MYC-Dependent Regulation and Prognostic Role of CIP2A in Gastric Cancer

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Background
Cancerous inhibitor of protein phosphatase 2A (CIP2A) is a recently identified human oncoprotein that stabilizes the c-Myc (MYC) protein. However, the clinical relevance of CIP2A to human cancers had not been demonstrated, but the mechanism of its regulation and its clinical role in cancer were completely unknown.

Methods
Tissue microarrays consisting of 223 gastric adenocarcinoma specimens were evaluated for the presence of CIP2A using immunohistochemistry, and the association of CIP2A expression with survival was assessed using Kaplan–Meier analysis. The effects of MYC and CIP2A on each other’s expression and on cell proliferation were investigated in several gastric cancer cell lines using small interfering RNAs to CIP2A and MYC and immunoblotting. To further evaluate the role of MYC in CIP2A regulation, an inhibitor of MYC dimerization, 10058-F4, and an inducible MycER model were used.

Results
Expression of CIP2A protein was associated with reduced overall survival for gastric cancer patients with tumors 5 cm or smaller, with a 10-year overall survival in the CIP2A-immunopositive group of 8.1% as compared with 37.6% in the CIP2A-negative group (difference = 29.5%, 95% confidence interval = 12.5% to 46.5%, \( P = .001 \)). In gastric cancer cell lines, CIP2A depletion led to decreased proliferation and anchorage-independent growth of the cells, as well as to reduced stability and expression of MYC protein. Interestingly, MYC depletion led to reduced expression of CIP2A mRNA and protein. Moreover, experiments with an MYC inhibitor and activator suggested that MYC directly promotes CIP2A gene expression. Finally, CIP2A and MYC immunopositivities were associated in gastric cancer specimens (\( P = .021 \)).

Conclusions
CIP2A immunopositivity is a predictor of survival for some subgroups of gastric cancer patients. CIP2A and MYC appear to be regulated in a positive feedback loop, wherein they promote each other’s expression and gastric cancer cell proliferation.


Although incidence of gastric cancer has steadily declined in the Western countries over several decades, it is still the second most common cause of cancer-related deaths worldwide (1). The high mortality is mostly due to late diagnosis of the disease. The great majority of gastric malignancies are adenocarcinomas that can be divided into two histological entities, intestinal and diffuse type, which exhibit distinct epidemiological and genetic patterns (2,3). Curative treatment of gastric cancer requires complete surgical removal of the neoplastic tissue, but even with curative intent, the 5-year survival is only about 20%–30% (4). Therefore, new diagnostic markers and treatment modalities are needed.

Overexpression of c-Myc (MYC), as determined by immunohistochemistry, is associated with poor survival among gastric cancer patients (5,6). However, the functional role of the MYC protein and the mechanism whereby its expression is regulated in gastric cancers are largely unexplored. In nontransformed cells, MYC is a very short-lived protein due to degradation by an ubiquitin-mediated proteosomal process (7–9). However, MYC protein stability is increased...
Cancerous inhibitor of protein phosphatase 2A (CIP2A) was recently reported by the authors to interact with and prevent dephosphorylation of the MYC oncoprotein by inhibiting PP2A-mediated dephosphorylation of MYC phosphoserine 62. CIP2A was reported to be overexpressed in head and neck and colon cancers, but the mechanisms of its regulation and its clinical role in cancer were unclear.

**Study design**

CIP2A expression in gastric tumor specimens was compared with patient survival data. Experiments were performed that used short interfering RNAs, a MYC inhibitor, or an inducible MYC construct in several gastric cancer cell lines to clarify the roles of MYC and CIP2A in each other’s expression and in cell proliferation.

**Contribution**

CIP2A immunopositivity was associated with substantially decreased 10-year overall survival among gastric cancer patients, and presence of CIP2A was associated with increased proliferation, anchorage-independent growth, and MYC protein stability in gastric cancer cells. In addition, MYC appeared to promote CIP2A mRNA and protein expression.

**Implications**

MYC and CIP2A appear to reinforce each other’s expression (or inhibition) in a positive feedback loop that would appear to be an attractive target for cancer therapeutics.

**Limitations**

Additional experiments will be necessary to definitively demonstrate a functional role for CIP2A in human tumorigenesis. Also, a direct effect of MYC on CIP2A transcription has not yet been shown, probably because MYC responsive elements lie outside the proximal CIP2A promoter.

**Context and Caveats**

**Prior knowledge**

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**Materials and Methods**

**Patients**

The study included 337 consecutive patients who underwent surgery for histologically verified gastric adenocarcinoma at the Department of Surgery, Helsinki University Central Hospital, between April 1983 and June 1999. Approval for this retrospective study was obtained from the Department of Surgery, Helsinki University Central Hospital, ethics committee and from the Finnish National Authority for Medicolegal Affairs. The International Union Against Cancer classification of 1992 was used for patient characteristics (Supplementary Table, available online). Overall survival data were obtained in October 2007 from patient records, the Finnish Cancer Registry, Population Register Centre, and Statistics Finland. Because of the retrospective nature of the study and with the permission from the Finnish National Authority for Medicolegal Affairs, written patient consent was not required.

**Preparation of Tumor Tissue Microarrays**

Tumor tissue microarrays were prepared that contained three representative tumor cores from each patient. Tumor samples were fixed in formalin, embedded in paraffin, and stored at the archives of the Department of Pathology, University of Helsinki. At the beginning of this study, in January 2006, three representative 0.6-mm cores were punched from the malignant areas of each tumor specimen using a tissue microarray instrument (Manual Tissue Arrayer 1; Beecher Instruments, Silver Spring, MD). The cores were arranged into six tissue array blocks, each containing between 80 and 180 tumor cores (15).

**Immunohistochemistry**

Tumor tissue microarray blocks were freshly cut into 4-µm-thick sections. Sections were fixed on slides and dried for 12–24 hours at 37°C. Sections were subsequently deparaffinized in xylene and rehydrated through gradually decreasing concentrations of ethanol to distilled water. For antigen retrieval, slides were treated in a Pretreatment Module (Lab Vision Corp., Fremont, CA) in Tris–HCl buffer (pH 8.5) for 20 minutes at 98°C. Sections were stained in an Autostainer 480 (Lab Vision Corp.) using the Dako REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse (Dako, Glostrup, Denmark). In brief, slides were treated for 5 minutes with 0.3% Dako REAL Peroxidase-Blocking Solution to block endogenous peroxidases. Subsequently, slides were incubated for 1 hour with a rabbit polyclonal CIP2A antibody (16) diluted 1:2000 in Dako REAL Antibody Diluent (Dako) or with mouse monoclonal MYC antibody 9E10 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500, followed by a 30-minute incubation with horseradish peroxidase (HRP)-conjugated Dako REAL EnVision rabbit anti-mouse antibody, and finally visualized by Dako REAL DAB+ Chromogen for 10 minutes. Between each step in the staining procedure, slides were washed with phosphate-buffered saline (PBS; 810 mM disodium phosphate [Na₂HPO₄], 150 mM monopotassium phosphate [KH₂PO₄], 270 mM potassium...
chloride [KCl], and 13.7 M sodium chloride [NaCl] dissolved in distilled water) containing 0.04% Tween-20 (Dako). Slides were counterstained with Mayer’s hematoxylin, washed in tap water for 10 minutes, and mounted in aqueous mounting medium (Aquamount; BDH, Poole, UK). For p53 staining, the slides were stained with a 1:300 dilution of mouse monoclonal DO-7 antibody (Dako), which recognizes both the mutant and the wild-type human p53 proteins as described previously (17).

Scoring of Immunoreactivity
CIP2A immunopositivity was graded in one to three tumor cores for each patient based on the intensity of the cytoplasmic immunoreactivity in the cancer cells, that is, 3 was strong, 2 moderate, 1 weak, and 0 negative. Samples were scored positive for MYC if either the cytoplasm or the nucleus of the cancer cells showed presence of MYC immunostaining. The p53 staining was scored as described previously (17).

All samples were scored independently by two researchers (by A. Hemmes and A. Ristimäki or by C. Böckelman and A. Ristimäki), one of whom is an experienced gastrointestinal pathologist (A. Ristimäki), without knowledge of clinical status and outcome data. Whenever the two scores were discordant, the specimens were reevaluated using a multiheaded microscope, and the consensus score was used for further analysis. The highest score among a given patient’s three samples was considered to be the representative one. In the final analysis, samples were considered to be CIP2A immunonegative (score 0) or CIP2A immunopositive (scores 1–3). We were able to process and score CIP2A in 223 samples in this manner, and CIP2A and MYC coexpression was determined in a subset of 47 specimens.

Cell Culture
The following five human gastric cancer cell lines were used: AGS (gastric adenocarcinoma) (18); MKN-28 (intestinal-type gastric adenocarcinoma); and KATOIII, MKN-45, and TMK-1 cells (all diffuse-type gastric adenocarcinomas) (19). AGS and KATOIII cells were obtained from American Type Culture Collection, and all other cell lines were a kind gift from Dr Hiroshi Yokozaki, Kobe University Graduate School of Medicine, Kobe, Japan. All cell lines were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 2 mM l-glutamine, and 13.7 M sodium chloride [NaCl] dissolved in distilled water and adjusted with HCl to pH 7.5) containing 0.1%-NP40 (Igepal Ca-630; Sigma-Aldrich) and then incubated with a 1:500 dilution in 5% milk in TBS-NP40 of mouse monoclonal anti-MYC antibody (Santa Cruz Biotechnology) at 4°C overnight, with a 1:5000 dilution of the rabbit polyclonal anti-CIP2A antibody at room temperature for 1 hour, or with a 1:1000 dilution of goat polyclonal anti-β-actin antibody (Santa Cruz Biotechnology) at room temperature for 1 hour. Other antibodies used for detection were a mouse monoclonal antibody to S62 phosphorylated MYC with 1:500 dilution (BioAcademia, Inc., Osaka, Japan), mouse monoclonal antibody to total MYC with 1:500 dilution (Nordic Biosite, Täby, Sweden), rabbit monoclonal anti-JUN with 1:1000 dilution (Cell Signaling Technology, Inc., Danvers, MA), and mouse monoclonal anti-p53 with 1:500 dilution (Santa Cruz Biotechnology). Subsequently, membranes were incubated with a 1:2000 dilution of HRP-conjugated sheep anti-mouse antibody (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), a 1:500 dilution of goat anti-rabbit antibody (Pierce Biotechnology, Inc., Rockford, IL), or a 1:2000 dilution of donkey anti-goat antibody (Santa Cruz Biotechnology), as appropriate to the species of the primary antibody, for 1 hour at room temperature. The proteins were visualized by enhanced chemiluminescence (ECL) with either the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Inc.) or the Proteome Grasp ECL Kit (Pierce Biotechnology, Inc.). The specificity of the CIP2A and MYC antibodies was verified by loss of the appropriate immunoreactive protein bands on immunoblots from siRNA-transfected cell lysates (see Figure 4, A and B, and Supplementary Figure 3, available online).

mRNA Analysis
Total mRNA was extracted from cells using the RNasy kit (Qiagen, Valencia, CA) and converted to cDNA by using the M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant cDNA synthesis kit (Promega Corporation, Madison, WI). cDNAs were subjected to quantitative real-time polymerase chain reaction (PCR) by using the Light Cycler (Roche Diagnostics, Mannheim, Germany) and SYBR Green PCR Master Mix kit (Roche Diagnostics). Primer sequences (Sigma-Prologio, St Louis, MO) used for PCR of CIP2A were as follows: CIP2A forward, 5′-CTGGTGAGATAATCTGACTTTTCA-3′ and CIP2A reverse, 5′-CGAAACATTTCACTAGCTTTTCA-3′. Expression of Junonji, a protein involved in chromatin regulation, that is also known as KDM3A, or previously, JMJD1A, was measured as a control for general transcription activity by PCR using the following primers: Junonji forward, 5′-CACCCCTGTGGTGGCAGACATTTC-3′ and Junonji reverse, 5′-GCCAATTTTGAGGCCACCACCT-3′. Transcript levels were normalized to levels of TATA-binding protein (TBP) or β-actin expression, which were determined by PCR of the same samples using the following primers: TBP forward, 5′-GAATATAATCCAAAGCCTTTTG-3′ and TBP reverse, 5′-ACTTACATCATACAGCTCCC-3′; actin forward, 5′-CGAGGACAGGCTGGCATTG-3′; and actin reverse: 5′-CATAGGATCTCCTTCTGACCCATG-3′.

siRNA Experiments
The siRNAs to inhibit CIP2A expression were obtained from Eurofins MWG operon (Ebersberg, Germany). Either of the following double-stranded oligonucleotides was transiently transfected
into AGS, MKN-28, and KATOIII cell lines as CIP2A siRNAs: CIP2A.1, 5′-CUGUGGUUGUGUUUGACUTT-3′, and CIP2A.2, 5′-ACCAUUGAUACCCUUAGATT-3′ (10). As a control, a scrambled siRNA with the sequence 5′-UAACAAUGAGAGCAGGGTC-3′ was used instead. HP-validated siRNAs for human MYC (Hs_Myc_7, Hs_Myc_5) were purchased from Qiagen Sciences (Germantown, MD).

AGS, MKN-28, and HT1080 cells at 30%–50% confluency in a six-well plate were transfected with 33 nm in 3 ml of medium in each well of either CIP2A or MYC siRNA in antibiotic-free RPMI-1640 medium supplemented with 10% FCS using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. To determine the effect of the siRNAs on mRNA levels, cells were harvested and mRNA was extracted 24 hours posttransfection. To determine the effect on protein expression, cells were harvested and lysates prepared for immunoblotting at 72 hours posttransfection.

**Cell Viability Assay**

One day before transfection of CIP2A.1 or CIP2A.2 siRNAs, MKN-28, AGS, and KATOIII cells were seeded in RPMI-1640 medium supplemented with 10% FCS at a density of 1 × 10^3 to 2 × 10^4 cells per well in 96-well plates. The cells were transfected with the following conditions: medium only, Lipofectamine 2000 reagent only, or 20 nM scrambled siRNA in 200 µL or 20 nM CIP2A.1 or CIP2A.2 siRNAs (with Lipofectamine 2000 reagent) in 200 µL of RPMI-1640 supplemented with 10% FCS. Cells were then cultured for 1, 3, 6, or 8 days posttransfection (for MKN-28 cells) or for 6 days posttransfection (for AGS and KATOIII cells) before relative numbers of viable cells were measured by fluorescence at the 544 and 590 nm wavelengths in a FLUOstar OPTIMA Microplate Reader (BMG Labtech, Inc., Durham, NC), using the resazurin-based CellTiter-Blue Assay (Promega Corporation) according to the manufacturer’s instructions.

**Cell Proliferation Assay**

One day before siRNA transfection, AGS cells were plated in six-well plates (1 × 10^4 cells per well) in triplicate. After adhering overnight, the cells were transfected with 33 nM scrambled or MYC1 siRNA per 3 mL of medium in each well using the Lipofectamine method. The number of cells in each well was counted after 6 days using a Z2 Coulter Counter Analyzer (Beckman Coulter, Fullerton, CA). Before cell counting, images of representative cell populations were captured.

**Soft Agar Assay**

At 48 hours after transfection, MKN-28 and KATOIII cells (1 × 10^4 per dish) were suspended in 1 mL of 0.25% agarose (GellyPhor; EuroClone Spa, Pero, Italy) supplemented with 2 mL complete RPMI-1640 culture medium (changed every third day). This suspension was layered over 1 mL of a base layer of 0.5% agarose in complete medium in six-well plates. After 8 or 12 days in agarose, cells were stained with Giemsa 1:20 in water (Sigma-Aldrich), photographs were taken with a Leica MZFLIII microscope (Leica, Hickersville, NY), and colonies were counted by analysis with ImageJ Software (Wayne Rasband, National Institutes of Health, Bethesda, MD). Cell groupings that were greater than 1200 pixels in diameter with 3.2× enlargement were counted as colonies.

**Cycloheximide Pulse Chase (Protein Stability) Experiments**

At 72 hours after transfection with scrambled or CIP2A.1 siRNA, AGS cells were treated with 100 µg/mL cycloheximide or left untreated, and cell lysates were prepared after indicated time periods of 0–120 minutes. Samples were then immunoblotted to visualize total endogenous MYC, CIP2A, and actin. Protein levels of MYC and actin were then quantified using ImageQuant TL image analysis software (Amersham Biosciences). The levels of MYC protein (normalized to actin) in cycloheximide-treated cells were then normalized to levels of MYC protein (normalized to actin) in non-cycloheximide-treated cells, using the value for non-cycloheximide-treated cells at the 0-hour time point as the value equal to 1. Relative MYC levels were then visualized on an X-Y scatter graph plotted with the best-fit exponential curve for the indicated time points.

**Preparation of Mouse Embryonic Fibroblasts**

Mouse embryonic fibroblasts were prepared from E13.5 R26-MycER^(T2) mice by a standard technique (20), as follows. Embryos were separated from their yolk sacs and decapitated with their internal organs removed. Carcasses were minced with a fixed-head cell scraper and trypsinized (5% trypsin in PBS) for 30 minutes at 37°C. Mouse embryonic fibroblasts were then pelleted by centrifugation, resuspended in standard growth media (Dulbecco's modified Eagle medium supplemented with 10% FCS, glutamine, and penicillin/streptomycin) and seeded directly on 150-mm dishes. Upon reaching confluence, the cells were harvested and aliquoted for freezing. Upon thawing, cells were denoted as “P1” (passage 1) and passed according to the 3T3 protocol. Cells in passages 3–5 were used for experiments. 4-Hydroxytamoxifen (4-OHT), which was used at 100 nM concentration to activate the MycER protein, was obtained from Sigma-Aldrich.

**Cell Cycle Experiments**

AGS cells (1 × 10^4 per well in a six-well plate) were synchronized by serum starvation in RPMI-1640 supplemented with 0.5% FCS for 48 hours. Cells were thereafter stimulated to reenter the cell cycle by changing the growth medium to RPMI-1640 supplemented with 10% FCS. Experiments in which cells were arrested in S phase by an established thymidine/aphidicolin protocol (21) were done in 70% confluent cultures grown in RPMI-1640 containing 10% FCS by incubating cells first in the presence of 2 mM thymidine (Calbiochem, Darmstadt, Germany) for 14 hours after which cells were washed three times with cold PBS and left untreated in RPMI-1640 medium containing 10% FCS for 12 hours. Thereafter, cells were treated with a 1.6 µg/mL concentration of aphidicolin (Calbiochem) for another 12 hours, and parallel cell plates were subsequently either analyzed for CIP2A and cell cycle marker expression levels or treated with 10058-F4, a MYC inhibitor (Sigma-Aldrich), for 6 hours.
Statistical Analysis

The association between the CIP2A positivity of tumors and the clinicopathologic characteristics of the patients was assessed using the $\chi^2$ test, and the association between CIP2A and MYC expression with Fisher exact test (SPSS version 16.0 for Mac; SPSS, Inc., Chicago, IL). Life tables of overall survival were calculated using the Kaplan–Meier method (22) in the whole material and in each of the preplanned subgroups identified in the Supplementary Table (available online). Survival curves were compared with the log-rank test (StatView for Windows, version 5.0; SAS Institute, Inc., Cary, NC; 23). Statistical significance between CIP2A-immunopositive and CIP2A-negative groups according to clinicopathologic parameters shown in the Supplementary Table (available online) was analyzed by an unpaired nonparametric test (Mann–Whitney) or with a $t$ test (SPSS, Inc.). All statistical tests were two-sided.

Results

Prognostic Role of CIP2A in Gastric Cancer

To address the clinical relevance of CIP2A protein expression in human malignancies, we analyzed whether CIP2A immunopositivity was associated with overall survival among gastric cancer patients. A set of 223 gastric carcinoma specimens was assembled from patients who had undergone surgery between 1983 and 1999. Three tumor cores for each of the 223 patients were included in tissue microarrays to ensure representative analysis of CIP2A protein expression as detected by immunohistochemistry (Figure 1A). Of the 223 specimens, 145 (65%) were immunopositive for CIP2A and 78 (35%) were immunonegative for this protein.

Over the course of follow-up, 91% (202/223) of the patients died; the median follow-up time was 1.2 years (range 0.02–20.8 years). Among all gastric cancer patients, presence of CIP2A immunostaining in tumors was associated with reduced survival; 10-year overall survival in the CIP2A-positive group was 7.5% compared with 17.2% in the CIP2A-negative group, which, however, was not a statistically significant difference (difference = 9.7%, 95% confidence interval [CI] = 0.1% to 19.3%, $P = .063$, log-rank test). In the subgroup of patients with small tumors ($\leq 5$ cm), there was an even stronger association between presence of CIP2A in tumors ($n = 110$; 74 positive and 36 negative) and reduced survival; among them, cumulative 10-year overall survival for patients with CIP2A-positive tumors was 8.1%, whereas that for patients with CIP2A-negative tumors was 18.0% (95% CI = 8.9 to 39.3) in CIP2A-negative patients and 7.0% (95% CI = 0.4 to 14.4) in CIP2A-positive patients (n = 80, $P = .017$). All statistical tests were two-sided.

CIP2A = cancerous inhibitor of protein phosphatase 2A; CI = confidence interval.
tumors was 37.6% (difference = 29.5%, 95% CI = 12.1% to 46.9%, \( P = .001 \); Figure 1, B). Furthermore, in a subgroup of patients with advanced disease (pT3 – T4; \( n = 172 \); 111 positive and 61 negative), cumulative 10-year overall survival for patients with CIP2A-positive tumors was 3.6%, whereas that for patients with CIP2A-negative tumors was 13.1% (difference = 9.5%, 95% CI = 0.4% to 18.6%, \( P = .044 \); Figure 1, C). In a third subgroup, p53-immunopositive patients (\( n = 80 \); 54 positive and 26 negative), cumulative 10-year overall survival was 1.9% among patients with CIP2A-positive tumors and 23.1% for those with CIP2A-negative tumors (difference = 21.2%, 95% CI = 4.6 to 37.8, \( P = .017 \); Figure 1, D). We did not observe a statistically significant survival difference in any other subgroup tested (see Supplementary Table, available online).

We also investigated the association of CIP2A immunopositivity with clinicopathologic variables. Presence of CIP2A in tumors was statistically significantly associated with old age at diagnosis (\( P = .013 \)), male sex (\( P = .001 \)), and intestinal type (vs diffuse type; \( P < .001 \) (Supplementary Table, available online). There was also a strong association between CIP2A immunopositivity and aneuploidy (\( P < .001 \)) and high S-phase fraction (SPF, \( P < .001 \)) (Supplementary Table, available online).

Together, these results demonstrate that CIP2A immunopositivity in tumors is associated with reduced overall survival in human cancer patients. Moreover, the association of CIP2A expression with markers of increased cellular malignancy (aneuploidy and high SPF) further supports a role for CIP2A in promoting cancer progression.

**Effect of CIP2A on Proliferation of Gastric Cancer Cells**

CIP2A expression was also studied by immunoblotting in a panel of human gastric cancer cell lines, including AGS cells and cells derived from intestinal-type (MKN-28) and diffuse-type (MKN-45, TMK-1, and KATOIII) gastric tumors (19). Roughly equivalent levels of CIP2A protein were expressed in all gastric cancer cell lines studied (Figure 2, A).

To understand the functional role of CIP2A, we studied the effects of CIP2A depletion on proliferation of gastric cancer cells in vitro. For these experiments, MKN-28 gastric cancer cells were transfected with CIP2A-specific siRNA or with a nonspecific scrambled control siRNA. Immunoblots of lysates from the transfected cells showed that a single transfection of CIP2A.1 siRNA resulted in potent reduction of CIP2A protein expression 6 days posttransfection that was still evident 8 days after transfection (Figure 2, B). When cell proliferation was assayed over this time period by using a fluorometric cell viability assay, there was a statistically significant and persistent decrease in proliferation of the MKN-28 cells: When MKN-28 cells that were transfected with scrambled siRNA – treated cells vs 7547 in CIP2A.1 siRNA – treated cells (difference = 2383, 95% CI = 1307 to 3458, \( P = .010 \)), and by day 8, there was a mean value of 37925 in scrambled siRNA–treated cells vs 28492 in CIP2A.1 siRNA–treated cells (difference = 11877, 95% CI = 9616 to 14138, \( P = .004 \)) (Figure 2, C). CIP2A.1 siRNA also inhibited proliferation of KATOIII cells: At...
day 6, the mean value of scrambled siRNA–treated cells was 16,100 vs 7089 in CIP2A.1 siRNA–treated cells (difference = 9011, 95% CI = 7514 to 10,507, P < .001) (Figure 2, D). The same effect on proliferation was seen in AGS cells: At 6 days, the mean value of scrambled siRNA–treated AGS cells was 25,433 vs 19,831 in CIP2A.1 or Scr. siRNAs. Data are presented as a percentage to the Scr. siRNA–transfected cells and shown as means with 95% CIs (n = 3) of the representative experiment. E) Effect of two independent MYC siRNAs on proliferation of AGS cells. In lieu of cell numbers, representative images of Scr. siRNA–, MYC.1 siRNA–, and MYC.2 siRNA–transfected AGS cells are shown. CHX = cycloheximide; CIP2A = cancerous inhibitor of protein phosphatase 2A; c-Myc = MYC; CI = confidence interval; siRNA = small interfering RNA; Scr. = scrambled.

The ability of cells to grow and form colonies on semisolid agar (anchorage-independent growth) is a hallmark of malignantly transformed cells. To study the role of CIP2A on malignant cell growth, MKN-28 cells were transiently transfected with CIP2A.1 siRNA or scrambled control siRNA and their capacity to form colonies on semisolid agar was evaluated at 8 and 12 days post-transfection. We found that CIP2A depletion in MKN-28 cells resulted in a statistically significant decrease in anchorage-independent growth of MKN-28 cells: 8 days after MKN-28 cells were transfected with scrambled or CIP2A.1 siRNAs, they were seeded at equal densities in soft agar, a mean of 11.6 colonies grew from control cells vs 5.5 colonies from cells with CIP2A.1 siRNA (difference = 6.1, 95% CI = 3.1 to 9.1, P = .017), and at 12 days, there was a mean of 24.8 colonies from control cells and 12.9 colonies from cells with CIP2A.1 siRNA (difference = 11.9, 95% CI = 4.4 to 19.3, P = .003) (Figure 2, E). In addition to MKN-28 cells, CIP2A.1 siRNA inhibited anchorage-independent growth in KATOIII cells (Supplementary Figure 2, available online).

Taken together, these results demonstrate that CIP2A can promote proliferation of either intestinal-type (MKN-28) or diffuse-type (KATOIII) gastric cancer cells. Furthermore, CIP2A appears to promote anchorage-independent growth of both cell types in soft agar assays.

Effect of CIP2A on MYC Stability in Human Gastric Cancer Cells

CIP2A has been shown to promote MYC serine 62 (S62) phosphorylation and thereby prevent proteolytic degradation of MYC in HeLa cervical carcinoma and in head and neck squamous cell carcinoma cells (10). To study whether CIP2A promotes MYC stability in human gastric cancer cells, steady-state levels of MYC protein expression were analyzed by immunoblotting 72 hours after transfection with CIP2A.1 siRNA or scrambled siRNA oligonucleotides. CIP2A depletion resulted in a clear inhibition of steady-state MYC protein levels in AGS gastric cancer cells (Figure 3, A), and depletion of CIP2A inhibited steady-state levels of S62-phosphorylated MYC protein (S62-p-MYC; Figure 3, B). Furthermore, CIP2A was shown to promote MYC expression in the MKN-28 gastric cancer cell line (see Figure 4, B). Next, the half-life of the endogenous MYC protein was analyzed in AGS cells that were treated with the protein synthesis inhibitor...
cycloheximide (100 µg/mL) for 0–120 minutes at 72 hours after transfection of these cells with CIP2A.1 siRNA or scrambled siRNA oligonucleotides. The time at which 50% of the MYC protein was remaining, as compared with the corresponding untreated cells, was measured to determine the half-life of the endogenous MYC protein in both of the cycloheximide-treated cell populations (CIP2A siRNA and scrambled siRNA transfected). In nontransformed cells, the half-life of the MYC protein is typically 20–30 minutes (7). By contrast, the half-life of the MYC protein in CIP2A-depleted cells was less than 50 minutes (Figure 3, C). These findings demonstrate that CIP2A promotes MYC stability in AGS cells.

To study the function of stabilized MYC in gastric cancer cells, MYC was depleted by siRNA and cell proliferation was studied by quantifying the cell number at 4–8 days after transfection. MYC depletion resulted in a clear inhibition of cell proliferation, first detected at 4 days and continuing for at least 8 days after siRNA transfection (data not shown). Six days after transfection, there was a statistically significant decrease in proliferation of MYC.1 siRNA–transfected cells (55% MYC.1 siRNA–transfected cells compared with 100% scrambled siRNA–transfected cells; difference = 45%, 95% CI = 28% to 62%, P = .004) (Figure 3, D). Another MYC siRNA resulted in equally efficient growth inhibition of AGS cells, verifying the specificity of the siRNA depletion (Figure 3, E). MYC depletion by the siRNAs used in Figure 3, D and E, was verified by immunoblot analysis (Figure 4, A).

Taken together, these results demonstrate that CIP2A promotes MYC stability in human gastric cancer cells (Figure 3, A–C). Maximal proliferation of these cells appears to be dependent on both CIP2A protein expression (Figure 2, C–E) and MYC protein expression (Figure 3, D and E).

**Identification of a Positive Feedback Mechanism Between MYC and CIP2A in Gastric Cancer**

Although CIP2A expression is increased in human cancer, the mechanisms by which CIP2A gene expression is regulated are completely unknown. Interestingly, when MYC siRNA–transfected AGS cells (Figure 3, E) were analyzed for CIP2A expression, a clear inhibition of CIP2A protein expression was noted (Figure 4, A). Although MYC depletion efficiently reduced the expression of CIP2A protein in both AGS and MKN-28 cells, it did not have any notable effects on p53 or JUN protein levels (Figure 4, A and B). MYC depletion also inhibited CIP2A protein expression in HT1080 fibrosarcoma cells (Supplementary Figure 3, C, available online), demonstrating that the observed effect is not specific to gastric cancer cells. Importantly, silencing of CIP2A reduced the expression levels of MYC in all of these cell lines (Figure 4, A and B, and Supplementary Figure 3, C, available online). In addition to CIP2A protein expression, we found that MYC siRNA efficiently inhibited CIP2A mRNA expression, with an mRNA expression of 1.25 for scrambled siRNA–treated cells compared with 0.18 for CIP2A.1 siRNA–treated cells (difference = 1.07, 95% CI = 1.00 to 1.15, P < .001) and 0.35 for MYC.1 siRNA–treated cells (difference = 0.90, 95% CI = 0.83 to 0.97, P < .001) as normalized to expression of actin in AGS cells (Figure 4, C). However, MYC depletion did...
not result in general suppression of transcription, as it did not substantially affect expression of either actin or jumonji mRNA, both of which served as unrelated controls (Figure 4, D).

The results given above demonstrate that CIP2A and MYC regulate each other’s expression in cultured cells. Next, to determine whether MYC and CIP2A were coexpressed in gastric cancer specimens, MYC and CIP2A were immunostained in adjacent tumor sections in tissue microarrays comprising 47 tumor specimens (Figure 5, A). Of these, four tumors were found to be double negative, two showed positive staining for only MYC, and seven showed positive staining for only CIP2A; most of the tumors (34/47; 72%) stained positive for both the MYC and the CIP2A proteins (Figure 5, B). Analysis of these results demonstrated a statistically significant association between MYC and CIP2A expression in vivo (P = .021).

**Effect of a MYC Inhibitor and a MYC Activator on CIP2A Expression**

The results given above demonstrate that siRNA-mediated depletion of MYC resulted in inhibition of CIP2A gene expression. MYC heterodimerizes with MAX to regulate expression of its downstream target genes (24). 10058-F4 is a recently characterized small-molecule inhibitor of MYC–MAX dimerization (25). In order to study whether MYC–MAX dimerization is required for MYC-mediated regulation of CIP2A expression, AGS cells were treated with increasing concentrations of 10058-F4, and CIP2A mRNA levels were examined after 24 hours. As shown in Figure 6, A, treatment of AGS cells with the concentration range (60–100 µM) of 10058-F4 that has previously been shown to inhibit MYC-mediated gene regulation (60–120 µM) (26,27) potently inhibited CIP2A mRNA expression. Importantly, 10058-F4 treatment resulted in inhibition of CIP2A mRNA expression within 6 hours, whereas expression of a control mRNA, jumonji, was not substantially inhibited even after 12 hours (Figure 6, B). The rapid time course of 10058-F4–elicited inhibition of CIP2A mRNA expression suggests that MYC directly affects CIP2A transcription. In addition to CIP2A mRNA regulation in AGS cells, inhibition of CIP2A protein expression was seen in both AGS and MKN-28 cell lines treated with 10058-F4 (Figure 6, C). Here, expression of nucleolin, an established MYC target gene, was used as a control (Figure 6, C). Finally, to confirm that 10058-F4–mediated or MYC siRNA–mediated effects on CIP2A gene expression were due to a direct effect on CIP2A transcription rather than an indirect cellular response, we performed additional experiments in mouse embryo fibroblasts (MEFs) that expressed a conditionally active MYC construct, MycER. In the MycER construct, the fusion of human MYC cDNA to a modified estrogen-binding domain renders MYC activity responsive to the addition of 4-OHT (28). Isolated MEFs homozygous for MycER expressed from the Rosa26 promoter (20) were treated with 4-OHT and analyzed for changes in CIP2A mRNA levels. As shown in Figure 6, D, MYC activation resulted in increased CIP2A mRNA expression that was detected at both the 6- and the 24-hour time points.

**Influence of Cell Cycle Activity on CIP2A Expression**

Increased expression of CIP2A has been observed in highly proliferating tumor tissues and cancer cell lines (10). To address whether CIP2A expression might be regulated by mitogenic signals or by cell cycle activity, in addition to MYC, AGS cells were serum-starved for 48 hours and then fed with medium containing 10% FCS before analysis of CIP2A protein expression. As shown in Figure 7, A, serum stimulation did not induce CIP2A protein expression, even though the cells reentered the cell cycle as evidenced by inhibition of cyclin E expression in S phase (9–12 hours after introduction of medium containing FCS) and recovery of cyclin E expression in G1 phase (24 hours). These results indicate...
that cell cycle activity does not regulate CIP2A expression. To verify this conclusion, AGS cells were blocked in S phase by an established thymidine/aphidicolin protocol (21), and expression of CIP2A protein in unsynchronized cells and cells blocked in S phase was compared. Consistent with the lack of cell cycle–associated effects on CIP2A expression in Figure 7, A, no difference was seen in CIP2A protein levels in these cell populations (aphidicolin block; Figure 7, B). In this experiment, S phase synchronization in thymidine/aphidicolin–treated cells was demonstrated by decreased cyclin E and increased cyclin B1 protein expression (Figure 7, B).
Thus, cell cycle activity does not regulate CIP2A expression. To address whether cell cycle activity is required for MYC regulation of CIP2A expression, CIP2A mRNA expression was examined in AGS cells that had been synchronized to S phase by the thymidine/aphidicolin protocol and then treated for 6 hours with 10058-F4. As shown in Figure 7, C, 10058-F4 treatment efficiently inhibited CIP2A expression in thymidine/aphidicolin–treated cells. Finally, to demonstrate that MycER–mediated induction of CIP2A mRNA expression is not dependent on cell cycle activity, 4-OHT–elicited CIP2A induction was studied in aphidicolin-treated MycER MEFs. As shown in Figure 7, D, aphidicolin treatment did not compromise CIP2A induction at the 6-hour time point after 4-OHT treatment (Figure 7, D). Taken together, these results verify that cell cycle activity is not linked with MYC-mediated regulation of CIP2A expression.

Discussion

CIP2A is a recently characterized human oncoprotein that functions to stabilize MYC by inhibition of protein phosphatase 2A activity (10). CIP2A has previously been shown to be expressed in human gastric cancer (16,29). However, the clinical role of CIP2A in human cancer progression has heretofore not been demonstrated. Here, we found that high expression of CIP2A protein is associated with reduced overall survival in certain subgroups of gastric cancer patients, which was especially strong in small tumors (Figure 1, B). On the other hand, the association of CIP2A status with prognosis among patients with pT3–T4 and p53–immunopositive gastric tumors (Figure 1, C and D) indicates that the role of CIP2A in tumor progression may be especially prominent in patients with advanced disease. However, CIP2A protein expression in gastric cancer specimens did not associate with p53 immunopositivity (Supplementary Table, available online), which suggests that there is no direct regulatory link between these two pathways. This conclusion is further supported by our results that showed that CIP2A depletion does not regulate p53 expression (Figure 4, A). In contrast to p53 staining, CIP2A immunopositivity did associate with aneuploidy and high SPF, which may reflect its role as a marker of aggressive tumor behavior.

Although overexpression of MYC has been suggested to be an early event during the genesis of both intestinal and diffuse types of gastric carcinomas (5,6,30), only a few reports thus far have indicated that MYC–targeted therapy can modulate gastric carcinogenesis (31). Consistent with previously published data (29,31), our results demonstrate that both CIP2A and MYC promote proliferation of gastric cancer cells. Furthermore, because CIP2A promotes MYC expression, and MYC in turn is required for maximal proliferation of gastric cancer cells, our results support the conclusion that CIP2A facilitates cell proliferation at least in part by promoting MYC stability. These results together with our recent studies of CIP2A in other cancer types (10) further suggest that CIP2A’s role in MYC protein stability regulation can be generalized to several human malignancies. However, we cannot exclude the possibility that CIP2A may promote malignant cell growth by other mechanisms, in addition to stabilizing MYC.

We demonstrate that depletion of either of these two human oncoproteins inhibits the expression of the other. Here, it is important to note that we obtained consistent results regarding MYC’s role in CIP2A regulation using three independent approaches (siRNA, 10058-F4, and MycER) to modulate MYC expression or activity. Our finding that CIP2A enhanced MYC expression and stability was confirmed by several independent experiments (Figures 3, A–C; Figures 4, A and B, and Supplementary Figure 3, C, available online). Our results strongly suggest that MYC-mediated stimulation of CIP2A expression is dependent on MYC–MAX dimerization (Figures 6, A–C). Also, because CIP2A transcription is affected within 6 hours of either inhibition or activation of MYC (Figures 6, B and D, and 7, C and D), it is likely that MYC directly regulates CIP2A gene expression. Based on these results, MYC–stimulated CIP2A expression appears to provide a positive feedback on MYC activity. A schematic model of the proposed positive feedback mechanism between these two human oncoproteins, in relation to induction of MYC activity by growth factors or oncogenic activating mutations of upstream signaling proteins such as Ras, is depicted in Figure 8 (see the figure legend for detailed description of the proposed mechanism).

In other systems, positive feedback loops have been shown to function as signal amplifiers or as mechanisms that maintain states of biological irreversibility (32). In that regard, stimulation of CIP2A expression by MYC could be envisioned as a mechanism for MYC to escape protein degradation, thereby resulting in increased strength and duration of MYC-mediated cellular responses, positively regulating expression of CIP2A and MYC. Extracellular stimuli, or activating mutations on oncogenes such as Ras, stimulate MYC protein expression. MYC, in addition to regulating its other target genes relevant for MYC-mediated cellular responses, positively regulates expression of CIP2A. Induction of CIP2A protein expression in turn promotes MYC protein stability by preventing MYC serine 62 (S62) dephosphorylation. Accumulation of stable S62-phosphorylated form of MYC further increases CIP2A expression, resulting in positive feedback loop regulation between MYC and CIP2A. Positive feedback between CIP2A and MYC may result in increased strength and duration of MYC-mediated cellular responses and MYC’s oncogenic activity, which is denoted by the increasing height and density of the triangle. CIP2A = cancerous inhibitor of protein phosphatase 2A; c-Myc = MYC.
enhancement of MYC-mediated cellular responses (Figure 8). Conversely, inhibition of a positive feedback circuit between CIP2A and MYC may explain the sustained inhibition of CIP2A protein expression in response to single transfection of CIP2A siRNA (Figure 2, B), which was also observed in our previous study (10). Moreover, the surprising efficacy of transiently transfected CIP2A siRNA for inhibition of head and neck squamous cell carcinoma growth over a 59-day observation period in vivo (10) could also be indicative of a self-perpetuating response between CIP2A and MYC well after the triggering stimulus (CIP2A siRNA) had been degraded (32). Based on these findings, it is plausible that the positive feedback loop between CIP2A and MYC could be a useful therapeutic target in gastric cancers and other CIP2A- and MYC-driven human malignancies.

Our study has some limitations. Although our current and previous results (10) indicate that CIP2A expression is important for supporting malignant growth of several established human cancer cell lines, they do not reveal whether CIP2A is required for cancer development in vivo. Establishment of a CIP2A-deficient mouse model should at least partly answer this question. Moreover, the contribution of CIP2A regulation to MYC-induced cell proliferation and cellular transformation needs to be clarified further. Interestingly, a recent study described distinct thresholds for MYC-induced biological output in different mouse tissues (20). It would be of particular interest to study whether CIP2A’s expression and MYC stabilizing function are involved in determining MYC’s output in vivo, and whether MYC-induced induction of CIP2A expression described in this study is required for MYC-mediated biological effects. Last, our study fails to identify MYC binding to the CIP2A promoter region, and our unpublished data show that the activity of a −2000/−50 CIP2A Luc promoter construct was not affected by MYC inhibition (data not shown). These results suggest that the MYC responsive element on the CIP2A gene is located outside the proximal promoter region, as in a large number of other MYC responsive genes.

In conclusion, our work demonstrates that CIP2A is a clinically relevant human oncoprotein. Moreover, the identification of CIP2A as an MYC target protein provides novel insights into understanding how MYC could regulate its own stability in malignant cells. Finally, results of this study suggest that inhibition of CIP2A-mediated MYC stabilization, and inhibition of other MYC stabilizing proteins such as USP28 (11), could provide novel means of inhibiting MYC protein expression in cancer cells for therapeutic purposes, without interfering with MYC gene expression in normal tissues.

References


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