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Interaction of Human Thiol-Specific Antioxidant Protein 1 with Erythrocyte Plasma Membrane†

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ABSTRACT: During the purification from human erythrocytes, human thiol-specific antioxidant protein 1 (hTSA1), one human member of the TSA/alkyl hydroperoxide reductase subunit C (AhpC) family, was fragmented to a molecular mass of 20 323.9300. The fragmented form, in contrast to the intact form, did not bind to the C-terminal peptide (Gln-185−Gln-197) antibody. On the basis of the molecular mass of the fragmented form, the cleavage site was calculated to be between Val-186 and Asp-187. The C-terminal region of hTSA1 appeared to be unnecessary for the antioxidant reaction. In addition to hTSA1, two isoenzymes (hORF06 and hTSA2) were detected in the soluble fraction, whereas only hTSA1 was detected in the membrane fraction. A membrane binding study shows that the intact form binds to erythrocyte plasma membrane but the fragment does not, which suggests that the deleted C-terminal legion (Asp-187−Gln-197) is required for the membrane binding. A model membrane study using phospholipid vesicle showed a strong association of hTSA1 with the phospholipid. Human TSA1 exhibited high catalytic activity for the reduction of the fatty acid hydroperoxide as indicated by K_m and V_{max} (89.9 μM for linoleic acid hydroperoxide, 28.64 μmol^{-1} min^{-1} mg^{-1}, respectively). In this paper, we are making the first report of the involvement of the C-terminal region of hTSA1 in membrane binding as evidence supporting the existence of the membrane-associated forms in the erythrocyte. On the basis of our observations, we suggest that hTSA1 can act as a very effective antioxidant to remove oxidative stresses not only in matrix as a free form but also in the membrane surface of red blood cells (RBC) as a membrane-associated form.

The incomplete reduction of molecular oxygen during respiration, lipid metabolism in peroxisomes, and aerobic metabolism cause the formation reactive oxygen species (ROS)[1] such as H_2O_2, alkyl hydroperoxides, and superoxide anion. ROS are potent oxidants capable of damaging cellular components, including DNA, protein, and membrane lipid. To protect against the toxicity of ROS, aerobic organisms are equipped with an array of defense mechanisms (*1*). Among these, a new type of peroxidase, thiol-specific antioxidant protein (TSA), has been known to eliminate H_2O_2 and alkyl hydroperoxides with the use of a thiol-reducing equivalent (*2*−*4*). The TSA family, also referred to as the TSA/AhpC family, is a large family of newly emerging peroxidases that are being discovered, from prokaryotes to eukaryotes (*1*−*12*). Instead of an active site of selenocysteine within glutathione peroxidase, they have one cysteine within TPx as a primary site of catalysis. In mammalian tissue, several types of TPx isoenzyme were identified (*10*).

Erythrocytes (red blood cells, RBC) are more exposed to oxidative stress than other cell types because of the abundance of heme iron and oxygen, which can promote the production of ROS. Therefore, RBC have been believed to have a very effective defense mechanism against cell damage by ROS such as H_2O_2 and alkyl hydroperoxides. Erythrocyte membrane damage can result in decreased activity of membrane-bound enzymes, inhibition of transmembrane transport systems, and leakage of cellular constituents to the plasma (*1*). Previously, we reported the predominant existence of one type of TSA/AhpC family (i.e., hTSA1), also called NKEF-B and HPRP (human protector protein), and its possible role as a major antioxidant in human RBC (*7*). Recently, on the basis of analysis of the amino acid sequences of tryptic peptides, calpromotin, which is of significance for KCl transport, was suggested to be identical to hTSA1 (*13*, *14*).

In this paper, we are making the first report of the involvement of the C-terminal region of hTSA1 in membrane binding as evidence supporting the existence of the membrane-associated forms in erythrocyte. We also discuss the physiological function of the membrane-associated form of hTSA1 on the basis of antioxidant activity to protect erythrocyte membrane damage caused by lipid hydroperoxides.

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[1] Abbreviations: ROS, reactive oxygen species; RBC, red blood cell; TSA, thiol-specific antioxidant; NKEF, natural killer enhancing factor; PMSF, phenylmethanesulfonyl fluoride; MCO, metal-catalyzed oxidation; DTT, dithiothreitol; GS, glutamine synthetase; Trx, thioredoxin; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline; GSH, glutathione; Px, peroxidase; PS, phosphatidylserine; PE, phosphatidylethanolamine; ANTS; 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX, *p*-xylylenebis(pyridinium)bromide; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; AhpC, alkyl hydroperoxide reductase subunit C; MALDI-TOF mass spectrometry, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

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EXPERIMENTAL PROCEDURES

Materials. Phosphatidylserine (PS) and phosphatidylethanolamine (PE) were obtained from Sigma. 8-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and *p*-xylylenebis-(pyridinium)bromide (DPX) were purchased from Molecular Probes. *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE) were from Avanti Polar Lipids. Peptide antibody derived from the C-terminal peptide of hTSA1 (^{187}NVDDSKEYFSKHN^{198}) was obtained from Dr. H. J. Chae (Chonam University).

Purification of hTSA1 from Human RBC. Human RBC was obtained from freshly drawn heparinized blood. Human TSA1 was purified as previously described (*7*). After the puncture of RBC with 10 volumes of lysis buffer (50 mM Tris, pH 7.4), the clear lysates were obtained by centrifugation at 18 000*g* for 30 min. To obtain the membrane fraction, the precipitate was washed five times with the same lysis buffer. The resultant membrane fraction was suspended with the same buffer to a concentration of 1.5 mM phospholipid and then saved for the membrane binding study and the purification of hTSA1. The clear lysates were precipitated with 70% ammonium sulfate. The dissolved precipitate was extensively dialyzed against DEAE column equilibrium buffer (50 mM Tris-HCl buffer, pH 7.6). After centrifugation, the clear supernatant was loaded into a DEAE column. The column was completely washed with equilibrium buffer and eluted with a linear KCl gradient (0−400 mM). Throughout the purification of hTSA1, a thiol-dependent antioxidant activity of hTSA1 was measured by monitoring its ability to inhibit the inactivation of *Escherichia coli* glutamine synthetase (GS) by a thiol metal-catalyzed oxidation (MCO) system composed of Fe^{3+}/O_2/DTT as previously described (*5*). Broad thiol-dependent antioxidant activities were eluted between 150 and 250 mM KCl. The active fractions were pooled and precipitated with 70% ammonium sulfate. The dissolved ammonium sulfate precipitates were three times applied to a Sephadex G-75 column previously equilibrated with 100 mM Hepes buffer (pH 7.4) containing 100 mM KCl. After analysis of the purity on an SDS−12% polyacrylamide gel, homogeneous fractions were used for this experiment.

Fatty Acid Hydroperoxidase Activity of hTSA1. With linoleic hydroperoxide as substrate, the fatty acid hydroperoxidase activity was measured in terms of the increase of ferrous ion oxidation in the presence of xylenol orange (i.e., FOX assay) (*15*). Fatty acid hydroperoxides were obtained by a lipoxygenase reaction. A 50 mM stock solution of fatty acid in ethanol/100 mM Tris, pH 8.0 (1:1 v/v), was added to 100 mM Tris buffer (pH 7.4) to give 0.1 mM fatty acid, and then after 5 min, soybean lipoxygenase (1000 units/mL) was added to convert the linoleic acid to its hydroperoxides. The complete conversion of linoleic acid to corresponding hydroperoxide by lipoxygenase during the 10 min reaction was confirmed by a reversed-phase HPLC with UV detection at 210 and 235 nm for linoleic acid and the hydroperoxide, respectively (*16*).

Fusion Assay. The fusion was monitored after addition of 50 μg of hTSA1 to a magnetically stirred cuvette containing 2 mL of the vesicle suspension (0.05 μmol of phospholipid/2 mL). The intermixing of aqueous vesicle contents upon fusion was measured by the ANTS/DPX fusion assay (*17*). One part of vesicles was prepared in a solution containing 25 mM ANTS and 60 mM NaCl, and another part of vesicles in a solution containing 90 mM DPX and 5 mM NaCl. Both solutions were buffered with 2 mM TES buffer, pH 7.0. The vesicles were separated from unencapsulated materials on a Sephadex G-75 column (1 × 18 cm). Mixing of aqueous contents of ANTS- and DPX-containing vesicles was monitored by measuring the decrease in ANTS fluorescence due to its quenching by DPX. The fluorescence intensity of a 1:1 mixture of ANTS and DPX vesicles for a given buffer in the absence of hTSA1 was taken as 100% (or 0% fusion). The 0% fluorescence level was set with a buffer alone. Excitation and emission wavelengths were 360 and 545 nm, respectively.

Lipid mixing accompanying the fusion was monitored by the probe dilution method (*18*). NBD-PE and Rh-PE were incorporated into each part of vesicles at 1 mol %. There is a significant NBD fluorescence quenching by Rh at this probe concentration. Upon dilution of these probes during fusion, the NBD fluorescence increases due to a decreased resonance energy transfer between NBD-PE and Rh−PE. The fluorescence scale was calibrated according to Ellen et al. (*17*). Excitation and emission wavelengths were 450 and 530 nm, respectively. The size of vesicles after fusion was also observed with a Jeol JEM 100 CX-II electron microscope.

Phospholipid binding of hTPx I to PS/PE (1:1) vesicles was determined by passing the mixture of hTSA1 and vesicles through a Sephadex CL-4B column (1 × 48 cm). One milliliter of solution containing hTSA1 /phospholipid vesicles was added to the same column after 1 h of incubation at 37 °C. Fractions (0.5 mL) were collected and analyzed for protein and phospholipid. Throughout this experiment, phospholipid and protein concentrations were determined by assay of elemental phosphorus (*19*) and by the Lowry method (*20*), respectively.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. Relative masses of the proteins were determined by MALDI-TOF with a PerSeptive Biosystems apparatus and sinapinic acid as the absorbing matrix. Calibration of the instrument was performed with bovine serum albumin.

Circular Dichroic Measurement. CD spectra were measured on the Jasco J 600 spectropolarimeter in a 0.1 cm path length cell at 37 °C. Protein solutions (absorbance between 0.2 and 0.4 at 280 nm) were scanned twice.

Other Methods. Immunoblot analysis of thiol peroxidase isoenzymes in various tissues was performed with rabbit polyclonal antibodies against each human TSA isoenzyme. Transfer of proteins from SDS−12% polyacrylamide gels to nitrocellulose and processing of nitrocellulose blots were carried out according to a standard protocol.

RESULTS

Purification and Identification of hTSA1 and Its Fragment. We previously reported the predominant existence of hTSA1 (HPRP or NKEF-B) in the human RBC matrix (*7*) and the amino acid sequence comprised 197 amino acids, as deduced from the gene cloned from human brain cDNA library (*6*). Like yeast TSA1 (yTSA1) (*5*, *10*), hTSA1 also has two

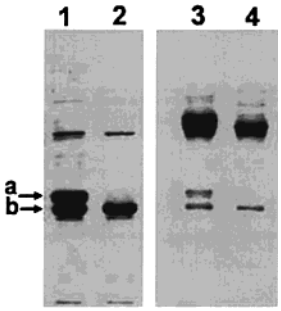

FIGURE 1: SDS−PAGE analysis of the purified hTSA1 from human red blood cell (RBC). Purified hTSA1 (lanes 1 and 3) and its small fragment (lanes 2 and 4) were analyzed by SDS−12% PAGE in the presence (lanes 1 and 2) or absence of DTT (lanes 3 and 4). Letters a and b indicate the 25-kDa intact hTSA1 and its 23.5-kDa fragment, respectively. The molecular masses were calculated from the low molecular mass protein size markers (94, 66, 45, 31, 21.5, and 14.4 kDa). Lanes 3 and 4 show the corresponding dimer forms upon oxidation.

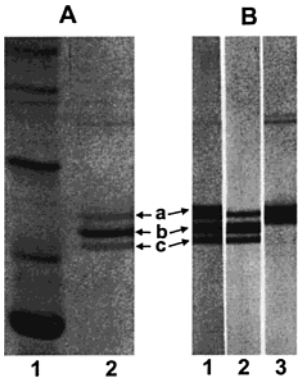

FIGURE 2: Characterization of the 23.5-kDa fragment by immunoblot. The mixture of intact hTSA1 (a) and its fragments (b and c) was analyzed on a reducing SDS−12% polyacrylamide gel (panel A) and its western blot (panel B) with various antibodies against yeast TSA (lane 1 of panel B), and hTSA1 (lane 2 of panel B), and the C-terminal peptide of hTSA1 (lane 3 of panel B). Intact hTSA1, 23.5-kDA, and 22.5-kDa fragments are represented as letters a, b, and c, respectively. The molecular masses were calculated from the low molecular mass protein size markers (lane 1 in panel A, from top, 66, 45, 31, 21.5, and 14.4 kDa)

conserved cysteines (*6*). This type of TSA, classified as a two-Cys subfamily, exists upon oxidation as a dimer in which the one conserved Cys is linked to the other Cys of the second subunit via intermolecular disulfide bonds (*21*). During the purification process, hTSA1 became cleaved to be the smaller form (Figures1, lane 1). Intact hTSA1 was completely converted to the smaller form upon repeated thawing−freezing (Figure 1, lane 2). Figure 1 also showed that, in addition to intact hTSA1, the smaller form could be dimerized upon oxidation, indicating that two conserved cysteines remained in the smaller form. Immunoblot analysis indicated that the smaller protein specifically cross-reacted with the polyclonal antibodies derived from yTSA1 and hTSA1, whereas in contrast to the intact form, it did not react with the peptide antibody derived from the C-terminal peptide of hTSA1 (^{187}NVDDSKEYFSKHN^{198}) (Figure 2). To investigate which site of hTSA1 was cleaved, the relative molecular mass of the smaller protein was measured by MALDI-TOF mass spectrometry. The molecular masses of intact and smaller forms were calculated to be 21 698.6098 and 20 323.9300 (20.3 kDa), respectively (Figure 3). The comparison of the molecular masses revealed that the

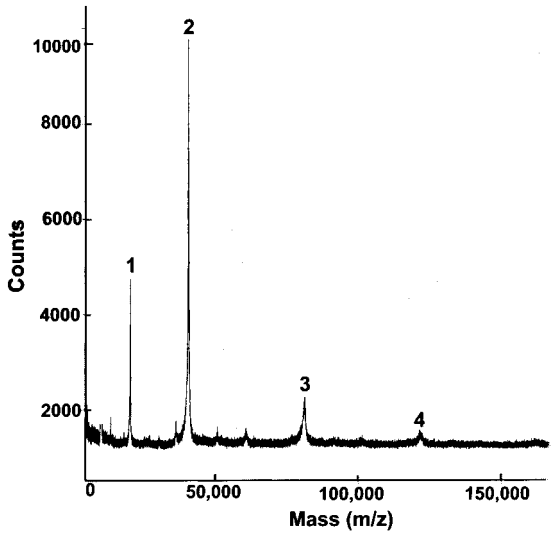

FIGURE 3: Analysis of relative molecular mass of the 23.5-kDa fragment using MALDI mass spectrometry. Peaks 1−4 indicate the mass of monomer (20 323.9300), the mass of the dimer (40 623.8377), the mass of tetramer (81 253.2892), and the mass of hexamer (121 977.7998), respectively.

cleavage occurred at the site between Val-186 and Asp-187. Taken together, these results imply that the smaller form, which is derived from hTSA1 upon purification process, is the C-terminal peptide-deleted form. Also, it is worthwhile to note on the MALDI mass spectrometry that, like the intact form (data not shown), the smaller form also exists as the multimers that consist of even numbers of the monomer (i.e., dimer, tetramer, hexamer, etc.), suggesting that high molecular weight multimers are made up of dimers. The multimer distribution pattern of the smaller form of hTSA1 was similar to that of intact hTSA1. This result suggests that the C-terminal region did not contribute to the multimerization of the dimer.

Biochemical Characterization of 20.3-kDa hTSA1. The resistance of the smaller protein (20.3 kDa) against physical stress such as repeated thawing−freezing implies that the structure may be very stable. It is very interesting to figure out a biochemical meaning of the existence of the fragile C-terminal peptide (^{187}DDSK EYES HHN^{197}). The antioxidant activity of 20.3 kDa hTSA1 was compared with that of the intact form. The plot of GS protection activity as a function of the protein concentration (data not shown) showed that the specific antioxidant activity of 20.3 kDa was the same as that given by the mixture of intact hTSA1 and its fragment (1:1 by molecular mass, approximately; see lanes 1 and 2 of Figure 1). This result suggests that the C-terminal domain is not necessary for the antioxidant activity.

To see the conformational stability of the 20.3 kDa fragment without the C-terminal peptide, the pH effect on the conformation was examined. The CD spectra as well as the tryptophan fluorescence spectra (λ_{max} 335 nm) were insensitive to the changing pH from 4 to 8 (data not shown), indicating that the 20.3 kDa fragment has a very stable structure. The conformational stability of the 20.3-kDa hTSA1 could explain the reason for the retained antioxidant activity of the 20.3-kDa protein.

Finally, in an attempt to see a possible fragmentation at the C-terminus as a physiological process, we tried to detect the larger N-terminal fragment in various tissues and cells

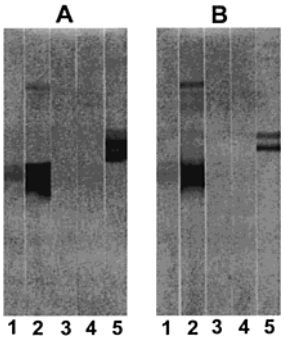

FIGURE 4: Western blot analysis for localization of TSA isoenzymes in human erythrocytes. One milligram of crude proteins from the matrix of human erythrocytes (panel A) and 300 μmol of the membrane fraction (panel B) were electrophoresed in an SDS−12% polyacrylamide gel and transferred to nitrocellulose paper. The whole blotted paper was cut into several strips to fit into mini-incubation tray having eight channels, and then immunoblot analysis was performed with various polyclonal antibodies against hTSA2 (lane 1), hTSA1 (lane 2), hMer5 (lane 3), hAOE372 (lane 4), and hORF06 (lane 5).

(human red blood cells and liver and bovine brain, heart, kidney, lung, skeletal muscle, retina, and spleen tissues) by immunoblot with the antibody against hTSA1 capable of binding to both intact and fragmented human and bovine TSA1. No detection of the fragment in the crude extracts (data not shown) excluded a possible presence of the fragment in vivo.

Cellular localization of hTSA1. It has been known that there are several TSA/AhpC isoenzymes in mammalian tissues (*6*, *10*, *22*). However, the cellular distribution of the isoforms remain poorly understood. To see the existence of various TSA isoenzymes and their localizations in human RBC, immunoblot analysis on its membrane and soluble fractions was carried out with each antibody. Figure 4 showed that hTSA1 (HPRP or NKEF-B) and hORF06 evidently existed in the matrix of RBC, but only smaller amount of hTSA2 (hNKEF-A or hPAG) (*11*, *12*) existed, and that hTSA1 was distributed in the membrane fraction.

To examine the binding nature of hTSA1 to the membrane fraction, after the consecutive three cycles of incubation in increasing NaCl concentration, the immunoblot analysis of each cycle of the membrane precipitate and the resultant supernatant was performed with antibody against hTSA1 capable of binding to intact and fragmented hTSA1. The immunoblot analysis of the three consecutive cycles (lanes 1, without NaCl; lanes 2, 150 mM NaCl; lanes 3, 500 mM NaCl in Figure 5) showed that a considerable amount of hTSA1 was still distributed in the final membrane fraction, which was incubated in high salt (i.e., 500 mM NaCl) (lanes 3 of Figure 5). Also, after the G-75 fraction containing both intact hTSA1 and its smaller fragment was incubated with the final membrane fraction, three successive cycles of incubation in increasing salt concentration (0, 150, and 500 mM) were carried out under the same method described above (lanes 4, 5, and 6 of Figure 5, respectively). Interestingly, we observed that the native form of hTSA1 preferred to bind to the membrane. This selective binding of intact hTSA1 to the membrane can be taken as evidence supporting the idea that the C-terminal region plays an important role in the membrane binding. The gradual release of the bound form to the free form by increasing salt concentration

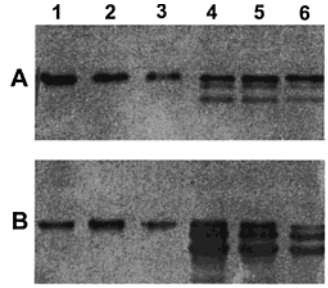

FIGURE 5: Immunoblot analysis for membrane binding of hTSA1. After three successive cycles of incubations with increasing salt concentration (0, 150, and 500 mM) were performed, each membrane precipitate (A1, A2, and A3, respectively) and supernatant (B1, B2, and B3, respectively) was subjected to SDS−12% PAGE for the immunoblot analysis with the antibody against hTSA1. In case of membrane precipitates, three additional washings with corresponding buffers were carried out to remove a nonspecifically bound hTSA1. Experimental procedures for A4, A5, A6, B4, B5, and B6 are the same as those of A1, A2, A3, B1, B2, and B3, respectively, except for external addition of hTSA1 comprosed of intact and fragmented hTSA1 (40 μg/mL) to the membrane fraction previously washed with 500 mM NaCl. Details are in the text.

suggests ionic interaction between the C-terminal peptide and membrane.

Recently, calpromotin has been suggested to be identical to hTSA1 (NKEF-B) (*13*, *14*, *23*−*25*). The association of calpromotin with the membrane in the presence of Ca^{2+} appeared to be involved in the activation of Ca^{2+}-dependent potassium transport (*14*). These reports suggested that Ca^{2+} could facilitate the binding of calpromotin to the plasma membrane. To test the possibility, we studied the effect of Ca^{2+} on the binding of hTSA1 to RBC membrane. Fresh RBC lysates containing its membrane fraction were incubated in a TBS (15 mM Tris-HCl and 150 mM NaCl, pH 7.4) buffer including or excluding 10 mM Ca^{2+} for 5 h at 4 °C, and we analyzed the membrane-bound hTSA1. In the case of incubation without Ca^{2+}, 10 mM EGTA was added for removing the free Ca^{2+} in the lysates. The quantitative analysis of the band intensity of the immunoblot for each membrane fraction using a polyclonal antibody against hTSA1 indicated that the amount of membrane-bound hTPx was increased about 2-fold upon Ca^{2+} treatment (data not shown). In accordance with the previous results (*24*), Ca^{2+} could facilitate the association of hTSA1 with the RBC membrane. Also, in this experiment, the fragmented hTSA1 was not detected.

Interaction of hTSA1 with Phospholipid Vesicle as a Model Membrane. To investigate the interaction nature of intact hTSA1 with RBC membrane, we performed membrane fusion and leakage tests with a phospholipid vesicle as a model membrane. Intact hTSA1 used for this study was homogeneously purified from the extract of RBC membrane fraction with 500 mM NaCl. The vesicles were made from pure PS or PS/PE (1:1 w/w), a major lipid component of the inner leaflet of RBC plasma membrane (*26*). Figure 6A shows the time course of fusion between phospholipid vesicles composed of pure PS (a) or PS/PE (b) after the addition of hTSA1 at pH 7.0. The apparent extents of lipid mixing (curve 1) and internal content mixing (curve 2) in both experiments with PS or PS/PE vesicles, which are indicative of the fusions between vesicles, suggested an interaction of hTSA1 with the membrane lipid. The apparent

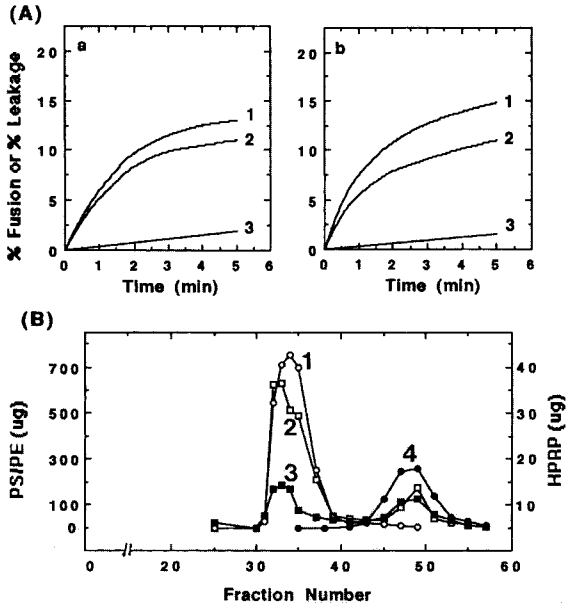

FIGURE 6: (A) Time-course intermixings of the lipid mixing (traces 1), the vesicle contents (traces 2) during the fusions of PS (a) and PS/PE (b) vesicles induced by hTSA1 (HPRP) at pH 7, and the leakage (traces 3) during the fusions of the control vesicles without hTSA1. Details are given under Experimental Procedures. (B) Gel filtration of hTSA1 and phospholipid (PS/PE) vesicles complex. Phospholipid vesicles were incubated with hTSA1 for 1 h at 37 °C, and chromatographed on a Sephadex CL-4B column. Effluent was monitored by phosphorus (left *y*-axis, PS/PE) and protein analysis (right *y*-axis, hTSA1). The elution buffer contained 100 mM Hepes, pH 7, and 100 mM KCl. Peak 1, vesicles only; peak 2; vesicles in the reaction mixture; peak 3, hTSA1 in the reaction mixture; peak 4, hTSA1 only.

extents of lipid mixing and internal content mixing without hTSA1 were not significantly increased (data not shown except for the internal content mixing without hTSA1, curve 3). The membrane fusion after the addition of hTSA1 was confirmed by direct observation with an electron microscope. The enlarged vesicles showing mutilamellar patterns were formed after the addition of hTSA1, which is direct evidence for the membrane fusion caused by hTSA1 (data not shown). Taken together, these observations reveal that hTSA1 has a capability to bind to the phospholipid layer of RBC membrane.

The phospholipid binding property of intact hTSA1 was also examined by a gel-filtration method (*27*). The protein was mixed with phospholipid vesicles (PS/PE) and then subjected to gel filtration. In the absence of phospholipid vesicles, hTSA1 was eluted in the included volume of the column (curve 4 of Figure 6B). Phospholipid vesicles were eluted in the excluded volume of the column (curve 1 of Figure 6B). When 0.2 mg of intact hTSA1 was incubated with 3 mg of phospholipid vesicles for 1 h at 37 °C, about 60% of hTSA1 was eluted in the void volume of the column (curve 3 of Figure 6B).

Fatty Acid Hydroperoxide Peroxidase Activity of hTSA1. Our results implicate that the C-terminal region of hTSA1 may act as an anchor to bind to the membrane, whereas the main domain (20.3 kDa) plays a role in catalyzing the antioxidant reaction. We previously reported that hTSA1 exerted a thiol peroxidase activity to remove H_2O_2 with the use of thioredoxin as the electron donor (*7*, *28*). Now, it is helpful for understanding the physiological meaning of the membrane-associated form of hTSA1 to investigate whether hTSA1 has a capability to remove lipid hydroperoxide. The lipid hydroperoxide peroxidase activity was measured in terms of the decrease of linoleic acid hydroperoxide by hTSA1 with the use of reducing equivalents such as DTT and thioredoxin (Trx). Figure 7 shows the lipid peroxidase activity as a function of the concentration of hTSA1. The peroxidase activity with Trx was 1.7-fold higher than the activity supported by DTT (Figure 7A), suggesting the Trx-linked lipid peroxidase activity. The increase of the NADPH consumption rate as a function of the amount of hTSA1 shown in Figure 7B also indicates the use of Trx system for reduction of fatty acid hydroperoxide. Peroxide-dependent kinetics revealed that the activity increased as a function of peroxide. However, above 0.15 mM peroxide, the peroxidase activity appeared to decline gradually (Figure 7C), showing a substrate inhibition pattern. This pattern was known to be a kinetic property of some of TSA/AhpC family (*28*). From the Lineweaver−Burk plot derived from the linear region,

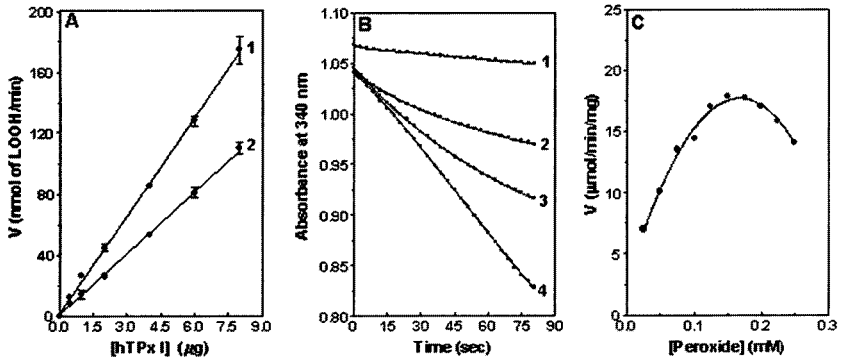

FIGURE 7: Fatty acid hydroperoxide peroxidase (FAP) activity of hTSA1. (A) Fatty acid hydroperoxide peroxidase activity of hTSA1 as a function of the concentration of hTSA1. The activity was measured in terms of the decrease of FAP. Remaining FAP during 10 min at 37 °C was determining by the FOX1 assay. Curves 1 and 2 show the peroxidase activities supported by the Trx system and DTT, respectively. The reaction mixture (20 μL) was comprised of 4 μg Trx, 4 μg of Trx reductase, 0.1 mM linoleic acid hydroperoxide, 100 mM Trs-HCl (pH 7.4), and the indicated amount of hTSA1. In the DTT-supported system, the Trx system was replaced by 0.2 mM DTT. (B) NADPH consumption rate as a function of the amount of hTSA1. The reaction mixture (0.4 mL) consisted of 10 μg of Trx, 10 μg of Trx reductase, 0.37 mM NADPH, 0.1 mM linoleic acid hydroperoxide, and varying amounts of hTSA1 (curve 1, without hTSA1; curve 2, with 5 μg of hTSA1; curve 3, with 10 μg of hTSA1; curve 4, with 20 μg of hTSA1). (C) Peroxidase activity of hTSA1 as a function of the concentration of linoleic acid hydroperoxide. The reaction mixture (20 μL) included 5 μg of Trx, 5 μg of Trx reductase, 4 μg of hTSA1, 0.37 mM NADPH, 100 mM Tris-HCl (pH 7.4), and the indicated concentration of linoleic acid hydroperoxide.

the apparent K_m and V_{max} values for linoleic acid hydroperoxide were calculated to be 89.9 μM and 28.64 μmol^{-1} min^{-1} mg^{-1}, respectively. This result suggested that hTSA1 exhibited high catalytic activity for the reduction of fatty acid hydroperoxide as indicated by K_m and V_{max} values. These kinetic values are still comparable to those of sheep lung selenium-independent GSH peroxidase toward prostaglandin G2 hydroperoxide (12 μM and 78 μmol^{-1} min^{-1} mg^{-1}, respectively) (*29*), although the kinetic values of hTSA1 should be underestimated because of the severe substrate inhibition shown in Figure 7C (*28*).

DISCUSSION

Red blood cells are much more exposed to oxidative stress as compared with other cells because over 95% of their total protein is hemoglobin acting as an oxygen carrier. The oxidation of hemoglobin results in the release of heme iron, which in turn can promote the production of membrane lipid hydroperoxide via the Fenton reaction (*30*). Therefore, RBC has been believed to be equipped with a very effective defense mechanism against the oxidative damages. We previously reported that hTSA1, called HPRP in our previous paper, is the predominant protein after hemoglobin in human RBC (*7*). In addition to hTSA1, two types of human TSA/AhpC isoenzymes (i.e., hORF06, and hTSA2) appeared to exist in RBC. Predominant existence of hTSA1 in the RBC reflects its important antioxidant function to maintain the integrity of RBC. In the present study, we first demonstrated a site-specific interaction between the C-terminal region of hTSA1 and the phospholipid layer of RBC membrane. Both its capabilities to bind to membrane lipid and to remove hydroperoxides such as fatty acid hydroperoxide suggest its physiological function to act as an antioxidant to protect the membrane from the oxidative damages.

Comparison of the amino acid sequences of C-terminal peptides within mammalian TSA1 proteins (human TSA1, P32119; rat TSA1, P35704; mouse TSA1, Q61171) revealed that each C-terminal region of the TSA1 group is perfectly conserved (i.e., ^{182}IKPNV DDSKE YFSKH N^{197}). Therefore, the perfectly conserved amino acid sequences within the C-terminal regions might be taken as evidence supporting the C-terminal-specific membrane binding of hTSA1.

There are many reports that the association of calpromotin with RBC membrane promotes the activation of Ca^{2+}-dependent potassium transport across the membrane. Calpromotin has been suggested to be either identical or extremely similar to hTSA1 (NKEF-B) on the basis of the pairwise identity of peptide fragments (*23*). Recently, calpromotin has been shown to be identical to hTSA1 on the basis of its thiol-specific antioxidant activity and physical property (*13*). Plishker et al. (*14*) reported that the activation of the potassium transport is associated with increased binding of calpromotin to human erythrocyte membrane. A key issue to be solved is how hTSA1 can bind to the membrane. We have shown that the C-terminal region of hTSA1 is necessary for the interaction with the phospholipid layers of the membrane and that calcium ion increases the site-specific association. These results could suggest the possibility that the C-terminal cleavage of hTSA1 by a protease such as calpain (a calcium-dependent protease) is a physiological process to release hTSA1 from the membrane to the cytosol as suggested in other reports (*14*, *23*, *24*). We tried to detect the C-terminal-cleaved hTSA1 in various tissue cells including RBC using immunoblot, but a significant amount of the smaller form could not be seen in the blots. Therefore, on the basis of our observations, we could propose another possible physiological process to facilitate the membrane association of hTSA1 that includes a calcium concentration-dependent equilibrium between free and membrane-bound forms. Some factors such as Ca^{2+} ions might change the equilibrium position favorable to the bound form.

There are several lines of evidence supporting the suggestion that the peroxidase activity of hTSA1 could be involved in the calcium-activated potassium transport in RBC. We have shown that hTSA1 has a general hydroperoxide peroxidase activity to remove hydroperoxides, such as a fatty acid hydroperoxide, and that the C-terminal region of hTSA1 involved in the interaction with the phospholipid layer of membrane is not necessary for the antioxidant reaction. The site-specific association of hTSA1 with the membrane increases in the presence of Ca^{2+} as previously described (*14*, *23*, *24*). In addition, there are many reports concerning inhibition of K^+ transport and increase of intracellular Ca^{2+} concentration by oxidative stress-induced plasma membrane damage. Oxidative stress-induced erythrocyte membrane damage is characterized by a strong inhibition of Na^+/K^+-ATPase and by an increased intracellular Ca^{2+} concentration (*30*−*35*). Therefore, on the basis of the antioxidant activity given by the membrane-associated form of hTSA1, we could speculate as to the reason the association of hTSA1 with the membrane can activate the potassium transport. That is, the oxidative stress in the membrane such as peroxidation of membrane lipids induces the increase of intracellular Ca^{2+} concentration by activating the Ca^{2+} pump, which, in turn, elevates the membrane-bound hTSA1. The increased membrane-bound form could reverse inactivation of the potassium pump by removing the oxidative stress.

In conclusion, on the basis of our observations, we suggest that hTSA1 can act as a very effective antioxidant to remove oxidative stresses not only in matrix as a free form but also in the membrane surface of RBC as a membrane-associated form. Future studies will be required to investigate how the C-terminal region of TSA1 binds to the membrane phospholipid layer.

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