Cell

CIP2A Inhibits PP2A in Human Malignancies

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DOI 10.1016/j.cell.2007.04.044

SUMMARY

Inhibition of protein phosphatase 2A (PP2A) activity has been identified as a prerequisite for the transformation of human cells. However, the molecular mechanisms by which PP2A activity is inhibited in human cancers are currently unclear. In this study, we describe a cellular inhibitor of PP2A with oncogenic activity. The protein, designated Cancerous Inhibitor of PP2A (CIP2A), interacts directly with the oncogenic transcription factor c-Myc, inhibits PP2A activity toward c-Myc serine 62 (S62), and thereby prevents c-Myc proteolytic degradation. In addition to its function in c-Myc stabilization, CIP2A promotes anchorage-independent cell growth and in vivo tumor formation. The oncogenic activity of CIP2A is demonstrated by transformation of human cells by overexpression of CIP2A. Importantly, CIP2A is overexpressed in two common human malignancies, head and neck squamous cell carcinoma (HNSCC) and colon cancer. Thus, our data show that CIP2A is a human oncoprotein that inhibits PP2A and stabilizes c-Myc in human malignancies.

INTRODUCTION

Cancer is a complex and diverse set of diseases related to the unharnessed growth, enhanced survival, and invasion of cells (Hanahan and Weinberg, 2000). Although there are various means to achieve cellular transformation, a limited number of elements can suffice to transform many different human cell types, suggesting that a discreet yet common set of pathways mediate this process (Hanahan and Weinberg, 2000; Zhao et al., 2004). Experimentally, it has been demonstrated that activation of Ras and telomerase, along with inactivation of the tumor suppressor proteins p53 and retinoblastoma protein (Rb) can immortalize a variety of human cell types, which can subsequently transform to a tumorigenic state only by inhibiting protein phosphatase 2A (PP2A) (Hahn et al., 1999; Janssens et al., 2005; Rangarajan et al., 2004; Zhao et al., 2004).

PP2A is a widely conserved protein serine/threonine phosphatase (PSP) that functions as a trimeric protein complex consisting of a catalytic subunit (PP2Ac or C), a scaffold subunit (PR65 or A), and one of the alternative regulatory B subunits (Figure 1A) (Janssens and Goris, 2001). In mammals, α and β isoforms exist for both the catalytic (C) and scaffolding (PR65/A) subunits; in addition, there are four B subunit families, each with several isoforms or splice variants. Such variability in PP2A holoenzyme composition results in an amazingly diverse enzyme with a vast array of substrate specificities.

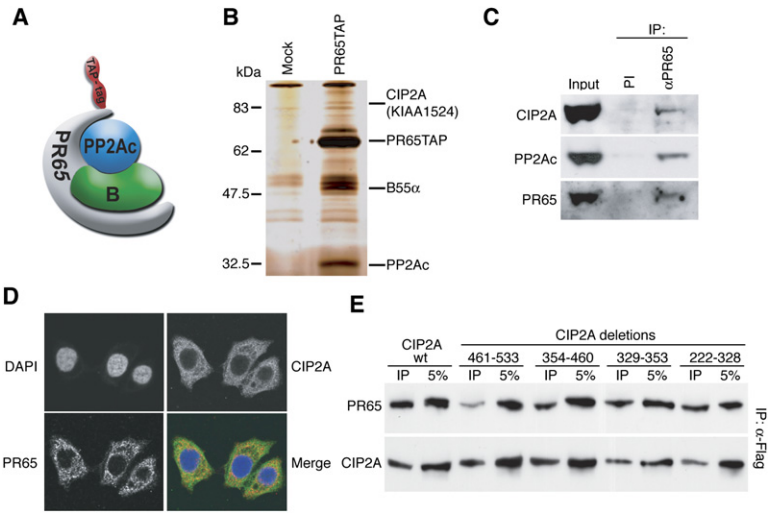


Figure 1. CIP2A Is a Novel PP2A-Interacting Protein

(A) Schematic representation of the PP2A complex composed of the PR65 scaffold subunit, PP2Ac catalytic subunit, and a regulatory B-subunit. TAP-tag, tandem affinity purification tag.

(B) Silver-stained gel of eluates from mock or PR65TAP stable cells following tandem affinity purification. The bands unique to the PR65TAP eluates were identified by tandem mass spectrometric analysis.

(C) Coimmunoprecipitation of endogenous PR65 from cytosolic HeLa extracts reveals an interaction between endogenous CIP2A, PR65 and PP2Ac proteins. PI, preimmune serum; input, input material.

(D) Confocal microscopy of endogenous PR65 and CIP2A in HeLa cells. Colocalization is indicated by the yellow staining in the merge image. Nuclear DNA was visualized with DAPI.

(E) Immunoblotting with PR65 antibody from coimmunoprecipates of Flag-CIP2A deletion constructs was used to evaluate the presence of the PP2A complex. The immmunoprecipitates were washed in 150 mM NaCl concentration. Shown is a representative result of three independent experiments. The lack of size differences between CIP2A deletions is due to the western blot running conditions disfavoring separation of the large size proteins. IP, immunoprecipitation; 5%, 5% of the input material used for immunoprecipitation.

Recent studies have identified target molecules for which dephosphorylation is critical for the tumor suppressor activity of PP2A (reviewed in Arroyo and Hahn, 2005; Janssens and Goris, 2001; Janssens et al., 2005). In addition to protein kinases, PP2A regulates the function of transcription factors involved in cellular transformation. While regulation of p53 function by PP2A (Moule et al., 2004; Okamoto et al., 2002) is not sufficient alone to promote cellular transformation (Arroyo and Hahn, 2005; Rangarajan et al., 2004), dephosphorylation of the oncogenic transcription factor c-Myc is critical for PP2A tumor suppressor activity. Inhibition of PP2A activity induces c-Myc serine 62 (S62) phosphorylation and c-Myc protein stabilization (Arnold and Sears, 2006; Yeh et al., 2004). Furthermore, it was shown that c-Myc S62 dephosphorylation inhibits cellular transformation and that inhibition of PP2A-mediated c-Myc dephosphorylation can suffice for SV40 small-t antigen in human transformation assays (Yeh et al., 2004). Taken together, the studies summarized above have established that inhibition of PP2A-mediated c-Myc S62 dephosphorylation is one of the prerequisites for human cell transformation. However, as the majority of evidence supporting the role of PP2A as a tumor suppressor has been obtained by using viral antigens or chemical inhibitors (Hahn et al., 1999; Janssens et al., 2005; Rangarajan et al., 2004; Yeh et al., 2004; Zhao et al., 2004), the in vivo mechanisms by which PP2A activity is inhibited in spontaneously transformed human cancer cells are unclear.

In this work, we identify KIAA1524, also termed p90 (Soo Hoo et al., 2002), as an endogenous PP2A-interacting protein from human cancer cells, which we named Cancerous Inhibitor of PP2A (CIP2A). Our results show that the protein inhibits PP2A activity toward c-Myc S62 and thereby stabilizes c-Myc protein. In addition, we demonstrate that KIAA1524/p90/CIP2A, called CIP2A hereafter, is required for the malignant cellular growth. Moreover, overexpression of the protein transforms immortalized human cells. Importantly, our results also demonstrate overexpression of CIP2A in two common human malignancies. Together, these results demonstrate that CIP2A is a human oncoprotein that inhibits PP2A in human malignancies.

RESULTS

Identification of CIP2A as an Endogenous PP2A-Associated Protein

To identify PP2A-interacting proteins from human cancer cells, cell clones stably expressing TAP-tagged PR65 protein, the 65 kDa scaffolding subunit of the PP2A complex (Figure 1A), were generated. TAP purification from cytoplasmic extracts of either mock transfected or PR65TAP-expressing cells revealed several proteins that only copurified with the PR65TAP (Figure 1B). Subsequently, the putative PR65-interacting proteins were identified by mass-spectrometric peptide sequencing. Among the proteins identified were both the PP2A catalytic subunit (PP2Ac) and a PP2A B55α subunit, thus validating the approach (Figure 1B). In addition, a novel putative PP2A-interacting protein, KIAA1524, was identified based on seven unique matching peptides and a combined Mascot score of 355 (Figure 1B and Table S1). KIAA1524 is a 90 kDa cytoplasmic protein shown to be expressed in gastric cancer tissue but with no previously identified cellular function (Soo Hoo et al., 2002). Sequence analysis of KIAA1524 reveals no significant homology to any protein with known function, although

the protein harbors several putative protein-protein interaction domains (Figure S1). In this work, we demonstrate that KIAA1524 inhibits PP2A in human malignancies and is therefore hereby designated as CIP2A.

To verify this interaction, coimmunoprecipitation analyses were performed from cytoplasmic extracts of HeLa cells using a PR65 antibody. Indeed, endogenous CIP2A and PP2Ac coimmunoprecipitated with the endogenous PR65 (Figure 1C). Moreover, confocal analysis of HeLa cells revealed colocalization of CIP2A with PR65 in the perinuclear region, in addition to cytoplasmic structures (Figure 1D). As expected based on the previously published data (Soo Hoo et al., 2002), CIP2A was primarily cytoplasmic, although weak nuclear staining was detected (Figure 1D and data not shown).

To strengthen the interaction data between CIP2A and the PP2A complex, a series of Flag-tagged CIP2A deletion constructs (Figure S1) were transiently transfected into HeLa cells for 48 hr, followed by immunoprecipitation with anti-Flag antibody. Importantly, no CIP2A antibody immunoreactivity was detected in anti-Flag antibody immunoprecipitates from the mock-transfected cells, confirming the specificity of anti-Flag antibody for CIP2A-deletion proteins in immunoprecipitation (data not shown). Interaction between the CIP2A mutants and the PP2A complex was assessed by western blot analysis of the PR65 subunit. In either low (150 mM NaCl) or moderate (300 mM NaCl) stringency conditions, Flag-CIP2A wild-type (CIP2Awt) clearly coimmunoprecipitated with the endogenous PR65 protein (Figure 1E and data not shown). Moreover, CIP2A lacking the amino acids between 461 and 533 was the only mutant out of 11 deletions (Figure S1) that demonstrated impaired binding to PR65 in either stringency condition (Figure 1E and data not shown).

These results identify CIP2A as an endogenous interaction partner for the PP2A complex. Moreover, the data show that amino acids between 461 and 533 on CIP2A appear to be important for the interaction.

CIP2A Promotes c-Myc Protein Stability

Suppression of PP2A activity by either overexpression of SV40 small-t antigen or by depletion of the B56γ subunit results in large changes in the genome-wide gene-expression profile (Moreno et al., 2004). To probe for the uncharacterized cellular functions of CIP2A, genome-wide gene-expression profiles of scrambled and CIP2A siRNA-transfected HeLa cells were compared after 72 hr. Remarkably, only a minor fraction of genes (76 out of 26091) included in the Sentrix Human-6 Expression BeadChip (Illumina, Inc.), showed a reproducible and statistically significant ($p < 0.05$, data not shown) difference in their expression levels between scrambled and CIP2A siRNA-transfected cells. As discussed above, PP2A activity has been shown to regulate the activity of two transformation-relevant transcription factors, p53 and c-Myc (Arroyo and Hahn, 2005). To characterize these two transcription factors in relation to the transcriptional profile of CIP2A-depleted cells, the list of 76 genes affected by CIP2A depletion was compared to target-gene databases published for p53 and c-Myc. Based on a p53 target-gene database (http://p53.bii.a-star.edu.sg/aboutp53/targetgene/index.php), only 1 out of 76 genes affected by CIP2A depletion has been published to harbor a p53 binding site in its promoter or to be transcriptionally regulated by p53. However, when compared with a c-Myc target-gene database (http://www.myc-cancer-gene.org/site/mycTargetDB.asp), 16% (12 out of 76) of genes affected by CIP2A depletion were found to bind c-Myc in their promoter region. These findings, together with the published role of PP2A regulation of c-Myc protein stability (Arnold and Sears, 2006; Yeh et al., 2004), suggest that CIP2A may regulate c-Myc function.

To study this assumption, c-Myc protein steady-state expression levels were evaluated from HeLa lysates 72 hr after transfection with CIP2A or scrambled siRNA. Depletion of CIP2A resulted in a clear downregulation of c-Myc protein expression (Figure 2A). Importantly, co-transfection of siRNA-resistant CIP2A cDNA rescued CIP2A protein levels and abolished c-Myc protein down-regulation (Figure 2A). Moreover, two independent CIP2A siRNAs suppressed c-Myc protein expression (Figure 2B). These results demonstrate that CIP2A supports c-Myc protein steady-state levels, whereas no marked differences were observed in the expression or phosphorylation status of other studied targets of PP2A or of PP2Ac (Figure 2C).

Although depletion of CIP2A effectively reduced c-Myc protein steady-state levels (Figures 2A—2C), c-Myc mRNA expression was not significantly altered by CIP2A depletion (Figure 2D), implying that CIP2A regulates c-Myc protein levels posttranscriptionally, as does PP2A (Arnold and Sears, 2006; Yeh et al., 2004). Indeed, the analysis of half-life of endogenous c-Myc protein revealed that CIP2A depletion reduced the stability of c-Myc protein after cycloheximide treatment (100 μg/ml) (Figure 2E). Quantification of c-Myc expression from three independent experiments revealed a statistically significant difference on c-Myc protein levels at 1–2 hr after cycloheximide treatment (Figure 2F).

Together, these results demonstrate that CIP2A promotes c-Myc protein stability in human cells.

CIP2A Inhibits c-Myc-Associated PP2A Activity

The results above demonstrate that CIP2A interacts with the PP2A complex and promotes c-Myc stability, suggesting that CIP2A may inhibit PP2A activity toward S62 on c-Myc (Arnold and Sears, 2006; Yeh et al., 2004). In support of that, we found that CIP2A depletion reduced c-Myc S62 phosphorylation relative to total c-Myc levels in HeLa cells (Figure 3A). To study whether CIP2A indeed inhibits c-Myc-associated PP2A activity, c-Myc immunoprecipitates from scrambled and CIP2A siRNA-transfected cells were subjected to an in vitro PP2A assay using 6,8-difluoro-4-methylumbelliferyl phosphate as the substrate (Pastula et al., 2003). The specificity of the assay conditions for detecting PP2A catalytic activity was

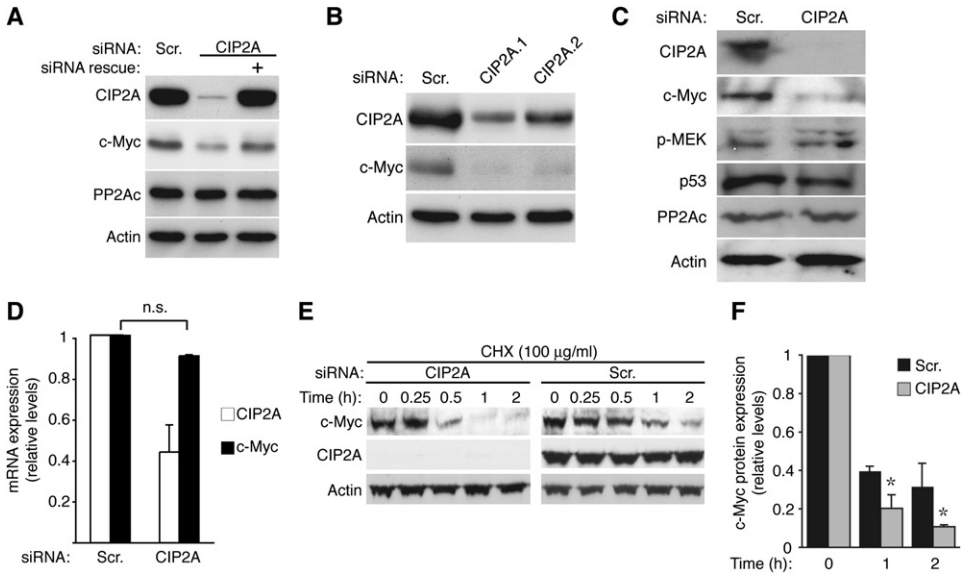


Figure 2. CIP2A Stabilizes c-Myc Protein

(A) Western blot analysis of CIP2A, c-Myc, and PP2Ac expression levels from HeLa cells 72 hr after transfection with scrambled siRNA (Scr), CIP2A.1 siRNA (CIP2A), or both CIP2A.1 siRNA and a siRNA-resistant CIP2A cDNA.

(B) Western blot analysis of CIP2A and c-Myc expression levels from HeLa cells 72 hr after transfection with two independent CIP2A-targeting siRNAs (CIP2A.1 or CIP2A.2) or scrambled siRNA.

(C) Expression and phosphorylation of PP2A substrate proteins and PP2Ac from HeLa cells 72 hr after transfection with scrambled or CIP2A.1 siRNA. Equal loading was verified with actin immoblotting. Shown is representative data from two to three independent experiments

(D) c-Myc and CIP2A mRNA expression levels 72 hr after transfection with scrambled or CIP2A.1 siRNA. Shown is mean +standard deviation (SD) of two microarray experiments. n.s., not significant by Student's t test. Similar results were obtained by northern blot analysis.

(E) c-Myc protein stability 72 hr posttransfection with scrambled and CIP2A.1 siRNA following cyclohexamide treatment (100 μg/ul) for the indicated time points. Equal loading was verified with actin immoblotting.

(F) Quantitation of c-Myc protein levels 1–2 hr after cyclohexamide treatment from three independent experiments identical to shown in (E). Shown is mean +SD of three experiments. *p <0.05, Student's t test.

demonstrated by inhibition of the phosphatase activity of the purified PP2Ac-PP65 dimer and of c-Myc immunoprecipitate, by low nanomolar concentration of the PP2A inhibitor okadaic acid (Figure S2). Interestingly, CIP2A depletion clearly increased PP2A activity in c-Myc immunoprecipitates, whereas no increase in MDM2-associated PP2A activity was observed in the same conditions (Figure 3B). Importantly, the CIP2A siRNA-elicited increase in c-Myc-associated PP2A activity was abolished by cotransfection of siRNA-resistant CIP2A cDNA (Figure 3B; siRNA rescue).

We previously demonstrated that the B56α subunit is required for PP2A complex recruitment to c-Myc (Arnold and Sears, 2006). To evaluate whether CIP2A affects the PP2A recruitment to c-Myc, c-Myc was immunoprecipitated from either CIP2A or scrambled siRNA-transfected cells and analyzed for PP2Ac by western blotting. CIP2A depletion did not affect the interaction between c-Myc and PP2A, since PP2Ac can be coimmunoprecipitated with c-Myc, regardless of CIP2A levels (Figure 3C; right panel). These results confirm that endogenous CIP2A and PP2Ac interact with c-Myc in HeLa cells and that CIP2A does not inhibit c-Myc-associated PP2A activity by preventing PP2Ac recruitment to c-Myc.

Coimmunoprecipitation of c-Myc and CIP2A from cellular extracts demonstrates a physical association between these proteins (Figure 3C) but does not reveal whether the interaction between CIP2A and c-Myc is direct. To further characterize CIP2A's interaction with c-Myc, purified Flag-CIP2A protein was used in an in vitro protein-protein interaction assay with recombinant GST-fused aminoterminal portion of c-Myc (aa 1–262). We found that Flag-antibody resin coimmunoprecipitated recombinant Flag-CIP2A and GST-Myc, whereas Flag-CIP2A did not coimmunoprecipitate with GST alone (Figure 3D), demonstrating that CIP2A directly binds to the c-Myc aminoterminus. To further study the determinants of CIP2A-c-Myc interaction, wild-type c-Myc (WT) and mutants, where the aminoterminal PP2A target S62 was mutated to either alanine (S62A) or aspartate (S62D), were coexpressed in 293 cells with Flag-CIP2A. Coimmunoprecipitation of Flag-CIP2A with V5-c-Myc was thereafter studied by western blotting. Interestingly, as compared to wild-type c-Myc, both S62 mutants displayed greatly reduced binding with CIP2A (Figure 3E), whereas an alanine substitution of the adjacent threonine 58 did not inhibit the c-Myc-CIP2A interaction (Figure S3).

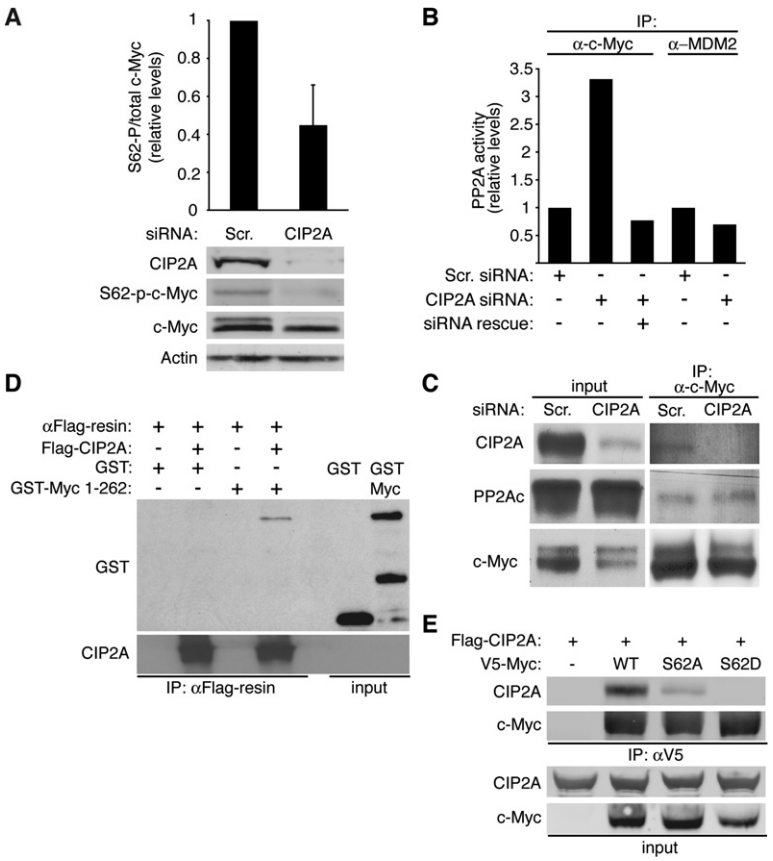


Figure 3. CIP2A Inhibits c-Myc-Associated PP2A Activity

(A) Western blot analysis of c-Myc S62 phosphorylation and total c-Myc levels 72 hr after treatment with scrambled or CIP2A.1 siRNA. Levels were quantitated using LI-COR software, and ratio of S62/total c-Myc was calculated. Shown is average +SD of three independent experiments.

(B) The PP2A substrates c-Myc and MDM2 were immunoprecipitated from scrambled siRNA (Scr), CIP2A.1 siRNA, or both CIP2A.1 siRNA and a siRNA-resistant CIP2A cDNA (CIP2A rescue)-treated cells and analyzed for PP2A activity. Shown are mean values measured in triplicate from a representative experiment of two to three independent experiments.

(C) Coimmunoprecipitation of c-Myc from cytosolic extracts of HeLa cells transfected with scrambled or CIP2A.1 siRNA for 72 hr. Left panel, input material; right panel, immunoprecipitates.

(D) In vitro binding assay with immobilized, recombinant Flag-CIP2A protein and recombinant GST protein or with GST-c-Myc 1-262 protein. Immunoblots of eluates with GST (top) or CIP2A (bottom). Input, GST proteins used for interaction assay. Shown is a representative blot of three independent experiments with similar results.

(E) CIP2A binds to c-Myc through recognition of c-Myc serine 62. 293 cells transiently cotransfected for 48 hr with V5/His-c-Myc, and Flag-CIP2A expression constructs were subjected to immunoprecipitation by anti-V5 antibody. Immunoprecipitates were analyzed by immunoblotting with the Flag (CIP2A) and His-antibodies (c-Myc). Lower panel shows input levels of Flag-CIP2A and V5/His-c-Myc.

Together these experiments provide solid biochemical evidence that CIP2A inhibits c-Myc-associated PP2A activity and protects c-Myc S62 from dephosphorylation. Moreover, direct binding of CIP2A to c-Myc aminoterminus provides the most feasible explanation for the observed selectivity of CIP2A toward c-Myc-associated PP2A activity (Figures 2C and 3B).

CIP2A Is Required for Tumor Growth

Inhibition of PP2A activity increases cell proliferation and supports cellular transformation (Arroyo and Hahn, 2005; Chen et al., 2004). Therefore, the role of CIP2A in regulating cell proliferation was next analyzed by thymidine-incorporation assay in HeLa cells. Compared to scrambled siRNA-transfected cells, CIP2A depletion resulted in significant inhibition of cell proliferation 72 hr after transfection (Figure 4A). However, CIP2A siRNA transfection did not induce a sub-G1 fraction in fluorescence-activated cell sorting (FACS) analysis of the cellular DNA content (Figure 4B), nor did it induce cleavage of the PARP protein (Figure 4C), demonstrating that CIP2A depletion does not induce programmed cell death.

To assess the contribution of CIP2A on the tumorigenic potential of HeLa cells, the effects of CIP2A depletion on the ability of these cells to form dense foci on a monolayer, as well as their ability to grow in an anchorage-independent manner, was analyzed. For this purpose, we first studied the efficiency of CIP2A depletion by a single transfection of siRNA 10 days after transfection and found very potent reduction of CIP2A protein expression still after 10 days (Figure 4D). Importantly, CIP2A depletion abrogated HeLa cell foci formation 10 days after transfection (Figure 4E) and also significantly impaired growth of HeLa cells in soft agar (Figure 4F). In accordance with results shown in Figure 2B, both CIP2A.1 and CIP2A.2 siRNAs identically reduced HeLa cell growth in soft agar (Figure S4). To assess the tumorigenic role of CIP2A in vivo, HeLa cells transfected with either CIP2A or scrambled siRNA for 72 hr were subcutaneously injected into athymic mice and tumor growth was monitored by measuring the size of palpable tumors. Importantly, depletion of CIP2A by siRNA reduced the overall tumor size (Figure 4G) and resulted in a significant inhibition of tumor weight at day 27 (Figure 4H).

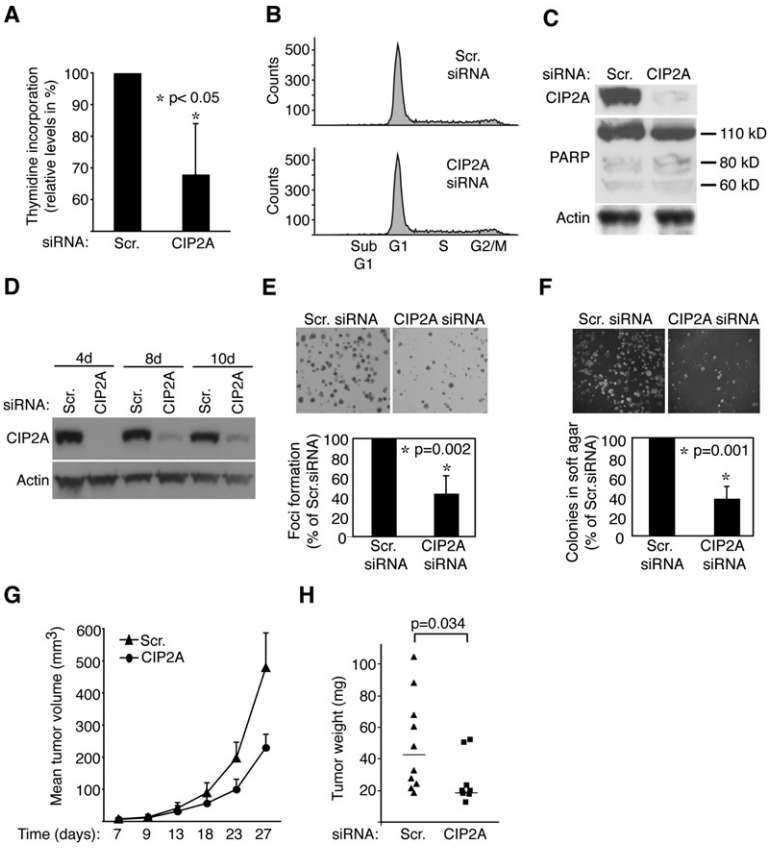


Figure 4. CIP2A Is Required for Malignant Growth and Tumorigenesis In Vivo

(A) Thymidine incorporation of HeLa cells transfected for 72 hr with scrambled or CIP2A.1 siRNA for cell proliferation. Shown is mean + S.D. of four experiments. *p < 0.05, Student's t test.

(B) Flow cytometric analysis of DNA content for cell-cycle progression from HeLa cells transfected with scrambled or CIP2A.1 siRNA for 72 hr. Results are from a representative experiment of four repetitions with similar results.

(C) CIP2A depletion does not induce poly(ADP-ribose) polymerase (PARP) cleavage in HeLa cells as detected by immunoblotting 72 hr after CIP2A.1 siRNA transfection. Shown is a representative result of three independent experiments. Expected molecular weights of the full-length (110 kDa) and caspase-cleaved forms of PARP proteins are shown on the right.

(D) Immunoblotting of CIP2A protein from HeLa cells 4, 8, and 10 days after transfection with scrambled or CIP2A.1 siRNA. Shown is a representative result of three independent experiments.

(E) Dense foci formation on a monolayer of HeLa cells transfected with scrambled or CIP2A.1 siRNA. Above, representative light miscroscopy images. Below, quantitation of number of foci 10 days after replating by Image J software. Shown is average +SD of four experiments. *p = 0.002, Student's t test.

(F) Colony growth on soft agar of HeLa cells transfected with scrambled or CIP2A siRNA. Above, representative phase-contrast miscroscopy images. Below, quantitation of number of colonies as measured 10 days after replating by Image J software. Shown is average +SD of four experiments. *p = 0.001, Student's t test.

(G) 3 × 10^6 HeLa cells transfected with the indicated siRNA were injected 72 hr after transfection subcutaneously into the flank of immunocompromised mice (scrambled siRNA n = 10 and CIP2A siRNA n = 8). Shown is mean + standard error of the mean (SEM) of tumor volumes in indicated time points. The experiment was performed twice with similar results.

(H) Weights (mg) of tumors from the experiment shown in (G) at day 27. *p = 0.034, Mann-Whitney U test.

Taken together, these data demonstrate that CIP2A expression maintains a transformed cellular phenotype and that CIP2A promotes in vivo tumor growth.

CIP2A Promotes Cell Transformation

To study if CIP2A has oncogenic properties when expressed in nonmalignant cells, spontaneously immortalized mouse embryo fibroblasts (MEFs) were infected with retroviral constructs encoding CIP2A and an oncogenic form of Ras (RasV12), and the foci formation of the infected cells on NIH-3T3 feeder layer was quantitated 14 days later. As expected, retroviral expression of RasV12 did induce foci growth of immortalized MEFs (Figure 5A). However, CIP2A expression alone did not induce foci formation but did increase foci formation of Ras-infected cells (Figure 5A), demonstrating that CIP2A has oncogenic properties when expressed with Ras in murine-immortalized fibroblasts.

The widely used human transformation model established by Hahn and colleagues (Hahn et al., 1999, 2002) was next utilized to analyze if CIP2A functions as an oncoprotein in human cells. Human embryonal kidney (HEK) fibroblasts, in which p53 and the Rb family of tumor suppressors have been inactivated and that express a catalytic subunit of human telomerase (hTERT), along with oncogenic Ras (referred to as HEK-TERV cells), still require inhibition of PP2A, for example by overexpression of small-t antigen, for complete transformation (Hahn et al., 1999, 2002). On the other hand, we have previously shown that overexpression of the T58A c-Myc mutant, which is resistant to PP2A-mediated dephosphorylation at S62, can replace small-t antigen in HEK-TERV cell transformation (Yeh et al., 2004). Importantly, lentiviral overexpression of CIP2A in HEK-TERV cells resulted in cell transformation, thus demonstrating that CIP2A has oncogenic potential (Figure 5B). It should be noted that although the transformation capacity of CIP2A seems somewhat reduced as compared to what has been published for small-t antigen, it is comparable to other conditions in which only a portion of the cellular PP2A complexes has been inhibited, for example by depletion of B56γ (Chen et al., 2004, 2005; W.C. Hahn, personal communication).

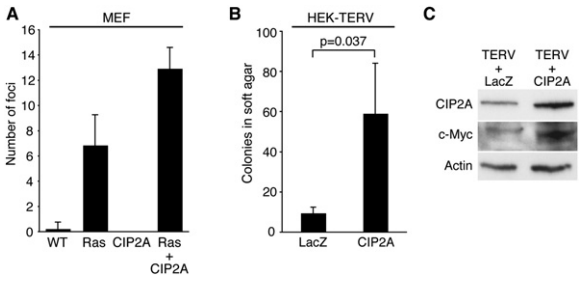


Figure 5. CIP2A Promotes Ras-Elicited Foci Formation in Mouse Embryo Fibroblasts and Transforms Human Cells

(A) Spontaneously immortalized mouse embryo fibroblasts (MEFs) were infected with recombinant retroviruses encoding RasV12, CIP2A, or combinations of those, and their ability to form multilayer foci was quantitated after 14 days. Shown is mean foci number of three replicate plates from a representative experiment. The experiment was performed twice with similar results.

(B) Anchorage-independent growth in soft agar of HEK-TERV cells expressing either lentiviral CIP2A or LacZ. Shown is mean +SD of number of colonies from 12 plates and from a representative experiment. *p = 0.037, Student's t test. The experiment was repeated twice with similar results by using HEK-TERV cells from independent lentiviral infections.

(C) Western blot analysis of endogenous c-Myc expression levels from HEK-TERV cells expressing either lentiviral CIP2A or LacZ. The experiment was repeated twice with similar results by using cells from independent lentiviral infections.

Moreover, in accordance with its role in regulation of c-Myc stability (Figure 2), CIP2A expression increased c-Myc protein expression levels in HEK-TERV cells (Figure 5C).

CIP2A Is Overexpressed in Human Malignancies

The results above indicate that CIP2A could be a putative novel target for cancer therapeutics; the presumption being that CIP2A expression is distinguishable between nontransformed and cancer tissues. According to our quantitative RT-PCR analysis, CIP2A mRNA was expressed at very low levels (< 1% of β-actin mRNA-expression levels) in the majority of the 21 nonmalignant samples, with the exception of bone marrow, prostate, testis, cerebellum, and brain (Figure 6A). To compare the protein levels of CIP2A between nonmalignant and malignant cells, whole-cell lysates of different cell types were immunoblotted for CIP2A. Consistent with Figure 6A, very low levels of CIP2A protein was detected in human epidermal keratinocytes (HEK), nontumorigenic MEFs, and immortalized NIH3T3 mouse fibroblasts (Figure 6B and data not shown). However, CIP2A protein was expressed at high levels in both HeLa cells and in HT-1080 fibrosarcoma cells, indicating that CIP2A expression may correlate with the tumorigenic potential of cells. Accordingly, analysis of CIP2A mRNA from low-passage cell lines derived from human head-and-neck squamous cell carcinomas (HNSCC), revealed a statistically significant overexpression of CIP2A mRNA in 36 HNSCC cell lines, as compared to normal human epidermal keratinocytes (Figure 6C). Importantly, immunohistochemical (IHC) staining of HNSCC tumors confirmed high expression of CIP2A protein in 11 out of 14 HNSCC tumor samples, whereas 9 nonmalignant tissues from the oral cavity were all negative for CIP2A immunoreactivity (Figure 6D). Furthermore, staining of the same tissue sample with the control IgG did not demonstrate any immunoreactivity (Figure 6D). In HNSCC tissue, CIP2A protein was detected in the tumor cells, whereas the stroma was mostly negative (see arrows in Figure 6D).

To further evaluate the observation of CIP2A expression in HNSCCs, CIP2A protein expression was analyzed in a mouse model of HNSCC (Lu et al., 2006). In this model, DMBA-initiated TGFβRII−/− mice develop HNSCC that displays pathology indistinquishable from that of human and has complete penetrance with some tumors progressing to metastases (Lu et al., 2006). Evaluation of CIP2A immunoreactivity by IHC in buccal tissue samples from wild-type mice, as well as mutant mice displaying either epithelial hyperplasia (4 weeks after DMBA initiation) or full-blown malignant transformation (15–25 weeks after DMBA initiation), revealed that both hyperplastic (5 out of 8) and HNSCC tissues (10 out of 12) clearly displayed enhanced expression of CIP2A when compared to DMBA-treated wild-type animals (Figure 6E). Moreover, in good agreement with the human HNSCC samples, CIP2A was predominantly expressed in tumor cells, whereas the stromal cells were negative for CIP2A (Figures 6D and 6E).

In addition to HNSCC, colon cancer is a human malignancy associated with increased Ras signaling and loss of TGF-β signaling (Grady and Markowitz, 2002). Therefore, CIP2A expression was analyzed from 43 human colon cancer samples and 5 control samples from normal colon by RT-PCR. In accordance with the HNSCC data, CIP2A mRNA was significantly overexpressed in human colon cancer tissues as compared to control tissues (Figure 6F).

Together, these results demonstrate that CIP2A is expressed at low levels in most of the nonmalignant tissues, but its expression is enhanced in malignant cells in vivo. Moreover, these results identify HNSCC and colon cancer as human malignancies associated with increased CIP2A expression.

CIP2A Enhances Malignant Growth and Tumorigenesis of HNSCCs

Our results indicate a role for CIP2A in the pathogenesis of human malignancies. To confirm that the cellular functions of CIP2A described in this study are not restricted to HeLa cells, three different low-passage HNSCC cell lines (Lansford et al., 1999) were transfected with CIP2A siRNA and analyzed for c-Myc protein levels by western blotting. CIP2A depletion resulted in a clear downregulation of c-Myc protein levels in all of the HNSCC cell lines (Figure 7A). Importantly, analysis of c-Myc immunoprecipitates revealed that CIP2A depletion also increased c-Myc-associated PP2A phosphatase activity in HNSCC cell lines (Figure 7B).

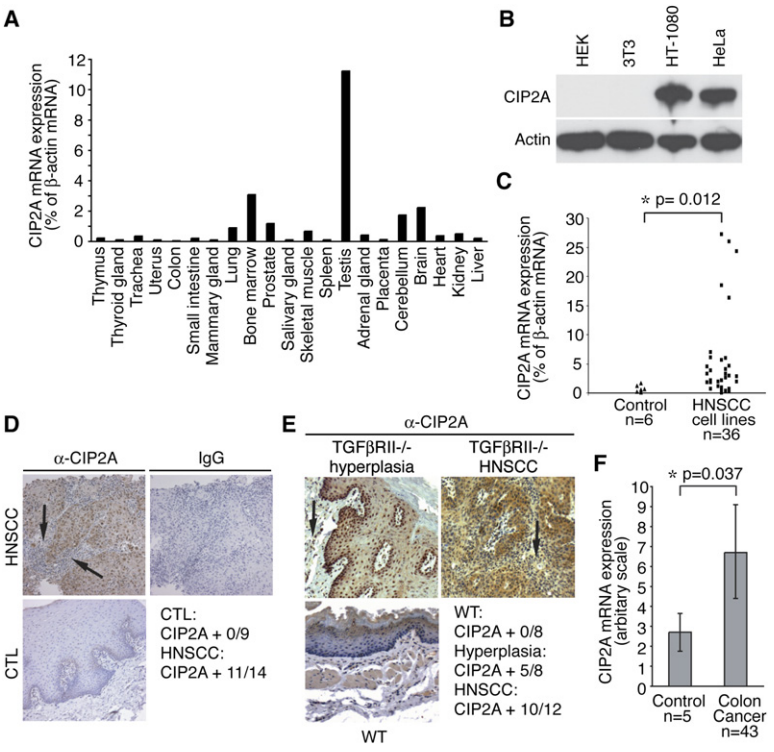


Figure 6. CIP2A Is Overexpressed in Head and Neck Squamous Cell Carcinomas

(A) CIP2A-mRNA expression was quantitated by quantitative RT-PCR analysis from normal tissue samples. CIP2A expression is presented relative to β-actin.

(B) Western blot analysis of CIP2A protein expression from human epidermal keratinocytes (HEK), mouse NIH3T3 fibroblasts, HeLa, and fibrosarcoma HT-1080 cell lines.

(C) mRNA expression analysis of CIP2A from HNSCC (n = 36) and human epidermal keratinocyte (control) cells (n = 6) as determined by quantitative RT-PCR. CIP2A-mRNA expression is presented relative to β-actin. *p = 0.012, Mann-Whitney U test.

(D) Immunohistochemical staining of HNSCC tissues (n = 14) and nonmalignant control tissues (CTL; n = 9) for CIP2A expression. Note that positive staining of tumor cells contrasts with the negative stromal compartment of the HNSCC (indicated by arrows). IgG, control rabbit serum; CIP2A +, positive CIP2A staining.

(E) Immunohistochemical staining of CIP2A in buccal tissue from *TGFβRII*+/+ (WT) mice or *TGFβRII*−/− mice 4 weeks (hyperplasia) and 20 weeks (HNSCC) after DMBA initiation. The arrows highlight the lack of stromal CIP2A staining. CIP2A+, positive CIP2A staining.

(F) Quantitative RT-PCR analysis of CIP2A-mRNA expression in colon cancer tissues and in nonmalignant colon tissues (control). Shown is mean expression of samples +S.D. *p < 0.05, Mann-Whitney U test.

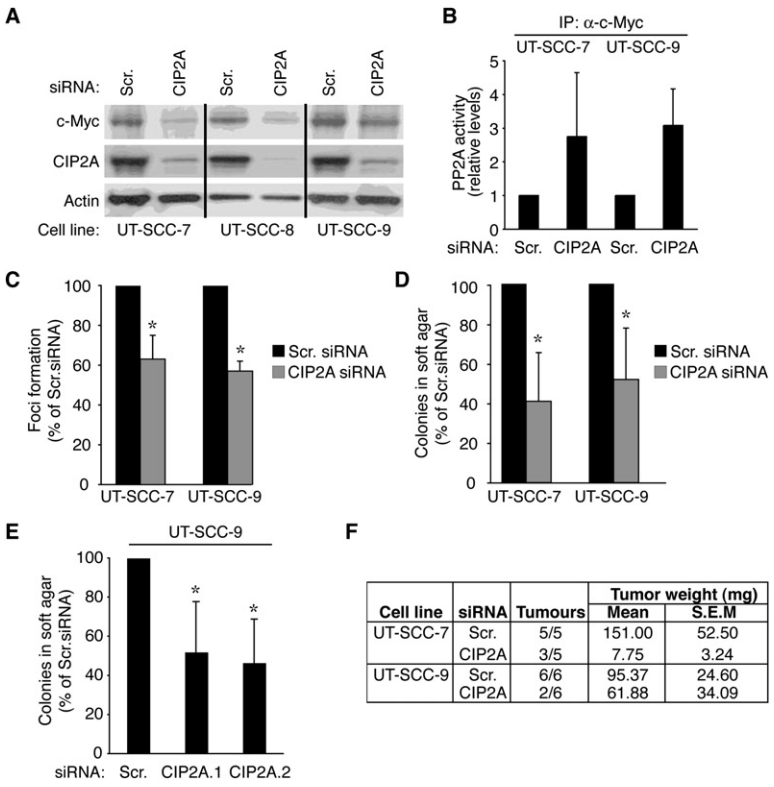
To examine the role of CIP2A in the regulation of HNSCC cell proliferation, UT-SCC-7 and UT-SCC-9 cell lines were subjected to CIP2A-siRNA transfection and dense foci formation of these cell lines was monitored for 10 days. In both cell lines, depletion of CIP2A resulted in a significant reduction in foci formation (Figure 7C). Importantly, CIP2A depletion also significantly reduced anchorage-independent growth of both UT-SCC-7 and UT-SCC-9 cells in soft agar 21 days after transfection (Figure 7D). Consistent with the demonstrated specificity of CIP2A siRNAs in HeLa cells (Figures 2A, 2B, 3B, and S4), two independent CIP2A siRNAs yielded a similar inhibition in soft-agar growth of UT-SCC-9 cells (Figure 7E).

Finally, to assess the role of CIP2A for in vivo tumor growth of UT-SCC cells, both UT-SCC-7 and UT-SCC-9 cells transfected with CIP2A or scrambled siRNAs were injected into the back of severe combined immunodeficiency (SCID) mice. Consistent with all the other data presented in this work indicating the importance of CIP2A for malignant cell growth and tumor progression, only three out of five and two out of six mice injected with CIP2A-siRNA transfected UT-SCC-7 and UT-SCC-9 cells, respectively, developed palpable tumors at day 65 when the experiment was terminated (Figure 7F). Moreover, CIP2A siRNA reduced the average size of tumors with both of the UT-SCC cells, as compared to scrambled siRNA transfected cells (Figure 7F).

These results confirm that the role of CIP2A in the regulation of c-Myc protein levels, c-Myc-associated PP2A activity, and in malignant cell growth are not restricted to HeLa cells but can be recapitulated in low-passage cancer cells derived from clinical tumor samples. Together with the transforming capacity of CIP2A (Figure 5B) and increased expression in HNSCC and in colon cancer (Figures 6D and 6F), these findings strongly support an oncogenic role of CIP2A in human malignancies.

DISCUSSION

Current evidence has established the requirement of PP2A inhibition for malignant transformation (reviewed in Arroyo and Hahn, 2005; Janssens et al., 2005). However, only a few reports thus far have presented potential mechanisms for the inhibition of PP2A activity in spontaneously developing human malignancies. In this regard, low-frequency mutations of PR65 proteins in human malignancies have been reported (Calin et al., 2000; Wang et al., 1998), and BCR/ABL-induced expression of the PP2A inhibitor SET was recently reported to promote malignant growth of chronic myelogenous leukemia cells (Neviani et al., 2005). Considering this evidence, it is clear that the discovery of CIP2A, a protein of previously unknown function that inhibits PP2A in human malignancies and has transforming activity, is important and expands the



Cell line	siRNA	Tumours	Tumor weight (mg)	
			Mean	S.E.M
UT-SCC-7	Scr.	5/5	151.00	52.50
	CIP2A	3/5	7.75	3.24
UT-SCC-9	Scr.	6/6	95.37	24.60
	CIP2A	2/6	61.88	34.09

Figure 7. CIP2A Promotes c-Myc Stability and Malignant Growth in HNSCC Cells

(A) Immunoblotting of c-Myc protein levels in HNSCC cells (UT-SCC) 72 hr after transfection with scrambled or CIP2A.1 siRNA.

(B) The indicated UT-SCC cell lines were transfected for 72 hr with scrambled or CIP2A.1 siRNA and then subjected to c-Myc immunoprecipitation and PP2A activity assay. Shown are mean values +SD from two independent experiments.

(C) Dense foci formation on a monolayer of UT-SCCs transfected with scrambled or CIP2A.1 siRNA 10 days after replating was quantitated by Image J software. Shown are average + SD of three experiments. *$p < 0.05$, Student's t test.

(D) Anchorage-independent growth in soft agar of UT-SCCs transfected with scrambled or CIP2A.1 siRNA at day 21. Shown is the average +SD of six replicate plates. *$p < 0.05$, Student's t test.

(E) Anchorage-independent growth in soft agar of UT-SCC-9 cells transfected with two independent CIP2A-targeting siRNAs (CIP2A.1 or CIP2A.2) or scrambled siRNA was quantitated at day 21. Shown is the average +SD of six replicate plates. *$p < 0.05$, Student's t test.

(F) UT-SCC-7 or UT-SCC-9 cells transfected for 72 hr with either scrambled or CIP2A.1 siRNAs were injected subcutaneously into the back of SCID mice and monitored for 59 days for the appearance tumors. Shown are the number of palpable tumors and mean +SEM of tumor weights.

general understanding of the mechanisms critical for cancer progression. In addition to its role in cancer, it is plausible that CIP2A regulation of c-Myc stability and cell growth could impact other conditions that rely on c-Myc function, for example, stem cell renewal (Gandarillas and Watt, 1997; Honeycutt and Roop, 2004). Therefore, the reported function of CIP2A in regulating c-Myc stability further expands the general importance of this work.

Based on previously published work, it is well established that c-Myc S62 is an in vivo target for PP2A and that phosphorylation of S62 protects c-Myc from proteolytic degradation both in mammals and in yeast (Arnold and Sears, 2006; Escamilla-Powers and Sears, 2007; Yeh et al., 2004). Therefore, our results are fully consistent with the proposed role of CIP2A as a PP2A inhibitor that stabilizes c-Myc protein (Arnold and Sears, 2006; Yeh et al., 2004). Importantly, our results indicate that among the PP2A substrates, CIP2A selectively targets the c-Myc-associated PP2A complex. These conclusions are supported by the data that CIP2A binds directly to c-Myc (Figure 3D) and that its depletion does not affect MEK1,2 phosphorylation or MDM2-associated PP2A activity (Figures 2C and 3B). Moreover, our findings demonstrate that the c-Myc S62 mutations, which have no effect on the interaction between PP2A and c-Myc (Arnold and Sears, 2006), drastically impaired the CIP2A-c-Myc interaction (Figure 3E). Therefore, it seems evident that CIP2A is not recruited to c-Myc via its interaction with PP2A but directly binds to c-Myc through recognition of the S62 site and subsequently inhibits c-Myc-associated PP2A activity. Taken together, our results suggest a hitherto unrecognized mechanism for localized inhibition of PP2A activity, whereby the in vivo specificity of PP2A is determined by direct binding of a PP2A inhibitor to the PP2A substrate. In the future, it would be of great interest to study if CIP2A binding to c-Myc only determines its in vivo specificity or if it is also a prerequisite for CIP2A's function as a PP2A inhibitor protein.

Our results demonstrate that depletion of CIP2A increases c-Myc-associated PP2A activity without affecting the PP2A-c-Myc interaction (Figures 3B and 3C). Moreover, the increase in c-Myc-associated PP2A activity was observed both in cells by enhanced dephosphorylation of c-Myc S62 (Figure 3A) and in vitro by using a PP2A substrate unrelated to c-Myc (Figure 3B). Together these results strongly indicate that the catalytic activity of PP2Ac per se is inhibited by CIP2A. Recently published structures of the PP2A holoenzyme suggest that the structural arrangement of the PP2A B-C subunit interface and the conformational flexibility of the PR65/A subunit make the PP2A complex susceptible to structural

changes that may inhibit substrate hydrolysis by PP2Ac or alter the substrate specificity (Cho and Xu, 2007; Xu et al., 2006). It is possible that allosteric regulation of the PP2A complex structure could mediate inhibition of PP2A activity by CIP2A binding. However, as the exact molecular mechanism of PP2A inhibition by CIP2A remains a matter of speculation at this point, future research is needed to untangle this possibility.

On the cellular level, our results show that CIP2A is not essential for cell-cycle progression or cell viability but supports malignant cell growth and c-Myc-mediated gene expression (Figure 4 and data not shown). Importantly, we demonstrate that CIP2A expression transforms HEK-TERV cells immortalized by Ras, TERT, and large-T antigen (Figure 5B) and that CIP2A is sufficient to induce tumorigenic conversion of spontaneously immortalized murine cells in combination with Ras (Figure 5A). These results strongly implicate CIP2A as a protein that cooperates with other oncoproteins in cellular transformation. Moreover, we show that CIP2A expression is already induced in premalignant HNSCC tissue in response to a combination of oncogenic Ras signaling and inhibition of the TGF-β tumor suppressor pathway (Figure 6F). These results further support the role of CIP2A as an oncoprotein involved in early cellular transformation and suggest functional cooperation between Ras and CIP2A at least in HNSCC development.

In addition to their biological significance, the results presented in this work strongly indicate that CIP2A inhibition could provide an opportunity for targeted degradation of c-Myc in cancer cells, making CIP2A a candidate target protein for future cancer therapeutics. Importantly, inhibition of CIP2A should decrease c-Myc levels in cancer cells regardless of the cause of c-Myc protein overexpression (Sears, 2004). Moreover, by focusing on a tumor-specific protein, such as CIP2A, the controversy surrounding the inability to directly regulate PP2A and/or c-Myc in normal tissue due to their requirement for normal cellular function would be alleviated (Gotz et al., 1998; Pelengaris et al., 2002).

Taken together, this study identifies CIP2A as a cellular PP2A inhibitor that inhibits proteolytic degradation of c-Myc. Moreover, we demonstrate that CIP2A is important for the maintenance of the malignant cellular phenotype and that overexpression of CIP2A transforms HEK-TERV cells. Importantly, CIP2A is overexpressed in two common human malignancies, HNSCC and colon cancer. Together, these results demonstrate that CIP2A acts as a human oncoprotein that inhibits PP2A in human malignancies. However, even though our current results suggest that inhibition of c-Myc-associated PP2A activity is one of the mechanisms by which CIP2A supports malignant cellular growth, we cannot rule out that CIP2A might exert its oncogenic properties also through other as yet unidentified mechanisms. Therefore, further elucidation of the molecular functions of CIP2A, both in its regulation of c-Myc stability and in other signaling events potentially regulated by the PP2A-CIP2A interaction, will be of great importance. In addition, identification of the physiological role of CIP2A and its potential involvement in nonmalignant cellular processes such as stem cell renewal or cellular differentation will pose an interesting future challenge.

EXPERIMENTAL PROCEDURES

Cell Cultures

HeLa, HT-1080, HEK-293, and 3T3 cells were obtained from ATCC. Spontaneously immortalized mouse embryo Fibroblasts was established as described previously (Dietrich et al., 2004). Human HNSCC cell lines were established at the time of operation from HNSCCs (Lansford et al., 1999). HEK-TERV cells were a generous gift from Professor W.C. Hahn (Chen et al., 2004, 2005). Normal HEK were established from skin samples (generous gift from Professor J. Peltonen) and cultured in Keratinocyte Basal Medium 2 (KBM-2), supplemented with SingleQuots (Cambrex Bioscience).

siRNA Transfections

One hundred nanomolars of double-stranded siRNA oligonucleotides (for siRNA sequences, see Supplemental Data) were transfected with Oligofectamine reagent (Invitrogen) according to the manufacturer's instructions. Four silent mutations were introduced in the CIP2A.1 siRNA-binding region of CIP2A flag construct to create the CIP2A rescue plasmid using QuikChange II site-directed mutagenesis kit (Stratagene) (for sequence information, see Supplemental Data). In rescue experiments, 2 μg of rescue plasmid was transfected 24 hr after siRNA transfection using Lipofectamine 2000 (Invitrogen).

Anchorage-Independent Growth and Foci Formation Assays

For HeLa cell foci formation and anchorage-independent growth assays, 4×10^5 cells were seeded on 10 cm plates 48 hr after siRNA transfection. Soft-agar assays were performed in medium containing 10% fetal bovine serum as described (Chen et al., 2005). The number and size of colonies were analyzed using ImageJ 1.33u software from microscopy images (×5 magnification). Anchorage-independent colonies were classified according to a number between 200—10,000 pixels.

For foci formation assays in MEFs, 200 or 500 cells of the retrovirally transduced MEFs were plated with 8.3×10^5 NIH3T3 cells as feeders per well in a 6-well plate. Cells were grown for 1–2 weeks. Methanol-fixed cells were stained with Giemsa. Foci numbers were calculated per 100 infected cells.

In Vivo Tumor Formation

For the mouse experiments, 3×10^6 siRNA transfected cells were injected subcutaneously into the flank or back of immunocompromised mouse. Tumor formation was evaluated every third day by palpation, and the size of the palpable tumors was measured by precision instrument or by weighing the tumors at the end of the experiment.

Virus Production

Lentiviral clones of CIP2A and lacZ were transfected into 293FT cells with ViraPower packaging mix (Invitrogen Life Technologies). Supernatants were harvested 48 hr after transfection, sterile filtered, and stored at −80°C. HEK-TERV cells were infected for 24 hr with viral supernatant at MOI 1000, and selection was begun 48 hr later with Zeocin (100 μg/μl) (Invitrogen Life Technologies). Clones were verified for transgene expression after 10 days and analyzed for anchorage-independent growth in soft agar. For retroviral infections, virus propagation and purification were performed as previously published (Morgenstern and Land, 1990).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, one table, and five figures and can be found with this article online at http://www.cell.com/cgi/content/full/130/1/51/DC1/.

ACKNOWLEDGMENTS

The expert technical assistance of Raisa Vuorinen and Sari Pitkänen is gratefully acknowledged. We thank T. Vahlberg for help in statistical analysis, A. Laiho and Tia Heinonen for the microarray analysis, and E. Mattila for the help with mouse experiments. Special thanks to T. Junttila and K. Elenius for the analysis of CIP2A mRNA in normal tissues and to H. Nordlund and M. Kulomaa for CIP2A protein production. We thank C.M Counter, W.C. Hahn, B. Hemmings, and J. Peltonen for materials. This study was supported by the Academy of Finland (projects 878179, 8212695, 8105778, and 45996), Sigrid Jusélius Foundation, the Cancer Research Foundation of Finland, Turku University Central Hospital (project 13336), and the European Union Framework Programme 6 (LSHC-CT-2003-503297; CANCERDEGRADOME). Grant support for Hugh Arnold was NIH T32-GM08617, for Rosalie Sears NIH R01-CA100855, and for Xiao-Jing Wang NIH DE15953.

Received: July 7, 2006
Revised: January 25, 2007
Accepted: April 26, 2007
Published: July 12, 2007

REFERENCES

Arnold, H.K., and Sears, R.C. (2006). Protein phosphatase 2A regulatory subunit B56alpha associates with c-myc and negatively regulates c-myc accumulation. Mol. Cell. Biol. *26*, 2832–2844.

Arroyo, J.D., and Hahn, H. (2005). Involvement of PP2A in viral and cellular transformation. Oncogene *24*, 7746–7755.

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.

Chen, W., Possemato, R., Campbell, K.T., Plattner, C.A., Pallas, D.C., and Hahn, W.C. (2004). Identification of specific PP2A complexes involved in human cell transformation. Cancer Cell *5*, 127–136.

Chen, W., Arroyo, J.D., Timmons, J.C., Possemato, R., and Hahn, W.C. (2005). Cancer-associated PP2A Aalpha subunits induce functional haploinsufficiency and tumorigenicity. Cancer Res. *65*, 8183–8192.

Cho, U.S., and Xu, W. (2007). Crystal structure of a protein phosphatase 2A heterotrimeric holoenzyme. Nature *445*, 53–57.

Dietrich, N., Thastrup, J., Holmberg, C., Gyrd-Hansen, M., Fehrenbacher, N., Lademann, U., Lerdrup, M., Herdegen, T., Jaattela, M., and Kallunki, T. (2004). JNK2 mediates TNF-induced cell death in mouse embryonic fibroblasts via regulation of both caspase and cathepsin protease pathways. Cell Death Differ. *11*, 301–313.

Escamilla-Powers, J.R., and Sears, R.C. (2007). A conserved pathway that controls c-Myc protein stability through opposing phosphorylation events occurs in yeast. J. Biol. Chem. *282*, 5432–5442.

Gandarillas, A., and Watt, F.M. (1997). c-Myc promotes differentiation of human epidermal stem cells. Genes Dev. *11*, 2869–2882.

Gotz, J., Probst, A., Ehler, E., Hemmings, B., and Kues, W. (1998). Delayed embryonic lethality in mice lacking protein phosphatase 2A catalytic subunit Calpha. Proc. Natl. Acad. Sci. USA *95*, 12370–12375.

Grady, W.M., and Markowitz, S.D. (2002). Genetic and epigenetic alterations in colon cancer. Annu. Rev. Genomics Hum. Genet. *3*, 101–128.

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.

Hanahan, D., and Weinberg, R.A. (2000). Hallmarks of cancer. Cell *100*, 57–70.

Honeycutt, K.A., and Roop, D.R. (2004). c-Myc and epidermal stem cell fate determination. J. Dermatol. *31*, 368–375.

Janssens, V., and Goris, J. (2001). Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. Biochem. J. *353*, 417–439.

Janssens, V., Goris, J., and Van Hoof, C. (2005). PP2A: the expected tumor suppressor. Curr. Opin. Genet. Dev. *15*, 34–41.

Lansford, C.D., Grénman, R., Bier, H., Somers, K.D., Kim, S.-Y., Whiteside, T.L., Clayman, G.L., Welkoborsky, H.-J., and Carey, T.E. (1999). Head and Neck Cancers. In Human Cell Culture, J. Masters and B. Palsson, eds. (Dordrecht: Kluwer Academic Press), pp. 185–255.

Lu, S.-L., Herrington, H., Reh, D., Weber, S., Bornstein, S., Wang, D., Li, A.G., Tang, C.-F., Siddiqui, Y., Nord, J., et al. (2006). Loss of transforming growth factor-beta type II receptor promotes metastatic head-and-neck squamous cell carcinoma. Genes Dev. *20*, 1331–1342.

Moreno, C.S., Ramachandran, S., Ashby, D.G., Laycock, N., Plattner, C.A., Chen, W., Hahn, W.C., and Pallas, D.C. (2004). Signaling and transcriptional changes critical for transformation of human cells by simian virus 40 small tumor antigen or protein phosphatase 2A B56gamma knockdown. Cancer Res. *64*, 6978–6988.

Morgenstern, J.P., and Land, H. (1990). Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. *18*, 3587–3596.

Moule, M.G., Collins, C.H., McCormick, F., and Fried, M. (2004). Role for PP2A in ARF signaling to p53. Proc. Natl. Acad. Sci. USA *101*, 14063–14066.

Neviani, P., Santhanam, R., Trotta, R., Notari, M., Blaser, B.W., Liu, S., Mao, H., Chang, J.S., Galietta, A., Uttam, A., et al. (2005). The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. Cancer Cell *8*, 355–368.

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.

Pastula, C., Johnson, I., Beechem, J.M., and Patton, W.F. (2003). Development of fluorescence-based selective assays for serine/threonine and tyrosine phosphatases. Comb. Chem. High Throughput Screen. *6*, 341–346.

Pelengaris, S., Khan, M., and Evan, G. (2002). c-MYC: more than just a matter of life and death. Nat. Rev. Cancer *2*, 764–776.

Rangarajan, A., Hong, S.J., Gifford, A., and Weinberg, R.A. (2004). Species- and cell type-specific requirements for cellular transformation. Cancer Cell *6*, 171–183.

Sears, R.C. (2004). The life cycle of C-myc: from synthesis to degradation. Cell Cycle *3*, 1133–1137.

Soo Hoo, L., Zhang, J.Y., and Chan, E.K. (2002). Cloning and characterization of a novel 90 kDa 'companion' auto-antigen of p62 overexpressed in cancer. Oncogene *21*, 5006–5015.

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.

Xu, Y., Xing, Y., Chen, Y., Chao, Y., Lin, Z., Fan, E., Yu, J.W., Strack, S., Jeffrey, P.D., and Shi, Y. (2006). Structure of the protein phosphatase 2A holoenzyme. Cell *127*, 1239–1251.

Yeh, E., Cunningham, M., Arnold, H., Chasse, D., Monteith, T., Ivaldi, G., Hahn, W.C., Stukenberg, P.T., Shenolikar, S., Uchida, T., et al. (2004). A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. Nat. Cell Biol. *6*, 308–318.

Zhao, J.J., Roberts, T.M., and Hahn, W.C. (2004). Functional genetics and experimental models of human cancer. Trends Mol. Med. *10*, 344–350.