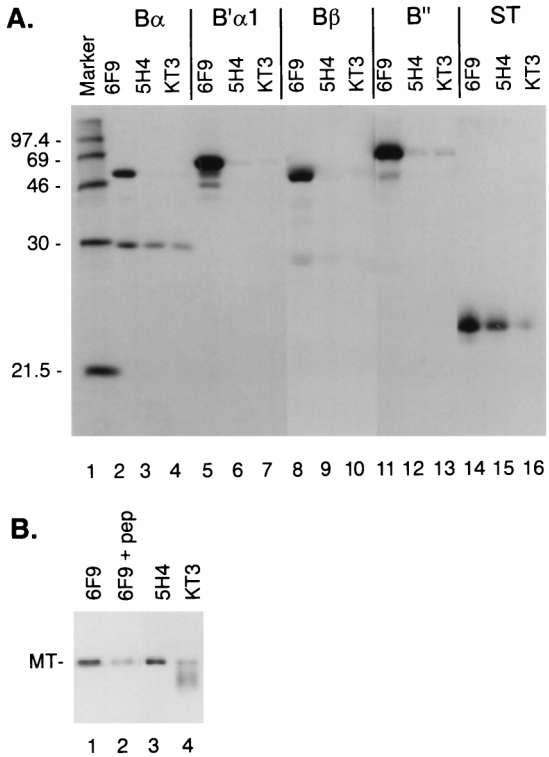

FIG. 8. Differential immunoprecipitation of complexes between core enzymes and different B subunits or T antigens by 6F9 and 5H4. (A) Different B subunits and SV40 small T antigen (ST) synthesized in vitro were immunoprecipitated with 6F9, 5H4, or KT3 (control) as indicated and analyzed by PAGE and fluorography. (B) Extracts of 293 cells infected with adenovirus expressing polyomavirus middle T antigen (MT) were immunoprecipitated with 6F9 in the absence (lane 1) or presence (lane 2) of competing peptide (pep), 5H4 (lane 3), and KT3 (lane 4). The precipitates were analyzed by PAGE and immunoblotting with an antibody against middle T antigen (see Materials and Methods).

cautions to avoid proteolysis (use of cold temperatures, short times, and effective protease inhibitors). The use of Triton X-100-containing buffer for immunoprecipitation had no influence on the yield of core enzyme since the same results were obtained in detergent-free buffer.

(iii) Both Tung et al. (59) and Zolnierowicz et al. (68) used rapid batchwise adsorption on DEAE-cellulose (DE-52) as one of the early steps of purification and obtained only holoenzyme at the end. They concluded that this step prevents core enzyme formation and that holoenzyme is the only form of enzyme in vivo. However, 75% of the total enzyme was lost during the early steps of purification (including the DE-52 batch procedure), and no attempt was made to check the makeup of the lost enzyme and whether it included core enzyme. In contrast, during consecutive immunoprecipitations with 5H4 or 1A12 and 6F9, almost all PP2A was recovered and accounted for.

(iv) It is possible that tissue culture cells differ from rabbit skeletal muscle, which was used as a source of PP2A in the studies mentioned above. It remains to be seen whether skeletal muscle contains both forms of PP2A when MAbs are used for its purification.

The finding that the core enzyme is abundant in cells is likely to have important implications for cell metabolism and growth control, because core enzyme differs from holoenzyme in substrate specificity and response to stimulatory and inhibitory substances (1, 4, 12, 22, 24, 54). In addition, the core enzyme could serve as a pool of enzyme ready to associate with different B subunits. For example, induction of Bα could lead to formation of new A-Bα-C that was shown to be associated with microtubules (56). Other B subunits, such as some forms of B′ (10, 20, 35, 57) and B″ (20), contain nuclear localization signals and may direct the core enzyme to the nucleus. In cells transformed by small DNA tumor viruses, the binding of T antigens to the core enzyme could occur without competition with B subunits. This implies that SV40 small T antigen could bind to core enzyme without displacement of B subunits such as B′ subunits, which have higher affinities than does SV40 small T antigen for the common binding site on the A subunit (22).

Further evidence for a model of PP2A holoenzyme. A model of trimeric PP2A has been previously developed based on site-directed mutagenesis studies. However, it has been argued that mutations (e.g., in the N-terminal region) which obliterate binding of the B subunit do not prove that the B subunit binds in this region, because these mutations might cause conformational changes in other parts of the A subunit (e.g., the C terminus) that are responsible for binding of the B subunit. The data presented here demonstrate that 5H4 and 1A12, whose epitopes are located in repeat 1, do not recognize the A subunit when a B subunit is bound to it. This shows directly that the B subunit binds to the N terminus of the A subunit. It was also demonstrated that 6G3, whose epitope is located at the C terminus of the A subunit, does not recognize the A subunit when the C subunit is bound to it. This finding shows that the C subunit binds to the C terminus of the A subunit. The properties of 6F9 also demonstrate that the N-terminal sequence preceding repeat 1 is not involved in B subunit binding, as previously suggested (43, 44). Thus, the present studies provide independent support for the model derived from site-directed mutagenesis experiments and reinforce the central role of the A subunit as a foundation on which the C and B subunits or T antigens interact.

How do B subunits bind to the A subunit? A fascinating question is how the B subunits from different gene families and the T antigens bind to the A subunit, considering that there is so little sequence similarity among them. Our previous work indicated that Bα requires repeats 1 through 10 for binding, and the present study independently shows that Bα, B′, and B″ each bind to repeat 1. It remains to be determined whether B′ and B″ also need all of repeats 2 through 10. Two extreme possibilities can be envisioned as to how binding might occur. (i) All B subunits and T antigens contain a common structural motif generated through protein folding. They interact with the same set or overlapping sets of amino acids in repeats 1 through 10. It is possible that some B subunits need fewer than 10 repeats, although they all bind to repeat 1. (ii) Alternatively, the various B subunit and T antigen binding domains are structurally unrelated to each other, and they each bind to different amino acids in repeats 1 through 10. It may be possible to distinguish between these models by assaying mutants of the A subunit for their ability to bind to the various B subunits. We have shown recently that the mutant SUB, in which seven amino acids in repeat 5 were replaced and which binds neither Bα nor T antigens, also does not bind B′ or B″ in vitro (unpublished data). McCright and Virshup demonstrated that B′ and B″ do not associate with the SUB mutant in vivo (35). Both observations favor the first model. On the other hand, the finding that B′ and B″, but not Bα, bind to the N terminus of the A subunit in the absence of the C subunit (35) is more consistent with the second model. Future studies will focus on elucidating the function of the core enzyme in vivo and on defining A subunit structural requirements for the interaction between core enzyme and specific B subunits.

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