Cancer-Associated PP2A Aα Subunits Induce Functional Haploinsufficiency and Tumorigenicity

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Abstract

The introduction of SV40 small t antigen or the suppression of PP2A B56γ subunit expression contributes to the experimental transformation of human cells. To investigate the role of cancer-associated PP2A Aα subunit mutants in transformation, we introduced several PP2A Aα mutants into immortalized but nontumorigenic human cells. These PP2A Aα mutants exhibited defects in binding to other PP2A subunits and impaired phosphatase activity. Although overexpression of these mutants failed to render immortalized cells tumorigenic, partial suppression of endogenous PP2A Aα expression activated the AKT pathway and permitted cells to form tumors in immunodeficient mice. These findings suggest that cancer-associated Aα mutations contribute to cancer development by inducing functional haploinsufficiency, disturbing PP2A holoenzyme composition, and altering the enzymatic activity of PP2A. (Cancer Res 2005; 65(18): 8183-92)

Introduction

The introduction of the SV40 early region (SV40 ER), the telomerase catalytic subunit (hTERT), and an oncogenic allele of H-RAS transforms many types of human cells (1, 2). The SV40 ER encodes two oncoproteins, the SV40 large T and small t antigens. Large T contributes to transformation by inactivating the retinoblastoma (pRB) and p53 tumor suppressor proteins (3, 4). SV40 small t forms complexes with and inhibits the serine-threonine protein phosphatase 2A (PP2A; ref. 5), and this interaction also plays a critical role in human cell transformation (4, 6, 7). Recently, we showed that suppressing a specific PP2A subunit, B56γ, converted immortal HEK cells expressing large T, hTERT, and H-RAS (HEK TER cells) into tumorigenic cells (8). These findings suggest that disruption of PP2A AC-B56γ complexes contributes directly to human cell transformation.

PP2A is a family of serine-threonine phosphatases implicated in the regulation of numerous signaling pathways (9). Each PP2A complex is composed of an A subunit bound to a catalytic C subunit and a regulatory B subunit. The PP2A B subunits determine PP2A substrate specificity and subcellular localization (9–11). The PP2A A and C subunits each exist as two isoforms. The B subunit isoforms are classified into four families with alternative nomenclatures: B (also known as B55 or PR55), B’ (also known as B56 or PR61), B’’ (consisting of PR72, PR130, PR59, and PR48), and B”’ (putative; consisting of PR93/S22NA and PR110/Striatin; ref. 12). Each of the B subunits binds to the A subunits to form distinct ABC holoenzyme complexes (13, 14). The diversity of PP2A heterotrimers suggests that particular regulatory subunits mediate specific physiologic functions; however, the roles of specific PP2A heterotrimers in the regulation of cell growth remain undefined.

The two PP2A A subunit isoforms, Aα (PPP2R1A) and Aβ (PPP2R1B), are 86% identical (15). Each isoform consists of 15 nonidentical repeats, and each repeat is composed of two α-helices connected by an intrarepeat loop (16). The B subunits bind within repeats 1 to 10, whereas the C subunit binds to repeats 11 to 15 (16). These PP2A A subunit isoforms exhibit differential expression patterns in normal tissues and in tumor cell lines, as well as different affinities for the PP2A C and B subunits and viral tumor antigens, suggesting that each of these isoforms have unique functions (17). At least four PP2A Aα isoform somatic mutations have been identified in human tumors, including a glutamic acid-to-aspartic acid (E64D) substitution in a lung carcinoma, a glutamic acid-to-glycine substitution (E64G) in a breast carcinoma, an arginine-to-trypothan substitution (R418W) in a malignant melanoma, and a frame-shift mutation at nucleotide position 652 in a breast carcinoma (18). Each of these PP2A Aα mutants are defective in their binding to specific B and/or C subunits (19) but involve only one PP2A Aα allele. In addition to these Aα point mutations, decreased expression of Aα has been reported in 43% of human brain tumors (20) and in the human breast cancer cell line MCF-7 (21).

To determine whether these PP2A Aα subunit mutations contribute to human cell transformation, we examined the effects of overexpressing specific Aα mutants and suppressing endogenous Aα expression on PP2A phosphatase activity, anchorage-independent growth, and tumor formation in immortalized but nontumorigenic HEK TER cells. Here we show that mutants of the PP2A Aα subunit associated with human tumors contribute to transformation by disrupting the constituency of specific PP2A complexes.

Materials and Methods

Plasmids. To create a FLAG epitope-tagged version of PP2A Aα, we did PCR using the pGRE5-2(T)63 vector (generously provided by D. Pallas, Emory University), the sense oligonucleotide GGGCGCGGATCCATGGACGACGACGACGACGCAGACGACGACGAC, and the antisense oligonucleotide GCCGGCGAATTCTCAGGCGAGAGACAGAAACGCGGCGGCCGACGGCGACGAC, and the

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the QuickChange Multi Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The B55α-specific (SHB55α) and B56γ-specific (SHB56γ) short hairpin RNAs (shRNAs) have been described (8). The vectors pMKO.1-puro-SHα and pMKO.1-puro-SHα1 were generated by introducing oligonucleotides corresponding to nucleotides 1798 to 1818 and 300 to 320 of PP2A α followed by a 6-bp loop and the corresponding antisense sequence, followed by five thymines into pMKO.1-puro (23). The FLAG-epitope tagged versions of the wild-type or mutant PP2A α subunits resistant to the PP2A α-specific shRNA (SHαα) were generated by site-directed mutagenesis and cloned into pmEG to create pmEG-RAα, pMIF-RE64D, pMIF-RE64G, and pMIG-RAβ56-4B1W.  

**Retroviral infections.** Wild-type and mutant versions of PP2A α were introduced into HEK cells expressing large T, hTERT, and H-RAS (HEK TER) using amphotrophic retroviruses to generate the cell lines HEK αα, E64D, E64G, and R418W as described (4). HEK cells expressing PP2A α-specific constructs are named HEK followed by the particular expression construct. HEK Lowα1 cells were generated by infecting HEK TER cells with SHαα-containing retrovirus produced from 5 × 10^5 293T packaging cells. To generate cells in which PP2A α was suppressed, HEK TER cells were infected with viral supernatants containing increasing titers of the SHA α allele, we expressed versions of wild-type and mutant PP2A α resistant to this shRNA vector in HEK Lowα1 cells, which yielded HEK Rαα-Lowα1, RE64D-Lowα1, RE64G-Lowα1, and R418W-Lowα1 cells. The HEK RAα-Lowα5, RE64D-Lowα5, RE64G-Lowα5, and R418W-Lowα5 cell lines were generated similarly.  

**Immunoblotting and immunoprecipitation.** Cells were suspended in 50 mM/L Tris-HCl (pH 7.5), 150 mM/L NaCl, 1 mM/L EDTA, and 0.5% NP40. Soluble proteins (100 μg) were subjected to 10% SDS-PAGE before immunoblotting. To detect c-Myc, we lysed cells directly on plates using 2× SDS buffer. The antibodies used included introduced PP2A α α (Covance, Richmond, CA); αα (BD Biosciences, San Diego, CA), B56α (Upstate, Lake Placid, NY); B56γ and c-Myc (9E10; Santa Cruz Biotechnology, Santa Cruz, CA); FLAG M2, FLAG M5, and β-actin (Sigma-Aldrich Co., St. Louis, MO); and mitogen-activated protein kinase (MAPK), phosphorylated MAPK (Thr187/Tyr202), AKT, and phosphorylated AKT (Ser473; Cell Signaling, Beverly, MA). Affinity-purified polyclonal antibodies were raised against B56α, B56γ, B56δ, and SV40 small t peptides as described (8). The B56γ polyclonal antibody was obtained by immunizing rabbits with a peptide (ELKRGLRRDGIIPT) corresponding to amino acids 454 to 467. For immunoprecipitation, cells were lysed in a 0.3% CHAPS lysis buffer. Cell lysates (2-5 μg) were incubated with the FLAG M5 or A α peptide (ELKRGLRRDGIIPT) antibody overnight at 4°C. The protein G beads were eluted in 2× SDS sample buffer followed by SDS-PAGE and immunoblotting.  

**Phosphatase activity.** The protein phosphatase activity in PP2A α or FLAG immune complexes was determined as described (8).  

**Cell cycle and apoptosis analysis.** Cells were fixed in 70% ethanol overnight and stained with propidium iodide (PI), and cell cycle distribution was determined using flow cytometry. To detect apoptotic cells, attached and floating cells were harvested, washed with PBS and resuspended in binding buffer containing FITC-conjugated Annexin V (Calbiochem, La Jolla, CA). Apoptotic cells were quantitated by fluorescence-activated cell sorting and analyzed by CellQuest software.  

**Anchorage-independent growth and tumor formation.** For anchorage-independent growth, 10^5 cells were plated in triplicate and incubated in triplicate for 0.4% Noble agar supplemented with 10% heat-inactivated fetal bovine serum. Anchorage-independent colonies were counted using a microscope 4 weeks after seeding (10-fold magnification). For tumorigenicity assays, 2 × 10^4 cells were injected s.c. into immunodeficient mice as described (1). The number tumors formed was determined 40 days after injection. BALB/cAnNcFox1m12m1 mice were purchased from Taconic (Albany, NY).  

**Proliferation assays.** To measure cell proliferation, 1 × 10^4 cells were plated in triplicate, and the cells were harvested at 24, 48, 72, and 96 hours. The number of viable cells was determined using a Z2 Particle Count and Size Analyzer (Beckman-Coulter, Miami, FL). For population doubling experiments, a seeding density of 1 × 10^3 cells in 10-cm plates was used. Triplicate plates were counted every 4 days.  

**Results**  

**Effects of expressing cancer-associated PP2A α mutants.** The reported cancer-associated PP2A mutations involve the αα and ββ/γγ subunits (18, 24–26) and affect only one αα allele. To determine whether these tumor-associated PP2A α mutants contribute to the transformed phenotype in a dominant manner, we generated FLAG epitope-tagged versions of several PP2A α mutants and introduced them into HEK TER cells, creating HEK TERβ (control vector), αα (wild type), E64D, E64G, and R418W cell lines. We detected these introduced PP2A α mutants using both FLAG epitope- and αα-specific antibodies (Fig. IA, top). In cells expressing the PP2A α mutants, we noted that the expression of PP2A β55α, B56γ, and Cα subunits was up-regulated 38%, 53%, and 44%, respectively, whereas the expression of B56γ remained unchanged. Because prior studies indicated that these αα mutants fail to bind other PP2A subunits in vitro (19), we examined whether these αα mutants retained the ability to bind the B and C subunits. When we isolated immune complexes using an antibody specific for the FLAG epitope tag, we found that the FLAG epitope-tagged, wild-type PP2A α bound Cα and each of the B subunits (Fig. IA, bottom). In contrast, whereas immune complexes formed with the E64D mutant contained similar amounts of the PP2A Cα, B56β, and B56c subunits, we found decreased amounts of the B55α subunit and did not detect the B56αc, B56γ, and B56δ subunits. The E64G α mutant exhibited decreased binding to PP2A αα and B56c subunits and failed to coprecipitate the B55c, B56c, B56β, B56γ, and B56δ subunits, whereas R418W mutant immune complexes contained decreased amounts of the B55α subunit and undetectable levels of the B56δ and Cα subunit (Fig. IA, bottom).  

We speculate that the partial stabilization of PP2A β55α, B56c, and Cα induced by the PP2A αα mutants (Fig. IA) may be due to residual subunit binding of these mutants or to an altered equilibrium between endogenous αα and the other β family subunits. These findings corroborate prior in vitro studies (19) and show that these αα mutants have functional defects forming PP2A complexes in human cells.  

We next examined the effect of expressing these PP2A α mutants on PP2A αα-attributable phosphatase activity. We found that FLAG immune complexes containing mutant E64D possessed 48% of the phosphatase activity of complexes containing wild-type αα, whereas mutants E64G and R418W showed no mutant-specific activity (Fig. IB, black columns). Thus, these mutants exhibit markedly deficient phosphatase activity. We also assessed the phosphatase activity detected in PP2A αα immune complexes, which represents the activity of both endogenous and introduced PP2A αα subunits. Compared with cells overexpressing the wild-type PP2A αα subunit, we detected 24%, 48%, and 54% less total PP2A activity in cells expressing the PP2A αα mutants E64D, E64G, and R418W, respectively (Fig. IB, gray columns). However, when compared with cells expressing control vector, total PP2A activity was increased 86% and 38% in cells expressing wild-type αα and mutant E64D, respectively, but no changes were found in cells expressing mutants E64G or R418W (Fig. IB, gray columns). Thus, whereas these PP2A αα mutants exhibit impaired PP2A activity and binding of C and B subunits, the introduction of αα mutants did not inhibit endogenous PP2A phosphatase activity.
Consistent with these observations, we found that introduction of either wild-type Aα or mutant E64D, E64G, or R418W alleles failed to affect short- or long-term cell proliferation rates (Fig. 1C). Moreover, we failed to observe anchorage-independent growth or tumor formation in these cell lines (Fig. 1D). Thus, these Aα mutants not only fail to form functional PP2A complexes but also fail to transform HEK TER cells, which express normal levels of the wild-type Aα subunit.

**Effects of suppressing PP2A Aα expression.** Because these cancer-associated PP2A Aα mutants failed to transform human cells, we speculated that such mutants might act as nonfunctional alleles that decrease the overall level of functional PP2A Aα. To assess the effect of suppressing Aα subunit expression on cell transformation, we generated a vector that drives the expression of a shRNA that targets PP2A Aα (SHAα) or a control vector encoding a shRNA specific for GFP (SHGFP). By infecting cells with increasing titers of retroviruses expressing SHAα or a control vector encoding a shRNA for GFP, we succeeded in generating a stable cell line expressing a range of Aα protein level (52%, 48%, 37%, 15%, and 16%) of cells expressing a control vector (Fig. 2A). We named those cells as LowAα3 (37%), LowAα4 (15%), and LowAα5 (16%). We found that the level of Aα suppression correlated with a similar level of reduction of Cα, B55α, B56γ, B56δ, and B56ε protein levels (Fig. 2A) as well as with the level of overall PP2A-specific phosphatase activity (Fig. 2B). This coordinate decrease in PP2A subunits upon suppression of Aα suggested that these subunits are unstable when not bound to PP2A Aα.

In parallel, we also generated control cell lines that infected with SV40 small t (TERST), SHB56γ, wild-type Aα, or the PP2A Aα mutants E64D, E64G, or R418W were plated in soft agar and counted 28 days after seeding. For tumor formation, 2 × 10⁴ cells were injected s.c. into immunodeficient mice. Data are reported as number of tumors formed per number of injection sites. Columns, means for three independent experiments; bars, ±SD.

**Figure 1.** Effects of expressing PP2A Aα mutants. A, immunoblot analysis of whole cell lysates derived from HEK TER cells expressing a control vector (TERV), wild-type PP2A Aα, or the Aα mutants E64D, E64G, or R418W using antibodies specific for the FLAG epitope tag Aα, B55α, B56γ, B56δ, and Cα subunits (top). Expression of β-actin is also shown. Immunoprecipitation of FLAG immune complexes was done on cell lysates (2 mg) from the indicated cells and followed by SDS-PAGE and immunoblotting using antibodies specific for PP2A subunits B55α, B56γ, B56δ, B56ε, B56δ, B56ε, and Cα (bottom). B, PP2A-specific phosphatase activity. Cell lysates (0.5 mg) were subjected to immunoprecipitation (IP) with FLAG- and Aα-specific antibodies. PP2A activity from FLAG immune complexes, which represents only introduced PP2A activity (black bars), or Aα immune complexes, which yields total (endogenous and ectopically introduced) PP2A activity (gray bars), was determined by measuring the release of phosphate from the substrate phosphopeptide [RRQ(pT)VA] and defined as pmol free PO₄ per mg of protein over time points indicated (bottom). Points, means for three independent experiments; bars, ±SD. C, effect of introducing wild-type or mutant PP2A Aα subunits on cell proliferation. Population doublings (PD) were calculated over 96 days (top). The doubling time of cells expressing either control vector, wild type PP2A Aα, E64D, E64G, or R418W was 22 ± 1.7, 22 ± 1.8, 21 ± 1.9, 23 ± 0.5, or 23 ± 1.4 hours, respectively.

Consistent with these observations, we found that introduction of either wild-type Aα or mutant E64D, E64G, or R418W alleles failed to affect short- or long-term cell proliferation rates (Fig. 1C). Moreover, we failed to observe anchorage-independent growth or tumor formation in these cell lines (Fig. 1D). Thus, these Aα mutants not only fail to form functional PP2A complexes but also fail to transform HEK TER cells, which express normal levels of the wild-type Aα subunit.

**Effects of suppressing PP2A Aα expression.** Because these cancer-associated PP2A Aα mutants failed to transform human cells, we speculated that such mutants might act as nonfunctional alleles that decrease the overall level of functional PP2A Aα. To assess the effect of suppressing Aα subunit expression on cell transformation, we generated a vector that drives the expression of a shRNA that targets PP2A Aα (SHAα) or a control vector encoding a shRNA specific for GFP (SHGFP). By infecting cells with increasing titers of retroviruses expressing SHAα or a control vector encoding a shRNA for GFP, we succeeded in generating a stable cell line expressing a range of Aα protein level (52%, 48%, 37%, 15%, and 16%) of cells expressing a control vector (Fig. 2A). We named those cells as LowAα3 (37%), LowAα4 (15%), and LowAα5 (16%). We found that the level of Aα suppression correlated with a similar level of reduction of Cα, B55α, B56γ, B56δ, and B56ε protein levels (Fig. 2A) as well as with the level of overall PP2A-specific phosphatase activity (Fig. 2B). This coordinate decrease in PP2A subunits upon suppression of Aα suggested that these subunits are unstable when not bound to PP2A Aα.

In parallel, we also generated control cell lines that infected with increasing amounts of the SHGFP vector (SHGFP cells). No differences in cell proliferation or anchorage-independent growth were found among these SHGFP-expressing cells (data not shown). In contrast, we observed that cells expressing low levels of PP2A Aα (LowAα4 and LowAα5 cells) proliferated poorly. To determine whether these cells were growth arrested, we analyzed the cell cycle distribution of such cultures. We found that a high percentage of LowAα5 cells were arrested in G1 and that a significant percentage of these cells (21%) exhibited a sub-G1 peak of DNA content (Fig. 2C, left), suggesting that such cells were apoptotic. We confirmed that this population represented apoptotic cells by costaining cells with Annexin V and PI (Fig. 2D). Under these
conditions, we found that 19% of the LowAα5 cells stained for Annexin V, whereas only 4% of SHGFP and LowAα1 cells stained for Annexin V (Fig. 2D). Furthermore, the three cell lines in which PP2A Aα levels were suppressed below 37% of normal levels (LowAα3, LowAα4, and LowAα5) showed a decreased rate of cell proliferation (Fig. 3A), failed to grow in an anchorage-independent manner and were unable to form tumors in immunodeficient hosts (Fig. 3B). Together, these findings indicate that suppression of PP2A Aα to levels less than one third of wild-type levels leads to apoptosis.

In contrast to HEK LowAα5, cells in which PP2A Aα was suppressed to ~50% of wild-type levels proliferated 21% (LowAα1) and 22% (LowAα2) faster than control cells (Fig. 3A). Such cells also grew in an anchorage-independent manner and formed tumors in immunodeficient mice (Fig. 3B), indicating that partial suppression of PP2A Aα induces functional consequences similar to that observed after the introduction of small t or the suppression of PP2A B56y. To eliminate the possibility that the transformed phenotype of LowAα1 and LowAα2 cells was due to off-target effects of RNA interference, we generated a second PP2A Aα-specific shRNA (SHAα1) that targets a different region of the PP2A Aα transcript. When introduced into HEK TER cells, the SHAα1 vector suppressed PP2A Aα levels by 51% (Fig. 3C), and such cells also grew in soft agar (Fig. 3D). These observations show that the degree of PP2A Aα subunit suppression induces different functional outcomes.

Cancer-associated PP2A Aα mutants fail to reverse Aα suppression-induced tumorigenicity. To investigate whether PP2A Aα levels were directly responsible for the tumorigenic phenotype, we examined whether restoring Aα in suppressed cells could reverse the transformed phenotype by generating an allele of PP2A Aα that is resistant to the effects of PP2A Aα-specific shRNA (SHAα1) that targets a different region of the PP2A Aα transcript. When introduced into HEK TER cells, the SHAα1 vector suppressed PP2A Aα levels by 51% (Fig. 3C), and such cells also grew in soft agar (Fig. 3D). These observations show that the degree of PP2A Aα subunit suppression induces different functional outcomes.
the PP2A α mutants RE64D, RE64G, and RR418W (data not shown).

The SHAα and SHGFP control vectors were introduced into HEK TER cells expressing the shRNA-resistant versions of wild-type or mutant PP2A α. The HEK TER cell lines expressing the control SHGFP were named HEK RAα-SHGFP, RE64D-SHGFP, RE64G-SHGFP, and RR418W-SHGFP, and cell lines expressing SHαα and therefore approximately half of the levels of endogenous PP2A α were named HEK RAα-LowAα1, RE64D-LowAα1, RE64G-LowAα1, and RR418W-LowAα1. We confirmed that these cell lines expressed decreased levels of endogenous PP2A α yet expressed high levels of the introduced wild-type or mutant PP2A α (Fig. 4D). We also created cell lines under conditions where only 16% of the endogenous PP2A α is present (HEK RAα-LowAα5, RE64D-LowAα5, RE64G-LowAα5, and RR418W-LowAα5).

Introduction of PP2A RAα or the RE64D mutant partially reversed the cell growth arrest observed in HEK LowAα5 cells, whereas the RE64G and RR418W mutants failed to rescue this cell growth arrest (Fig. 4C). In cells expressing 50% of endogenous PP2A α levels (LowAα1 cells), expression of RAα inhibited cell proliferation and reversed the ability of such cells to grow in soft agar or form tumors (Fig. 4C–D). These findings support the view that the effects observed in cells expressing PP2A α-specific shRNA are not the result of off-target effects of RNA interference. In contrast, the RE64D, RE64G, and RR418W mutants failed to exhibit any inhibitory effects on cell proliferation or transforming activity (Fig. 4C–D). Moreover, expression of wild-type RAα restored the levels of B55α, B56γ, B56ε, and Cα subunits in cells expressing 50% of the endogenous levels of ααα (Fig. 4D). Introduction of the RE64D mutant restored the expression levels of PP2A α and B56ε but failed to stabilize the expression of the other PP2A B subunits, whereas the mutants RE64G and RR418W failed to restore the levels of PP2A C or any of the B subunits examined (Fig. 4D).

These observations confirm that these PP2A α mutants are nonfunctional alleles and that partial depletion of PP2A α levels contributes directly to transformation.

Loss of PP2A AC-B56γ complexes in cells expressing haploinsufficient levels of ααα. We previously showed that perturbation of PP2A complexes containing the B56γ subunit correlates with cell transformation (8). To investigate whether suppression of PP2A ααα also altered PP2A B56γ complexes, we isolated PP2A α immune complexes and analyzed their composition. Whereas we detected the same amount of PP2A B55α, B56α, B56δ, and B56ε subunits bound to ααα in cells expressing 50% of endogenous PP2A α (LowAα1) compared with control cells, we failed to find AC-B56γ complexes in either LowAα5 or LowAα1 cells even when twice as much cell lysate was analyzed (Fig. 5B, left). Moreover, when we analyzed tumorigenic HEK cells expressing PP2A ααα mutants in the setting of partially suppressed PP2A ααα (Fig. 5A, right), we found that the PP2A AC-B56γ holoenzyme was also absent in PP2A α immune complexes (Fig. 5B, right). These results are consistent with the notion that haploinsufficient PP2A α levels create competition among PP2A B subunits for the

Figure 3. Effects of PP2A ααα suppression on cell transformation. A, cell proliferation. Long-term proliferation over 96 days (left) and short-term proliferation over 96 hours (right) for the indicated cell lines. Points, means; bars, ± SD. B, anchorage-independent growth and tumor formation. Columns, means; bars, ± SD. C, immunoblot analysis of PP2A subunits (ααα, B55α, B56γ, B56ε, and Cα subunits) in cells expressing a second PP2A ααα-specific shRNA (SHAα1). D, anchorage-independent growth of TERSHAα1 cells. Columns, means; bars, ± SD.
available Aα subunits, which results in loss of PP2A complexes containing B56γ subunit.

**Activation of AKT in tumorigenic cells.** Because the AKT pathway has been implicated in small t–mediated transformation (27, 28), we analyzed the status of AKT phosphorylation in tumorigenic cells expressing reduced levels of the PP2A Aα or B56γ subunits. We found that the basal levels of phosphorylated AKT (Ser473) in cycling cells were elevated 4.3-, 7.5-, and 5.9-fold in LowAα1, SHB56γ, and TERST cells, respectively, compared with control or nontumorigenic cells (Fig. 6). Moreover, we observed phosphorylated AKT in cells expressing mutant PP2A Aα along with reduced endogenous Aα subunit (LowAα1 background, Fig. 6), yet failed to detect phosphorylated AKT in cells expressing either wild-type PP2A Aα or a PP2A Aα allele resistant to SHAα (RAα), cell proliferation. PP2A Aα alleles resistant to SHAα (RAα, RE64D, RE64G, and RR418W) or a control vector (pMIG) were introduced into HEK TER cells. Endogenous PP2A Aα was suppressed by introduction of different retroviral titers of SHAα or control vector (SHGFP), generating the cell lines indicated. Cells (1 x 10⁴) were plated and counted at the indicated time points. Points, means for three independent experiments; bars, ± SD. D, immunoblot analysis of cells coexpressing RAα, RE64D, RE64G, or RR418W and SHAα or control vector with antibodies specific for PP2A Aα, B55γ, B56γ, B56ε, and Cα (top). Anchorage-independent growth and tumor formation in nude mice (bottom). Endogenous PP2A Aα is the lower band detected by the PP2A Aα-specific antibody. Columns, means for three independent experiments; bars, ± SD.

**Figure 4.** PP2A Aα mutants fail to reverse the transforming potential induced by PP2A Aα suppression. A, generation of silent mutations in the PP2A Aα sequence targeted by SHAα. The wild-type PP2A Aα subunit sequence GACAAGACACCTTGCAAGGT, which corresponds to nucleotides 1798 to 1818, were substituted with GACAAATAGTACGTTACAAAG (mutated bases are underlined). B, immunoblot analysis of PP2A Aα expression in 293T cell lysates coexpressing SHAα and either wild-type PP2A Aα or a PP2A Aα allele resistant to SHAα (RAα), C, cell proliferation. PP2A Aα alleles resistant to SHAα (RAα, RE64D, RE64G, and RR418W) or a control vector (pMIG) were introduced into HEK TER cells. Endogenous PP2A Aα was suppressed by introduction of different retroviral titers of SHAα or control vector (SHGFP), generating the cell lines indicated. Cells (1 x 10⁴) were plated and counted at the indicated time points. Points, means for three independent experiments; bars, ± SD. D, immunoblot analysis of cells coexpressing RAα, RE64D, RE64G, or RR418W and SHAα or control vector with antibodies specific for PP2A Aα, B55γ, B56γ, B56ε, and Cα (top). Anchorage-independent growth and tumor formation in nude mice (bottom). Endogenous PP2A Aα is the lower band detected by the PP2A Aα-specific antibody. Columns, means for three independent experiments; bars, ± SD.
levels in other cell lines lacking PP2A complexes containing PP2A B56γ. Together, these results suggest that cell transformation induced by PP2A dysfunction involves the activation of the AKT pathway.

Discussion

Several lines of evidence now indicate that the transformation of human cells by the SV40 ER requires the interaction of the small t oncoprotein with the PP2A family. We previously showed that disruption of PP2A complexes containing the B56γ subunit by small t alters PP2A activity and cooperates with large T, hTERT, and H-RAS to convert human cells to tumorigenicity. Using PP2A Aα mutants and RNA interference, we now show that cancer-associated Aα mutations contribute to cell transformation by functionally rendering cells deficient in Aα, and in turn, altering the composition of PP2A complexes.

Although abundant evidence supports the notion that small t plays an important role in experimental human cell transformation (4, 7, 30), the relevance of the interaction of small t with PP2A to spontaneous tumor development was unclear. Mutations in the PP2A Aα and Aβ subunits and reduction of Aα expression have been reported in some human cancers (18, 20, 24–26), but the contribution of these alterations to transformation had not been examined. Here we focused on the roles of PP2A Aα mutations in human cell transformation. Because human cancer-associated Aα mutations occur predominantly in the presence of an intact wild-type allele, we determined whether these Aα mutations are directly oncogenic. However, we failed to observe transforming activity when we introduced these PP2A Aα mutants into immortal HEK TER cells, and the introduction of such mutants failed to decrease overall cellular PP2A-attributable phosphatase activity. Thus, it is unlikely that these PP2A Aα mutants act as oncogenic or dominantly interfering mutants.

Because we confirmed that these cancer-associated PP2A Aα mutants are deficient in their ability to bind the B and/or C subunits, we speculated that these PP2A Aα mutations act as inactive alleles and thereby reduce the levels of functional Aα protein. To test this hypothesis, we suppressed endogenous PP2A Aα expression using two different PP2A Aα-specific shRNAs. We found that cells expressing approximately half the level of endogenous PP2A Aα together with large T, hTERT, and H-RAS...
formed colonies in soft agar and tumors as xenografts. We also produced human cells expressing a loss-of-function allele of PP2A Aα in the setting of half the normal level of endogenous PP2A Aα, recapitulating the configuration of the PP2A Aα locus in tumors that harbor such mutations and confirming that such genetic alterations induce cell transformation.

Recent work in animal models suggests that haploinsufficiency of certain loci may also lead to tumorigenesis, suggesting an alternative form of tumor suppression (31). For example, deletion of one allele of the Fbxw7/cdc4, Chk1, and Dmp1 tumor suppressor genes predisposes mice to tumor development (32–34), and Dmp1 (−/−) mice are more sensitive to treatment with carcinogens and γ-irradiation (32). Somatic mutation of PP2A Aα in human tumors occurs primarily in only one allele, and reduced expression of Aα has been observed in human gliomas and MCF-7 cells (20, 21). Thus, our results show that reduction of PP2A Aα gene dosage by suppression of Aα expression or a single PP2A Aα allele mutation creates a functionally haploinsufficient state for Aα that contributes directly to tumorigenicity.

Whereas suppression of endogenous PP2A Aα by 50% leads to cell transformation, further suppression of Aα expression resulted in cell cycle arrest and apoptosis. These findings are consistent with experiments in which PP2A Aα levels were reduced in rat cells (35) or Drosophila S2 cells (36) and are reminiscent of findings in murine embryonic stem cells lacking PP2A Cα (37). In each case, suppression of PP2A Aα or Cα levels induced apoptosis, indicating that a minimal level of PP2A Aα or Cα is required for cell survival. Prior work suggests that PP2A controls apoptosis at several levels (reviewed in ref. 38). For example, PP2A interacts with and dephosphorylates Bcl-2 at Ser70, resulting in inactivation of Bcl-2 (39). In addition, association of the PP2A B56α subunit with Bcl-2 plays a role in ceramide-induced apoptosis (40), and ablation of all PP2A B56 subunits in Drosophila S2 cells leads to apoptosis (36). Our findings imply that loss of PP2A complexes containing Aα and specific B subunits induces apoptosis. At present, the specific PP2A complexes involved in the regulation of apoptosis remain undefined.

Recently, the α4 protein, which binds directly to the PP2A C subunit (41), was shown to play an important role in the control of cell survival. The α4 protein was required for murine embryonic stem cell viability, and loss of α4 resulted in apoptosis (42). Although the binding of α4 protein with the PP2A C subunit results in the displacement of the PP2A A and B subunits (41), the effect of suppressing PP2A Aα on the formation of α4-C complexes remains unclear. However, our observations suggest that α4 fails to prevent apoptosis induced by PP2A Aα depletion.

We also showed that the integrity of PP2A core enzyme is essential for stabilizing the PP2A holoenzyme. Suppression of PP2A Aα subunit expression resulted in the corresponding degradation of Cα and all B subunits examined. The down-regulation of PP2A Cα and other B subunits is due to accelerated protein degradation, because the mRNA level of Cα and other B subunits remained unchanged (data not shown). Our observations suggest that free PP2A C and B subunits including B55α, B56γ, B56δ, and B56ε are unstable in human cells. These observations corroborate similar observations in rat (35) and Drosophila S2 cells (36). Taken together, these experiments suggest that PP2A B subunits exist primarily as part of a heterotrimer with PP2A A and C.

Overexpression of wild-type Aα lead to stabilization of the B55α, B56ε, and Cα subunits. Elevated levels of these subunits were also observed with expression of the Aα mutants, despite the impaired binding properties of the mutants. Residual binding of the E64D and E64G mutants to other B subunits likely accounts for the stabilization observed in HEK E64D and E64G cells. Although we found that the R418W mutant binds only weakly to the B55α subunit and fails to bind the B56ε or Cα subunits, we suspect that this mutant retains binding to either PR72 or PR93/110 family subunits. Such binding may alter the equilibrium among the B subunits and free additional endogenous PP2A Aα subunit to stabilize the B56ε and Cα subunits. However, these PP2A Aα mutants were unable to stabilize the B and C subunits under conditions of partial PP2A Aα suppression, indicating that the equilibrium among PP2A Aα and the other PP2A subunits is disrupted when the expression of PP2A Aα falls below wild-type levels.

The finding that PP2A AC-B56ε complexes were undetectable in cells expressing 50% endogenous Aα levels suggests that functional
haploinsufficiency of α contributes to cell transformation through disruption of B56y-containing complexes. The PP2A B56 family consists of five isoforms (10, 43). Although sharing 68% sequence identity (44), these five PP2A B56 isoforms differ in genomic organization, chromosomal localization, tissue distribution, and developmental regulation (45), suggesting that they mediate different PP2A functions. Under limiting amounts of the PP2A α subunit, we found that competition among the various PP2A B subunits leads to loss of AC-B56 complexes. In addition, we speculate that the loss of PP2A AC-B56 complex also occurs in human cancers that bear α mutations. Because these PP2A α mutations are defective in binding to B56 family members, expression of these PP2A α mutants effectively decreases by half the amount of αc available for PP2A B56y subunit binding. Whereas we had previously established that disruption of PP2A B56 complexes participates in cell transformation (8), the relationship of these findings to human cancers remained unclear. Here we have linked loss of B56y-containing PP2A complexes to cancer-associated PP2A α mutations and suggest that the altered abundance of these B56y complexes accounts for the function of these mutations in tumors.

Our understanding of PP2A function has been guided in part by studies of SV40 small t, which exerts its effects by altering the activity of PP2A, thereby preventing dephosphorylation of multiple protein kinases including MAPK (46, 47) and AKT (27, 28) and inducing c-Myc stabilization (29). A recent study defined a PP2A-dependent role for polyoma small t in disrupting ARF-mediated activation of p53 (48). In addition, SV40 small t induces a conformational change in the PP2A α subunit that mediates PP2A binding to the androgen receptor (49). Although the interaction of small t with PP2A complexes is involved in regulating many signaling pathways, only some of those pathways are responsible for the transforming function of small t. Here we showed that the introduction of small t or suppression of PP2A B56y or α all lead to loss of AC-B56y complexes, constitutive phosphorylation of AKT, and cell transformation, suggesting a common mechanism for PP2A alterations and human cell transformation. Because monoallelic PP2A α mutations occur at low frequency in human tumor samples, these observations provide strong evidence that haploinsufficiency of PP2A α disrupts PP2A complexes containing PP2A B56y and contributes directly to the development of human cancers.

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Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22–24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on “The Effects of Radiation on Aqueous Solutions,” which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is “Physical Measurements for Radiobiology” and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray’s lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, “The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration,” November, 1952, page 891; substitute for the last paragraph:

The data in Table 8 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O2 consumption, it is seen that the amount of glucose “cleavage products” exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if \( M = +0.27 \) and \( L = -0.16 \) and the normal differential is 65 per cent M and 35 per cent L, then

\[
0.65 \times (+0.27) + 0.35 \times (-0.16) = +0.12
\]

a figure identical to the observed +0.12 for normal leukocytes.
Cancer-Associated PP2A Aα Subunits Induce Functional Haploinsufficiency and Tumorigenicity

Wen Chen, Jason D. Arroyo, Jamie C. Timmons, et al.


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