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The three Type 2A protein phosphatases, PP2Ac, PP4c and PP6c, are differentially regulated by Alpha4

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Abstract

Alpha4 is a non-canonical regulatory subunit of Type 2A protein phosphatases that interacts directly with the phosphatase catalytic subunits (PP2Ac, PP4c, and PP6c) and is upregulated in a variety of cancers. Alpha4 modulates phosphatase expression levels and activity, but the molecular mechanism of this regulation is unclear, and the extent to which the various Type 2A catalytic subunits associate with Alpha4 is also unknown. To determine the relative fractions of the Type 2A catalytic subunits associated with Alpha4, we conducted Alpha4 immunodepletion experiments in HEK293T cells and found that a significant fraction of total PP6c is associated with Alpha4, whereas a minimal fraction of total PP2Ac is associated with Alpha4. To facilitate studies of phosphatases in the presence of mutant or null Alpha4 alleles, we developed a facile and rapid method to simultaneously knockdown and rescue Alpha4 in tissue culture cells. This approach has the advantage that levels of endogenous Alpha4 are dramatically reduced by shRNA expression thereby simplifying interpretation of mutant phenotypes. We used this system to show that knockdown of Alpha4 preferentially impacts the expression of PP4c and PP6c compared to expression levels of PP2Ac.

Keywords

Alpha4; Protein phosphatases; Protein phosphatase 2A (PP2A); Lentiviral expression

1. Background

Alpha4 is a highly conserved protein with similarity to Tap42 from *Saccharomyces cerevisiae* [1,2]. In yeast, Tap42 plays an integral role in the Target of Rapamycin (TOR) pathway that regulates cellular growth and metabolism in response to growth factors and nutrients [2,3]. Consistent with this role in growth and metabolism, Alpha4 is upregulated in a number of cancers and transformed cell lines correlating with increased rates of cellular migration and proliferation [4,5]. Additionally, knockout of Alpha4 induces apoptosis in a p53-dependent manner [6], and knockdown of Alpha4 in cancer cell lines decreases proliferation and migration [4,5].

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Alpha4 was first discovered as a phosphoprotein in B-cells that interacted with and regulated the Type 2A family of serine/threonine phosphatases [7–11]. Its role in regulating the Type 2A phosphatases has not been fully elucidated, but one of its functions includes protection of the phosphatase catalytic subunit from degradation [12–14]. This protective effect requires both an intact Alpha4C-terminal region, as well as an ability to bind to PP2Ac [13].

Alpha4 is multidomain protein in which the N-terminus contains the residues responsible for binding to PP2Ac and the C-terminus has been shown to bind to the E3 ubiquitin ligase, Mid1 [12,15,16]. Recent crystal structures show the N-terminal domain of Alpha4 bound to a partially unfolded and catalytically inactive fragment of PP2Ac [17], consistent with the observation that Alpha4-associated PP2Ac has greatly diminished activity [10,14,18,19]. This crystal structure also illustrates a possible mechanism for the phosphatase protective effects of Alpha4, as it shows that the binding of Alpha4 with PP2Ac blocks access to the lysine residue in PP2Ac that is the target of polyubiquitination leading to degradation [17]. As the protective effect of Alpha4 relies upon direct interaction with the catalytic subunit, the ability of Alpha4 to affect levels of the Type 2A phosphatase catalytic subunits may correlate with the level of interaction between Alpha4 and catalytic subunits. To date, no studies have been published investigating the fraction of any of the phosphatase catalytic subunits that interacts with Alpha4 in cells. In studies presented here, we determined the fractions of PP2Ac, PP4c, and PP6c that are associated with Alpha4 and determined if this correlates with the effects of Alpha4 on expression levels of PP2Ac, PP4c, and PP6c.

Most prior studies conducted assessed short-term effects of Alpha4 knockdown, conditional knockout, or overexpression on phosphatase expression levels. A study investigating Alpha4 knockout showed significant declines in PP2Ac, PP4c, and PP6c expression [14], but studies of transient knockdown or over-expression of Alpha4 have not reported any significant changes in PP2Ac expression [5,14,20]. We decided to investigate chronic, rather than transient, changes in Alpha4 expression levels via creation of stable knockout and knockout with re-expression cell lines. We hypothesized that this approach better recapitulates the perturbations seen in diseases where Alpha4 is misregulated, as is the case in many cancers [4,5,21,22]. To accomplish this, we established a protocol that allowed for simultaneous knockdown and expression using a single lentiviral vector to create stable rescue cell lines. We then used these stable cell lines to investigate the effects of long-term knockdown and expression of Alpha4 on expression levels of PP2Ac, PP4c and PP6c.

2. Materials and methods

2.1. Plasmids

We used a second generation lentiviral transfection system consisting of three plasmids: a packaging plasmid (psPAX2; gift from Didier Trono, Addgene #12260), an envelope plasmid (pMD2.G; gift from Didier Trono, Addgene #12259), and a transfer plasmid (pLKO.1-TRC; gift from David Root, AddGene #10878) [23]. The scrambled shRNA in pLKO.1 was a gift from David Sabatini (Addgene #1864) [24]. The shRNAs directed to the 3'UTR (NM_001551.x-1110s1c1) and coding regions of Alpha4 (NM_001551.2-752s21c1) were from Sigma-Aldrich. The pcDNA5TO expression vector containing Flag-tagged human Alpha4 has been described [25].

2.2. Antibodies and reagents

The rabbit polyclonal Alpha4 antibody was from Bethyl Laboratories (Cat# A300–471A). The mouse monoclonal PP2Ac antibody was from BD Biosciences. The sheep PP4c and PP6c antibodies have been described [11]. The mouse monoclonal HSP90 antibody was from Santa Cruz Biotechnology. The mouse tubulin antibody was from Sigma-Aldrich. Puromycin was from Mediatech, Inc (Manassas, VA). Protein A agarose was obtained from Genscript. FuGENE was from Promega (Madison, WI). The PCR primers used for amplifying human Flag-Alpha4 from pcDNA5/TO with added MfeI restriction site for ligation into the PLKO.1 vector were F: GGCAAGGCTT-GACCGACAATTGCATGAAGAATCTGC and R: GTGGTGCAATTG-GAGCCCCAGCTGGTTCTTTCCGC (Sigma).

2.3. Cell culture and transfection

HEK293T and HeLa cell stocks were obtained from the ATCC. A549 cell stocks were a gift from Dr. John V. Williams (University of Pittsburgh Medical Center). All cell lines were grown in DMEM supplemented with 10% FBS fetal bovine serum and incubated at 37 °C in 5% CO₂.

2.4. Immunodepletion

Wild-type HEK293T cells were seeded at a density of 3×10^6 cells in 10 cm tissue culture plates and allowed to grow to near confluency over 48 h. The cells were lysed with 400 µl of ice cold lysis buffer (10 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1% Igepal) containing freshly added protease and phosphatase inhibitors (1 µM PMSF, 1 µg/ml leupeptin, 0.7 µg/ml pepstatin, 2 µg/ml aprotinin, 1 mM Na₃VO₄, 30 mM NaF, 20 mM Na₄O₇P₂, 60 mM β-glycerophosphate disodium, pH 7.2). Cells lysates were clarified by centrifugation at 17,000g for 15 min. Successive rounds of immunodepletion were performed at 4 °C. Protein A resin was washed three times in a PBS buffer containing 1% BSA and resuspended in a 50% slurry with PBS buffer containing 1% BSA. Immunodepletions were conducted using 300 µl of clarified cell lysate and 20 µL of the pre-washed Protein A resin slurry in the presence or absence of 3 µl Alpha4 antibodies (1:100 dilution). The first round of immunodepletions was conducted for 4 h and then lysates were centrifuged at 1400g for 5 min. The supernatants were collected and 40 µL aliquots were taken for analysis. Alpha4 antibodies or an equal volume of buffer were added to the remaining lysates at a 1:100 dilution and incubated overnight. The next morning 20 µL of the pre-washed Protein A resin slurry was added and incubated for 1 h and then lysates were centrifuged at 1400g for 5 min. Supernatants were collected and 40 µL aliquots were taken for analysis. All samples for analysis were solubilized in SDS sample buffer and heated to 95 °C for 10 min.

2.5. Lentiviral production

HEK293T cells were seeded at a density of 7×10^5 cells/well in 6-cm tissue culture plates. Lentiviral plasmids (250 ng pMD2.G, 750 ng psPAX2, 1 µg PLKO.1 vector plasmid) were transfected into HEK293T cells, using FuGENE and following the manufacturer's protocol, for packaging into viral particles. Media was exchanged after 15 h and virus-containing

supernatant was harvested and pooled at 24 h and 48 h. Supernatant was clarified by centrifugation at 1000g for 5 min and stored at -20°C .

2.6. Creation of stable cell lines

Cells were seeded at a density of 5×10^5 cells/well in 6-well tissue culture plates and allowed to grow overnight before infection with lentivirus using 0.5 ml of viral supernatant. Media was replaced after 24 h and cells were treated with puromycin for selection of stably infected cells. Puromycin concentrations used for selection were 7 $\mu\text{g/ml}$ (A549), 3 $\mu\text{g/ml}$ (HEK293T), and 1 $\mu\text{g/ml}$ (HeLa). Polyclonal stable cell lines were selected in puromycin for 10–14 days before being passaged and frozen down in liquid N_2 . All experiments were performed with cells brought up from frozen stocks.

2.7. Cell lysis

Cells were seeded at 4×10^5 cells/well in 6-well plates in DMEM supplemented with 10% FBS and incubated at 37°C in 5% CO_2 for 72 h. Plates were placed on ice, rinsed $2 \times$ with 1 ml of cold PBS, and then lysed with 200 μL of cold lysis buffer (20 mM MOPS, pH 7.0, 5 mM EDTA, 2 mM EGTA, 1 mM DTT) containing freshly added protease and phosphatase. Cell lysates were clarified by centrifugation at 17,000g for 20 min at 4°C . Protein concentrations of supernatants were determined using a Bradford assay (BioRad Protein concentration reagent). Supernatants were diluted, typically to 1 mg/ml, aliquoted and stored at -20°C in SDS sample buffer.

2.8. Western analysis

Typically 15–20 mg of protein were separated by SDS-PAGE using 4–12% Bis-Tris NOVEX NU_PAGE gels (Lifetech). Proteins were transferred to 0.45 μm nylon-supported nitrocellulose membranes (GE Life Science, Amersham) and membranes were stained with PonceauS to verify transfer and protein loading. Membranes were blocked overnight in Odyssey Buffer (Li-COR; Lincoln, NE) and then probed with primary antibodies to the proteins of interest overnight at 4°C . All antibodies were diluted in a 1:1 solution of Tris buffered saline with Tween (TTBS: 50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6) and Odyssey blocking buffer (Li-COR). Antibody dilutions were as follows: anti-Alpha4 (1:1000), anti-PP2Ac (1:1000), anti-PP4c (1:500), anti-PP6c (1:500), anti-HSP90 (1:1000), and anti-tubulin (1:1000). Membranes were washed 3 times with TTBS then probed with appropriate fluorescently labeled secondary antibodies (LiCOR; diluted 1:20,000 in TTBS) for 30 min. Membranes were washed 3 times then images were obtained using the Odyssey Imaging platform.

3. Results

3.1. Association of Alpha4 with Type 2A phosphatases

To determine the fraction of each phosphatase catalytic subunit (PP2Ac, PP4c, and PP6c) bound to Alpha4 in HEK293T cell lysates, we conducted successive rounds of immunodepletion using an Alpha4-specific antibody. The resulting supernatants were probed for PP2Ac, PP4c, and PP6c (Fig. 1). Levels of PP6c were significantly reduced in the immunodepleted supernatants as compared to controls, while neither PP2Ac nor PP4c

showed any significant reductions in levels following immunodepletion (Fig. 1). These results indicate that a significant fraction of the total cellular pool of PP6c is associated with Alpha4, whereas very small percentages of the cellular pools of PP2Ac and PP4c are associated with Alpha4 under the conditions tested.

3.2. Creation of stable dual promoter cell lines

One of the goals of this study was to efficiently and stably knockdown Alpha4 to enable investigation of long-term effects of Alpha4 repression, and to investigate the ability of Alpha4 and Alpha4 mutants to rescue the knockdown. The first step in this process was to verify the ability of the shRNA to effectively decrease Alpha4 levels. To test this, lentiviral particles expressing either scrambled shRNA or shRNA targeted to the 3'UTR of Alpha4 were used to infect HEK293T, HeLa, and A549 cells, and stable lines were selected using puromycin. The knockdown efficiency of Alpha4 was approximately 85% in the HEK293T and HeLa cell lines and approximately 80% in the A549 cell line (Fig. 2A–C).

With the efficacy of the knockdown established, we created a dual promoter plasmid to allow for simultaneous knockdown of endogenous Alpha4 and expression of Flag-Alpha4. Flag-Alpha4, expressed from a pCMV promoter, was inserted into a PLKO.1-TRC vector containing scrambled or Alpha4-targeted shRNA driven by a U6 promoter. The Alpha4 fragment was cloned from an Alpha4/pcDNA5TO expression vector using PCR primers containing MfeI restriction sites (Fig. 2D). The PCR product was digested with MfeI and ligated into an EcoRI digested PLKO.1-TRC vector, resulting in the dual promoter plasmid (Fig. 2D). These constructs were used to establish stable HEK293T cell lines expressing scrambled shRNA, 3'UTR Alpha4 shRNA, 3'UTR Alpha4 shRNA plus Flag-Alpha4 cDNA, and scrambled shRNA plus human Flag-Alpha4 cDNA. The cDNA construct of Flag-Alpha4 does not contain the native 3'UTR, allowing the 3'UTR directed shRNA to knockdown endogenous Alpha4, while leaving Flag-Alpha4 unaffected. The levels of knockdown appeared equivalent in knockdown and knockdown/expression cell lines (Fig. 2D).

3.3. Alpha4 knockdown and expression differentially effect the PP2Ac family of phosphatases

Using the dual promoter stable cells lines, we investigated the effect of stable knockdown and re-expression of Alpha4 on the expression levels of endogenous PP2Ac, PP4c, and PP6c in HEK293T cells under both standard growth conditions of DMEM supplemented with 10% FBS and after overnight serum starvation. In all cases, levels of phosphatase catalytic subunit are compared relative to levels in the scrambled shRNA control cells. Under standard growth conditions, PP2Ac expression levels were significantly reduced in the Alpha4 knockdown cells compared to scrambled control cells (0.808 ± 0.139 S.D.), with levels completely rescued in the simultaneous knockdown and expression cells (1.154 ± 0.280 S.D.) (Fig. 3A). Knockdown of Alpha4 led to greater reductions in PP6c levels (0.686 ± 0.084 S.D.) which were also rescued with re-expression of Alpha4 (0.888 ± 0.167 S.D.) (Fig. 3C). PP4c levels exhibited a similar decline as PP6c levels in Alpha4 knockdown cells (0.643 ± 0.270 S.D.), but unlike PP2Ac and PP6c re-expression of Alpha did not lead to rescue of PP4c levels (0.511 ± 0.114 S.D.) (Fig. 3B).

Similar results were seen when cells were stressed by overnight serum starvation before harvesting. PP2Ac expression levels in the Alpha4 knockdown cells were still reduced compared to control cells, but to a slightly lesser extent (0.908 ± 0.082 S.D.) (Fig. 3D). The cells stably expressing both the Alpha4 shRNA and exogenous Alpha4 showed an increase in PP2Ac expression levels compared to control cells (1.397 ± 0.356 S.D.) (Fig. 3D). The expression levels of PP6c were reduced in the knockdown cells when serum starved (0.713 ± 0.160 S.D.), and expression of exogenous Alpha4 completely rescued the effects of knockdown (0.964 ± 0.264 S.D.) (Fig. 3F). PP4c also showed greatly reduced expression in the Alpha4 knockdown cells (0.624 ± 0.230 S.D.), but this could not be rescued by expression of exogenous Alpha4 (0.591 ± 0.335 S.D.) (Fig. 3E).

4. Discussion

Knockout of Alpha4 causes dramatic reductions in expression levels of all three Type 2A phosphatases, leading to the hypothesis that Alpha4 plays a role in stabilizing nascent catalytic subunit and allowing for proper folding [14]. This hypothesis is consistent with observations from recently determined crystal structures showing Alpha4 bound to partially unfolded PP2Ac [17]. If Alpha4 is a protein specific foldase, it would explain the dramatic effects of Alpha4 knockout on the expression of phosphatase catalytic subunits, independent of the fraction of phosphatase associated with Alpha4. It also follows from this hypothesis that only a small amount of Alpha4 would be necessary to stabilize levels of phosphatase expression. The results in our experiments are consistent with these previous results and hypotheses in that they demonstrate that knockdown of Alpha4 has a less dramatic effect upon phosphatase levels than knockout of Alpha4. However, knockdown of Alpha4 allowed the discovery of differences in the effects Alpha4 has on the three phosphatases.

Only PP6c showed significant specific interaction with Alpha4 in the immunodepletion experiments, suggesting that knockdown of Alpha4 would have the most significant impacts upon PP6c expression. Consistent with this observation, Alpha4 knockdown greatly decreased expression of PP6c and re-expression rescued these effects. Although, the immunodepletion experiment did not detect a significant fraction of PP2Ac associated with Alpha4, knockdown of Alpha4 did lead to decreased PP2Ac expression levels. The effects of Alpha4 on PP4c expression were the most complex. Knockdown of Alpha4 had a very substantial negative effect on PP4c expression level, even though little interaction between Alpha and PP4c was observed in the immunodepletion experiment. In addition, expression of Flag-Alpha4 was unable to rescue decreased expression.

A confounding observation from the immunodepletion experiment was the high level of non-specific binding for PP4c within the experiment, whereas PP2Ac showed negligible levels of nonspecific binding (Fig. 1B,C.) These differences in nonspecific binding indicate a fundamental difference between these closely related proteins. The high levels of nonspecific binding could indicate that PP4c has a propensity to partially unfold under the conditions tested, thus increasing the likelihood of nonspecifically binding to the resin. As recent crystal structures reveal that Alpha4 binds to partially unfolded catalytic subunits and stabilizes them, this increased propensity for unfolding could account for the greater effect of Alpha4 knockdown on PP4c expression than hypothesized from the immunodepletion

experiment. The inability of expression of Flag-Alpha to rescue PP4c expression is perplexing, as it was able to rescue both PP2Ac and PP6c expression levels. A plausible explanation is that the Flag-Alpha4 is inappropriately localized leading to alterations in complex formation and interactions with PP4c.

Most studies on Alpha4 have focused on its interactions with PP2Ac and have attributed alterations in cellular functions to changes in PP2Ac expression or activity. This study indicates that Alpha4 plays a significant role in regulating the closely related Type 2A phosphatases, PP4c and PP6c, and these should be considered when investigating the effects of Alpha4 on cellular functions. This work also highlights differential effects of Alpha4 on the three Type 2A phosphatases (PP2Ac, PP4c and PP6c), which should be considered when investigating the cellular effects of Alpha4. In addition, in creating the dual promoter knockdown/expression system, we established a simple and efficient means to test the effects of Alpha4 mutations in the absence of endogenous Alpha4.

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Fig. 1. Association of phosphatase catalytic subunits with Alpha4.

Cell lysates were cleared by centrifugation and the cleared supernatants were split into equal aliquots for immunodepletion experiments. Supernatants were incubated with either 20 μ l Protein A resin alone or with Alpha4 antibody (1:100 dilution) and 20 μ l Protein A resin. Two successive rounds of immunodepletion were conducted and aliquots were removed after centrifugation for analysis. Samples were separated via SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. A) Western blots of input and supernatants from successive rounds of immunodepletion probed for Alpha4, tubulin, PP2Ac, PP4c, and PP6c. B–D) Graphs show % of phosphatase catalytic subunit depleted from the supernatant as compared to input levels ($((\text{Input-Sup})/\text{Input}) \times 100$) for both control (Protein A resin alone) and immunodepletion conditions (Protein A resin + Alpha4 antibody), as quantified using Odyssey Imaging Software (Li-COR). A total of 3 independent experiments were conducted. Statistical significance was calculated using two-way RM ANOVA analysis. Graphs show mean and individual data points. *, $p < 0.05$.

Fig. 2. Creation of stable dual promoter cell lines.

A–C) Graphs showing Alpha4 expression of KD cell lines relative to scrambled control shRNA in HEK293T (A), HeLa (B), and A549 (C). At least 3 independent experiments were used for quantifications. Graphs show mean $\pm$ SD. Statistical significance was calculated using one-sample *t*-test against a hypothetical mean of 1. **, $p < 0.001$; ***, $p < 0.0001$. Representative western blots of cell lysates from HEK293T (A), HeLa (B), and A549 (C) stable cells expressing either scrambled shRNA or 3' UTR targeted shRNA probed for Alpha4 and HSP90 (load control) are shown in the insets. D) Schematic representation of creation of simultaneous knockdown and expression vector using PLKO.1-TRC as a backbone. U6, RNA promoter; cPPT, central polypurine tract, Puro R, Puromycin resistance gene; EcoRI, EcoRI restriction site; pCMV, CMV promoter. Representative western blot showing Alpha4 expression profiles in stable cell lines expressing scrambled shRNA (SCR), scr shRNA + Flag-Alpha4 (OE), 3' UTR shRNA + Flag-Alpha4 (RES), 3' UTR shRNA (KD) shown on right.

Fig. 3. Effects of stable Alpha4 knockdown and expression on Type 2A family phosphatase expression.

HEK293T cells were grown in DMEM + 10% FBS (A,B,C) or subjected to overnight serum starvation before being lysed (D,E,F). Cell lysates were then cleared by centrifugation and the cleared lysates separated by SDS-PAGE and transferred to nitrocellulose. A, D) Representative western blots probed for PP2Ac, Alpha4, and HSP90 (loading control) (left). Quantification of PP2Ac expression levels relative to scrambled control cells (right). B, E) Representative western blots probed for PP4c, Alpha4, and HSP90 (loading control) (left). Quantification of PP4c relative to scrambled control cells (right). C, F) Representative western blots probed for PP6c, Alpha4, and HSP90 (loading control) (left). Quantification of PP6c expression levels relative to scrambled control cells (right). At least 5 independent

experiments were performed for all analyses. Expression levels of phosphatase catalytic subunits were normalized using specified protein loading control. Relative expression levels of phosphatase catalytic subunits in the knockdown and knockdown/expression cell lines compared to control cells were calculated for each independent experiment. Graphs show mean $\pm$ SD and individual data points. Statistical significance was calculated using one sample t-tests against a hypothetical mean of 1 (as the control cells have a value of 1). *, $p < 0.05$; **, $p < 0.01$.

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