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Alpha4 protein as a common regulator of type 2A-related serine/threonine protein phosphatases

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Abstract The catalytic activity of the C subunit of serine/threonine phosphatase 2A is regulated by the association with A (PR65) and B subunits. It has been reported that the alpha4 protein, a yeast homolog of the Tap42 protein, binds the C subunit of serine/threonine phosphatase 2A and protein phosphatase 2A-related protein phosphatases such as protein phosphatase 4 and protein phosphatase 6. In the present study, we showed that alpha4 binds these three phosphatases and the association of alpha4 reduces the activities of these phosphatases in vitro. In contrast, PR65 binds to the C subunit of serine/threonine phosphatase 2A but not to protein phosphatase 4 and protein phosphatase 6. These results suggest that the alpha4 protein is a common regulator of the C subunit of serine/threonine phosphatase 2A and protein phosphatase 2A-related protein phosphatases.

Key words: Alpha4; Protein phosphatase 2A; Protein phosphatase 4; Protein phosphatase 6; PR65

1. Introduction

Serine/threonine protein phosphatases have important roles in signal transduction pathways by removing phosphate from various proteins. These protein phosphatases are classified into two classes, as type 1 and type 2 phosphatases [1]. The type 2 phosphatases is a family of proteins including protein phosphatase 2 (PP2)A, PP2B and PP2C. Recently, many novel serine/threonine protein phosphatases such as protein phosphatase 4 (PP4) and protein phosphatase 6 (PP6) have been cloned [2].

In mammalian tissues, PP2A exists as a heterotrimer consisting of A (PR65), B (B′, B″, PR130 or PR72) and C (PP2A-C) subunits. PP2A-C is a catalytic subunit having a molecule mass of 36 kDa and two isoforms, α and β, have been identified [1]. PP2A-C usually forms a dimer with PR65 and the B subunit forms a heterotrimer of ABC through the association with PR65 [1,3,4]. The substrate specificity and subcellular localization of PP2A-C are influenced by the interaction of the regulatory subunits such as PR65 and the B subunit [1–8]. In mammalians, PP4, or PPX, and PP6 have been identified as PP2A-related protein phosphatases [2,9,10]. The overall sequence of PP4 and PP6 is closely related to that of PP2A-C with 66% and 59% identity at the protein level, respectively [9,10]. PP4 is located at the centrosome in the nucleus and little is found in the cytoplasm [2,11]. PP6 is a functional homolog of the budding yeast Sit4. Sit4 is required for the G1 to S transition of the cell cycle [12]. This implies the function of PP6 in the cell cycle regulation [10].

The PP2A-related protein phosphatases such as Sit4, Pph21 and Pph22 in yeast are associated with the Tap42 protein [13,14]. The association of Tap42 with the yeast PP2A-related protein phosphatases is sensitive to an immunosuppressant rapamycin [14]. The alpha4 protein was identified as a 52 kDa phosphoprotein associated with Ig receptor-associated MB-1 protein in B lymphocytes [15,16]. Alpha4 is a homolog of the yeast Tap42 protein. Recently, it has been reported that alpha4 is associated with PP2A-C and type 2A-related protein phosphatases (PP4 and PP6) in mammalian cells [17–20]. Namely, alpha4 associates with PP2A-C in a rapamycin sensitive manner in COS7 and Jurkat cells and in the immunosuppressant-independent way in other cell types such as human embryonic kidney 293 (HEK293) cells [17–20]. It has been shown that the association of alpha4 with PP2A-C inhibits the catalytic activity in vitro [20]. However, the effects of the association of alpha4 on the catalytic activity of type 2A-related protein phosphatases have not been elucidated. Thus, in the present study, we examined the effects of the association of alpha4 on PP2As (PP4 and PP6) in vitro, using a *p*-nitrophenyl phosphate (*p*NPP) assay.

2. Materials and methods

2.1. Reagents and antibodies

Protein G Sepharose was purchased from Pharmacia Biotech. Rapamycin was purchased from Calbiochem. *p*-Nitrophenyl phosphoric acid disodium salt was obtained from Nacalai Tesque. Dulbecco's modified Eagle's minimal essential medium (DMEM) was from Life Technologies. Anti-FLAG monoclonal M2 antibody was obtained from Eastman Kodak. Anti-myc monoclonal antibody was produced using MYC 1-9E10.2 hybridoma cells (ATCC). Anti-HA monoclonal antibody (12CA5) was from Boehringer-Mannheim.

2.2. Plasmids and cDNA

pME18S HA vector (HA vector) was gifted from Dr Masa-aki Muramatsu (Helix Research Institute, Chiba, Japan). pCMV FLAG vector (FLAG vector) was gifted from Dr Joseph Avruch (Diabetes Unit, Massachusetts General Hospital, Harvard Medical School). pcDNA3.1 (-)/Myc-His vector (MYC vector) was purchased from Invitrogen. Rat PP2A-C β isoform cDNA was gifted from Dr Takayoshi Kuno (Department of Pharmacology, Kobe University). The mouse and human EST cDNA clones were obtained from the UK

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Abbreviations: PP2A, protein phosphatase 2A; PP4, protein phosphatase 4; PP6, protein phosphatase 6; *p*NPP, *p*-nitrophenyl phosphoric acid; TOR, target of rapamycin

The nucleotide sequence of mouse PR65 has been submitted to DDBJ/EMBL/GenBank and assigned accession number AB021743.

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HGMP Resource Center. Mouse PR65 and human PP4 were cloned by PCR using the EST clones AA764624 and AA405553 as templates, respectively. Human PP6 was cloned from a human skeletal muscle Quick-Clone cDNA library (Clonetech), using PCR. Sequence analysis of PCR clones was performed on an Applied Biosystem 377 DNA sequencer. Alpha4 cDNA was obtained as described previously [16] and inserted into the pcDNA 3.1 (-)/Myc-His vector with the carboxyl-terminal myc epitope (Alpha4-MYC). DNA fragments of alpha4 encompassing 1–909 bp, 1–738 bp and 1–591 bp were amplified by PCR, using cDNA of wild-type alpha4 as a template, and were inserted into the pcDNA3.1 (-)/Myc-His vector to generate deletion mutants of amino acid 1–303 (Alpha4 mut 303-MYC), amino acid 1–246 (Alpha4 mut 246-MYC) and amino acid 1–197 (Alpha4 mut 197-MYC), respectively. cDNA encoding PR65 was introduced into the pME18S HA vector with the amino-terminal HA epitope (HA-PR65). cDNAs encoding PP2A-C, PP4 and PP6 were ligated into the pCMV FLAG vector with the amino-terminal FLAG epitope (FLAG-PP2A, FLAG-PP4 and FLAG-PP6), respectively.

2.3. Cell culture and cDNA transfection

HEK293 cells were maintained and cultured in DMEM with 10% fetal calf serum. Transfection of plasmids in mammalian cells was carried out by the lipofection method using LipofectAMINE (Life Technologies). Cells were harvested 36–48 h after transfection.

2.4. Immunoprecipitation and immunoblot

Cells were lysed in ice-cold buffer A (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 20 mM β-glycerophosphate, 1 mM dithiothreitol, 50 μM *p*-amidinophenyl methanesulfonyl fluoride, 2 μg/ml aprotinin) and the extracts were centrifuged at 10 000×*g* for 20 min at 4°C. Aliquots of the supernatant were subjected to immunoprecipitation with antibodies bound on protein G Sepharose beads at 4°C. The beads were washed twice with ice-cold buffer B (50 mM HEPES, pH 7.4, 0.1% Triton X-100, 500 mM NaCl). The bound proteins were eluted directly into SDS sample buffer, subjected to SDS-PAGE and transferred to a PVDF membrane. Immunoblotting was performed using the ABC peroxidase method (Vectastain).

2.5. pNPP assay

HEK293 cells, transfected with various plasmids as indicated, were lysed in ice-cold buffer C (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 120 mM NaCl, 1 mM EDTA, 6 mM EGTA, 1 mM dithiothreitol, 50 μM *p*-amidinophenyl methanesulfonyl fluoride, 2 μg/ml aprotinin). Transiently expressed FLAG-PP2A-C, FLAG-PP4 and FLAG-PP6 were immunoprecipitated from the cell lysates with anti-FLAG antibody bound on protein G Sepharose beads at 4°C. The complexes of HA-PR65/FLAG-PP2A-C, myc-alpha4/FLAG-PP2A-C, myc-alpha4/FLAG-PP4 and myc-alpha4/FLAG-PP6 were precipitated with either the anti-HA or anti-myc antibodies bound on protein G Sepharose beads. Beads were washed twice with ice-cold buffer B. Beads were incubated with 1 ml of the reaction mixture, which contains 40 mM Tris-HCl, pH 8.0, 20 mM KCl, 30 mM $MgCl_2$, 2 mM dithiothreitol, 1 mg/ml bovine serum albumin and 5 mM *p*NPP, for 20 min at 30°C. The reaction was terminated by centrifugation at 10 000×*g* for 30 s at room temperature and the change in absorbance at 400 nm of the supernatant was measured [21]. The expression of alpha4, PR65, PP2A-C, PP4 and PP6 were confirmed by immunoblotting after immunoprecipitation from the aliquots of the cell lysates as described above.

3. Results

3.1. Association of PR65 with PP2A-C but not with type 2A-related serine/threonine protein phosphatases

We cloned mouse PR65 (accession number AB021743) that is almost identical to human PR65α (99% identity at the protein level). Using this mouse PR65, the association of the regulatory subunit with PP4 and PP6 was examined in the transfected cells. FLAG-PP2A-C, FLAG-PP4 and FLAG-PP6 were transiently co-expressed with HA-PR65 in HEK293 cells. FLAG-PP2A-C was co-immunoprecipitated with HA-PR65 using anti-HA antibody (Fig. 1A, lane 4 and Fig. 1B, lane 4), whereas FLAG-PP4 and FLAG-PP6 were not co-immunoprecipitated with HA-PR65 using anti-HA antibody (Fig. 1A lane 6 and Fig. 1B, lane 6).

3.2. Association of alpha4 with PP4 and PP6

The association of alpha4 with PP4 and PP6 was examined. FLAG-PP4 and FLAG-PP6 were co-expressed with myc-alpha4 in HEK293 cells. FLAG-PP4 and FLAG-PP6 were co-immunoprecipitated with myc-alpha4 using anti-myc antibody (Fig. 2A, upper panel, lane 4 and Fig. 2B, upper panel, lane 4) and myc-alpha4 was co-immunoprecipitated with FLAG-PP4 and FLAG-PP6 using anti-FLAG M2 antibody (Fig. 2A, lower panel, lane 4 and Fig. 2B, lower panel, lane 4). Then we examined the effects of rapamycin on the association of alpha4 with PP4 and PP6. The dissociation of alpha4 from PP4 and PP6 was not observed in rapamycin-treated cells (Fig. 2A, lane 5 and Fig. 2B, lane 5).

Next, to examine the region of alpha4 required for the association of PP2A-C and type 2A-related protein phosphatases, three deletion mutants of alpha4 were constructed. FLAG-PP2A-C, FLAG-PP4 and FLAG-PP6 were co-expressed with wild-type or mutants of myc-alpha4 in HEK293 cells. FLAG-PP2A-C, FLAG-PP4 and FLAG-PP6 were co-immunoprecipitated with wild-type myc-alpha4, Alpha4 mut 303-MYC and Alpha4 mut 246-MYC using anti-myc antibody (Fig. 3, left panels, lanes 3–5). In addition, wild-type myc-alpha4, Alpha4 mut 303-MYC and Alpha4 mut 246-MYC were co-immunoprecipitated with FLAG-PP2A-C, FLAG-PP4 and FLAG-PP6 using anti-FLAG M2 antibody

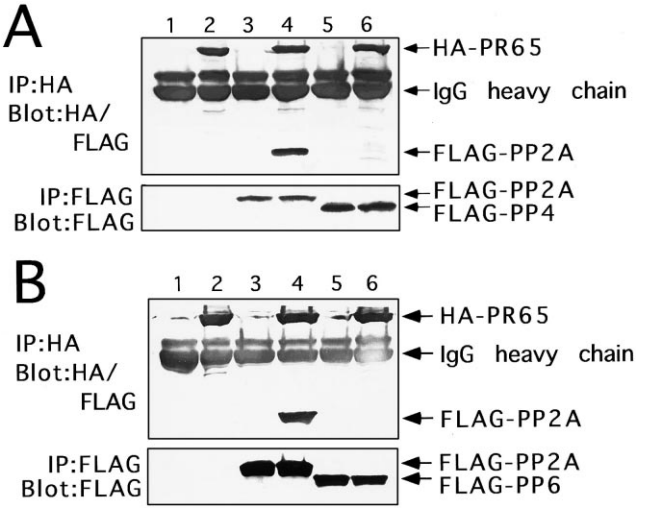

Fig. 1. Association of PR65 with PP2A-C. HEK293 cells cultured in 60 mm dishes were transfected with 500 ng FLAG-PP4 (A: lanes 5 and 6) and 100 ng FLAG-PP6 (B: lanes 5 and 6). In addition, cells were transfected with the same plasmid DNAs in A and B as follows: lane 1, mock transfection; lane 2, 100 ng FLAG vector; lanes 3 and 5, 2 μg HA vector; lanes 2, 4 and 6, 2 μg HA-PR65; lanes 3 and 4, 100 ng FLAG-PP2A. After extraction, immunoprecipitates with either anti-HA antibody (A and B: upper panels) or anti-FLAG M2 antibody (A and B: lower panel) were subjected to 10% SDS-PAGE and an immunoblot was carried out using a mixture of anti-HA and anti-FLAG M2 antibodies (A and B: upper panels) or anti-FLAG M2 antibody (A and B: lower panels) as the first antibodies.

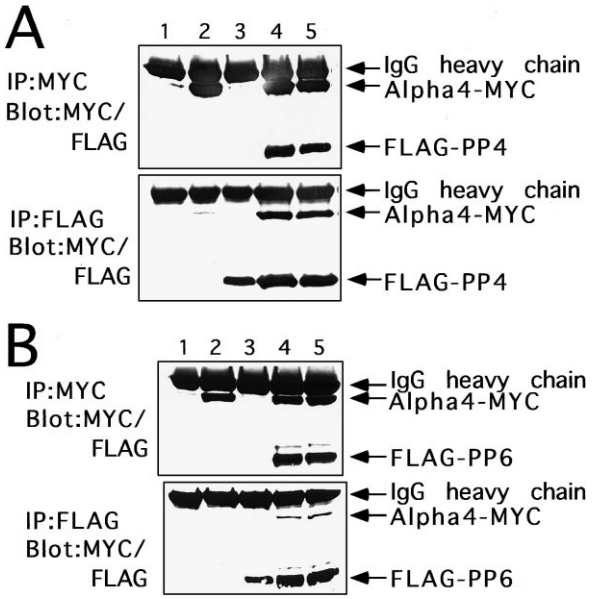

Fig. 2. Association of alpha4 with PP4 and PP6. HEK293 cells cultured in 60 mm dishes were transfected with 500 ng FLAG vector (A: lane 2), 500 ng FLAG-PP4 (A: lanes 3–5), 100 ng FLAG vector (B: lane 2) and 100 ng FLAG-PP6 (B: lanes 3–5). In addition, cells were transfected with the same plasmid DNAs in A and B as follows: lane 1, mock transfection; lane 3, 2 μg MYC vector; lane 2, 4 and 5, 2 μg Alpha4-MYC. In lane 5 (A and B), cells were treated with 100 nM rapamycin for 30 min at 37°C. After extraction, immunoprecipitates with either anti-myc antibody (A and B: upper panels) or anti-FLAG M2 antibody (A and B: lower panels) were subjected to 10% SDS-PAGE and an immunoblot was carried out using a mixture of anti-myc and anti-FLAG M2 antibodies (A and B: upper and lower panels) as the first antibodies.

(Fig. 3, right panels, lanes 3–5). However, FLAG-PP2A-C, FLAG-PP4 and FLAG-PP6 were not co-immunoprecipitated with Alpha4 mut 197-MYC (Fig. 3, left panels, lane 6). Consistent with this, Alpha4 mut 197-MYC was not co-immunoprecipitated with FLAG-PP2A-C, FLAG-PP4 or FLAG-PP6 (Fig. 3, right panels, lane 6).

3.3. Inhibition of the catalytic activity of PP2A-C and type 2A-related protein phosphatases by alpha4

We examined the effects of the association of alpha4 on the phosphatase activity of PP2A-C, PP4 and PP6 using a *p*NPP assay. FLAG-PP2A-C was immunoprecipitated with anti-FLAG M2 antibody bound on protein G Sepharose beads and FLAG-PP2A-C in complex with HA-PR65 were co-immunoprecipitated with anti-HA antibody bound on protein G Sepharose. Both forms of PP2A-C dephosphorylated *p*NPP (Fig. 4A, lower panel, lanes 4 and 5). In consistent with our previous report [18], FLAG-PP2A-C in complex with myc-alpha4, co-precipitated with anti-myc antibody bound on protein G Sepharose, exhibited a diminished phosphatase activity toward *p*NPP (Fig. 4A, lower panel, lane 6). Immunoblot analysis revealed that the amount of PP2A-C in the complex form with alpha4 (Fig. 4A, upper panel, lane 6) was higher than either that of the free form of PP2A-C (Fig. 4A, upper panel, lane 4) or that in complex form with HA-PR65 (Fig. 4A, upper panel, lane 5). FLAG-PP4 and FLAG-PP6, immunoprecipitated with anti-FLAG antibody bound on protein G Sepharose, dephosphorylated *p*NPP (Fig. 4B,C, lower panel, lane 3). The catalytic activity of PP6 is weaker than that of PP2A-C and PP4 (Fig. 4A, lower panel, lane 4 and Fig. 4B,C, lower panel, lane 3). FLAG-PP4 and FLAG-PP6 in complex with myc-alpha4 also showed a significant diminished phosphatase activity toward *p*NPP (Fig. 4B,C, lanes 4). The amounts of FLAG-PP4 and FLAG-PP6 in complex with alpha4 (Fig. 4B,C, upper panel, lane 4) were almost equal to that of the free form of FLAG-PP4 and FLAG-PP6 (Fig. 4B,C, upper panel, lane 3).

4. Discussion

In the present study, we reported that PR65 binds to PP2A-C but not to PP4 or PP6, whereas alpha4 binds to PP2A-C and type 2A-related protein phosphatases. It has been shown that PR65 binds to PP2A-C but not to PP4 [11] and that alpha4 binds to PP2A-C and type 2A-related protein phosphatases [19]. Taken together, these results suggest that PR65 is a regulatory subunit specific to PP2A-C and that alpha4 is a

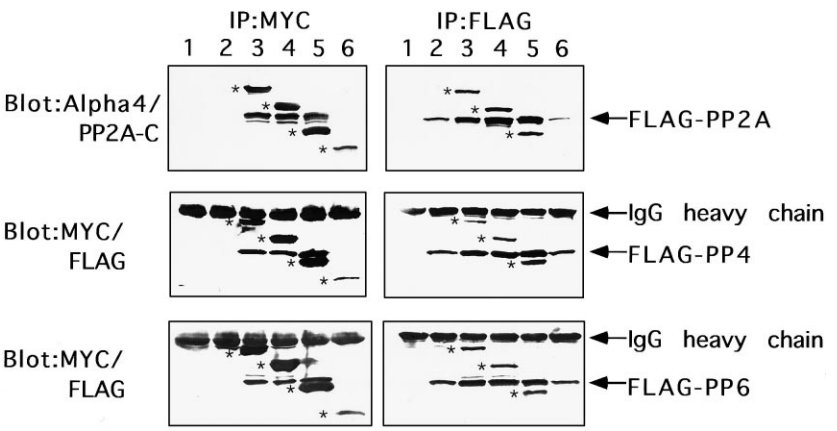

Fig. 3. Association of PP2A-C, PP4 and PP6 with wild-type and deletion mutants of alpha4. HEK293 cells in 60 mm dishes were transfected with 100 ng FLAG-PP2A (upper panels, lanes 2–6), 500 ng FLAG-PP4 (middle panels, lanes 2–6), 100 ng FLAG-PP6 (lower panels, lane 2–6) and the other plasmid DNAs (all panels: lane 1, mock transfection; lane 2, 2 μg MYC vector; lane 3, 2 μg Alpha4-MYC; lane 4, 2 μg Alpha4 mut 303-MYC; lane 5, 2 μg Alpha4 mut 246-MYC; lane 6, 4 μg Alpha4 mut 197-MYC). After extraction, immunoprecipitates with either anti-myc antibody (left panels) or anti-FLAG M2 antibody (right panels) were subjected to 12% SDS-PAGE and animmunoblot was carried out using a mixture of anti-alpha4 and anti-PP2A-C polyclonal antibodies (upper panels) or a mixture of anti-myc and anti-FLAG M2 monoclonal antibodies (middle and lower panels). Asterisks indicate the positions of the wild-type and three deletion mutants of alpha4.

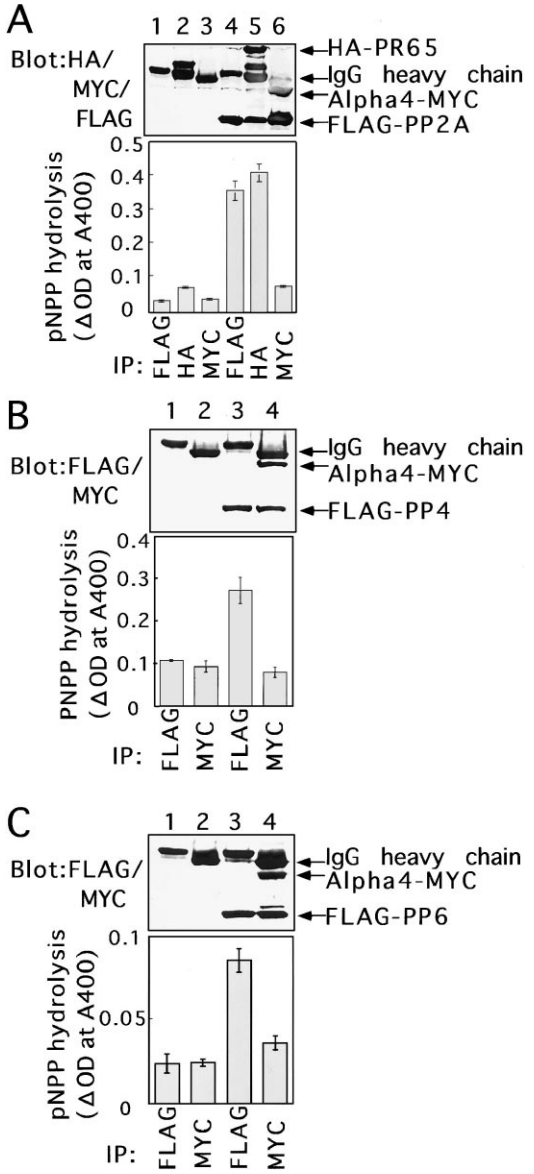

Fig. 4. Inhibition of the catalytic activities of type 2A-related protein phosphatases by alpha4. HEK293 cells cultured in 150 mm dishes were transfected with 4 μg FLAG-PP2A (A: lanes 4–6), 10 μg FLAG-PP4 (B: lanes 3 and 4), 5 μg FLAG-PP6 (C: lanes 3 and 4), 20 μg HA-PR65 (A: lane 5) and 20 μg Alpha4-MYC (A: lane 6, B and C: lane 4). The other lanes were treated with mock transfections (A: lanes 1–3, B and C: lanes 1 and 2). After extraction, cell lysates were subjected to immunoprecipation with anti-FLAG M2 antibody (A: lanes 1 and 4, B and C: lanes 1 and 3), anti-HA antibody (A: lanes 2 and 5) or anti-myc antibody (A: lanes 3 and 6, B and C: lanes 2 and 4) and then immunoprecipitates were subjected to a *p*NPP assay as described in Section 2. Values represent the means ± S.D. from triplicate experiments. Aliquots of the immunoprecipitates were subjected to 10% SDS-PAGE and an immunoblot was carried out using a mixture of anti-HA, anti-myc and anti-FLAG M2 monoclonal antibodies (A) or a mixture of anti-myc and anti-FLAG M2 monoclonal antibodies (B and C).

common regulator among PP2A-C and type 2A-related protein phosphatases.

It has been reported that alpha4 binds directly to PP2A-C but not together with PR65 [17]. We also found that PP2A-C is associated with either alpha4 or PR65 and that PP2A-C, alpha4 and PR65 do not form a ternary complex (data not shown). This also indicates that alpha4 does not form a complex with PP2A-C through the association with PR65.

We examined the region of alpha4 which is important for the binding to PP2A-C, PP4 and PP6. All of the three phosphatases bound the alpha4 fragment of the amino-terminal 246 amino acids but not to the alpha4 fragment of the amino-terminal 197 amino acids. Our previous report has shown that the region encompassing 109 amino acids in the middle portion of alpha4 (94–202 amino acids) was necessary and sufficient for the binding to PP2A-C [18]. These results suggest that PP2A-C, PP4 and PP6 bind to the same region of alpha4 and the region including amino acid 198–202 is critical for the binding of PP2A-C and type 2A-related protein phosphatases.

Finally, It was examined how the association of alpha4 with type 2A-related protein phosphatases affects the catalytic activity. We have reported that alpha4 inhibits the phosphatase activity of PP2A-C toward ^{32}P-phosphorylated eIF-4E binding protein 1 (4E-BP1), a component of the translation initiation complex [20]. It has been reported that 4E-BP1 is a downstream effector of growth factor receptors [22] and the mammalian target of rapamycin (mTOR) [23,24] and mTOR exhibits a kinase activity toward 4E-BP1 in vitro [25]. However, the phosphatase activity of PP6 was hardly detected toward ^{32}P-phosphorylated 4E-BP1. Thus, we employed *p*NPP as a substrate to investigate the phosphatase activity of PP2A-C, PP4 and PP6 in this study. The association with alpha4 inhibited the catalytic activity of PP2A-C, PP4 and PP6. (Fig. 4). These results suggest that alpha4 may be a negative regulator of type 2A-related serine/threonine phosphatases. However, the physiological substrates of PP4 and PP6 have not yet been identified. It has been demonstrated that the modulation of PP2A-C activity by the association with PR65 is dependent on the size of the substrate [26]. *p*NPP is not a protein substrate but a small organic molecule. Although we show here that alpha4 is the common negative regulatory subunit of PP2A-C, PP4 and PP6 in vitro, further investigations will be required to find physiologically relevant protein substrates and how alpha4 regulates the catalytic activity of type 2A-related protein phosphatases.

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