Identification of specific PP2A complexes involved in human cell transformation

Wen Chen,[1,4] Richard Possemato,[1,2,4] K. Thirza Campbell,[1] Courtney A. Plattner,[3] David C. Pallas,[3] and William C. Hahn[1,2,*]

[1]Department of Medical Oncology, Dana-Farber Cancer Institute, Departments of Medicine, Brigham and Women's Hospital and Harvard Medical School, 44 Binney Street, Boston, Massachusetts 02115
[2]Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115
[3]Department of Biochemistry and Winship Cancer Institute, Emory University School of Medicine, Atlanta, Georgia 30322
[4]These authors contributed equally to this work.
*Correspondence: william_hahn@dfci.harvard.edu

Summary

The SV40 small t antigen (ST) interacts with the serine-threonine protein phosphatase 2A (PP2A). To investigate the role of this interaction in transformation, we suppressed the expression of the PP2A B56γ subunit in human embryonic kidney (HEK) epithelial cells expressing SV40 large T antigen, *hTERT*, and *H-RAS*. Suppression of PP2A B56γ expression inhibited PP2A-specific phosphatase activity similar to that achieved by ST and conferred the ability to grow in an anchorage-independent fashion and to form tumors. Overexpression of PP2A B56γ3 in tumorigenic HEK cells expressing ST or human lung cancer cell lines partially reversed the tumorigenicity of these cells. These observations identify specific PP2A complexes involved in human cell transformation.

Introduction

The study of the interactions between DNA tumor virus-encoded proteins and host cell proteins has uncovered a number of intracellular pathways that permit efficient viral replication. Some of these same molecular interactions also program the malignant state in susceptible cells and mimic alterations found in human cancers. In particular, the study of the viral oncoproteins encoded by the SV40 early region (SV40 ER) has revealed critical host cell pathways whose perturbation plays an essential role in the experimental transformation of mammalian cells. Recent work has reinvestigated the roles of two SV40 ER oncoproteins, the large T antigen (LT) and the small t antigen (ST), in human cell transformation (Ali and DeCaprio, 2001; Rundell and Parakati, 2001). Coexpression of these two oncoproteins together with the telomerase catalytic subunit, *hTERT*, and an oncogenic version of the *H-RAS* oncoprotein, suffices to transform human cells (Yu et al., 2001; Hahn et al., 2002). LT inactivates two key tumor suppressor pathways by binding to the retinoblastoma protein (pRB) and p53 (Ali and DeCaprio, 2001). Human cells expressing LT, *hTERT*, and *H-RAS* are immortal; however, such cells fail to grow in an anchorage-independent manner or to form tumors in animals without the additional introduction of ST (Yu et al., 2001; Hahn et al., 2002). These observations reconfirm earlier studies suggesting that ST plays a critical role in human cell transformation (Bikel et al., 1987; de Ronde et al., 1989).

Using mutant versions of ST, several groups have shown that the ability of ST to transform human cells requires interactions with PP2A, a family of abundantly expressed serine-threonine phosphatases (Pallas et al., 1990; Mungre et al., 1994; Rundell and Parakati, 2001; Yu et al., 2001; Hahn et al., 2002). PP2A is a heterotrimer composed of catalytic C subunit, a structural A subunit, and one of several regulatory B-type subunits. Several lines of evidence suggest that the interaction of PP2A with ST results in the displacement of at least one PP2A B subunit and the inhibition of PP2A activity (Pallas et al., 1990; Sontag et al., 1993; Mumby, 1995). However, it remains unclear whether the interaction of ST with PP2A displaces some or all of the B subunits.

Adding to this complexity, the PP2A A and C subunit each exist as two isoforms, and the B subunits fall into four largely unrelated families named B (also known as B55 and PR55), B′ (also known as B56 and PR61), B″ (also known as PR72, PR130, PR59 or PR48) and B‴ (putative; also known as PR93/SG2NA or PR110/Striatin). Each of these B families is composed of

SIGNIFICANCE

The introduction of the SV40 early region, the telomerase catalytic subunit (*hTERT*), and an oncogenic allele of *H-RAS* directly transforms several different types of primary human cells. SV40 ST, which forms complexes with and inhibits PP2A, is one of the SV40 early region oncoproteins that plays a critical role in human cell transformation. Here, we demonstrate that suppressing the expression of a specific PP2A subunit, B56γ, is sufficient to convert immortal HEK cells into cells capable of forming tumors in immunodeficient mice. Moreover, overexpression of PP2A B56γ3 displaces ST and partially reverses ST-induced tumorigenicity. These findings suggest that ST targets PP2A enzymatic complexes containing the B56γ subunit, and that alterations in expression of this subunit contribute to cancer development.

several different members, all of which bind to the A subunit in what is believed to be a mutually exclusive manner to form distinct ABC holoenzyme complexes (Cohen, 1989; Schönthal, 2001; Li and Virshup, 2002).

Several isoforms have been identified within each of the PP2A B subunit families. For example, the PP2A B56γ gene belongs to the PP2A B56 (also called PR61 or B′ subunit) family that consists of α (*PPP2R5A*), β (*PPP2R5B*), γ (*PPP2R5C*), δ (*PPP2R5D*), and ϵ (*PPP2R5E*) isoforms (Csortos et al., 1996; McCright et al., 1996). The PP2A B56γ gene locus resides at 14q32.2 and encodes three differentially spliced variants, PP2A B56γ1, B56γ2, and B56γ3 (Muneer et al., 2002). Each of these alternative splice forms shares the first 12 exons of the PP2A B56γ gene. The longest form, PP2A B56γ3, consists of 14 exons, whereas exon 13 is absent in the medium length form, PP2A B56γ2. The shortest form, PP2A B56γ1, ends in an extra alternatively spliced exon 12a. The PP2A B56γ subunit isoforms are abundantly expressed in human heart and skeletal muscle (McCright and Virshup, 1995) and localize to the nucleus when expressed in green monkey kidney cells (McCright et al., 1996) and murine NIH 3T3 cells (Tehrani et al., 1996). Members of the PP2A B56 family bind the adenomatous polyposis coli (APC) tumor suppressor (Seeling et al., 1999; Polakis, 2000) and cyclin G (Okamoto et al., 1996; Bennin et al., 2002; Okamoto et al., 2002). However, despite these important biochemical and cell biological characterizations, the role(s) of the PP2A B56γ isoforms in human cell transformation remain incompletely understood.

In order to understand the signaling pathways perturbed by the interaction of ST with PP2A, we studied the effects of altering the interaction of particular B subunits with the PP2A A and C subunits. Here, we demonstrate that suppression of the PP2A B56γ subunit mimics the introduction of ST and permits cell transformation. Moreover, expression of wild-type PP2A B56γ3 partially reverses cell transformation in experimental models dependent on ST as well as in human lung cancer cell lines. Taken together, these observations suggest that alterations in PP2A play an important role in malignant transformation.

Results

Effects of ST on PP2A phosphatase activity

In prior studies, we identified a set of genetic elements that, when introduced into primary human cells, converted these cells to tumorigenicity (Hahn et al., 1999). Human embryonic kidney (HEK) cells expressing LT, *hTERT*, oncogenic *H-RAS*, and ST (HEK TERST) form tumors in immunodeficient mice, whereas cells expressing only LT, *hTERT*, and oncogenic *H-RAS* (HEK TERV) are immortal but not tumorigenic (Hahn et al., 2002). Since previous studies had shown that ST interacts with and inhibits PP2A (Pallas et al., 1990; Sontag et al., 1993; Mumby, 1995), we determined whether pharmacologic inhibition of PP2A was sufficient to cooperate with LT, *hTERT*, and *H-RAS* to transform human cells by treating HEK TERV cells with the polyether toxin okadaic acid (OA), a highly selective inhibitor of protein phosphatase type 1 (PP1) and 2A (PP2A). In mammalian cells, PP1 and PP2A account for more than 90% of total protein serine/threonine phosphatase activity (Fernandez et al., 2002). OA inhibits PP2A (IC_{50} 0.2–1 nM) much more potently than PP1 (IC_{50} 20 nM) (Bialojan and Takai, 1988; Cohen, 1989; Fernandez et al., 2002). We determined that treatment of HEK TERV cells with 6 nM OA induced 50% cell death at 24 hr (see Experimental Procedures). Thus, in these studies, we used OA at picomolar concentrations to study the effects of OA on PP2A-specific phosphatase activity, cell proliferation, and anchorage-independent growth.

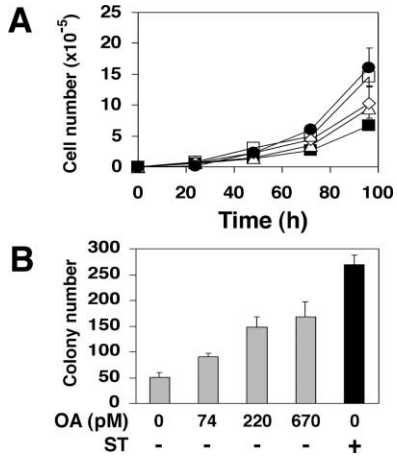

Figure 1. Inhibition of PP2A by okadaic acid (OA) leads to cell transformation

A: OA stimulates HEK TERV cell proliferation. 10^4 cells were treated with OA and counted at the time points shown. OA was used at the following concentrations: 0 (■), 74 pM (△), 220 pM (◇), and 670 pM (□). HEK TERST cells are represented by (●). Data are expressed as the mean ± standard deviation (SD) for 3 experiments.

B: Anchorage-independent growth of OA-treated TERV cells. HEK TERV or HEK TERST cells were seeded into 0.4% Noble agar and treated with OA as indicated. The number of colonies that formed after 28 days is reported as the mean ± SD for 3 experiments. Gray bars represent HEK TERV cells treated with OA. The black bar represents HEK TERST cells. Treatment of HEK TERST with 670 pM OA failed to alter growth of these cells in soft agar.

Subconfluent cultures of HEK TERV cells were treated with OA at concentrations of 74 pM, 220 pM, and 670 pM, and PP2A activity was measured using the consensus peptide RRA(pT)VA. Under these conditions, we detected a dose-dependent reduction in PP2A activity immediately after treatment that persisted for at least 24 hr (data not shown). Correlating with this inhibition of PP2A phosphatase activity, we noted that treatment with these same concentrations of OA led to an increase in the rate of cell proliferation (Figure 1A). Indeed, HEK TERV cells treated with OA at 670 pM proliferated at nearly the same rate as cells expressing ST (TERST). Moreover, treatment of HEK TERV cells with OA conferred the ability to grow in an anchorage-independent fashion (Figure 1B). Taken together, these observations indicate that the pharmacologic inhibition of PP2A leads to similar functional outcomes induced by the expression of ST.

Downregulation of PP2A B56γ in HEK cells

Previous studies suggested that ST displaces the PP2A B55α subunit (Pallas et al., 1990; Sontag et al., 1993; Mumby, 1995). To identify whether ST interacts with specific PP2A heterotrimers in addition to those containing PP2A B55α, we developed antisense RNA reagents to members of the PP2A B55 and B56 families and introduced them into HEK TER cells to create HEK TERAS55α (B55α antisense construct) and HEK TERAS56γ3 (B56γ3 antisense construct) cells. Using primers specific for the antisense constructs, we confirmed that these antisense

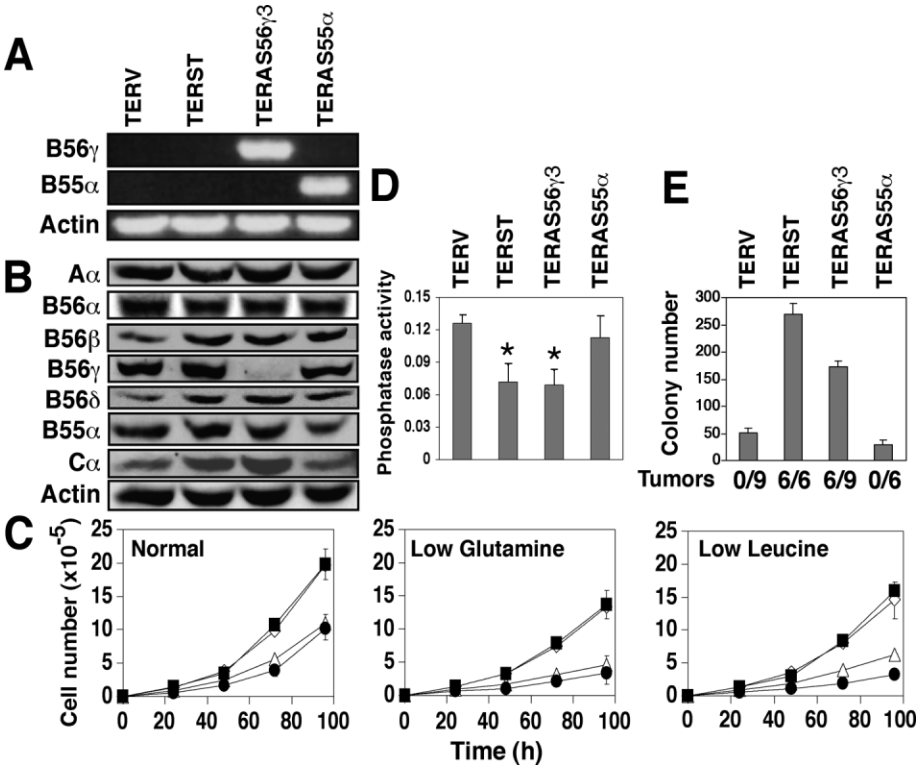

Figure 2. Suppression of PP2A B56γ by PP2A B56γ3 antisense RNA

A: Total RNA was isolated from HEK TERV (control vector), HEK TERST (ST-expressing), HEK TERASB56γ3 (PP2A B56γ3 antisense-expressing), and HEK TERAS55α (PP2A B55α antisense-expressing), and RT-PCR was performed using primers specific for the PP2A B56γ and B55α antisense-RNA constructs. RT-PCR for β-actin confirmed the presence of equal amounts of RNA in each sample.

B: Immunoblotting of whole cell lysates (100 μg) derived from the cells described in **A** performed with antibodies specific for PP2A Aα, B55α, B56α, B56β, B56γ2 and B56γ3 (indicated as B56γ), B56δ, and Cα. Immunoblotting for β-actin confirmed that equal amounts of protein were loaded for each sample.

C: HEK TERV, TERST, TERASB56γ3, and TERAS55α cells were grown in medium supplemented with standard amino acid concentrations (normal), low concentrations of glutamine (0.375 mM), or low concentrations of leucine (80 μM) as indicated. Cells were counted at the indicated time points for HEK TERV (●), TERST (■), TERAS56γ3 (◇), and TERAS55α cells (△). Data are expressed as the mean ± SD for 3 experiments.

D: Serine/threonine phosphatase activity for HEK TERV, TERST, TERAS56γ3, and TERAS55α cells. Protein phosphatase activity was performed on immune complexes isolated by an antibody (6F9) specific for PP2A A. Results are reported as pmol free PO_4 generated/μg protein/min and are expressed as the mean ± SD for 3 experiments. Significant differences (indicated as *) were found in TERST cells ($p < 0.001$) and TERASB56γ3 cells ($p < 0.001$) comparing to TERV cells using student *t* test.

E: For anchorage-independent growth, 10^5 cells were seeded into 0.4% Noble agar and colonies counted after 4 weeks. The mean ± SD for 3 experiments is shown. For tumorigenicity assays, 2×10^6 cells were injected subcutaneously into immunodeficient mice and are reported as number of tumors formed/number of injection sites.

constructs were expressed (Figure 2A). The PP2A B55α antisense construct suppressed B55α protein expression by 40%, while the PP2A B56γ3 antisense construct nearly completely suppressed B56γ protein expression (Figure 2B). Since the antibody specific for B56γ used in these experiments recognizes only the PP2A B56γ2 and B56γ3 isoforms, we were not able to determine whether the B56γ1 isoform was also suppressed. However, since the B56γ1, B56γ2, and B56γ3 splicing variants share exons 1–12, we speculate that introduction of the PP2A B56γ3 antisense RNA also targets and suppresses the B56γ1 isoform.

Since each of the other members of the PP2A B56 family including B56α, B56β, B56δ and B56ε shares approximately 68% amino acid identity with B56γ subunit (McCright and Virshup, 1995), it remained possible that this PP2A B56γ3 antisense RNA also perturbed other PP2A B56 subunits. Using antibodies specific for the PP2A B56α, β, and δ isoforms, we found that the expression of the antisense PP2A B56γ3 RNA failed to alter the expression of PP2A B56α, B56β, or B56δ (Figure 2B). These observations indicate that expression of an antisense RNA complementary to the PP2A B56γ3 subunit specifically suppresses members of the PP2A B56γ family in human cells.

Functional effects of suppressing PP2A B56γ expression

We then examined the effects of suppressing PP2A B56γ expression on PP2A activity, cell proliferation, and cell transformation. We had previously shown that expression of SV40 ST stimulates cell proliferation, confers resistance to nutrient deprivation, and permits anchorage-independent growth and tumor formation in immunodeficient mice (Hahn et al., 2002). Using the HEK TERAS56γ3 cells, we found that suppression of PP2A B56γ by antisense RNA led to an increase in cell proliferation, particularly when cells were cultured under conditions where the concentration of specific essential amino acids was limiting (Figure 2C). When compared to cells expressing a control vector, cells expressing the antisense PP2A B56γ3 RNA grew similarly to cells expressing ST and exhibited the ability to continue to proliferate in low levels of glutamine (0.375 mM) or leucine (80 μM) (Figure 2C). Consistent with these observations, we noted that the phosphatase activity of PP2A immune complexes was inhibited 43% in cells expressing ST and 45% in cells expressing the PP2A B56γ3 antisense RNA (Figure 2D).

To assess the impact of PP2A B56γ subunit suppression in cell transformation and tumorigenicity, we then tested whether these cells were able to grow in an anchorage-independent manner or as subcutaneous tumors in immunodeficient mice. As shown in Figure 2E, HEK TERAS56γ3 cells formed colonies in soft agar and tumors in immunodeficient mice in a manner similar to HEK TERST cells. In contrast, expression of the PP2A B55α antisense RNA showed no effects on PP2A phosphatase activity, cell proliferation, and cell transformation (Figure 2).

In order to further address the specificity of these antisense constructs in suppressing PP2A B55α and B56γ protein levels,

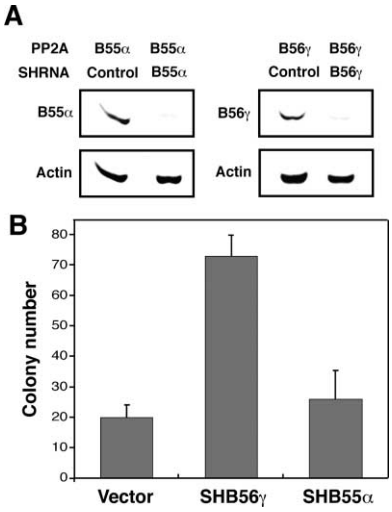

Figure 3. Suppression of PP2A B subunit expression by shRNA

A: Expression of PP2A B55α-specific and B56γ-specific shRNA (SHRNA) in 293T cells expressing the indicated PP2A subunit (PP2A). For each sample, 100 μg of whole cell lysate was separated by SDS-PAGE and immunoblotted with antibodies specific for the indicated proteins.

B: Anchorage-independent growth of HEK TER cells expressing a control vector (Vector), B56γ-specific shRNA (SHB56γ), or PP2A B55α-specific shRNA (SHB55α). The mean ± SD for 3 experiments is shown. HEK TERST cells formed larger colonies than HEK TERSHB56γ cells.

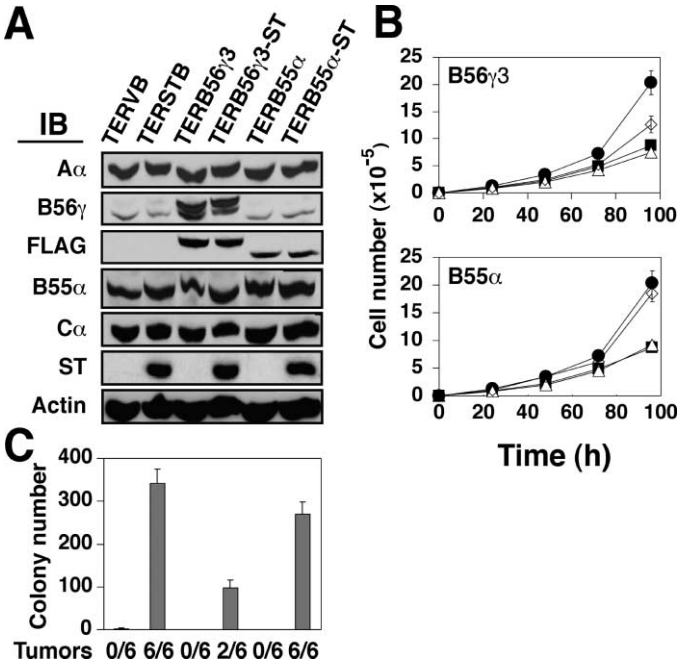

Figure 4. Overexpression of PP2A B56γ3 attenuates ST-induced tumorigenicity

A: Immunoblotting for PP2A Aα, B56γ2, and B56γ3 (showed as B56γ), FLAG, B55α, Cα, ST, and β-actin in HEK TERVB, TERSTB, TERB56γ3, TERB56γ3-ST, TERB55α, and TERB55α-ST cells. V and B refer to control vectors. For these experiments, FLAG-epitope-tagged versions of PP2A B56γ and B55α were used.

B: Cell proliferation for HEK TERVB (■), TERSTB (●), TERB56γ3 (△, upper panel) and TERB55α (△, lower panel), TERB56γ3-ST (◇, upper panel) and TERB55α-ST (◇, lower panel). The data are shown as the mean ± SD for 3 experiments.

C: Anchorage-independent growth and tumorigenicity of the cell lines indicated in **A**. The data are shown as the mean ± SD for 3 experiments. Tumors are reported as number of tumors formed/number of injection sites.

we generated vectors that drive the expression of small hairpin RNAs (shRNA) specific for these genes (Masutomi et al., 2003; Stewart et al., 2003). We confirmed that these shRNAs suppressed the expression of particular PP2A B subunits by introducing these PP2A B55α- and B56γ-specific shRNAs together with either a control vector or an expression vector encoding the corresponding PP2A subunit into 293T cells (Figure 3A). These shRNA vectors were then used to infect HEK TER cells in order to assess cell transformation. HEK TER cells infected with the B55α-specific shRNA (HEK TERSH55α) formed few colonies in soft agar, indistinguishable from those formed by HEK TER cells infected with a control shRNA vector. In contrast, HEK TER cells infected with a vector carrying a PP2A B56γ-specific shRNA (HEK TERSH56γ) formed many more colonies, albeit fewer than formed by HEK TERST (Figure 3B). These observations suggest that expression of ST and loss of B56γ subunit expression have similar biochemical effects on PP2A and induce similar functional consequences.

Introduction of the PP2A B56γ3 subunit attenuates transformation potential

Since we postulated that ST displaces the PP2A B56γ subunit, we attempted to verify the functional interaction of ST and PP2A complexes containing B56γ by overexpressing B56γ3 in HEK TERST cells. After introduction of a retroviral vector encoding a FLAG-epitope-tagged version of PP2A B56γ3, we noted an increase in the expression level of PP2A B56γ3 (Figure 4A). Functionally, the coexpression of PP2A B56γ3 in TERST cells (named TERB56γ3-ST cells) reduced cell proliferation (Figure 4B), anchorage-independent growth, and tumorigenicity in immunodeficient animal hosts (Figure 4C), while coexpression of PP2A B55α in TERST cells (named TERB55α-ST) failed to induce any change in these parameters (Figures 4B and 4C). Taken together, these observations suggest that interactions among ST, PP2A B56γ3, and the PP2A A-C subunits regulate tumorigenic potential in these cells.

In consonance with these functional experiments, we found that expression of ST in HEK 293T cells using adenoviral vectors displaces PP2A B56α, B56γ1, or B56γ3 PP2A AC complexes to a similar degree (Figure 5A). Specifically, expression of ST reduced the amount of C subunit found associated with PP2A B56α, B56γ1, and B56γ3 to 56 ± 11%, 55 ± 17%, and 70 ± 17% of the levels observed in the absence of ST expression, respectively. In addition, the expression of ST induced a small decrease in the expression level of each PP2A B56 subunit, perhaps due to degradation of some displaced B56 subunits. Similarly, we found that the amount of ST associated with the PP2A AC complex was diminished in whole cell lysates derived from HEK TER cells coexpressing PP2A B56γ3 and ST (HEK TERB56γ3-ST cells) (Figure 5B). Moreover, when we isolated immune complexes with an antibody specific for the PP2A A subunit, we noted that overexpression of PP2A B56γ3 or PP2A B55α decreased the amount of ST associated with the PP2A A subunit. Similar results were obtained when we mixed purified proteins in vitro (data not shown). Specifically, in cells expressing both ST and PP2A B55α (HEK TERB55α-ST), we noted a

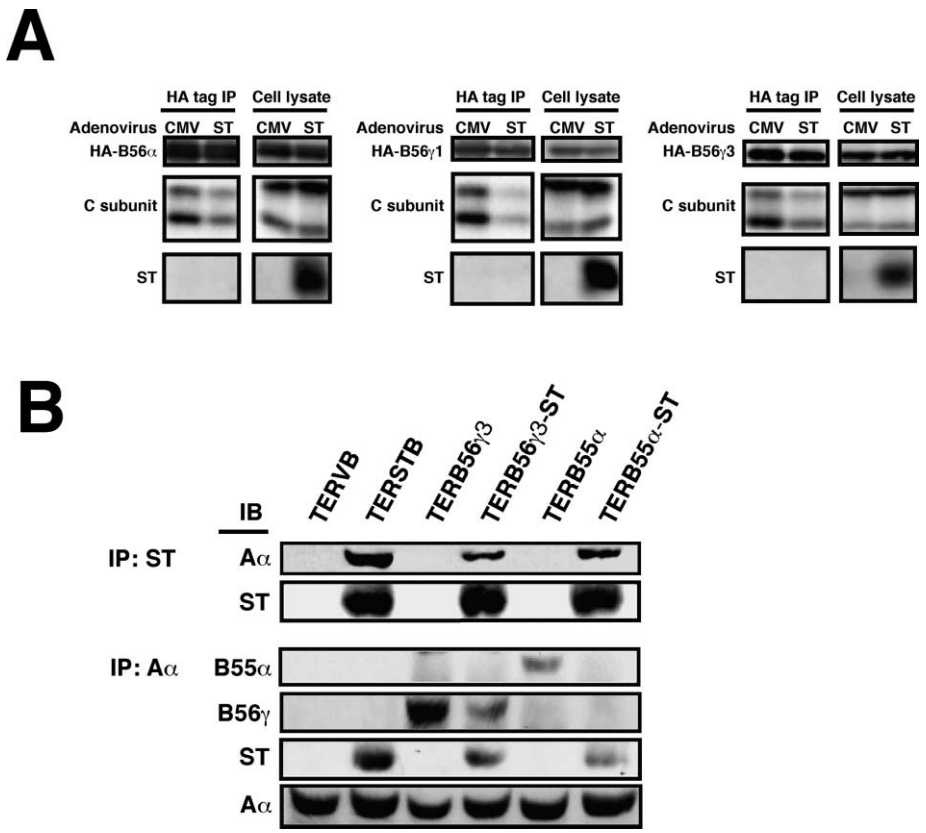

Figure 5. Expression of ST displaces PP2A B subunits

A: Introduction of ST using adenoviral vectors displaces members of the PP2A B56 family from the PP2A A and C subunits. Human 293 cells were transfected with specific HA-epitope-tagged PP2A B56 subunits and then infected 24 hr later with either adenovirus expressing ST (ST) or control adenovirus containing only the CMV promoter (CMV). Immune complexes containing HA-tagged-B56 were isolated and immunoblotted in parallel with whole cell lysates for the presence of HA-tagged-B56 isoforms, associated C subunit, and ST. Expression of ST in human 293 cells reduced the amount of C subunit coimmunoprecipitated with each of the HA-tagged PP2A B56 isoforms tested. Quantitation of 4 separate experiments showed that the introduction of ST decreased the amount of PP2A C subunit found associated with PP2A B56α, B56γ1, and B56γ3 to 56 ± 11%, 55 ± 17%, and 70 ± 17% (mean ± SD) of control levels, respectively. The C subunits migrate sometimes as singlets and sometimes as doublets. This pattern of migration in SDS-PAGE has been noted previously for endogenous and epitope-tagged PP2A C subunits (Campbell et al., 1995; Turowski et al., 1995) and does not appear to be due to degradation.

B: Interactions among ST, PP2A A, C, and B subunits. The indicated cells were lysed, and immunoprecipitation was performed with the ST-specific mAb Pab 430 (top) or the PP2A A subunit-specific mAb 6F9 (bottom). Immunoblotting was subsequently performed with antibodies specific for PP2A Aα, Cα, B55α, B56γ, and ST. Coexpression of PP2A B56γ3 and ST displaced 38% of ST and 63% of B56γ from PP2A Aα, while coexpression of B55α and ST displaced 66% of ST and nearly all of the PP2A B55α from PP2A Aα as determined by densitometry using the amount of PP2A Aα in the immune complex to normalize expression levels. A representative experiment of 3 independent experiments is shown.

nearly complete loss of B55α subunit binding to PP2A A and a 66% decrease in the amount of ST bound to PP2A A. Similarly, in cells expressing both ST and PP2A B56γ3, we noted a 63% decrease in the amount of PP2A B56γ3 bound to PP2A A and a 38% decrease in the amount of ST bound to PP2A A. Taken together, these findings suggest that coexpression of PP2A B56γ3 with ST attenuates the tumorigenic potential induced by ST at least in part by competing with ST for A subunit binding. Moreover, these observations are consistent with the view that although ST competes with several PP2A B subunits for binding to the PP2A AC heterodimer, only a subset of these complexes participate in human cell transformation.

PP2A A subunit mutations are found in a subset of human cancers. Specifically, PP2A Aβ subunit mutations were found in 15% of primary lung tumors, 6% of lung tumor derived cell lines, and 15% of primary colon tumors (Wang et al., 1998), and alterations in both PP2A Aα and Aβ subunit isoforms have been reported in a variety of primary human cancers (Calin et al., 2000). Expression of these PP2A mutants in vitro demonstrated that each of these PP2A A subunit mutants is defective in binding either the PP2A B or the PP2A B and C subunits (Ruediger et al., 2001a, 2001b). These observations suggest that regulation of PP2A activity by loss or mutation of particular A and B subunits may play an important role in tumorigenesis. To address whether alterations of PP2A B subunit expression also occur in spontaneously arising human tumors, we selected 10 lung cancer cell lines and measured the expression of the PP2A Aα, Cα, B56γ, and B55α proteins. Although we did not detect significant alterations in the level of mRNA for each of these PP2A subunits in these lung cancer cell lines using real time RT-PCR as compared to immortalized human airway epithelial cells (Lundberg et al., 2002) (data not shown), we failed to detect the PP2A B56γ proteins in all lung cancer cells (Figure 6A). In contrast, PP2A B56γ3 was readily detectable in HEK TER cells.

To determine whether introduction of the PP2A B56γ subunit affected the tumorigenic phenotype of these lung cancer cell lines, we introduced the PP2A B56γ3 subunit into three lung cancer cell lines (H1395, H1437, H446) by retroviral-mediated gene transfer (Figure 6B). Expression of PP2A B56γ3 suppressed cell proliferation (Figure 6C) and reduced the number of colonies that formed in soft agar (Figure 6D). In contrast, the introduction of the PP2A B55α subunit in parallel cultures failed to alter the proliferation rate or the ability of these cell lines to grow in an anchorage-independent manner (Figures 6C and 6D). Moreover, introduction of PP2A B56γ3 into BJ fibroblast cell lines immortalized with *hTERT* that express endogenous PP2A B56γ3 failed to alter the proliferative potential of these cells (Figure 6E). Expression of PP2A B56γ3 in the breast cancer cell line MCF-7 and the prostate cancer cell line DU-145 also suppressed cell proliferation and inhibited growth in soft agar (data not shown). These observations suggest that the level of B56γ3 expression plays a critical role in the transformed behavior of these human cancer cell lines.

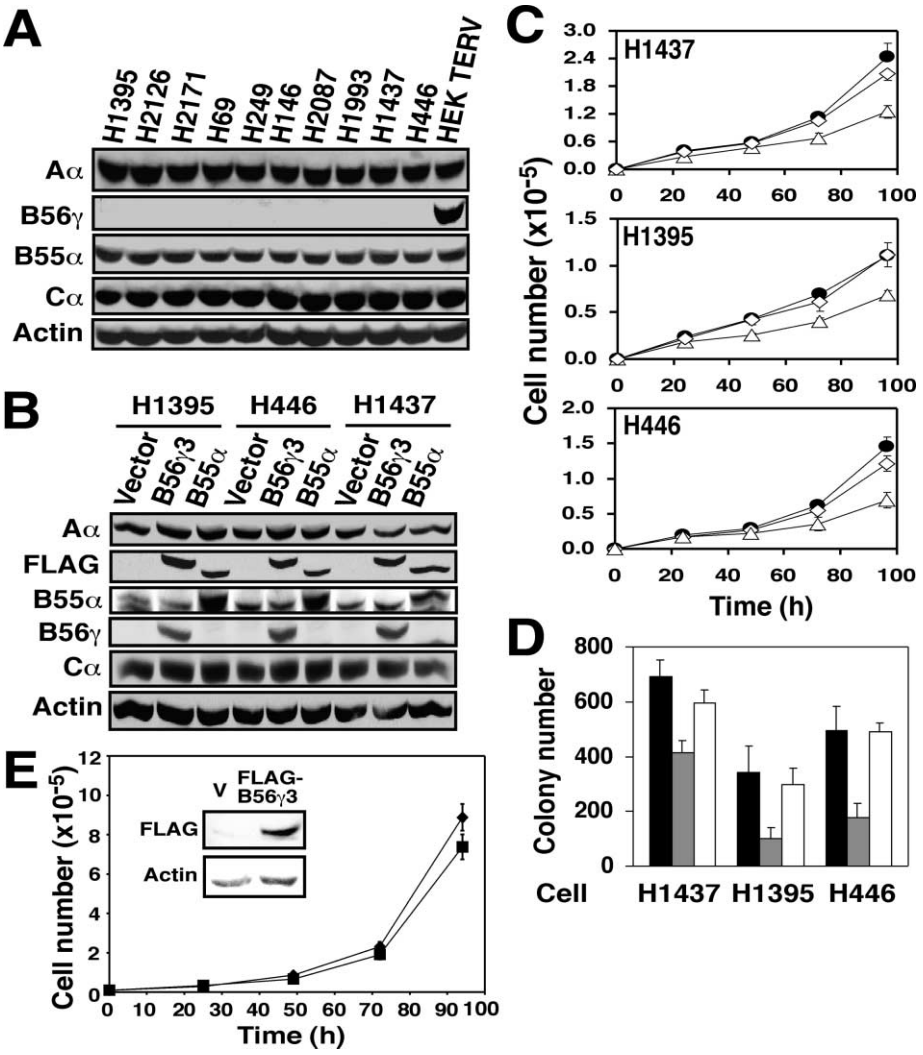

Figure 6. Introduction of PP2A B56γ3 into lung cancer cells inhibits cell proliferation and anchorage-independent growth

A: Immunoblotting of the PP2A subunits, Aα, B55α, B56γ, and Cα in lung cancer cell lines and HEK TERV cells.

B: The H1395, H446, and H1437 lung cancer cell lines were infected by a control retrovirus (Vector), a retrovirus encoding FLAG-epitope tagged-PP2A B56γ3 (B56γ3), or a retrovirus encoding FLAG-epitope tagged-PP2A B55α (B55α) to generate the cell lines indicated. Immunoblotting with antibodies specific for PP2A Aα, Cα, B55α, B56γ3, FLAG, and β-actin is shown.

C: Cell proliferation of lung cancer cell lines expressing a control vector (●), PP2A B56γ3 (△), and PP2A B55α (◇).

D: Anchorage-independent growth of lung cancer cell lines expressing a control vector (black bars), PP2A B56γ3 (gray bars), or PP2A B55α (white bars). The data are expressed as the mean ± SD for 3 experiments.

E: Proliferation of immortalized BJ fibroblasts expressing a control vector (◆), and PP2A B56γ3 (■). Immunoblotting with antibodies specific for FLAG and β-actin are also shown (inset).

Discussion

Several groups have demonstrated that the interaction of SV40 ST with PP2A contributes to the transformation of human cells (Mumby, 1995; Yu et al., 2001; Hahn et al., 2002). ST forms stable complexes with PP2A (Pallas et al., 1990), and this interaction leads to partial enzymatic inhibition of PP2A (Yang et al., 1991). The binding of ST with the PP2A A and C subunits has been shown to displace the PP2A B55α subunit (Pallas et al., 1990; Sontag et al., 1993). Here we confirm that ST competes with the PP2A B55α subunit for binding to PP2A A and C subunits; however, this interaction fails to reverse ST-dependent cell transformation. In contrast, ectopic expression of PP2A B56γ3 disrupts ST-PP2A AC complexes and partially reverses ST-dependent anchorage-independent growth and tumor formation. Furthermore, suppression of PP2A B56γ subunit expression functionally mimics the introduction of SV40 ST in this model of human cell transformation.

Taken together, these observations suggest that the effects of ST in human cell transformation derive, at least in part, from interactions of ST with PP2A complexes containing the B56γ subunit. While the relationship between ST and PP2A B56γ3 expression correlates with several surrogate measures of cell transformation, the mechanism by which these alterations in PP2A function lead to cell transformation remain undefined. For example, loss of PP2A B56γ expression may alter the phosphorylation of specific PP2A substrates or the subcellular localization of the PP2A holoenzyme. Alternatively, since PP2A B56γ binds both APC and cyclin G, suppression of this PP2A B subunit may directly perturb specific signaling pathways. Although further work is required to understand how alterations in PP2A B56γ function contribute to cancer development in spontaneously arising human tumors, these observations identify specific PP2A complexes that merit further investigation.

The functional effects of expressing ST or suppressing the expression of PP2A B56γ involve inhibition of PP2A enzymatic function. OA treatment of immortalized, nontumorigenic cells expressing LT, *hTERT*, and *H-RAS* but lacking ST stimulates cell proliferation and permits anchorage-independent growth. Although OA not only inhibits PP2A, but also PP1, PP2B, PP4, and PP5 (Millward et al., 1999), the concentration of OA required for inhibiting PP1, PP2B, and PP2C is higher than 1 μM (Millward et al., 1999), well above the concentrations used herein. Since OA is a known potent tumor promoter (Suganuma et al., 1988;

Fujiki, 1992), these observations suggest that some of its tumor promotion activities depend upon the inhibition of PP2A.

Many potential PP2A substrates have been reported, including several classes of protein kinases, transcription factors, and cell cycle regulators. Indeed, PP2A modulates the activity of more than 30 protein kinases in vitro, and PP2A forms stable complexes with several kinases including p70 S6, CaM kinase IV, RAF-1, and p21-activated kinase (PAK1) (Millward et al., 1999). The existence of multiple families and isoforms of PP2A regulatory subunits is consistent with the hypothesis that different PP2A complexes perform specific and distinct physiological functions and that particular B subunits may confer substrate specificity upon the PP2A AC core enzyme (Sola et al., 1991; Ferrigno et al., 1993; Cegielska et al., 1994). Moreover, the interaction of PP2A with specific kinases may result in activation or inactivation of the kinase, depending upon the particular substrate (Gomez and Cohen, 1991; Mueller et al., 1995; Cegielska et al., 1998; Ricciarelli and Azzi, 1998). Recent work implicates the pathway regulated by phosphatidylinositol-3-kinase (PI3K) as an important target of ST (Yuan et al., 2002; Zhao et al., 2003). Specifically, expression of ST in human foreskin keratinocytes, fibroblasts, and mammary epithelial cells leads to increased and sustained AKT activation, which is necessary for human cell transformation. However, since activation of AKT alone fails to suffice to achieve cell transformation (Zhao et al., 2003), these observations indicate that several PP2A targets participate in cell transformation.

In addition, specific PP2A B subunits direct particular PP2A complexes to distinct intracellular locations (Strack et al., 1998; Dagda et al., 2003). For example, PP2A complexes containing PP2A B56α, B56β, and B56ϵ localize to the cytoplasm, whereas PP2A complexes composed of PP2A B56δ or B56γ are concentrated in the nucleus (Sontag et al., 1995; McCright et al., 1996). Moreover, PP2A enzymes containing PP2A B55α are found associated with microtubules (Sontag et al., 1995). Thus, ST may also influence PP2A function by targeting PP2A complexes restricted to particular subcellular compartments or by altering the localization of specific PP2A complexes.

Although alterations in PP2A phosphatase activity may influence cell transformation, PP2A B56γ may also contribute to tumor development through direct interactions with the tumor suppressor APC and/or cyclin G. By binding APC, PP2A B56γ inhibits APC-axin complex formation, leading to destabilization of the β-catenin protein (Polakis, 2000). Moreover, expression of PP2A B56 subunits including α, β, γ3, δ, and ϵ isoforms in HEK 293 cells alters the expression of β-catenin (Seeling et al., 1999). In addition, PP2A B56γ also binds cyclin G (Okamoto et al., 1996; Bennin et al., 2002; Okamoto et al., 2002), a p53 target gene (Okamoto and Beach, 1994; Okamoto et al., 1996), suggesting that the PP2A and p53 pathways are interconnected by cyclin G. Further work is necessary to determine the functional consequences of these interactions in malignant transformation.

Since B56 family members share 68% identity in amino acid sequence (McCright and Virshup, 1995), it remains possible that each member of the PP2A B56 family contributes to the control of cell growth and cancer development. Moreover, the PP2A B56γ3 antisense RNA used in these studies may also target other PP2A B56 subunits. However, using antibodies specific for PP2A B56α, B56β and B56δ, we have eliminated the possibility that this PP2A B56γ3 antisense RNA targets these three subunits, and using specific shRNA, we have successfully suppressed PP2A B56γ protein expression in HEK TER cells and observed anchorage-independent growth in such cells. Nevertheless, it remains possible that more than one PP2A B56 family member may contribute to human cell transformation.

Indeed, prior studies of PP2A B56γ mRNA expression in human melanoma have led to conflicting observations (Francia et al., 1999; Deichmann et al., 2001). One group reported downregulation of PP2A B56γ mRNA in tissue samples derived from human primary melanoma and melanocytic nevi using subtractive suppression hybridization (Deichmann et al., 2001). In contrast, another group found that PP2A B56γ mRNA is expressed in both human melanoma cell lines and normal epidermal melanocytes using in situ hybridization (Francia et al., 1999). In addition, in B16 mouse melanoma cells, a truncated isoform of PP2A B56γ1 is expressed in a clone that exhibits increased invasiveness (BL6 cells), implying that mutations in B56γ1 promote cell invasiveness and neoplastic progression (Ito et al., 2000). Consistent with these observations, we failed to detect PP2A B56γ protein in 10 lung cancer cell lines. Since cancer-associated mutations of the PP2A A subunit appear to interfere with the binding of particular PP2A B-type subunits (Ruediger et al., 2001a, 2001b), it is also possible that such mutations act to disrupt the binding of PP2A B56γ to the PP2A A subunit. Taken together, these observations suggest that perturbation of the interaction of PP2A B56γ with the A subunit is the critical event in human cell transformation.

Although further study is required to determine whether specific PP2A B56γ subunit mutations occur in many human tumors, these observations implicate the intracellular pathways regulated by PP2A holoenzymes containing B56γ as important participants in cellular transformation. Further elucidation of these pathways will identify mutants that act as analogs of ST and will facilitate the generation of models that will increasingly mimic the phenotypes of naturally arising human tumor cells and that will serve as useful platforms for drug discovery.

Experimental procedures

Retroviral infection and cell lines

Amphotropic retroviruses were produced by transfection of the HEK 293T producer cell line with a specific retroviral vector and a vector encoding a replication-defective helper virus, pCL-Ampho (Imgenex, San Diego, CA), using Fugene 6 (Roche Molecular Biochemicals, Indianapolis, IN). HEK TER cells were generated from human embryonic kidney (HEK) cells as described (Hahn et al., 2002). To generate the PP2A B56γ3 and B55α antisense constructs, wild-type B56γ3 and B55α in the pCMV vector were used as the templates for PCR to obtain full-length of B56γ3 and B55α fragments amenable for cloning into the retroviral vector pMIG in the anti-sense (AS) orientation. pMIG-ASB56γ3, pMIG-ASB55α, pMIG-FLAG-B56γ3, pMIG-FLAG-B55α, and corresponding control retroviral vectors were introduced into HEK TER cells and selected for green fluorescent protein (GFP)-expressing cells using a fluorescent-activated cell sorter (FACS). After 2 rounds of infection, 70% to 90% of the cells expressed GFP, and 50% of these GFP-expressing cells were retained by FACS. To make retroviruses expressing shRNAs, oligonucleotides were inserted in the vector pMKO.1-puro (Masutomi et al., 2003; Stewart et al., 2003) modified by replacing the puromycin resistance gene with hrGFP (pMKO.1-GFP). The shRNAs were generated by cloning nucleotides 1156–1175 of PP2A B55α and nucleotides 423–442 of PP2A B56γ, followed by a 6 bp loop and the corresponding antisense sequence followed by five thymidines into pMKO.1-GFP. To generate HEK TERB56γ3-ST and TERB55α-ST cell lines, pWZL-blast-ST was introduced into TERB56γ3 and TERB55α cells. The TERVB and TERSTB cell lines were generated by introducing the pWZL-blasticidin retroviral vector into TERV and TERST cells. These cell lines were selected with blasticidin (2 μg/ml).

The human lung cancer cell lines H1395, H1437, and H446 were infected with pMIG-FLAG-B56γ3 or pMIG-FLAG-B55α, respectively, and subsequently selected by FACS. Immortalized human fibroblasts (BJ) expressing hTERT were propagated as described (Hahn et al., 2002).

Immunoblotting and immunoprecipitation

Cells were suspended in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, protease inhibitor cocktail [Roche] and 0.5% Nonidet P-40) and cleared of insoluble material by centrifugation. The supernatants (100 μg) were subjected to SDS-PAGE in 7.5%–15% gradient acrylamide gels and immunoblotting performed. The antibodies used included anti-PP2A A 6F9 (Covance, Richmond, CA), anti-PP2A C (Upstate, Lake Placid, NY), anti-PP2A B56α, anti-B56β (Santa Cruz, CA), and Flag M2 (Sigma-Aldrich Co., St. Louis, MO). Affinity-purified polyclonal antibodies were raised against a PP2A B55α peptide (CFSQVKGAVDDDV) corresponding to the N-terminal amino acids 13–25, a PP2A B56γ peptide (CPQAQKDPKKDR) corresponding to amino acids 471–480 of B56γ3 and residues 431–441 of B56γ2, a PP2A B56δ peptide (QSQPPSSNKRPSNS) corresponding to amino acids 49–62, and a SV40 ST peptide (CDIIGQT TYRDLKL) corresponding to the C-terminal amino acids 161–174 (Zymed Laboratories, South San Francisco, CA). For immunoprecipitation, each antibody (ST: Pab 430; PP2A Aα:6F9) was incubated with whole cell lysates (2 mg) overnight at 4°C. 30 μl of prewashed 1:1 slurry of protein A Sepharose (for Pab 430) or protein G Sepharose (for 6F9) was then added and incubated for additional 2 hr. The protein A or G beads containing PP2A AC-ST or PP2A AC-B complexes were washed three times with lysis buffer and eluted in 2× SDS loading buffer, followed by SDS-PAGE and immunoblotting.

Assaying PP2A B56 displacement by ST using adenoviruses

HEK 293 cells were grown in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% fetal calf serum (IFS). We transfected HEK 293 cells with vectors encoding 4× hemagglutinin (HA)-tagged PP2A B56α, B56γ1, or B56γ3 (McCright et al., 1996) using the GenePORTER transfection reagent (Gene Therapy Systems, San Diego, CA). 24 hr after transfection, cells were infected with either an adenovirus expressing wild-type ST under the control of the CMV promoter or a control adenovirus (Howe et al., 1998) at an MOI of 2–3. After 24 hr in DMEM supplemented with 2.5% IFS, cells were lysed in 1% NP-40, 10% glycerol, 20 mM Tris (pH 8), and 137 mM NaCl containing 0.036 TIU Aprotinin and 2 mM phenylmethylsulfonyl fluoride (PMSF) and cleared at 13,000 × g. Half of the soluble fraction was incubated with 3 μg anti-HA mAb (12CA5) and 20 μl of Protein A beads at 4° for 2 hr, washed 4 times (once with lysis buffer and 3× with PBS), and then analyzed by 12% SDS-PAGE. We visualized the HA-tagged PP2A B56 subunits, PP2A C subunit, and ST by immunoblotting with anti-HA tag mAb 16B12 (1:10,000; BabCO), anti-PP2A C subunit mAb (1:10,000; Transduction Laboratories, Lexington, KY), and the rabbit anti-SV40 ST serum (1:1000), respectively. Quantitation was performed using a Biorad Fluor-S Max chemilumimager.

Protein phosphatase activity

Cells were suspended in a CHAPS lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, protease inhibitor cocktail, and 0.3% CHAPS) and cleared of insoluble material by centrifugation. Cell lysates (500 μg) were incubated with the 6F9 anti-PP2A A antibody (5 μg) overnight at 4°C, followed by an additional 2 hr incubation with prewashed 1:1 protein G Sepharose slurry. The protein G Sepharose beads were then washed 3× with 50 mM Tris-HCl, 50 mM NaCl, 2 mM EDTA, and 0.1% CHAP and 2× with 50 mM imidazole (pH 7.2), 0.2 mM EGTA, 0.02% β-mercaptoethanol, and 0.1 mg/ml BSA to remove nonspecific proteins and free phosphate. Serine/threonine phosphatase activity in these immune complexes was determined by measuring the ability of such immune complexes to dephosphorylate a synthetic phosphothreonine peptide RRA(pT)VA specific for PP2A (Promega, Madison, WI). The reaction was initiated by adding phosphate free water, RRA(pT)VA and reaction buffer to the washed beads. After incubation with gentle agitation for 30 min at 25°C, free phosphate was measured colorimetrically (600 nm). In each case, a standard curve with free phosphate was used to determine the amount of free phosphate generated. Phosphatase activity was defined as pmol free PO_4 generated/μg protein/min. EGTA and EDTA were included in the lysis buffer to inhibit PP2B and PP2C, respectively. Purified PP2A core enzyme in the presence or absence of 5 nM OA was used to confirm the specificity of these reaction conditions.

Anchorage-independent growth and tumor formation

Growth of cells in soft agar and in immunodeficient animals was performed as previously described (Hahn et al., 1999). Anchorage-independent colonies were counted with the MultiImage imaging counter (Alpha Innotech Corp, San Leandro, CA).

Proliferation assays

HEK cells were maintained in minimal Eagle medium α (MEMα) supplemented with 10% IFS. To generate proliferation curves, cells were plated in triplicate and counted in a Z2 Particle Count and Size Analyzer (Beckman-Coulter, Miami, FL). To test the growth of cells at a limiting glutamine or leucine concentration, glutamine-free or leucine-free medium was supplemented with dialyzed IFS and 0.375 mM glutamine or 80 μM leucine representing 10% of the usual glutamine or leucine concentration. To measure IC_{50} of OA in these cells, 10^4 TERV cells were treated with either 2.0 nM, 4.0 nM, 8.0 nM, or 16.0 nM OA, and cell number was counted at 24 hr. Using this approach the IC_{50} for toxicity of OA to these cells was determined to be 6 nM.

Acknowledgments

We thank Drs. Craig Kamibayashi, John Minna, and David Virshup for providing the pCMV-B56γ3, pCMV-B55α, and pCEP-4HA B56γ3 plasmids, Dr. Bayar Thimmapaya for adenoviruses, and Dr. Matthew Meyerson for RNA derived from lung cancer cell lines. We thank Dr. Rene Bernards for sharing unpublished observations. This work was supported in part by grants from the U.S. National Cancer Institute K01 CA94223 (W.C.H.), R01 CA57327 (D.C.P.), PO1 CA50661 (W.C.H.), a Howard Hughes Medical Institute predoctoral fellowship (R.L.P.), the Doris Duke Charitable Foundation (W.C.H.), a New Investigator Award from the U.S. Department of Defense DAMD17-01-1-0049 (W.C.H.), a Dunkin Donuts Rising Star Award (WCH), and a Kimmel Foundation Scholar Award (W.C.H.).

Received: August 28, 2003
Revised: December 3, 2003
Accepted: December 23, 2003
Published: February 23, 2004

References

Ali, S.H., and DeCaprio, J.A. (2001). Cellular transformation by SV40 large T antigen: Interaction with host proteins. Semin. Cancer Biol. *11*, 15–23.

Bennin, D.A., Don, A.S., Brake, T., McKenzie, J.L., Rosenbaum, H., Ortiz, L., DePaoli-Roach, A.A., and Horne, M.C. (2002). Cyclin G2 associates with protein phosphatase 2A catalytic and regulatory B′ subunits in active complexes and induces nuclear aberrations and a G1/S phase cell cycle arrest. J. Biol. Chem. *277*, 27449–27467.

Bialojan, C., and Takai, A. (1988). Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. Biochem. J. *256*, 283–290.

Bikel, I., Montano, X., Agha, M.E., Brown, M., McCormack, M., Boltax, J., and Livingston, D.M. (1987). SV40 small t antigen enhances the transformation activity of limiting concentrations of SV40 large T antigen. Cell *48*, 321–330.

Calin, G.A., di Iasio, M.G., Caprini, E., Vorechovsky, I., Natali, P.G., Sozzi, G., Croce, C.M., Barbanti-Brodano, G., Russo, G., and Negrini, M. (2000). Low frequency of alterations of the alpha (PPP2R1A) and beta (PPP2R1B) isoforms of the subunit A of the serine-threonine phosphatase 2A in human neoplasms. Oncogene *19*, 1191–1195.

Campbell, K.S., Auger, K.R., Hemmings, B.A., Roberts, T.M., and Pallas, D.C. (1995). Identification of regions in polyomavirus middle T and small t antigens important for association with protein phosphatase 2A. J. Virol. *69*, 3721–3728.

Cegielska, A., Shaffer, S., Derua, R., Goris, J., and Virshup, D.M. (1994). Different oligomeric forms of protein phosphatase 2A activate and inhibit simian virus 40 DNA replication. Mol. Cell. Biol. *14*, 4616–4623.

Cegielska, A., Gietzen, K.F., Rivers, A., and Virshup, D.M. (1998). Autoinhibition of casein kinase I epsilon (CKI epsilon) is relieved by protein phosphatase and limited proteolysis. J. Biol. Chem. *273*, 1357–1364.

Cohen, P. (1989). The structure and regulation of protein phosphatases. Annu. Rev. Biochem. *58*, 453–508.

Csortos, C., Zolnierowicz, S., Bako, E., Durbin, S.D., and DePaoli-Roach, A.A. (1996). High complexity in the expression of the B′ subunit of protein phosphatase 2A0. Evidence for the existence of at least seven novel isoforms. J. Biol. Chem. *271*, 2578–2588.

Dagda, R.K., Zaucha, J.A., Wadzinski, B.E., and Strack, S. (2003). A developmentally regulated, neuron-specific splice variant of the variable subunit B-beta targets protein phosphatase 2A to mitochondria and modulates apoptosis. J. Biol. Chem. *278*, 24976–24985.

de Ronde, A., Sol, C.J., van Strien, A., ter Schegget, J., and van der Noordaa, J. (1989). The SV40 small t antigen is essential for the morphological transformation of human fibroblasts. Virology *171*, 260–263.

Deichmann, M., Polychronidis, M., Wacker, J., Thome, M., and Naher, H. (2001). The protein phosphatase 2A subunit Bgamma gene is identified to be differentially expressed in malignant melanomas by subtractive suppression hybridization. Melanoma Res. *11*, 577–585.

Fernandez, J.J., Candenas, M.L., Souto, M.L., Trujillo, M.M., and Norte, M. (2002). Okadaic acid, useful tool for studying cellular processes. Curr. Med. Chem. *9*, 229–262.

Ferrigno, P., Langan, T.A., and Cohen, P. (1993). Protein phosphatase 2A1 is the major enzyme in vertebrate cell extracts that dephosphorylates several physiological substrates for cyclin-dependent protein kinases. Mol. Biol. Cell *4*, 669–677.

Francia, G., Poulsom, R., Hanby, A.M., Mitchell, S.D., Williams, G., McKee, P., and Hart, I.R. (1999). Identification by differential display of a protein phosphatase-2A regulatory subunit preferentially expressed in malignant melanoma cells. Int. J. Cancer *82*, 709–713.

Fujiki, H. (1992). Is the inhibition of protein phosphatase 1 and 2A activities a general mechanism of tumor promotion in human cancer development? Mol. Carcinog. *5*, 91–94.

Gomez, N., and Cohen, P. (1991). Dissection of the protein kinase cascade by which nerve growth factor activates MAP kinases. Nature *353*, 170–173.

Hahn, W.C., Counter, C.M., Lundberg, A.S., Beijersbergen, R.L., Brooks, M.W., and Weinberg, R.A. (1999). Creation of human tumour cells with defined genetic elements. Nature *400*, 464–468.

Hahn, W.C., Dessain, S.K., Brooks, M.W., King, J.E., Elenbaas, B., Sabatini, D.M., DeCaprio, J.A., and Weinberg, R.A. (2002). Enumeration of the simian virus 40 early region elements necessary for human cell transformation. Mol. Cell. Biol. *22*, 2111–2123.

Howe, A.K., Gaillard, S., Bennett, J.S., and Rundell, K. (1998). Cell cycle progression in monkey cells expressing simian virus 40 small t antigen from adenovirus vectors. J. Virol. *72*, 9637–9644.

Ito, A., Kataoka, T.R., Watanabe, M., Nishiyama, K., Mazaki, Y., Sabe, H., Kitamura, Y., and Nojima, H. (2000). A truncated isoform of the PP2A B56 subunit promotes cell motility through paxillin phosphorylation. EMBO J. *19*, 562–571.

Li, X., and Virshup, D.M. (2002). Two conserved domains in regulatory B subunits mediate binding to the A subunit of protein phosphatase 2A. Eur. J. Biochem. *269*, 546–552.

Lundberg, A.S., Randell, S.H., Stewart, S.A., Elenbaas, B., Hartwell, K.A., Brooks, M.W., Fleming, M.D., Olsen, J.C., Miller, S.W., Weinberg, R.A., and Hahn, W.C. (2002). Immortalization and transformation of primary human airway epithelial cells by gene transfer. Oncogene *21*, 4577–4586.

Masutomi, K., Yu, E.Y., Khurts, S., Ben-Porath, I., Currier, J.L., Metz, G.B., Brooks, M.W., Kaneko, S., Murakami, S., DeCaprio, J.A., et al. (2003). Telomerase maintains telomere structure in normal human cells. Cell *114*, 241–253.

McCright, B., and Virshup, D.M. (1995). Identification of a new family of protein phosphatase 2A regulatory subunits. J. Biol. Chem. *270*, 26123–26128.

McCright, B., Rivers, A.M., Audlin, S., and Virshup, D.M. (1996). The B56 family of protein phosphatase 2A (PP2A) regulatory subunits encodes differentiation-induced phosphoproteins that target PP2A to both nucleus and cytoplasm. J. Biol. Chem. *271*, 22081–22089.

Millward, T.A., Zolnierowicz, S., and Hemmings, B.A. (1999). Regulation of protein kinase cascades by protein phosphatase 2A. Trends Biochem. Sci. *24*, 186–191.

Mueller, P.R., Coleman, T.R., and Dunphy, W.G. (1995). Cell cycle regulation of a Xenopus Weel-like kinase. Mol. Biol. Cell *6*, 119–134.

Mumby, M.C. (1995). Regulation by tumour antigens defines a role for PP2A in signal transduction. Semin. Cancer Biol. *6*, 229–237.

Muneer, S., Ramalingam, V., Wyatt, R., Schultz, R.A., Minna, J.D., and Kamibayashi, C. (2002). Genomic organization and mapping of the gene encoding the PP2A B56gamma regulatory subunit. Genomics *79*, 344–348.

Mungre, S., Enderle, K., Turk, B., Porras, A., Wu, Y.Q., Mumby, M.C., and Rundell, K. (1994). Mutations which affect the inhibition of protein phosphatase 2A by simian virus 40 small-t antigen in vitro decrease viral transformation. J. Virol. *68*, 1675–1681.

Okamoto, K., and Beach, D. (1994). Cyclin G is a transcriptional target of the p53 tumor suppressor protein. EMBO J. *13*, 4816–4822.

Okamoto, K., Kamibayashi, C., Serrano, M., Prives, C., Mumby, M.C., and Beach, D. (1996). p53-dependent association between cyclin G and the B′ subunit of protein phosphatase 2A. Mol. Cell. Biol. *16*, 6593–6602.

Okamoto, K., Li, H., Jensen, M.R., Zhang, T., Taya, Y., Thorgeirsson, S.S., and Prives, C. (2002). Cyclin G recruits PP2A to dephosphorylate Mdm2. Mol. Cell *9*, 761–771.

Pallas, D.C., Shahrik, L.K., Martin, B.L., Jaspers, S., Miller, T.B., Brautigan, D.L., and Roberts, T.M. (1990). Polyoma small and middle T antigens and SV40 small t antigen form stable complexes with protein phosphatase 2A. Cell *60*, 167–176.

Polakis, P. (2000). Wnt signaling and cancer. Genes Dev. *14*, 1837–1851.

Ricciarelli, R., and Azzi, A. (1998). Regulation of recombinant PKC alpha activity by protein phosphatase 1 and protein phosphatase 2A. Arch. Biochem. Biophys. *355*, 197–200.

Ruediger, R., Pham, H.T., and Walter, G. (2001a). Alterations in protein phosphatase 2A subunit interaction in human carcinomas of the lung and colon with mutations in the A beta subunit gene. Oncogene *20*, 1892–1899.

Ruediger, R., Pham, H.T., and Walter, G. (2001b). Disruption of protein phosphatase 2A subunit interaction in human cancers with mutations in the A alpha subunit gene. Oncogene *20*, 10–15.

Rundell, K., and Parakati, R. (2001). The role of the SV40 ST antigen in cell growth promotion and transformation. Semin. Cancer Biol. *11*, 5–13.

Schönthal, A.H. (2001). Role of serine/threonine protein phosphatase 2A in cancer. Cancer Lett. *170*, 1–13.

Seeling, J.M., Miller, J.R., Gil, R., Moon, R.T., White, R., and Virshup, D.M. (1999). Regulation of beta-catenin signaling by the B56 subunit of protein phosphatase 2A. Science *283*, 2089–2091.

Sola, M.M., Langan, T., and Cohen, P. (1991). p34cdc2 phosphorylation sites in histone H1 are dephosphorylated by protein phosphatase 2A1. Biochim. Biophys. Acta *1094*, 211–216.

Sontag, E., Fedorov, S., Kamibayashi, C., Robbins, D., Cobb, M., and Mumby, M.C. (1993). The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the map kinase pathway and induces cell proliferation. Cell *75*, 887–897.

Sontag, E., Nunbhakdi-Craig, V., Bloom, G.S., and Mumby, M.C. (1995). A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle. J. Cell Biol. *128*, 1131–1144.

Stewart, S.A., Dykxhoorn, D.M., Palliser, D., Mizuno, H., Yu, E.Y., An, D.S.,

Sabatini, D.M., Chen, I.S., Hahn, W.C., Sharp, P.A., et al. (2003). Lentivirus-delivered stable gene silencing by RNAi in primary cells. RNA *9*, 493–501.

Strack, S., Zaucha, J.A., Ebner, F.F., Colbran, R.J., and Wadzinski, B.E. (1998). Brain protein phosphatase 2A: Developmental regulation and distinct cellular and subcellular localization by B subunits. J. Comp. Neurol. *392*, 515–527.

Suganuma, M., Fujiki, H., Suguri, H., Yoshizawa, S., Hirota, M., Nakayasu, M., Ojika, M., Wakamatsu, K., Yamada, K., and Sugimura, T. (1988). Okadaic acid: An additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter. Proc. Natl. Acad. Sci. USA *85*, 1768–1771.

Tehrani, M.A., Mumby, M.C., and Kamibayashi, C. (1996). Identification of a novel protein phosphatase 2A regulatory subunit highly expressed in muscle. J. Biol. Chem. *271*, 5164–5170.

Turowski, P., Fernandez, A., Favre, B., Lamb, N.J., and Hemmings, B.A. (1995). Differential methylation and altered conformation of cytoplasmic and nuclear forms of protein phosphatase 2A during cell cycle progression. J. Cell Biol. *129*, 397–410.

Wang, S.S., Esplin, E.D., Li, J.L., Huang, L., Gazdar, A., Minna, J., and Evans, G.A. (1998). Alterations of the PPP2R1B gene in human lung and colon cancer. Science *282*, 284–287.

Yang, S.I., Lickteig, R.L., Estes, R., Rundell, K., Walter, G., and Mumby, M.C. (1991). Control of protein phosphatase 2A by simian virus 40 small-t antigen. Mol. Cell. Biol. *11*, 1988–1995.

Yu, J., Boyapati, A., and Rundell, K. (2001). Critical role for SV40 small-t antigen in human cell transformation. Virology *290*, 192–198.

Yuan, H., Veldman, T., Rundell, K., and Schlegel, R. (2002). Simian virus 40 small tumor antigen activates AKT and telomerase and induces anchorage-independent growth of human epithelial cells. J. Virol. *76*, 10685–10691.

Zhao, J.J., Gjoerup, O.V., Subramanian, R.R., Cheng, Y., Chen, W., Roberts, T.M., and Hahn, W.C. (2003). Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase. Cancer Cell *3*, 483–495.