Biochemical and Biophysical Research Communications 425 (2012) 730–734

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# RNA-binding properties and RNA chaperone activity of human peroxiredoxin 1

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ARTICLE INFO

*Article history:*
Received 24 July 2012
Available online 1 August 2012

*Keywords:*
Human peroxiredoxin 1
RNA-binding activity
RNA chaperone
RNA-binding protein
Cold stress

ABSTRACT

Human peroxiredoxin 1 (hPrx1), a member of the peroxiredoxin family, detoxifies peroxide substrates and has been implicated in numerous biological processes, including cell growth, proliferation, differentiation, apoptosis, and redox signaling. To date, Prx1 has not been implicated in RNA metabolism. Here, we investigated the ability of hPrx1 to bind RNA and act as an RNA chaperone. *In vitro*, hPrx1 bound to RNA and DNA, and unwound nucleic acid duplexes. hPrx1 also acted as a transcription anti-terminator in an assay using an *Escherichia coli* strain containing a stem–loop structure upstream of the chloramphenicol resistance gene. The overall cellular level of hPrx1 expression was not increased at low temperatures, but the nuclear level of hPrx1 was increased. In addition, hPrx1 overexpression enhanced the survival of cells exposed to cold stress, whereas hPrx1 knockdown significantly reduced cell survival under the same conditions. These findings suggest that hPrx1 may perform biological functions as a RNA-binding protein, which are distinctive from known functions of hPrx1 as a reactive oxygen species scavenger.



## 1. Introduction

Peroxiredoxins (Prxs) are thiol-based antioxidant proteins that eliminate various peroxide substrates. All Prx enzymes undergo a cycle of oxidation and reduction through their conserved Cys residues during catalysis [1]. Six isoforms of Prx exist in mammalian cells (Prx1–6), which are classified into three subgroups (2-Cys, atypical 2-Cys, and 1-Cys) based on the number and position of conserved Cys residues [2]. In eukaryotic cells, the Prxs act as both antioxidant proteins and regulators of $H_2O_2$-mediated cell signaling. Bacterial Prxs appear to be required only to protect cells against damaging effect of reactive oxygen species (ROS) [3]. And the 2-Cys Prxs has dual functions in modulating ROS concentrations and preventing protein aggregation may play pivotal roles in cellular response to pathogens and external stresses [4,5].

Human peroxiredoxin 1 (hPrx1) is a member of the 2-Cys peroxiredoxin (2-Cys Prx) family. hPrx1 is known to be involved in a variety of biological processes [6]. hPrx1 enhances the cytotoxicity of natural killer cells and inhibits the oncogenes c-myc and c-Abl [7–10]. hPrx1 is phosphorylated by cyclin-dependent kinase in M phase of the cell cycle. Phosphorylation of hPrx1 at Thr90 markedly reduces its peroxidase activity [11]. It has been also demonstrated that hPrx1 is secreted in hPrx1-overexpressing, transfected human lung adenocarcinoma cell cultures [12].

To date, Prxs have not been implicated in RNA metabolism. Here, we investigated the ability of hPrx1 to bind RNA and to act as an RNA chaperone. To study the function of Prxs as RBPs in a mammalian system, we selected hPrx1. We demonstrated that hPrx1 exhibits RNA-binding activity and acts as an RNA chaperone.

## 2. Materials and methods

### 2.1. Cell culture and transfection

Human HeLa cervical cancer cells were maintained in DMEM containing 10% fetal bovine serum and 100 U/ml penicillin/streptomycin. The cells were grown at 37 °C in a humidified atmosphere containing 5% $CO_2$. Transfection was performed with 20 nM siRNA or pCS4-myc-hPrx1 plasmid using Lipofectamine 2000 according to the manufacturer's protocol. The sequence of the hPrx1 siRNA (si-hPrx1) was 5′-GCCGAAUUGUGGUGUCUUAUU-3′.

### 2.2. Purification of protein from Escherichia coli cells

The pRSET-A::hPrx1 plasmid was transformed into *E. coli* BL21 (DE3) pLysS. Transformants were grown in Luria–Bertani (LB) medium at 37 °C, and recombinant protein production was induced with 0.5 mM IPTG for 4 h. After centrifugation, the cell pellet was suspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl) and ruptured by sonication. The soluble fraction was loaded onto a settled Ni–NTA resin column (QIAGEN, USA), which was washed

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http://dx.doi.org/10.1016/j.bbrc.2012.07.142

with lysis buffer. His-tagged hPrx1 was eluted with 150 mM imidazole and dialyzed against 20 mM Tris (pH 7.5).

### 2.3. Nucleic acid-binding analysis

Recombinant hPrx1 or GST was incubated with single-stranded DNA (ssDNA) (M13mp18, NEB), double-stranded DNA (dsDNA) (M13mp18 RF I DNA, NEB), *in vitro*-transcribed *luc* mRNA, or total RNA from HeLa cells in 10 mM Tris–HCl (pH 7.5) on ice for 15 min. For DNase and RNase protection assays, DNase or RNase was added to mixtures containing DNA– or RNA–hPrx1 complexes. The samples were separated in a 0.8% agarose gel and stained with ethidium bromide to visualize gel shifts.

### 2.4. In vitro DNA melting assay with molecular beacons

The molecular beacon used in this study was a 78-nucleotide hairpin molecule labeled with a fluorophore and quencher: tetramethyl-rhodamine-AGGGTTCTTTGTGGTGTTTTTATCTGTGCTTCCCTATGCACCGCCGACGACAGTCGCTAACCTCTCGCTAAGAACCCT-DABCYL. Fluorescence measurements were performed in 96-well plates using a Spectra Max Gemini XPS spectrofluorometer (Molecular Devices), with excitation and emission at 555 and 575 nm, respectively.

### 2.5. In vivo transcription anti-termination

RL211 *E. coli* cells, which contain a *cat* gene cassette positioned downstream of the *trpL* terminator [13], were transformed with pINIII vector, pINIII–CspA (an RBP), or pINIII–hPrx1; spotted onto LB plates containing 50 μg/ml ampicillin and 1 mM IPTG with or without 30 μg/ml chloramphenicol (Cm); and grown for one or two nights.

### 2.6. Reverse-transcription PCR

Total RNA was isolated from transfected cells using TRIzol reagent and was treated with DNase I. cDNA was synthesized from 4 μg of total RNA using a Primescript 1st Strand cDNA Synthesis kit (Takara) and random hexamer primers, in a volume of 20 μl. PCR was performed using 20-μl reactions containing 1 μl of cDNA, Ex Taq polymerase (Takara), and gene-specific primer sets. PCR products were subjected to electrophoresis in a 1.2% agarose gel and stained with ethidium bromide for visualization.

### 2.7. Western blot analysis

Cells were lysed in lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 50 mM EDTA, 1% Triton X-100). The cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto a nitrocellulose membrane, immunoblotted with antibodies, and visualized using an enhanced chemiluminescence detection system.

### 2.8. Immunofluorescence staining

HeLa cells on glass cover slips were incubated at 25 °C for the indicated times and fixed with 4% formaldehyde in PBS for 15 min. The cells were washed three times with PBS, permeabilized by incubation with 0.1 M glycine in PBS for 3 min, and washed a further three times with PBS. After treatment with 0.2% Triton X-100 for 20 min, the cells were blocked by incubation with 5% BSA in PBS for 1 h and then incubated overnight with anti-hPrx1 antibody. After three washes with PBS, Alexa Fluor 546-labeled anti-mouse IgG (Invitrogen) was applied as the secondary antibody, nuclei were stained with DAPI, and the cells were observed under a confocal fluorescence microscope.

### 2.9. Nuclear and cytoplasmic fractionation

Nuclear and cytoplasmic fractions were extracted using a NE-PER kit (Pierce, Rockford, IL) according to the manufacturer's protocol. The nuclear and cytoplasmic fractions were analyzed by Western blotting with anti-hPrx1 antibody. A histone-specific antibody was used as a nuclear marker. A tubulin-specific antibody was used as a cytoplasmic marker.

### 2.10. Cell viability assay (MTT reduction assay)

HeLa cells were transfected with pCS4-myc vector, pCS4-myc-hPrx1, or siRNA. At 48 h after transfection, the cells were incubated at 30 or 25 °C for 12 h, and cell viability was assessed by MTT reduction assay. Briefly, 50 μl of MTT (3 mg/ml) were added to the cells, followed by incubation for 2 h at 37 °C. The medium was removed, and 100 μl of isopropanol were added to each well. MTT reduction was determined by measuring the absorbance at 570 nm. Cell viability at 30 and 25 °C was compared with that at 37 °C (which was set to 100%).

## 3. Results and discussion

### 3.1. Upregulation of RNA-binding proteins in Prx-deficient mutant cells

To investigate the biological function of Prx1, we compared the protein expression profiles in yeast Prx1 (YPrx1) deletion mutant cells (Δ*yprx1*) and wild-type yeast cells. The expression level of RNA-binding proteins, including Tif2, Nop15, Nab2, and Yra1, was increased in the mutant cells (Table 1). The upregulation of a number of RBPs in yeast Prx1 (YPrx1) knockout mutants suggested that the loss of Prx1 induced a compensatory mechanism. This is a common feature in adaptive responses to environmental stressors such as heat, cold, and oxidation. For example, a thioredoxin-dependent mechanism compensates for a deficient glutathione system in some cells [14]. In *E. coli* mutants lacking CspA, the major cold shock protein in *E. coli*, upregulation of the CspA homologs CspB and CspG compensates for the loss of CspA during cold shock adaptation [15]. Thus, the upregulation of RBPs as a result of Prx1 deficiency suggests that Prx1 may be linked to the function of RBPs. To provide more evidence in support of Prx1 as a novel RNA-binding protein, we used a mammalian system and hPrx1, the human homologue of YPrx1.

### 3.2. RNA-binding properties of hPrx1

The ability of hPrx1 to bind nucleic acids was examined in binding assays using ssDNA dsDNA, and RNA. As shown in Fig. 1A, recombinant hPrx1 bound to and shifted ssDNA and dsDNA in a concentration-dependent manner. In contrast, when up to 20 μg of GST protein were added to the binding reaction as a negative control, there was no shift. RNA-binding activity was assayed by performing gel shift assays using total RNA from HeLa cells and *in vitro*-transcribed *luc* mRNA. When $\geqslant$10 μg of hPrx1 were added to the reaction, hPrx1–RNA complexes were observed, whereas no shift was observed with GST (Fig. 1A). Moreover, hPrx1 protected ssDNA and RNA from degradation by DNase and RNase, respectively, indicating that hPrx1 binds strongly to DNA and RNA (Fig. 1B). These findings suggest that hPrx1 has RNA-binding properties.

**Table 1**
Proteins upregulated in Δ*yprx1* yeast cells compared with wild-type yeast cells.[a]

| Protein name | Accession No. | MW (kDa) | Function |
|---|---|---|---|
| Tif2 | P10081.3 | 43 | DEA(D/H)-box RNA helicase that couples ATPase activity to RNA binding and unwinding |
| Hyp2 | P23301.3 | 17 | Translation initiation factor eIF-5A |
| Nop15 | P53927.1 | 24 | pre-rRNA processing |
| Eft2 | P32324.1 | 93 | Translation elongation factor 2 |
| Nab2 | P32505.1 | 58 | Nuclear polyadenylated RNA-binding protein |
| Yra1 | Q12159.2 | 25 | Nuclear protein with RNA annealing activity |
| Tma20 | P89886.1 | 20 | Associates with ribosomes and has a putative RNA binding domain; interacts with Tma22p |

[a] Proteins that were shown by 2-DE analysis to be upregulated in Δ*yprx1* cells were identified by MALDI-TOF. Only proteins implicated in RNA metabolism are shown.

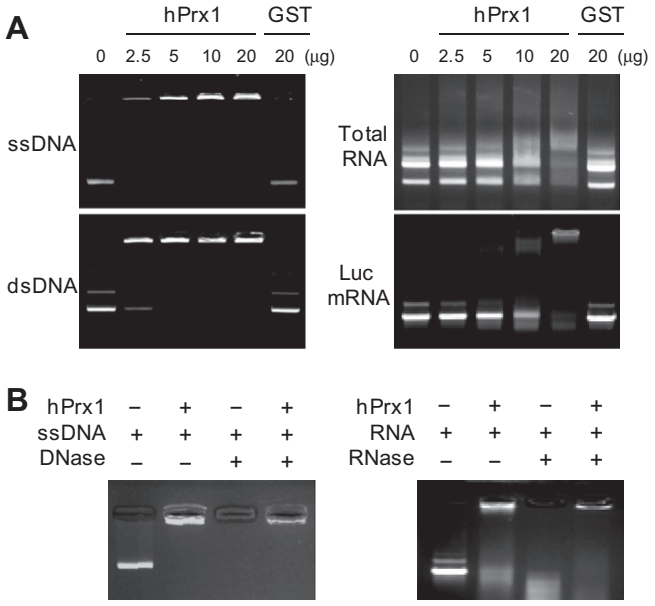


**Fig. 1.** Nucleic acid-binding activity of hPrx1. (A) Recombinant hPrx1 protein was incubated with ssDNA, dsDNA, or total RNA from HeLa cells, or with *in vitro*-transcribed *luc* mRNA, and the complexes were analyzed using agarose gel shift assays. (B) Protection of hPrx1–nucleic acid complexes from DNase and RNase. The hPrx1–nucleic acid complexes were treated with DNase or RNase and analyzed by gel shift assays performed as in (A).

### 3.3. RNA chaperone activity of hPrx1

To investigate the function of hPrx1 as an RNA chaperone, we examined its helix-destabilizing activity in nucleic acid melting and anti-termination assays. The nucleic acid melting assay uses an *in vitro* molecular beacon system in which fluorescence is produced when a protein binds to and melts the stem–loop structure of the molecular beacon substrate. Because of the close proximity of the fluorescent tag and the quencher, fluorescence is efficiently quenched when the two parts of the substrate remain annealed to each other. When a protein melts the substrate, the fluorescent tag and quencher separate spatially, and the fluorescence intensity increases. This system has been used to demonstrate the RNA chaperone activity of several RBPs, including CspA, Hfq, and CspE [16–18]. When hPrx1 was added to the molecular beacon, a marked increase in fluorescence intensity was detected, indicating that hPrx1 melted the secondary structure of the molecular beacon substrate (Fig. 2A).

To examine the helix-destabilizing activity of hPrx1 *in vivo*, we performed a transcription anti-termination assay using RL211 *E. coli*. In the RL211 *E. coli* system, transcription termination can be caused by the formation of a stem–loop structure in a nascent transcript, followed by a poly(U) stretch. This prevents RNA polymerase from reaching the downstream reporter gene (chloramphenicol acetyl transferase, CAT), rendering the cells sensitive to chloramphenicol (Cm). When RNA chaperones are overexpressed in these cells, the terminator stem is melted, CAT is transcribed, and the cells become resistant to Cm [19].

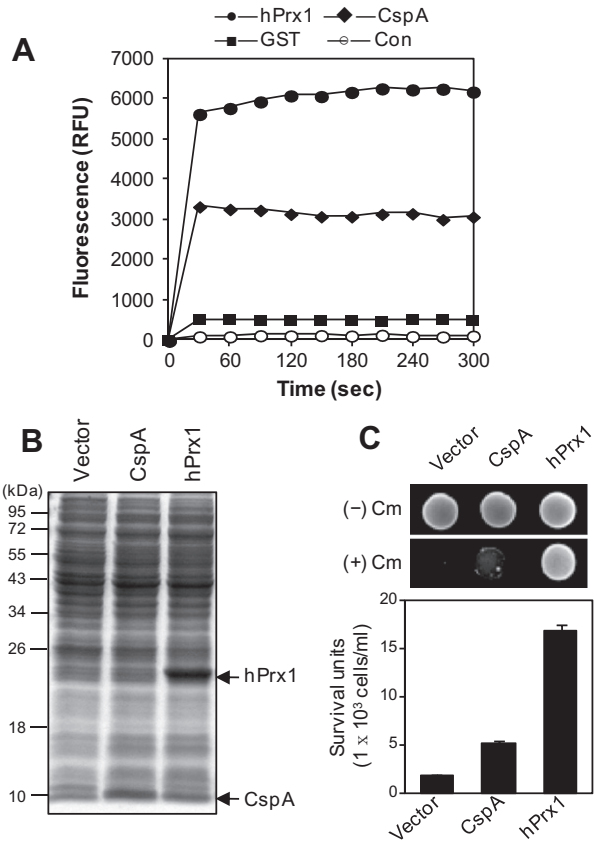


**Fig. 2.** RNA chaperone activity of hPrx1. (A) Nucleic acid melting activity of hPrx1. The fluorescence of a molecular beacon was monitored as hPrx1, CspA, GST, or buffer alone (without the molecular beacon) was added. The excitation and emission wavelengths were 555 and 575 nm, respectively. (B) Protein expression of hPrx1 and CspA in RL211 *E. coli* cells was verified by SDS–PAGE. (C) Transcription anti-termination activity of hPrx1. RL211 *E. coli* cells containing a *cat* gene cassette positioned downstream of the *trpL* terminator were transformed with pINIII vector, pINIII–hPrx1, or pINIII–CspA. Transformed cells were incubated on plates containing 1 mM IPTG with or without chloramphenicol (Cm). The results of cell growth after one night are presented (top). Relative cell survival was quantified as the number of colony-forming units (bottom).

For the assay, RL211 *E. coli* cells were transformed with CspA and hPrx1 overexpression plasmids (Fig. 2B) and plated on medium containing Cm. As shown in Fig. 2C, cells harboring the control vector did not grow on Cm-containing plates, whereas cell harboring the CspA or hPrx1 overexpression plasmid were able to grow on Cm-containing plates, demonstrating the abilities of CspA and

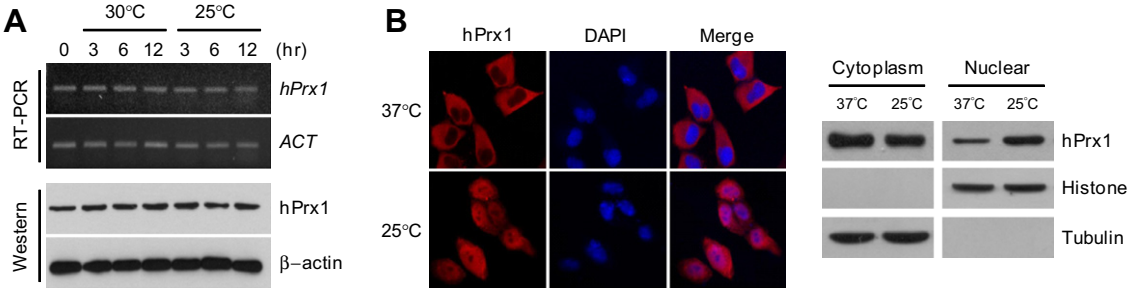


**Fig. 3.** Change of hPrx1 level in response to cold stress. (A) The expression levels of hPrx1 mRNA and protein in cells exposed to cold stress. (B) HeLa cells were exposed to cold stress (25 °C) for 9 h, and the subcellular localization of hPrx1 was assessed by immunostaining (left) and fractionation (right). DAPI and histone, nuclear markers; tubulin, cytoplasmic marker.

hPrx1 to destabilize the stem–loop structure of the transcription terminator. Based on the comparative cell survival, the efficiency of hPrx1 in inducing chloramphenicol resistance gene expression was at least three times that of CspA, a well-known RBP (Fig. 2C). These results indicate that hPrx1 exhibits helix-destabilizing activity and is capable of melting RNA secondary structures.

### 3.4. Cold stress increases hPrx1 level in the nucleus

RBPs that act as RNA chaperones, such as bacterial Csp proteins, are necessary for cellular adaptation to low temperatures, although only some of them are cold-shock inducible. Two major cold-inducible RBPs in human cells, CIRP and RBM3, can be induced by exposure to moderate cold shock [20]. To examine whether hPrx1 is induced by cold stress, we determined the hPrx1 mRNA and protein levels in HeLa cells exposed to mild cold shock (30 °C) and severe cold shock (25 °C). Neither hPrx1 mRNA nor hPrx1 protein was increased by cold stress (Fig. 3A). However, hPrx1 translocated from the cytoplasm into the nucleus in response to cold stress, as shown by an increased level of hPrx1 in the nuclear fraction (Fig. 3B). Altogether, these findings indicate that hPrx1 is responsive to cold stress in a nucleus-specific way.

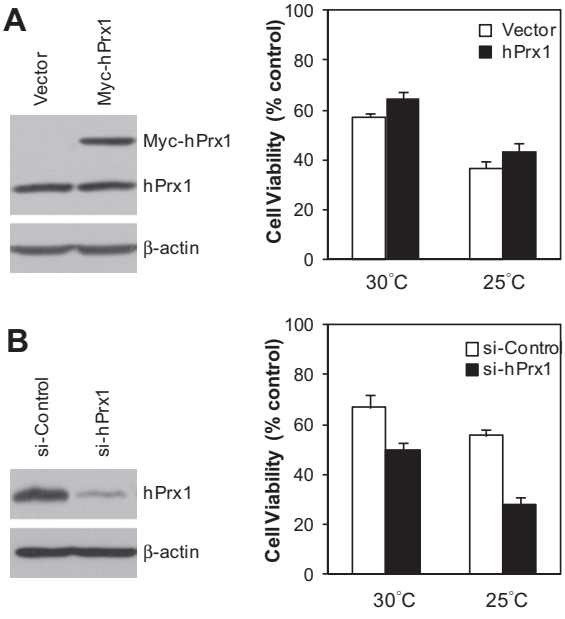


**Fig. 4.** The effect of hPrx1 on cell viability under conditions of cold stress. (A) After transfection with pCS4-myc-vector (Vector) or pCS4-myc-hPrx1 (Myc-hPrx1), myc-hPrx1 overexpression was confirmed by Western blot analysis (left). Transfected cells were incubated at 30 or 25 °C for 12 h, and cell viability was assessed by MTT reduction assay (right). The results are shown as relative viability compared with cells incubated at 37 °C. (B) Western blot analysis was performed to detect hPrx1 protein expression in control siRNA-transfected (si-Control) and hPrx1 siRNA-transfected (si-hPrx1) HeLa cells (left). Cell viability assays were performed as in (A). Cell viability data are expressed as the means ± SD of three independent experiments.

### 3.5. hPrx1 enhances the viability of cells exposed to cold stress

To investigate whether hPrx1 is involved in cold adaptation, we examined its effect on cell viability under conditions of cold stress, using the MTT reduction assay. After transfection with a control vector or myc-hPrx1, HeLa cells were incubated at 30 or 25 °C for 12 h. Compared with control vector-transfected cells, the cells overexpressing hPrx1 exhibited increased cell viability during mild (30 °C) and severe (25 °C) cold stress (Fig. 4A). Conversely, hPrx1 knockdown by siRNA reduced the viability of cold-exposed HeLa cells to about 20–30% of control siRNA-transfected cell viability; the effect was especially strong at 25 °C (Fig. 4B). These results indicate that hPrx1 is involved in the adaptation of cells to cold stress.

### 3.6. Summary

Here, we demonstrated the RNA-binding property of hPrx1 using a series of experiments, including nucleic acid binding assays, nucleic acid melting assays, and anti-termination assays. This novel finding suggests that hPrx1 may perform biological functions as a RBP, which are distinctive from known functions of hPrx1 as a ROS scavenger.

## Acknowledgments

This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0006424) and a grant from the Next-Generation BioGreen 21 Program (SSAC, grant #: PJ009068), Rural Development Administration, Republic of Korea.

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