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Double-stranded RNA Mediates Selective Gene Silencing of Protein Phosphatase Type 1 Delta Isoform in HEK-293 Cells

HIROYUKI MORIMOTO[a,*], HIROHIKO OKAMURA[b], KAYA YOSHIDA[b], SEIICHIRO KITAMURA[a] and TATSUJI HANEJI[b]

[a]*Department of Anatomy, School of Dentistry, The University of Tokushima, Kuramoto, Tokushima 770-8504, Japan;* [b]*Department of Histology and Oral Histology, School of Dentistry, The University of Tokushima, Kuramoto, Tokushima 770-8504, Japan*

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The reversible phosphorylation of proteins mediates cellular signals in eukaryotic cells. RNA interference inhibits the expression of genes and proteins in a sequence-specific manner and provides a tool to study the functions of target molecules. The effect of RNA interference on protein phosphatase isoforms in HEK-293 cells was examined. Protein phosphatase 1 delta (PP1δ) sequence-specific double-stranded RNA (dsRNA) inhibited mRNA and protein expression of the PP1δ. This RNA interference did not affect the expression of α and γ1 isoforms of PP1. Transfection of antisense RNA specific for PP1δ also suppressed the expression of PP1δ. It was further demonstrated by an *in vitro* RNA cleavage assay that extracts of HEK-293 cells catalyzed the processing of dsRNA. This cell line had much stronger mRNA expression of Dicer, an RNase III-like enzyme, than did human osteoblastic MG63 cells. The present results show that RNA interference is a useful tool to distinguish between PP1 isoforms.

Keywords: Protein phosphatases; Isoform; Double-stranded RNA; RNAi; HEK-293 cells

INTRODUCTION

Phosphorylation of structural and regulatory proteins is a major intracellular control mechanism in eukaryotes. The phosphorylation state of proteins is a dynamic process controlled by both protein kinases and protein phosphatases.[1–3] Two major families of protein phosphatases are present; protein serine/threonine phosphatases and protein tyrosine phosphatases. Based on biochemical parameters, the ser/thr protein phosphatases were initially divided into 2 classes, type 1 and type 2. Protein phosphatases type-1 (PP1s) are inhibited by 2 heat-stable proteins and preferentially dephosphorylate the β-subunit of phosphorylase kinase, whereas type-2 phosphatases are insensitive to the heat-stable inhibitors and preferentially dephosphorylate the α-subunit of phosphorylase kinase.[1–4] PP1s are involved in many cellular processes including glycogen metabolism, muscle contraction, protein synthesis, and intracellular transport. In mammals, there are 4 highly homologous isoforms of PP1s (PP1α, PP1δ, PP1γ1 and PP1γ2). These isoforms share a high identity and are encoded by 3 distinct genes, with PP1γ1 and PP1γ2 being produced from the alternative splicing of the same primary transcript.[5] In a previous study, we reported the subcellular localization of PP1 isoforms in mouse osteoblastic MC3T3-E1 cells.[6] The interaction of PP1s with organelles and membranes is mediated by specific targeting subunits. Targeting subunits direct PP1s to particular subcellular locations and selectively enhance the dephosphorylation of certain substrates.[1–4]

In many eukaryotes, double-stranded (ds) RNA inhibits gene expression in a sequence-specific manner by triggering degradation of mRNA.[7,8] This effect, referred to as RNA interference (RNAi), offers a way to inactivate genes of interest and thus provides a powerful tool to study gene function. For dsRNA-mediated inhibition of gene expression, RNAi has been studied most extensively in *Caenorhabditis elegans* and *Drosophila melanogaster*. However, RNAi with long dsRNA in cultured mammalian cells generally has been less successful. These failures are explained by the action of a latent enzyme activated in part of the interferon (IFN) defense pathway and an enzyme activated by long

*Corresponding author. Tel.: +81-88-633-7320. Fax: +81-88-633-7320. E-mail: morimoto@dent.tokushima-u.ac.jp

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dsRNA.[9,10] RNAi can be demonstrated in early mouse development, mouse embryonic carcinoma (EC) cells, and embryonic stem (ES) cells.[11–13] These cells contained much higher levels of Dicer, an RNase III-like enzyme, than the extracts prepared from cultures of differentiated cells including REF52, NIH3T3, or HeLa cells. The Dicer responsible for the generation of short interfering RNAs (siRNA) has been identified.[14] siRNAs are the sequence-specific posttranscriptional mediators of RNAi and are important to trigger silencing in mammalian cells.[15,16] Because of the high sequence homology, PP1s cannot be distinguished by the enzyme activity assay. The function of individual isoforms of PP1s is also unknown, and no type-selective inhibitors of PP1s have been identified. In the present study, we provided RNAi that specifically inhibited PP1δ gene expression in human cells.

MATERIALS AND METHODS

Materials

Dulbecco's and alpha modified Eagle's minimum essential medium (D-MEM and α-MEM) were purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS, USA). Plastic dishes were from Iwaki (Chiba, Japan). Antibodies for PP1α, PP1γ1, and PP1δ, that had been characterized previously,[17] were gifts from Drs. M. Nagao and H. Shima.

Cell Culture and Transfection of Cells

Human embryonic kidney cell line (HEK-293 cells), provided by Dr. E. R. Weiss, were cultured in D-MEM. MG63 cells, purchased from the American Type Culture Collection (Rockville, MD, USA), were cultured in α-MEM. Each cell line was cultured in medium containing 10% (v/v) FBS and was maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The 293 cells were transfected with 5 μg of single-stranded RNA (ssRNA) and dsRNA by using the calcium phosphate precipitation method. Six hours after transfection, the cells were exposed to 1.5% (w/v) glycerol for 1 min, rinsed 2 times with PBS and incubated for another 48 h. For a mock transfection, we transfected 293 cells without RNAs by using the same calcium phosphate precipitation method. Cell modification was monitored by an Olympus IMT-2 phase-contrast microscope.

RNA Synthesis

Total cellular RNA was prepared from 293 cells by using ISOGEN (Nippon Gene, Tokyo, Japan) followed by phenol extraction and ethanol precipitation. To prepare the specific PP1δ DNA, RT-PCR was performed by using Ready To Go RT-PCR Beads (Amersham Pharmacia Biotech, Uppsala, Sweden) with Oligo dT primer and the following specific forward and reverse primers:

PP1δ F (1850–1870): 5′-gcacattgtccaatccagtg-3′
PP1δ R (2302–2322): 5′-aaaatgtcccactgaccagc-3′

T7 RNA polymerase promoter was added to the PCR product, and the templates of RNA transcription were generated by PCR using Lig'n Scribe (Ambion, Austin, TX, USA). PP1δ antisense and sense RNAs were transcribed by using RiboMAX Large Scale RNA Production System (Promega, Madison, WI, USA). The RNAs were extracted with phenol/chloroform, ethanol precipitated, and dissolved in TN buffer (10 mM Tris-HCl, pH 7.5, 20 mM NaCl). For annealing, equimolar concentrations of sense and antisense RNA samples were heated at 95°C for 2 min, transferred to 65°C for 20 min, and then gradually cooled down over 6 h to room temperature. Formation of the dsRNA was monitored on 1.5% agarose gel. In addition, RNAs were digested with RNase III (Ambion) for 30 min at 37°C in the assay buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM DTT).

Northern Blotting

Total cellular RNA was prepared from 293 cells, denatured, separated by electrophoresis on 0.8% agarose /6.7% formaldehyde gel, and transferred to a nitrocellulose membrane (Hybond-N, Amersham Pharmacia). DIG-labeled antisense PP1δ RNA was used as a probe. Hybridization and luminescence detection was performed with DIG Northern Starter Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

SDS-PAGE and Western Blotting

The 293 cells were washed twice with PBS, and scraped into lysate buffer containing 1 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 5 mM EGTA, 50 mM NaF, and 1 mM Na_3VO_4 in PBS. The protein concentrations were evaluated by using Protein Assay Reagent (Bio-Rad, Hercules, CA, USA) and diluted to a protein concentration of 1 mg/ml with lysate buffer before the addition of Laemmli's 5 × sample buffer. Twelve micrograms of each sample and prestained molecular weight markers (Gibco BRL) were separated by SDS-PAGE and transferred to polyvinylidene difluoride transfer membranes (Immobilon, Millipore, Bedford, MA, USA). The membranes were incubated for 2 h at 20–22°C in a blocking solution containing 5% nonfat skim milk in PBS containing 0.1% Tween 20

(PBS-Tween). Then they were washed briefly in PBS-Tween and incubated overnight at 4°C in PBS-Tween containing antibodies to PP1s diluted 1:2000. The membranes were washed 4 times within 30 min in PBS-Tween. The washed membranes were then incubated for 1 h at 20–22°C in PBS-Tween containing horseradish peroxidase (HRP)-conjugated anti-rabbit IgG. The membranes were washed again and the proteins recognized by the antibodies were visualized by using an ECL detection kit (Amersham Pharmacia).

dsRNA-processing Assay

To prepare the cytoplasmic extracts for dsRNA-processing assays, 293 cells were washed extensively with PBS and scraped into lysate buffer. The cells were then disrupted on ice with a Dounce homogenizer (tight pestle), and nuclei were prepared by centrifugation for 10 min at 2000 rpm. The supernatant was centrifuged again at 10,000 rpm for 10 min. The extract was supplemented with glycerol to 10% and stored at −80°C. dsRNA-processing assays (20 μl) contained 10 μl of cytoplasmic extract and 1 μg of the dsRNA in 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM $MgCl_2$, and 5 mM DTT. Reaction mixtures were incubated for 60 min at 37°C and the RNA was subsequently ethanol precipitated and analyzed on 2.0% agarose gels following staining with ethidium bromide.

RT-PCR Analysis of Dicer mRNA

Total RNAs were isolated from 293 and MG63 cells. cDNA was synthesized as described above. RT-PCR was performed on the cDNA by using the following sense and antisense primers:

Dicer L, 5′-tgggtcctttctttggactg-3′
Dicer R, 5′-caaggcgacatagcaagtca-3′
GAPDH L, 5′-accacagtccatgccatcac-3′
GAPDH R, 5′-tccaccaccctgttgctgta-3′

Amplification products were separated on 2.0% agarose gel, stained with ethidium bromide, and visualized with an UV transilluminator. Photographs were taken with a Polaroid DS-300 camera.

RESULTS

Double-stranded RNA Synthesis

PP1δ-specific sense and antisense single-stranded RNAs were synthesized by using RNA polymerase. Following transcription reactions, sense and anti-sense transcripts were annealed to yield dsRNA. The quality of dsRNA was analyzed by non-denaturing agarose gel electrophoresis using ssRNAs as a marker. Sense and anti-sense RNAs showed the same migration patterns of electrophoresis on the agarose gel (Figure 1A, lanes 1 and 2). Because of the conformational changes dsRNA showed many migration patterns different from that of the ssRNAs; i.e., the migration shifted to the long-base pair side (Figure 1A, lane 3). Next, RNAs synthesized as above were treated with the recombinant RNase III which is a double-strand-specific RNase. Treatment of recombinant RNase III generated 21–23 nt RNA products from a 472 bp dsRNA substrate (Figure 1B, lane 3). Degradation of ssRNA was observed in the sample treated with RNase III (Figure 1B, lanes 1 and 2), however, accumulation of the characteristic 21–23 nt RNA products were not detected (Figure 1B, lanes 1 and 2).

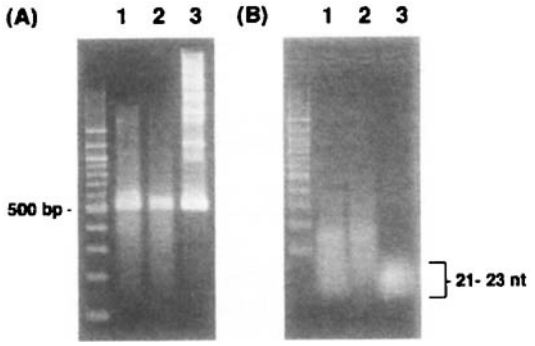

FIGURE 1 Production of dsRNA. PP1δ sequence-specific sense and antisense RNAs were synthesized. Equal amounts of sense and antisense RNA were annealed to make dsRNA. The quality of RNA preparation was analyzed on 2% non-denature agarose gels (A). RNAs were incubated with RNase III (B). Lane 1, sense RNA; lane 2, antisense RNA; and lane 3, dsRNA.

mRNA Expression Analysis of PP1δ

HEK-293 cells were transfected with RNAs (sense, antisense, and double-stranded RNAs). To analyze the changes in PP1δ gene expression at the transcriptional level, we purified total RNA by a standard method and subjected the mRNA to Northern blot analysis by using a mRNA probe corresponding to PP1δ. Figure 2 shows that the antisense 5′RNA (lane 2) and dsRNA (lane 4) inhibited the PP1δ mRNA expression in the transfected cells (upper panel). However, mock infection (lane 1) and sense 3′RNA (lane 3) had no effect on the PP1δ mRNA expression

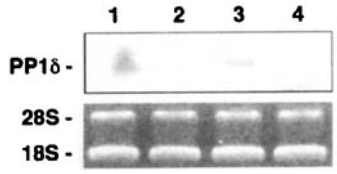

FIGURE 2 Northern blot analysis of PP1δ in HEK-293 cells. The cells were treated with mock (lane 1), antisense RNA (lane 2), sense RNA (lane 3), or double-stranded RNA (lane 4). Total RNAs were isolated from the cells. Upper panel, PP1δ; and lower panel, 18s and 28s rRNA as a loading control.

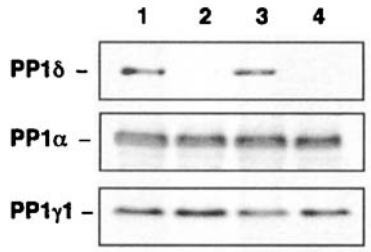

FIGURE 3 Western analysis of PP1s in HEK-293 cells. The cells were treated with mock (lane 1), antisense RNA (lane 2), sense RNA (lane 3), and double-stranded RNA (lane 4). Cell extracts were prepared, separated in 12.5% SDS-PAGE and transferred to Immobilon membranes. The membranes were probed with anti-PP1δ (upper panel), anti-PP1α (middle panel), and anti-PP1γ1 (lower panel) antibodies.

(upper panel). The amounts of 18S and 28S rRNAs (Figure 2, lower panel) and β actin mRNA (data not shown) were unaffected by any RNA transfection.

Protein Expression of PP1 Isoforms

The changes in the expression of PP1s in the PP1δ RNAi-treated 293 cells were examined by Western analysis. Figure 3 shows the results obtained from Western blot analysis of PP1 isoforms in the cells transfected with the various RNAs. The amount of PP1δ did not change in the cells treated with mock (lane 1) and sense 3′RNA (lane 3). However, the intensity of the PP1δ band decreased in the cells treated with antisense 5′RNA (lane 2) and dsRNA (lane 4) (upper panel). The staining intensity of PP1α (middle panel) and PP1γ1 (lower panel) did not change after any RNA treatment.

dsRNA Cleavage Analysis and Dicer Expression

Cytosol was prepared from the 293 cells and its dsRNA-processing activity *in vitro* was examined. Figure 4 shows that the dsRNA was cleaved with the treatment of cytoplasmic extract of 293 cell (lane 2). The cleavage was not detected in the preparation incubated without the cytoplasmic extract (lane 1). An extract of 293 cells did not contain dsRNA

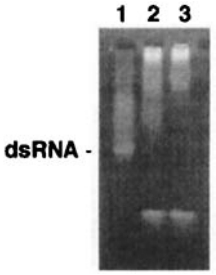

FIGURE 4 *In vitro* cleavage of dsRNA. dsRNAs were incubated in the absence (lane 1) or presence (lane 2) of a cytoplasmic extract of HEK-293 cells. An extract of HEK-293 cells without dsRNA was loaded in lane 3. The RNAs were ethanol precipitated and analyzed on a 2% agarose gel.

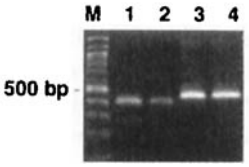

FIGURE 5 Expression of Dicer and GAPDH mRNA in HEK-293 and MG63 cells. Total RNA was isolated from 293 and MG63 cells. cDNA was synthesized and mRNA was amplified by PCR and electrophoresed in 2% agarose gels. Amplified product of Dicer (lanes 1 and 2) and that of GAPDH (lanes 3 and 4) are derived from HEK-293 cells (lanes 1 and 3) and MG63 cells (lanes 2 and 4).

(lane 3). The presence of Dicer in 293 cells was determined by semi quantitative RT-PCR method (Figure 5). The 293 cells expressed much higher levels of Dicer mRNA than MG63 cells (lanes 1 and 2). Both cell lines expressed similar amount of GAPDH mRNA as an internal control (lanes 3 and 4).

DISCUSSION

In the present study, 472-bp dsRNA for PP1δ (PP1δ, 1850–2322 bp) was used to induce RNAi. Our results indicate that PP1δ RNA specifically inhibited PP1δ gene expression in HEK-293 cells, i.e., it did not influence the expression of PP1α and PP1γ1. The RNAi was sequence specific, and the suppression of PP1δ was observed both at mRNA and protein levels. The homology of amino acid sequence among the PP1 isoforms (PP1α, PP1δ and PP1γ1) is more than 90%.[5] Although the cDNAs were derived from the distinct genes, the DNA sequences of these 3 isoforms have high homology. Isoform-specific sequences are mainly localized in 30 C-terminal and 40 N-terminal amino acid residues.[5] The long dsRNA used in the present study was selected to be PP1δ isoform specific, as similar sequences do not exist in PP1α and PP1γ1 genes. RNAi can lead to specific silencing of genes highly homologous in sequence to the transfected dsRNA

Dicer, an RNase III-like enzyme, cuts long dsRNA molecules into shorter siRNAs. Although recombinant RNase III also cleaved ssRNA, it did not generate 21–23 nt RNA fragments. The degradation of ssRNA was consistent with the previous report.[18] The recombinant RNase III cut the dsRNA to 21–23 nt RNAs indicating that dsRNA works as RNAi in the cells with Dicer. The siRNA or synthetic siRNA triggers silencing in cultured cells.[19,20] In mouse EC and ES cells, oocytes, and early embryos, specific RNAi was induced in response to the long dsRNA.[11–13] However, long dsRNA-mediated inhibition of mRNA expression has been less successful in other mammalian systems. These results are explained by the fact of the presence of a latent enzyme activated in IFN defense pathway and an enzyme activated by long dsRNA. In the undifferentiated EC and ES cells, these

enzymes are deficient. Induction of IFN genes by dsRNA or viral infection in these cells might be impaired.[9,10] Mouse EC and ES cells have Dicer activity localized in their cytoplasm.[13] In the present study, we demonstrated that human embryonic kidney cell line HEK-293 cells had dsRNA-processing activity and contained much higher levels of Dicer mRNA than human osteoblastic cell line MG63 cells. When the dsRNA was treated *in vitro* with the cytosol of HEK-293 cells, 21–23 nt RNA fragments were not observed. It might be possible that other types of RNase existed in the cytosol of HEK-293 cells.

Recently, it was reported that PP1α dephosphorylated double-stranded RNA-activated protein kinase (PKR) and inhibited its enzyme activity.[21] The PKR is an IFN-induced gene product that directly induces the dsRNA-mediated nonspecific inhibition of protein synthesis, which causes the failure of RNAi.[21] The dsRNA-induced RNAi in human cells described in the present study may have been derived from the interaction between PP1s and PKR. The antisense RNA also suppressed the expression of the PP1δ. The sequence of antisense RNA we selected might be the suitable material for antisense-mediated gene knock down. It was reported that both 5′RNA and dsRNA could suppress the specific genes. However, dsRNA was substantially more effective than antisense RNA to produce interference.[7] In the present study, the dose of antisense RNA might be high enough to produce antisense-mediated gene interference. In contrast to 5′RNA, the dsRNA whose formation was confirmed by RNase III treatment produced dsRNA-mediated gene interference (RNAi).

Protein phosphatases are involved in many cellular processes. The function of PP1s has been studied by using inhibitors including okadaic acid, calyculin, microcystin, tautomycin, and cantharidin. In previous studies, we demonstrated that inhibition of protein phosphatases by okadaic acid-treatment resulted in apoptosis,[22,23] expression of Fas antigen,[24,25] and osteoblast differentiation.[26] The PP1γ1 gene expression was inhibited in human cells by using antisense oligonucleotide.[27] It was reported that PP1γ1 inhibition resulted in the failure of cell division in a late stage of cytokinesis. However, RNAi of PP1δ did not alter the cell division (data not shown). PP1δ binds to pRb,[28,29] focal adhesion kinase,[30] splicing factor,[31] nucleophosmin,[32] and nucleolin.[33] Because the PP1 isoforms have high sequence homology and cannot be distinguished by activity assay, PP1δ-specific inhibition is important in understanding the regulation of these binding substrates and to reveal the function of PP1s in many cellular processes.

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