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Peroxiredoxin II is essential for preventing hemolytic anemia from oxidative stress through maintaining hemoglobin stability

Ying-Hao Han [a,b,1], Sun-Uk Kim [a,1], Tae-Ho Kwon [a,c,1], Dong-Seok Lee [a,d], Hye-Lin Ha [a], Doo-Sang Park [a], Eui-Jeon Woo [a], Sang-Hee Lee [e], Jin-Man Kim [e], Ho-Byoung Chae [f], Sang Yeol Lee [f], Bo Yeon Kim [g], Do Young Yoon [c], Sue Goo Rhee [h], Eitan Fibach [i], Dae-Yeul Yu [a,*]

[a] KRIBB, Daejeon, Republic of Korea
[b] College of Life Science and Technology, Heilongjiang Bayi Agricultural University, Daqing, HeiLongJiang Province, China
[c] Bio/Molecular Informatics Center, Konkuk University, Seoul, Republic of Korea
[d] College of Natural Sciences, Kyungpook National University, Daegu, Republic of Korea
[e] College of Medicine, Chungnam National University, Daejeon, Republic of Korea
[f] Environmental Biotechnology Research Center, Gyeongsang National University, Jinju, Republic of Korea
[g] World Class Institute, KRIBB, Cheongwon, Republic of Korea
[h] Division of Molecular Life Sciences, Ewha Womans University, Seoul, Republic of Korea
[i] Dept. of Hematology, Hadassah University Hospital, Ein-Kerem, Jerusalem, Israel

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ABSTRACT

The pathophysiology of oxidative hemolytic anemia is closely associated with hemoglobin (Hb) stability; however, the mechanism of how Hb maintains its stability under oxidative stress conditions of red blood cells (RBCs) carrying high levels of oxygen is unknown. Here, we investigated the potential role of peroxiredoxin II (Prx II) in preventing Hb aggregation induced by reactive oxygen species (ROS) using Prx II knockout mice and RBCs of patients with hemolytic anemia. Upon oxidative stress, ROS and Heinz body formation were significantly increased in Prx II knockout RBCs compared to wild-type (WT), which ultimately accelerated the accumulation of hemosiderin and heme-oxygenase 1 in the Prx II knock-out livers. In addition, ROS-dependent Hb aggregation was significantly increased in Prx II knockout RBCs. Interestingly, Prx II interacted with Hb in mouse RBCs, and their interaction, in particular, was severely impaired in RBCs of patients with thalassemia (THAL) and sickle cell anemia (SCA). Hb was bound to the decameric structure of Prx II, by which Hb was protected from oxidative stress. These findings suggest that Prx II plays an important role in preventing hemolytic anemia from oxidative stress by binding to Hb as a decameric structure to stabilize it.

1. Introduction

Peroxiredoxins (Prxs), a family of thiol-containing peroxidases, were identified primarily by their peroxidase activities, and contribute to the control of endogenously produced peroxides in eukaryotes [1]. In addition to their antioxidant activity, Prxs have been implicated in numerous cellular functions, such as proliferation, differentiation [2] and intracellular signaling [3].

Erythrocytes contain a large amount of Hb, which delivers oxygen to all tissues and organs in the body. During the oxygen transport, Hb undergoes autoxidation to produce superoxide [4], which is dismutated to hydrogen peroxide by superoxide dismutase (SOD). Hydrogen peroxide is known to be detoxified by antioxidant enzymes, such as catalase, glutathione peroxidase and Prxs [5]. Prx II is a member of the Prx family that is abundantly expressed in all types of cells. Especially, Prx II is the third most abundant protein and thought to be one of the main players for protection of RBCs from oxidative stress through hemoglobin autoxidation [4]. We have reported that Prx II knockout mice showed Heinz body formation and oxidative hemolytic anemia [6]. Therefore, the redox balance regulated by Prx II in RBCs may be expected to be associated closely with hematological pathologies, such as decreased RBC life span and Hb instability. In addition to the peroxidase function, yeast and human Prxs containing 2-cysteine residues have been shown to act as molecular chaperones under increased oxidative stress [7]. Therefore, current research has been focused on investigating their protection activity in relation to Hb stability and its underlying molecular mechanisms.

In this study, we examined the function of Prx II in the protection of Hb stability in Prx II knockout mice and patients with

* Corresponding author. Address: Aging Research Center, KRIBB, 125 Gwahak-ro, Yuseong-gu, Daejeon 305 806, Republic of Korea. Fax: +82 42 860 4609.
E-mail address: dyyu10@kribb.re.kr (D.-Y. Yu).
[1] These authors contributed equally to this work.

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hereditary hemolytic anemia. Our results show that loss of Prx II in mouse RBCs resulted in an elevated ROS level and protein, mainly Hb, aggregation and hypersensitive response to increased ROS. In addition, we also show impaired binding of Prx II to Hb in patients with THAL and SCA RBCs, resulting in the significant increase in the Hb aggregation by ROS attack. These effects were closely associated with a Hb-Prx II interaction. Our findings indicate that the decameric form of Prx II can bind to Hb and protect Hb from oxidative-induced denaturation and aggregation in human and mouse RBCs.

2. Materials and methods

2.1. Mice and patients

Mice of the 129/SvJ background were maintained in a specific pathogen-free authorized facility in the Korean Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, Korea). All animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee, KRIBB.

Peripheral blood were transported in a box with dry ice by using airplane from Israel to Korea and stored at −70 °C until use. Informed consent was obtained in all cases according to the institutional Helsinki Committee regulations.

2.2. Co-immunoprecipitation

The protein samples were incubated with agarose beads (Santa Cruz) for 30 min at 4 °C to pre-absorb any polypeptides that might bind nonspecifically to the beads as described previously [8]. After removing the beads by centrifugation, the supernatant was incubated with anti-Prx II (Lab Frontier, Korea) and Hb antibodies (Santa Cruz) for 1 h and subsequently with protein A-linked agarose beads for 1 h at 4 °C. Proteins were separated by 12% or 15% SDS–PAGE.

2.3. Assay for ROS-induced Hb aggregation

Peripheral blood RBCs (6×10^6) were lysed in 5 mM of phosphate buffer (PB; pH8.0) under native conditions, and an Hb solution was obtained as described previously [9]. Hb was treated with several concentrations of hydrogen peroxide (H_2O_2) for 30 min at 37 °C in the presence or absence of Prx II proteins. Turbidity due to Hb aggregation was analyzed by spectrophotometry (Nano Drop Technologies) at 360 nm as described previously [7].

2.4. Preparation of purified human hemoglobin (phHb)

Human hemoglobin 0.5 g (Sigma Aldrich) was dissolved in 5 ml of loading buffer (20 mM Tris–HCl, pH 8.5, 10 mM NaCl, 5 mM β-mercaptoethanol, 2% polyethylene glycol 4000 (PEG 4K). The Hb solution was placed onto a HiTrap Q FF Column (5 × 1 ml, GE Healthcare) and eluted by elution buffer (20 mM Tris–HCl, pH 8.5, 1 M NaCl, 5 mM β-mercaptoethanol, 2% PEG 4 K). The eluted Hb solution was further purified by dialysis (30 mM Tris–HCl, pH 7.5, 10 mM NaCl, 2 mM β-mercaptoethanol).

2.5. Preparation of recombinant WT and mutant Prx II proteins

The human Prx II (hPrx II) gene was cloned from a human liver library by PCR. N and C terminally truncated hPrx II mutants lacking 14 and 26 amino acids (ΔN-ter and ΔC-ter, respectively) and mutants where the cysteines at positions 51 and 172 were replaced by serine residues. Mutants (C51S and C172S, respectively) were generated by standard PCR-mediated site-directed mutagenesis with pPROEX HTb (Invitrogen). WT and 4 kinds of mutant hPrx II proteins were expressed in *Escherichia coli* BL21 (DE3) and purified using a Ni–NTA column (1 × 4 cm, Ni–NTA superflow; Qiagen), and then cleaved by TEV. Obtained WT and 4 kinds of mutant hPrx II proteins were purified as described previously [10]. To examine the oligomerization states of the WT hPrx II proteins (dimer, decamer, and high molecular form), the WT Prx II proteins were analyzed by size exclusion chromatography (SEC), SEC on HPLC (Dionex) was performed with a Superdex 200 10/30 GL column (GE Healthcare) equilibrated at a flow rate of 0.5 ml/min at 25 °C with a 50 mM HEPES pH 7.0 buffer containing 100 mM NaCl, and the apparent molecular weight of WT hPrx II were confirmed by native-PAGE as described previously [11].

2.6. Statistical analyses

Statistical analysis was performed using ANOVA test. A P value of less than 0.05 was considered to be significant.

3. Results

3.1. Prx II-deficient RBCs fail to resist oxidant-induced Hb aggregation

RBCs are known to require a potent antioxidative defense system. We previously showed that loss of Prx II causes oxidative hemolytic anemia, as evidenced by Heinz body formation in peripheral RBCs and splenomegaly [6]. To further understand the essential role of the Prx II in RBC redox balance, in the present study, we examined the possible connection between ROS level and Hb aggregation rate in Prx II$^{-/-}$ RBCs in response to *in vivo* treatments by aniline hydrochloride (AH) respectively. Prx II$^{-/-}$ RBCs showed higher ROS levels than WT RBCs in response to *in vivo* treatment with AH which reflects oxidative injury in RBCs and induces Heinz body formation [12] (Fig. 1A). In Prx II$^{-/-}$ mice, this event was accompanied by a decrease in hematological parameters, such as hematocrit and Hb content, and increased reticulocyte count in Prx II$^{-/-}$ mice (Table 1). The incidence of Heinz body-containing RBCs had a higher relative increment in Prx II$^{-/-}$ mice (Fig. 1B). These results suggest that the role of Prx II is closely associated with protection of RBCs from ROS-induced Hb aggregation.

3.2. Prx II interacts with Hb and prevents oxidative Hb aggregation

To examine whether Prx II is involved in the protection of Hb against ROS, we prepared cytosolic Hb solution from WT and Prx II$^{-/-}$ RBCs under native conditions, treated them with various concentrations of H_2O_2 in the presence or absence of recombinant human Prx II (rhPrx II), and analyzed Hb aggregation spectrophotometrically (Fig. 2A). Both Hb extracts were aggregated by treatment with H_2O_2, but Prx II$^{-/-}$ Hb were hypersensitive to H_2O_2-induced aggregation compared to WT Hb. However, the addition of rhPrx II restored the aggregation level of Prx II$^{-/-}$ Hb to the level of WT Hb (Fig. 2A). To investigate the underlying mechanism, first we studied the interaction of Prx II with Hb under native conditions using co-immunoprecipitation and Western Blot analyses (Fig. 2B). RBC protein lysates prepared from WT and Prx II$^{-/-}$ RBCs were immunoprecipitated and immunoblotted with anti-Prx II and Hb antibodies. Unlike the result from Prx II$^{-/-}$ RBCs, intense immunoreactive bands against α-Hb and β-Hb were found in WT cytosolic proteins precipitated by the anti-Prx II antibody. Consistent with the result, the Prx II immunoreactive band was only found in proteins from WT cytosolic proteins precipitated by antibodies for α-Hb and β-Hb. These results suggest that Prx II is a novel interacting partner to Hb in RBCs, the interaction of these two proteins

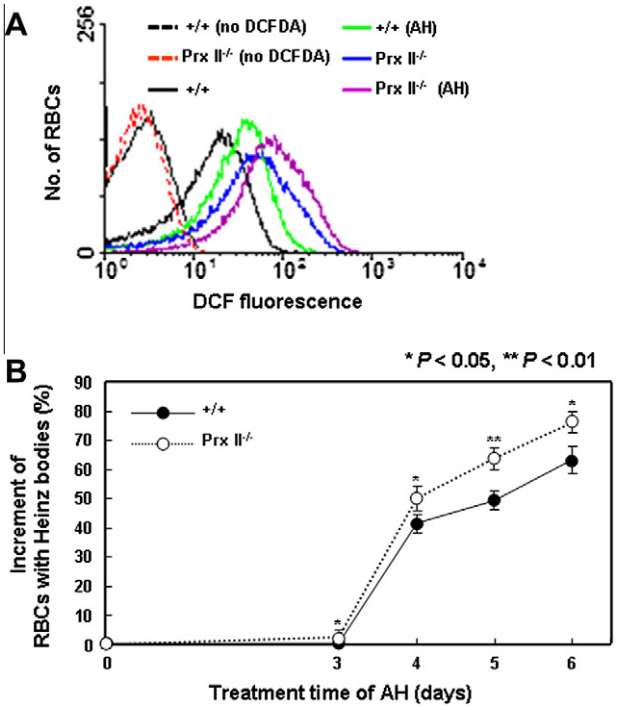

Fig. 1. ROS levels and Heinz body formation in Prx II$^{-/-}$ and WT RBCs. (A) Relative ROS levels in WT (+/+) and Prx II$^{-/-}$ RBCs were measured by FACS for DCF fluorescence intensity after intraperitoneal injection of aniline hydrochloride (AH). The mice of the two genotypes were injected intraperitoneally with 160 mg/kg (body weight) of AH in the first and second day; the RBCs were collected at third day. (B) RBCs were collected from AH injected mice ($n = 3$), to count the number of RBCs with Heinz bodies, peripheral blood smears were prepared on the indicated days, stained with Cresyl violet, and observed under light microscopy. To show the increment value, we used following formula; Percentage of increment = percentage after AH treatment – percentage before AH treatment/100% – percentage before AH treatment.

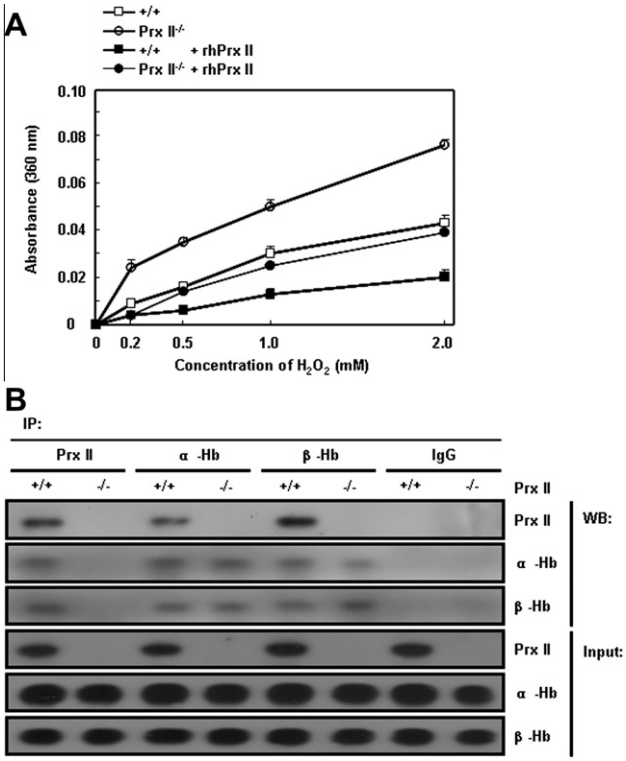

Fig. 2. Prx II interacts with Hb and protects against ROS-induced Hb aggregation. (A) Hb extracted from the two genotype RBCs was treated with the indicated concentrations of H_2O_2 in the presence or absence of rhPrx II. The data are representative of three independent experiments. (B) Cytosolic proteins extracted from WT (+/+) and Prx II$^{-/-}$ RBCs were IP with antibodies for α-Hb, β-Hb and Prx II, and immune complexes were analyzed by WB. Input lanes contain 10% materials used in IP.

could be construed as a novel form of Prx II protection against Hb instability.

3.3. Decameric Prx II predominantly bound to Hb

The mammalian Prx II found in humans and mice shares over 92% amino acid sequence identity [13], and erythrocyte Prx II exists in solution as a dynamic equilibrium of dimers (44 kDa) and decamers (220 kDa) [14]. Hyperoxidation at the peroxidatic cysteine to cysteine sulfinic acid traps the enzyme as decameric toroids, at least *in vitro* [15]. Based on these reports, we investigated whether these forms contribute to Hb stabilization under oxidative condition. According to the experiment of SEC of rhPrx II, clearly distinguishable molecular weights were fractionated (Fig. 3A). Staining with Coomassie Blue dye on a native-PAGE demonstrated that approximately three types of molecular sizes were mainly detected in each fraction (Fig. 3B): high MW (F–H, >300 kDa), middle-size MW (F–M, around 220 kDa) and low MW (F–L, <220 kDa). The protein sizes of hPrx II in native-PAGE were similar to those estimated by SEC. However, a single protein band with a 22 kDa MW was observed upon SDS–PAGE of all the SEC-separated fractions (Fig. 3B lower panel). To determine the protective activity of the Prx II multimers on Hb under oxidative condition, Hb was treated with H_2O_2 in the presence or absence of the three multimer types of hPrx II and Hb aggregation was determined using spectrophotometry. As shown in Fig. 3C, Hb aggregation was greatly inhibited by the addition of F-M hPrx II. However, F-H and F-L hPrx II had no protective activity against H_2O_2-induced Hb aggregation. Interestingly, an immunoprecipitation assay showed that F-M hPrx II predominantly bound to Hb compared to the F-H and F-L forms (Fig. 3D). These results suggest that Prx II protects Hb against H_2O_2 induced aggregation mainly via decameric oligomerization and the subsequent physiological interaction.

To determine the binding domain of Prx II to Hb, we constructed the vectors expressing truncated or point mutated Prx II (Fig. 3E), purified the respective recombinants from *E. coli*, and carried out IP and Western Blotting analyses. Interestingly, C-terminus truncated (ΔC-ter) or two types of point mutated (C51S and C172S) rhPrx II were also able to interact with Hb, whereas the interaction

Table 1
Changes in blood parameters of wild type and Prx II$^{-/-}$ mice at 3rd day after injection of aniline hydrochloride (AH).

Genotypes	Treatment of AH	WBC (10^3/μl)	RBC (10^6/μl)	Hb (g/dL)	HCT (%)	MCV (fL)	Retics (%)
+/+	–	5.9 ± 0.8	10.6 ± 0.4	16.2 ± 0.3	52.3 ± 1.6	49.7 ± 1.8	4.3 ± 3.7
+/+	+	4.5 ± 1.4	9.2 ± 1.4	14.3 ± 1.8	45.3 ± 5.9	49.4 ± 1.5	8.4 ± 0.9
Prx II$^{-/-}$	–	5.3 ± 2.8	9.7 ± 0.7	14.8 ± 1.0	47.0 ± 2.6	48.4 ± 1.4	6.0 ± 0.1
Prx II$^{-/-}$	+	4.0 ± 1.6	7.6 ± 0.8*	12.4 ± 1.5	39.1 ± 5.0	51.5 ± 2.0	12.7 ± 1.1*

WBC, indicates white blood cells; RBC, red blood cell; Hb, hemoglobin; Hct, hematocrit; MCV, mean cell volume; Retics, reticulocytes.
* The data were statistically analyzed by *t*-test (*$P < 0.05$, $n = 3$).

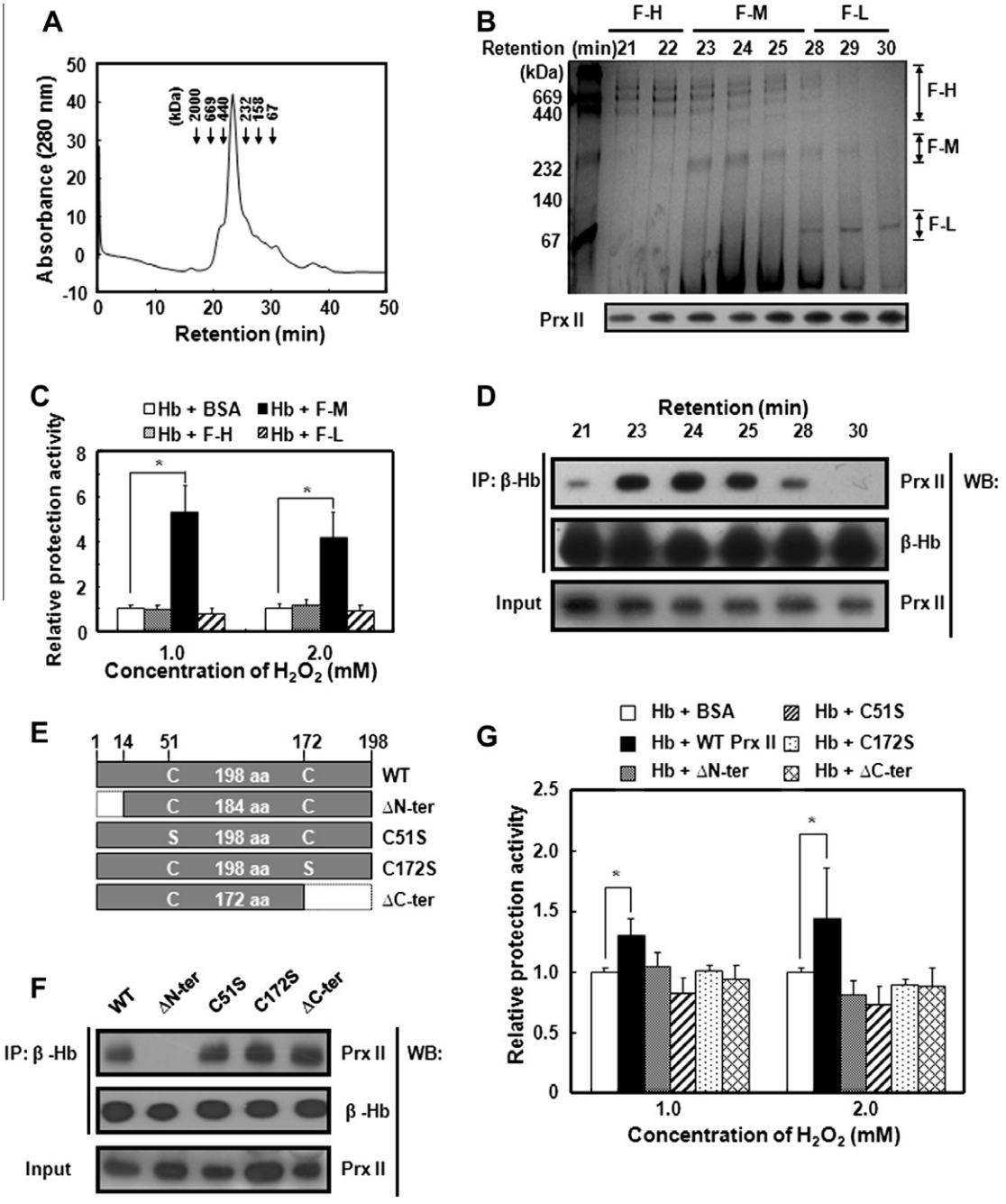

Fig. 3. Decameric form of Prx II is effective for stabilization of Hb. (A) Multiple forms of hPrx II protein complexes were analyzed by SEC. (B) Each fraction was subjected to staining with Coomassie Blue after protein separation by 10% native PAGE (upper panel), and immunoblotting with a hPrx II antibody (lower panel). (C) Human Hb was treated with 1.0 and 2.0 mM H_2O_2 in the presence of three fractions of Prx II protein. (D) Three fractions of Prx II protein and human Hb were mixed in a PB and then immunoprecipitated with β-Hb antibodies and WB were performed. Input lanes contain 10% materials used in IP. (E) Four mutant hPrx II constructs were prepared by the PCR method. (F) Four recombinant mutant proteins or a WT of Prx II proteins and human Hb were mixed in PB and then IP and WB were performed. (G) Human Hb was treated with 1.0 and 2.0 mM H_2O_2 in the presence of recombinant WT human Prx II and mutant Prx II proteins. Data means ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$.

was completely ablated by N-terminus truncation (ΔN-ter) of rhPrx II (Fig. 3F), suggesting that the binding site of Prx II to Hb is located in its N-terminus.

Next we compared the ability of different types of rhPrx II in protection of Hb against oxidative stress. As shown in Fig. 3G, WT rhPrx II displayed an excellent protecting capacity for Hb against H_2O_2 compared to bovine serum albumin (BSA), whereas the ΔN-ter rhPrx II did not show any protective action. Despite ΔC-ter, C51S and C172S rhPrx II could interact with Hb, but these mutants could not ameliorate the H_2O_2-induced Hb aggregation. Collectively, these results suggest that Hb stabilization under oxidative stress requires both the N-terminal binding site and the peroxidase activity-associated domains of Prx II.

3.4. Impaired binding of Prx II to Hb fails to protect against ROS-induced Hb aggregation in RBCs from thalassemia and SCA patients

Inherited RBC diseases with oxidative hemolytic anemia have a severe oxidant imbalance [16], which could make their Hb vulnerable to oxidative-mediated aggregation. To investigate the possible roles of Prx II in oxidant imbalance in human RBCs, we measured the Prx II contents in RBCs collected from patients with

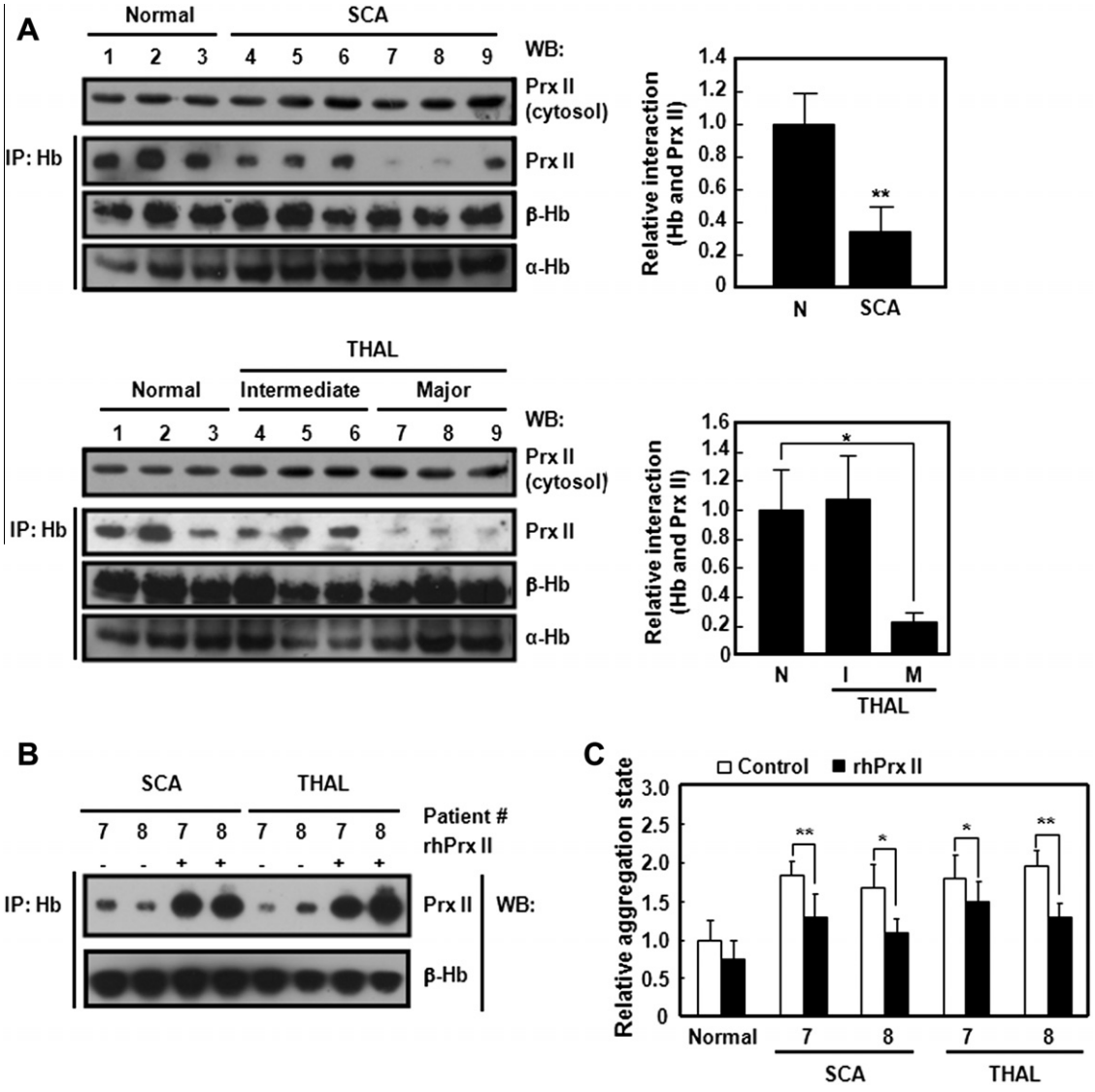

Fig. 4. Reduced interaction of Prx II with Hb in RBCs from patients with SCA and THAL. (A) Cytosolic proteins were extracted from RBCs of patients suffering from SCA (upper left panel) and THAL (lower left panel). The Prx II level (upper) and its interaction with Hb (lower) were analyzed by IP and WB analyses. In the upper left panel, cytosolic proteins were extracted from human normal and SCA RBCs. In the lower left panel, protein lysates were from normal (N), THAL intermediate (I) and THAL major(M) RBCs. IP products were normalized with α-Hb and β-Hb expression level in immune-complex by WB. For quantification, the expression levels of Prx II against α-Hb and β-Hb in immune-complex were measured by densitometric analysis (right upper and lower). (B) Hb extracted from RBCs of SCA and THAL patients and rhPrx II were mixed in PB and then immunoprecipitation with β-Hb antibodies and WB were performed. (C) Hb prepared from SCA and THAL RBCs was treated with the 5 mM H_2O_2 in the presence or absence of rhPrxs. Data means ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$.

THAL and SCA and investigated the interaction between Prx II and Hb using IP and Western Blotting analyses (Fig. 4A). Although the cytosolic Prx II contents between normal and patient RBCs were similar, the interaction of Prx II with Hb was severely impaired in the patient RBCs (Fig. 4A). Consistent with this, we found two SCA (#7, #8) and three THAL (#7–9) patient samples with severely impaired interaction between Prx II and Hb. To examine whether restoration of Prx II binding to Hb can recover its Hb-stabilizing ability against oxidative stress, cytosolic Hb solution was obtained from THAL and SCA patients with severely impaired interaction between Prx II and Hb and subjected to IP, Western Blotting, and H_2O_2-induced Hb aggregation assays. IP analysis using Hb antibodies showed that the binding of Prx II to Hb was restored by addition of rhPrx II into the patient Hb solution (Fig. 4B). Interestingly, the patient RBCs were more vulnerable to H_2O_2-induced Hb aggregation compared to normal RBCs, whereas the rhPrx II-added groups ameliorated the aggregation of patient Hbs to a control level (Fig. 4C). These results suggest that Prx II plays a pivotal role in the progression or pathogenesis of hemolytic anemia through participation in Hb stabilization under oxidative conditions.

4. Discussion

Hb is the most abundant protein in RBCs. Maintaining Hb stability is crucial for normal physiology. We have reported that loss of Prx II resulted in Heinz body formation, splenomegaly and oxidative hemolytic anemia [6]. In the present study, we show that loss of Prx II accelerates Heinz body formation by oxidative stress in mice. It is well known that Heinz bodies are RBC inclusions composed mainly of denatured Hb [17], indicating that increased Heinz body formation in Prx $II^{-/-}$ mice was mainly caused by denatured Hb aggregation. Our findings suggest that Hb protection from oxidative stress by Prx II was closely associated with its interactive status.

Prx II uses two cysteine residues (Cys51 and Cys172) for reducing hydrogen peroxide, and its oxidation (disulfide bond), and

hyperoxidation (sulfinate form) is reduced by Trx and Srx respectively [18]. Two mutant rPrx II proteins (C51S and C172S) bound to Hb, but did not prevent H_2O_2-induced Hb aggregation. The C-terminal domain of Prx II protein contains the YF motif and a C-terminal truncated human Prx II protein did not show chaperon activity *in vitro* [7]. Prx II bound to the membrane via its C-terminal extension, but ΔC-ter (includes removal of Cys172) mutant Prx II still interacted with Hb, suggesting that Prx II binds to Hb, but not through the cysteine or C-terminal domain. In contrast, N-terminal truncated human Prx II protein neither bound to Hb nor protected it from ROS attack, although it contains two normal cysteines. This result demonstrates that the N-terminal domain of Prx II is necessary for its binding to Hb. Recent studies also reported that oxidant stress-dependent oligomeric status of erythrocyte Prx II and cross-linked with hemoglobin [19]. Using Hex 6.1 (http://hex.loria.fr/) [20], the potential interaction status between high MW form of Prx II and Hb was predicted as shown in Supplementary Fig. 1. Presently, we are trying to further understand the interaction of Prx II to Hb by X-ray crystallography.

Recently, it was reported that Prx II functions are modulated in response to oxidative stress in diseased RBCs. Cytosolic Prx II was observed in the erythrocyte membrane of hereditary spherocytosis (HS) patients, which is associated with a higher susceptibility of HS erythrocytes to oxidative stress [21]. Prx II is increased in β-THAL mouse RBCs, but the binding of Prx II to the membrane is markedly reduced. These changes contribute to the accumulation of oxidative damage, which seems to be mainly caused by transition of Prx II into the oxidized/dimeric form and subsequent dissociation from RBC membrane [22]. In contrast, increased binding of Prx II to the membrane was observed in dense SCA cells [23]. Thus, it is important to define the role(s) of Prx II with different subcellular location, structure and expression level under oxidative condition. These results indicated that subcellular distribution of Prx II is closely associated with the redox-sensitive structural modification in RBCs. Thus, the impaired binding of Prx II to Hb in THAL and SCA patient RBCs may be resulted from the accumulation of oxidized/dimeric changes of Prx II.

We demonstrated here, for the first time, that the binding of Prx II to Hb is effective for stabilizing Hb against excessive oxidative damage in mouse and human RBCs. Prx II deficiency in mouse RBCs or the reduced binding to Hb in THAL and SCA RBCs make Hb susceptible to ROS-induced aggregation. It might be suggested that determining the binding status between Prx II and Hb could provide a diagnostic marker and a target for designing therapeutic strategies for hemolytic anemia patients, including THAL and SCA.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.113.

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