Serine/Threonine Protein Phosphatase Type 1γ1 Is Required for the Completion of Cytokinesis in Human A549 Lung Carcinoma Cells

(Aiying Cheng, Nicholas M. Dean, and Richard E. Honkanen)

From the Department of Biochemistry and Molecular Biology, University of South Alabama, Mobile, Alabama 36688 and the Department of Pharmacology, ISIS Pharmaceuticals, Carlsbad, California 92008

In lower eukaryotic organisms, the loss of serine/threonine protein phosphatase type 1 (PP1) results in growth arrest after the onset of mitosis. In humans, four highly homologous isoforms of PP1 (PP1α, PP1β, PP1γ1, and PP1γ2) have been identified. Determining the roles of these phosphatases, however, has proven difficult due to the lack of subtype-specific inhibitors. In this study, we developed chimeric antisense 2'-O-(2-methoxy)ethylphosphothioate oligonucleotides targeting human PP1γ1 that specifically inhibit PP1γ1 gene expression. Two potent antisense oligonucleotides (ISIS 14435 and 14439; IC50 ~ 50 nM) were then employed to elucidate the cellular functions of PP1γ1 during cell cycle progression. In A549 cells, the inhibition of PP1γ1 expression resulted in a dose-dependent inhibition of cellular proliferation, with growth arrest occurring after ~36–48 h, when PP1γ1 mRNA expression was inhibited by >50%. Fluorescence-activated cell sorter analysis revealed that ISIS 14435/14439-induced growth arrest was associated with an increase in the number of cells containing 4N DNA. Immunostaining of treated cells revealed that the inhibition of PP1γ1 expression had no apparent effect on the formation of mitotic spindles. However, decreased expression was associated with the failure of cell division in a late stage of cytokinesis and the formation of dikaryons.

In eukaryotic cells, the reversible phosphorylation of proteins determines the biological activity of many protein complexes and is recognized as a major mechanism controlling cell cycle progression. Therefore, the protein kinases that mediate progression through the cell cycle have been extensively investigated. Because many of the protein kinases that play key roles in the regulation of cell cycle progression are Ser/Thr kinases, it seems logical that the regulation of serine/threonine protein phosphatase (PPase)1 activity will also contribute to growth control processes. Indeed, the addition of semiselective cell division inhibitors, and PP1

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testis, while PP1α, PP1β, and PP1γ are expressed in most, if not all, tissues. The functions of the individual isoforms are unknown, and no type-selective inhibitors of PP1 have been identified to date. Furthermore, due to the high degree of homology, it is impossible to draw conclusions about the roles of mammalian PP1 isoforms from comparative studies in lower eukaryotes. To study the role of individual PP1 isoforms in the regulation of cell cycle progression, in the present study we developed antisense oligonucleotides targeting PP1γ that specifically inhibit PP1γ gene expression in human cells. Using these antisense oligonucleotides, we demonstrate that the expression of PP1γ is necessary for A549 cell proliferation and that the inhibition of PP1γ expression leads to the formation of dikaryons following the failure of cytokinesis.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Tissue culture medium, Lipofectin®, and TRIZol® were purchased from Life Technologies, Inc. DECAprime™ II DNA labeling, MAXIscriptr™ in vitro transcription, and HybSpeed™ RPA kits were purchased from Ambion Inc. (Austin, TX). PP1γ-specific antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibody against α-tubulin, fluorocine isothiocyanate (FITC)-conjugated phallidin, and FITC- and horseradish peroxidase-conjugated secondary antibodies were obtained from Sigma. [α-32P]dATP and [α-32P]PIUTP were purchased from NEN Life Science Products.

**Cell Culture—**Human A549 lung carcinoma cells obtained from the American Type Culture Collection were grown in Dulbecco’s modified Eagle’s medium containing 1 g of glucose/10 ml (DMEM) and 10% heat-inactivated fetal bovine serum. Cells were routinely passaged when 90–95% confluent.

**Oligonucleotide Synthesis—**2′-O-(2-Methoxy)ethylphosphorothioate oligonucleotides were synthesized and purified as described previously (36). The sequence of the oligonucleotides tested is provided in Table I.

**Assays for Oligonucleotide Inhibition of PP1γ Expression—**A549 cells were seeded in 60-mm dishes and cultured in DMEM containing 10% fetal bovine serum. When the cultures were ~70% confluent, they were treated with the indicated oligonucleotides as described previously (36). Briefly, cells were washed with DMEM. A solution (1 ml) of DMEM containing 15 μg/ml DOTMA/DOPE (Lipofectin®) and the oligonucleotides at the indicated concentration were then added. After incubating the cells for 4 h at 37 °C, the cells were washed and cultured in fresh DMEM containing 10% fetal calf serum for 18–20 h. The cells were harvested, and total RNA was isolated with TRIZol reagent according to the methods provided by the manufacturer. The total RNA (20 μg) was then separated on 1% agarose gels containing formaldehyde and transferred to Duralon-UV™ membrane (Stratagene, La Jolla, CA). Following UV cross-linking, the membranes were hybridized with a [32P]-labeled PP1γ cDNA probe employed was generated from the full-length coding region of human PP1γ and was [32P]-labeled by polynucleotide chain reaction amplification in the presence of [α-32P]dATP using a DECAprime II™ labeling kit (Ambion) according to the protocol of the manufacturer. Hybridization was performed at 42 °C for 16 h in 50% formamide, 0.1 M Pipes, 5× Denhardt’s solution, 100 μg/ml denatured herring sperm DNA, 1 × 106 cpm/ml [32P]-labeled probe, and 10% dextran sulfate. Following hybridization, the filter was washed twice in 2× SSC, 0.1% SDS for 20 min at room temperature and then twice in 0.1× SSC, 0.1% SDS for 20 min at 55 °C. Hybridization was visualized by autoradiography, and the filters were then stripped and reprobed with a [32P]-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe to confirm equal loading. Quantification of hybridization signals was achieved by analysis of the scanned autoradiograms using the NIH Image program (ImagePC).

**Analysis of Cell Growth—**A549 cells were seeded in 60-mm tissue culture plates at a density of 5.0 × 103 cells/dish. On the next day, the cells were treated with PP1γ-specific antisense oligonucleotides (ISIS 14435 and 14439) or scrambled mismatch control oligonucleotides (Table I) at a final concentration of 500 nM as described above. On each of the next 4 days, the cell cultures were treated briefly by trypsin to detach the cells from the dish (three wells from each test group). The number of cells was determined by counting using a hemacytometer. Cell viability was determined by trypan blue staining. The percentage of viable cells was calculated by dividing the number of cells excluding trypan blue by the total number of cells.

**RNase Protection Assay—**DNA fragments of PP1α, PP1β, and PP1γ that varied in size were amplified by polymerase chain reaction and subcloned into pGEM5Zf (+) T-vectors (Promega), and the integrity of each construct was verified by DNA sequencing. DNA templates for in vitro transcription, which contained T7 and SP6 promoter regions, were generated by polymerase chain reaction amplification with nested primers contained in the plasmid. DNA template for cyclophilin was purchased from Ambion Inc. [32P]-Labeled antisense RNA probes were prepared with MAXIscriptr™ in vitro transcription kit following the instructions of the manufacturer. Total RNA (5 μg) from A549 cells was then analyzed employing a HybSpeed™ RPA kit according to the methods of the manufacturer with a slight modification (i.e. the coprecipitation step was omitted, and the total RNA, [32P]-labeled probes, and hybridization buffer were combined prior to reducing the volume of the reaction to 10 μl by evaporation). Protected RNA probes were then separated on 5% denatured polyacrylamide gel and visualized by autoradiography.

**Immunoblotting of PP1γ—**Western analysis was performed essentially as described previously using positional antibodies against PP1γ (37). Briefly, A549 cells grown in T-75 flasks were washed twice with ice-cold phosphate-buffered saline (PBS) and scraped from the plate in 1 ml of PBS. The cells were collected by centrifugation and then lysed by sonication in 0.1 ml of radioimmune precipitation buffer (10 mM Tris-HCl, pH 7.4) containing 1% (v/v) Nonidet P-40, 0.1% SDS, 0.1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, and protease inhibitor mixture (Sigma). Protein was determined using a Bio-Rad protein quantitation assay, with bovine serum albumin as a standard. Samples were prepared for electrophoresis by adding an equal volume of 2× sample buffer (120 mM Tris-HCl, pH 7.4, 200 mM dithiothreitol, 20% glycerol, 4% SDS, and 0.02% bromphenol blue) and boiling for 5 min. Protein samples (60 μg) were then separated on 10% SDS-polyacrylamide gel electrophoresis and electroblotted onto Immobilon-™ membranes (Millipore Corp.). The membrane was then blocked for 1 h with Tris-HCl, pH 7.6, containing 150 mM NaCl and 5% nonfat milk. PP1γ was detected with an anti-PP1γ antibody (Santa Cruz Biotechnology, Inc.) diluted 1:1000 in Tris-HCl (pH 7.6) containing 150 mM NaCl, 0.2% Tween 20 (TBST) and 2% nonfat milk for 18 h. The membrane was then washed, and the primary antibody was detected employing a BioMax Western ECL Western blot detection reagents (Amerham Pharmacia Biotech, Buckinghamshire, United Kingdom), following the protocols of the manufacturer.

**Fluorescence-activated Cell Sorter Analysis—**A549 cells were seeded in 10-cm culture dishes (1.5 × 105 cells/dish) and grown in DMEM with 10% fetal calf serum for 22–24 h (70–80% confluent). The cells were then treated with oligonucleotides as described above. DNA content per cell was measured by flow cytometry in propidium iodide-stained cells. Cells (~107) were harvested using trypsin/EDTA and suspended in 1 ml of 50 μg/ml propidium iodide in PBS. Cells in suspension were mixed with an equal volume of Vindelov’s propidium iodide solution (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 μg/ml ribonuclease, 0.1% IGEPAL CA-630, and 50 μg/ml propidium iodide), incubated for 1 h at 4 °C, and analyzed by flow cytometry. Flow cytometry analysis was performed on

| Table I | Design strategy for PP1γ oligonucleotides |
|---|---|
| Oligonucleotide no. | Sequence (5′ → 3′) | cDNA target |
| ISIS 14430 | CCATCGGCTTCCAGCAGCGC | AUG codon |
| ISIS 14431 | CATATTTGAGTGGTCTC | Coding |
| ISIS 14432 | TGCCAGTTACGTTCCCTC | Coding |
| ISIS 14433 | TCTCACTACAGAGAATAC | Coding |
| ISIS 14434 | GCAATATCAAG | Coding |
| ISIS 14435 | GCTTGCTTTGTGATCATCC | Coding |
| ISIS 14436 | GATTCAAGACCCTTGGCGC | 3′-UTR |
| ISIS 14437 | AGTAGCTGCGAGGTTGCTT | 3′-UTR |
| ISIS 14438 | CCAAGAAGAGGCGATGTTG | 3′-UTR |
| ISIS 14439 | AGTGGACGGTTCTAGGGCTC | 3′-UTR |
| ISIS 14440 | AAACGATAATCGCTGACTC | 3′-UTR |
| ISIS 14441 | CACGGTGATGTACAGCTGCA | 3′-UTR |
| ISIS 15026 | CCTACGTTGGTTCAATACCC | Scrabemled for 1443S |
| ISIS 15030 | GCTCGGGTGTCTATAACCC | Scrabemled for 1443S |
| ISIS 15680 | GCTCAGGCGCTTGCCAGT | Scrabemled for 14439 |
| ISIS 15682 | AGGATAGTTGGCCCAGCTG | Scrabemled for 14439 |

Twelve 2′-O-(2-Methoxy)ethylphosphorothioate oligonucleotides 20 bases in length targeted to different regions of human PP1γ were synthesized. Mismatch-scrambled control oligonucleotides corresponding to antisense oligonucleotide ISIS 14435 and 14439 were synthesized. Sequences are shown 5′ to 3′, and the approximated location on the PP1γ cDNA is indicated.
FIG. 1. Inhibition of PP1γ1 mRNA and protein expression by treatment with antisense oligonucleotides. A, relative positioning of the predicted hybridization sites within the human PP1γ1 mRNA of 12 antisense oligonucleotides that were evaluated for their ability to inhibit PP1γ1 mRNA expression in cultured A549 tumor cells. B, identification of antisense oligonucleotides that inhibit the expression of PP1γ1 mRNA. A549 cells were treated with the indicated antisense oligonucleotides at a concentration of 300 nM. RNA was prepared 24 h later and analyzed for PP1γ1 and GAPDH mRNA levels by Northern blot analysis. Control cells were treated with a random oligonucleotide. C, PP1γ1 mRNA levels from Northern blot analysis (B) expressed as a percentage of the levels of PP1γ1 mRNA in control cells following normalization to GAPDH. D, inhibition of PP1γ1 mRNA levels by ISIS 14435. A549 cells were treated with increasing concentrations (25–500 nM) of ISIS 14435 (left panel) ISIS 14439 (center panel), or a 300–500 nM concentration of the indicated oligonucleotides (right panel). ISIS 15026 and ISIS 15030 are mismatched control
a Becton Dickinson fluorescence-activated cell sorter as described previously (4).

**Indirect Immunofluorescence Microscopy**—Cells were plated onto 60-mm dishes containing sterile coverslips in 4 ml of DMEM at a concentration of 1 × 10⁶ cells/dish and grown until the cultures were ~70% confluent. The cells were then treated with oligonucleotides as described above. After 18 h, the cell coverslips were then fixed by immersion of the coverslips in −20 °C methanol for 6–8 min and processed for immunofluorescence microscopy using previously published methods (4, 38). To collect mitotic cells, the cells from 60-mm dishes were harvested by "mitotic shake off" and deposited by centrifugation onto polylysine-coated coverslips. The coverslips were then incubated for 24–48 h. Time Lapse Video Microscopy—A549 cells were plated and treated with oligonucleotides in 60-mm dishes as described above. Twenty-four hours after the treatment, cell cycle progression was monitored by time lapse video microscopy using an Axiosvert 35 M microscope (Carl Zeiss, Inc.). A heated stage was employed to maintain the cells at a constant temperature of 37.0 °C, and CO₂ was maintained at 5% during the course of the experiments. Images were recorded at a rate of 1 frame/s for 24–48 h.

**RESULTS**

**Antisense-mediated Inhibition of PP1γ mRNA Expression**— Twelve oligonucleotides, 20 bases in length, predicted to hybridize to different regions of human PP1γ mRNA were synthesized (Table I). The oligonucleotides tested were designed to target specific regions in the protein coding region, the 5′-untranslated, or the 3′-untranslated regions of human PP1γ mRNAs (Fig. 1A) and were “chimeric” 2′-O-(2-methoxyethyl)phosphothioate oligonucleotides, containing eight central phosphorothioate oligodeoxy residues (“oligodeoxy gap”) flanked by six 2′-O-(2-methoxy) residues on the 3′- and 5′-ends. These modifications have been shown previously to enhance the potency of antisense oligonucleotides targeting mRNAs encoding other proteins (36, 39). Because phosphorothioate oligonucleotides commonly act through an RNase H-dependent mRNA cleavage mechanisms in cells (40), the ability of each oligonucleotide to specifically inhibit the expression of PP1γ was determined by Northern blot analysis probing for levels of PP1γ mRNA. For the initial screen, PP1γ mRNA was detected using a PP1γ-specific cDNA probe that forms a hybrid with oligonucleotide to specifically inhibit the expression of PP1γ mRNA levels in A549 cells following treatment with increasing concentrations of ISIS 14435 (C) or 14439 (D). Western blot analysis of PP1γ protein levels in A549 cells 24 h after treatment with ISIS 14435 or a mismatch control (ISIS 15026). Cells were treated with ISIS 14435 at a concentration of 0–500 nM or with 500 nM ISIS 15026, and protein extracts were prepared 24 h later. Western analysis was then performed as described under “Experimental Procedures,” with each lane loaded with 60 μg of protein. G, quantification of PP1γ protein levels in A549 cells following treatment with increasing concentrations of ISIS 14435 determined by NIH Image analysis of the exposed film shown above. H, Western blot analysis of PP1γ protein levels in A549 cells following a single treatment with ISIS 14435. Cells were treated with ISIS 14435 at a concentration of 500 nM or with 500 nM mismatch control oligonucleotides (ISIS 15026), and protein extracts were prepared at the time of treatment (0) and then each day for the next 4 days (1–4). Each lane contained 60 μg of protein.
expression of PP1γ1, we next explored the roles played by PP1γ1 in human cells. To study the effects of antisense oligonucleotides targeting PP1γ1 on cell growth and viability, A549 cells were treated one time with ISIS 14435 or its mismatched control at a concentration of 0–500 nM. Cell number and viability were then determined daily over a 4-day period. As seen in Fig. 3A, treatment resulted in a dose-dependent repression of A549 cell proliferation, with nearly complete inhibition of proliferation noted by 48 h after treatment with 500 nM ISIS 14435. In contrast, treatment with mismatched control oligonucleotides had no apparent effect on A549 cell growth (Fig. 3B). Similar results were obtained with ISIS 14439 (data not shown), and viability studies (Table II) indicated that 89% of cells treated cells were capable of excluding trypan blue 48 h after treatment. Therefore, the decrease in cell number was probably due to growth inhibition rather than cell death.

Inhibition of PP1γ1 Expression Inhibits a Late Stage in Cytokinesis—Studies in lower eukaryotic cells suggest that PP1 activity is necessary for the completion of mitosis, and the treatment of mammalian cells with okadaic acid or fostriecin at concentrations that partially inhibit the activity of PP1 in vitro results in the appearance of 4N cells that arrest in the G2/M phase of the cell cycle (4, 8, 9). To determine if ISIS 14435-mediated growth arrest occurs during a specific stage of the cell cycle, we employed fluorescence-activated cell sorter analysis of propidium iodide-stained A549 cells 48 h after treatment with antisense oligonucleotides targeting PP1γ1. These studies revealed that ~31% of the cells in which the expression of PP1γ1 was suppressed contained twice the normal content of DNA (4N) (Fig. 4). In comparison, only 14–16% of the control cells (cells treated with either mismatch control oligonucleotides or Lipofectin alone) were 4N.
**PP1γ1 Is Required for Cytokinesis**

A549 cells were treated with ISIS 14435, ISIS 14439, or mismatched controls (ISIS 15026, ISIS 15030, ISIS 15680, ISIS 15682) at a concentration of 500 nM in the presence of DOTMA/DOPE liposomes (Lipofectin®). Additional control cells were treated with Lipofectin alone. Cell viability was estimated 48 h later by the ability of cells to exclude trypan blue dye as described under “Experimental Procedures.”

| Treatment | Viability % |
|-----------|-------------|
| Lipofectin | 94.68 ± 1.67 |
| ISIS 14435 | 95.15 ± 1.27 |
| ISIS 15026 | 95.73 ± 1.55 |
| ISIS 15030 | 88.70 ± 5.84 |
| ISIS 14439 | 95.03 ± 2.11 |
| ISIS 15680 | 96.98 ± 1.81 |
| ISIS 15682 | 95.71 ± 1.44 |

![Fig. 4. DNA content flow cytometric histograms of PP1γ1 antisense oligonucleotide-treated A549 cells](image)

**DISCUSSION**

In eukaryotic organisms, cell cycle progression is regulated to a large extent by the reversible phosphorylation of proteins. Therefore, deciphering the complex mechanisms controlling the activity of the cellular kinases and phosphatases that participate in the control of cell cycle progression is a major challenge for understanding both normal and aberrant cellular proliferation. Determining the roles of individual PPases in human cells, however, has proven difficult due to the lack of truly specific inhibitors. To overcome this difficulty, in the present study we developed antisense oligonucleotides (ISIS 14435 and ISIS 14439) that potently inhibit the expression of human PP1γ1 without having an effect on the expression of other structurally related PPases (i.e., PP1α, PP1β, PP2A, PP4, or PP5). These antisense oligonucleotides provided us with the ability to study the roles of a single PP1 isoform in human cells.

In mammals, there are four isoforms of PP1 (PP1α, PP1β, PP1γ1, and PP1γ2), whereas in lower eukaryotes, PP1 homo-
logues are apparently encoded by fewer genes. Studies in yeast indicate that PP1 plays an essential role in the normal progression through mitosis. In fission yeast, S. pombe, two genes (sds21 and dis2/ bws1) encode PPases homologous to the catalytic subunit of mammalian PP1 (27–29). Mutants containing the semidominant dis2–11 allele of the major PP1 isoform enter, but fail to exit, mitosis (27). Loss-of-function mutations in both of these genes arrest cell growth in mitosis at the restrictive temperature, with the cells having condensed, unseparated chromosomes and short mitotic spindles (28, 42). In

![Image](image_url)
PP1γ1 Is Required for Cytokinesis

To characterize the cellular roles of individual human PP1 isoforms and to determine if PP1γ1 is necessary for the completion of mitosis in human cells, we treated A549 cells with ISIS 14435 and 14439 at concentrations that inhibited the expression of PP1γ1. These studies indicated that the inhibition of PP1γ1 expression inhibits cell proliferation. Fluorescence-activated cell sorting analysis of the treated cells revealed an increase in the number of cells containing 4N DNA. However, immunostaining to detect microtubules revealed no apparent abnormalities in the formation of mitotic spindles. In addition, there was no indication of growth arrest in mitosis or prior to the completion of anaphase. Therefore, it is likely that an isoform other than PP1γ1 is necessary for the transition into or out of mitosis. Nonetheless, in A549 cells, when PP1γ1 expression is suppressed cell division often fails. This failure is characterized by a delay in cytokinesis after the formation of the contractile ring and a deep cleavage furrow. Analysis of >1000 cells in which PP1γ1 expression was inhibited by >85% revealed that ~41% of the growth-arrested cells then progress to become dikaryons, which preliminary studies suggest occurs when the contractile ring “relaxes” and two apparently normal nuclei reform. Therefore, although additional studies are needed to clarify the role of PP1γ1 in human cells, it appears that PP1γ1 has an essential function that is necessary for the completion of cytokinesis.

Although the molecular mechanisms regulating cytokinesis are poorly understood, other studies also suggest that the aberrant phosphorylation of proteins can impede the completion of cytokinesis. In NIH3T3 cells, the overexpression of p70 S6 kinase (p70^65K) produces a similar phenotype (i.e. incomplete cytokinesis leading to the formation of dikaryons cells (45)). Similarly, in Chinese hamster ovary cells, the overexpression of protein kinase Cδ, but not protein kinase Ca, protein kinase CβII, or protein kinase Cζ, produces cells with two nuclei following the inhibition of cell division in telophase (46). When the activation of cyclin-dependent kinase 1 (CDK1) is maintained through the introduction of a mutant form of cyclin B that is not degraded, cytokinesis is also inhibited (47). Although the inhibition of cytokinesis induced by prolonged CDK1 activity may be due to the inhibition of midzone microtubule formation or the phosphorylation of myosin II regulatory light chain (48), the precise reason(s) that aberrant or maintained activation of protein kinase Cδ or p70^65K inhibits cytokinesis are unclear. The mechanism(s) by which the inhibition of the PP1γ1 expression is translated to a failure in cytokinesis is also not clear. All of the above mentioned kinases are serine/threonine kinases, and the phosphorylation of cytoskeletal components necessary for the completion of cytokinesis. Alternatively, PP1γ1 activity may be required at a more distal site, such as the ubiquitin degradation pathway that mediates cyclin B degradation. Clearly, additional studies will be required to determine the substrates utilized by PP1γ1 and how the regulation of PP1γ1 activity is coordinated with that of cellular proteins that regulate the onset and progression of cytokinesis.

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