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

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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.

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1. Background

Alpha4 is a highly conserved protein with similarity to Tap42 from *Saccharomyces cervisiae* [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].

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 Alpha4 C-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

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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 overexpression 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 Mfe1 restriction site for ligation into thePLKO.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 Pittsburg Medical Center). All cell lines were grown in DMEM supplemented with 10% FBS fetal bovine serum and incubated at 37 °C in 5% CO_2.

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 Na_3VO_4, 30 mM NaF, 20 mM $Na_4O_7P_2$, 60 mM β-glycerophosphate disodium, pH 7.2). Cells lysates were clarified by centrifugation at 17,000*g* 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 1400*g* 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 1400*g* 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 1000*g* 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 μg/ml (A549), 3 μg/ml (HEK293T), and 1 μg/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× 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 17,000*g* 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 μg 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

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