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ORIGINAL RESEARCH COMMUNICATION

Redox Regulation of Actin by Thioredoxin-1 Is Mediated by the Interaction of the Proteins *via* Cysteine 62

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Abstract

Actin is a highly conserved protein in eukaryotic cells, and has been identified as one of the main redox targets by redox proteomics under oxidative stress. However, little is known about the mechanisms of regulation of the redox state of actin. In this study, we investigated how thioredoxin-1 (Trx1) affected the redox state of actin and its polymerization under oxidative stress in SH-SY5Y cells. Trx1 decreased the levels of reactive oxygen species (ROS) in the cells, and cysteine residues at positions 32, 35, and 69 of the Trx1 protein were active sites for redox regulation. Actin could be kept in a reduced state by Trx1 under H_2O_2 stimulation. A physical interaction was found to exist between actin and Trx1. Cysteine 62 in Trx1 was the key site that interacted with actin, and it was required to maintain cellular viability and anti-apoptotic function. Taken together, these results suggested that Trx1 could protect cells from apoptosis under oxidative stress not only by increasing the total antioxidant capability and decreasing the ROS levels, but also by stabilizing the actin cytoskeletal system, which cooperatively contributed to the enhancement of cell viability and worked against apoptosis. *Antioxid. Redox Signal.* 13, 565–573.

Introduction

ACTIN IS THE MAIN COMPONENT of the microfilament cytoskeleton and exists as monomeric G-actin that can polymerize into filamentous F-actin upon extracellular stimuli. The constant and rapid reorganization of the actin microfilament system is highly regulated (2). It is reported that the actin cytoskeleton plays an important role in nitric oxide-induced oxidative stress (10). H_2O_2 has profound and differential effects on both monomeric and filamentous actin, and can lead to the formation of disulfide-linked antiparallel homodimers (12). However, little is known about the mechanisms of assembly and disassembly of actin under different redox conditions, as well as the regulation of this process by associated proteins.

Many proteins contain cysteines that are sensitive to oxidation. These proteins are potential sensors of oxidative stress, and their own oxidation state is sometimes reversible through their interactions with the Trx1 system (Trx1, TrxR, and NADPH). It has been demonstrated that a direct redox control of actin is one of the most important processes regulating the dynamics of the microfilament system. Cys 272 and Cys 374 in actin are important in the disassembly of filaments as the environment becomes more oxidative (12). This suggests that actin has the structural determinants for its redox regulation. Trx1 is a 12 kD multifunctional protein with redox-active dithiol/disulfide in the active site:-CYS-GLY-PRO-CYS-. A classic function is to act as a hydrogen donor under oxidative stress. By interacting with different target molecules, Trx1 can act as an antioxidant, a controller of growth and anti-apoptotic agents (8, 15).

These studies have led to the view that the Trx1 system is largely involved in redox regulation of actin. Our results indicate that actin is susceptible to oxidative stress and that Trx1 controls the redox status of actin by interacting with cysteine 62 of actin. Under oxidative stress, cell viability and anti-apoptosis capability are improved by Trx1.

Materials and Methods

Chemicals and reagents

Dulbecco's Modified Eagle medium (DMEM), fetal bovine serum, Lipofectamine 2000, and TRITC-phalloidin were

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purchased from Invitrogen (Carlsbad, CA). Ethylenediaminetetraacetic acid (EDTA), phenylmethanesulfonyl fluoride (PMSF), methylthiazolyldiphenyl-tetrazolium bromide (MTT), propidium iodide (PI), monoclonal Anti-actin (A2228) antibody, and annexin-V antibody were purchased from Sigma (St. Louis, MO). Tris-HCl, HEPES, and NaCl were purchased from Merck Company (Whitehouse Station, NJ) Trx1 antibody (2429s) was purchased from Cell Signaling Technology Company (Danvers, MA).

Cell culture and transfection

Human neuroblastoma SH-SY5Y cells were cultured at 37°C and 5% CO_2 in 25 cm^2 culture flask (Costar, Lowell, MA) in DMEM containing 10% fetal bovine serum (Invitrogen). For transfection, cells were plated on 6-well plate (Costar) at a density of 70%–80% confluence and transfected in Opti-DMEM (Invitrogen) with Lipofectamine 2000. After 6 h, the medium was changed. The cells were cultured in fresh medium for 24 h and then harvested. For oxidative stress experiments, cells were cultured in medium with H_2O_2 for 30 min, and then washed with PBS.

Plasmids construction

The human Trx1 cDNA sequence (NM_003329 Homo sapiens thioredoxin TXN) was amplified by RT-PCR and sequenced to confirm its integrity. All primers were designed by DNAMAN 6.0 software. The plasmids pcDNA3-Trx1, pcDNA3-Trx1 (C32/35S) and pIRES-neo-TrxR were a kind gift from Professor Xun Shen (Chinese Academy of Sciences, China). PCR were performed for Trx1 mutation sites using the following primers: Trx1(C62S) F(5′-CAAGCAACATCC TGAGAGTCATCCACATCTAG-3′),Trx1(C62S) R(5′-GTAGA TGTGGATGACTCTCAGGATGTTGCTTC-3′);Trx1(C69S) F (5′-CCTGCATTTGACTTCAGACTCTGAAGCAACATGC-3′),Trx1(C69S) R(5′-GGATGTTGCTTCAGAGTCTGAAGT CAAATGCACG-3′);Trx1(C73S) F(5′-CAAATGTTGGCGT GGATTTGACTTCACGG-3′),Trx1(C73S) R(5′-GTGTGAA GTCAAATcCACGCCAACATTCC-3′). All the Trx1 mutation plasmids were constructed by the Quickchange Site Directed Mutagenesis Kit (200523-5, Stratagene, Santa Clara, CA).

Western blot analysis

Cells were lysed in lysis buffer (50 m*M* Tris pH 7.5, 250 m*M* NaCl, 0.1% SDS, 2 m*M* DTT, 0.5% NP-40, 1 m*M* PMSF, and protease inhibitor cocktail) on ice for 30 min. Protein fractions were collected by centrifugation at 16,000 *g* at 4°C for 10 min, then subjected to SDS/PAGE and transferred to PVDF membrane. Membranes were blocked with PBS-0.1% Tween with 5% BSA and incubated with specific antibody overnight. Horseradish peroxidase-labeled secondary antibody was added and visualized by using the enhanced chemiluminescence kit (Pierce, Rockford, IL).

Immunofluorescence staining of cells

The cells were fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized by 0.1% Triton X-100 in PBS for 10 min, then blocked with PBS containing 5% BSA for 30 min. The cells were subsequently incubated with Trx1 antibody overnight at 4°C, and flooded by FITC-labeled secondary antibody (Molecular Probes, Carlsbad, CA) for 120 min, then incubated with TRITC-phalloidin for 120 min. The fluorescence images were observed on a microscope equipped with AquaCosmos Microscopic Image Acquisition and Analysis System (Hamamatsu Photonics K.K., Hamamatsu City, Japan), using excitation wavelengths of 488 nm and 590 nm, respectively.

F-actin/G-actin in vivo assay

The ratio of F actin versus G actin in cells was analyzed using the F-actin/G-actin *in vivo* assay kit (BK037, Cytoskeleton Company, Denver, CO) based on the manufacturer's protocol. Briefly, the cells were lysed with cell lysis and F-actin stabilization (LAS) buffer and homogenized using 30-gauge syringes. The cell lysates were centrifuged at 100,000 *g* for 60 min at 37°C. The supernatants (G-actin) were separated from the pellets (F-actin) and immediately placed on ice. The pellets were resuspended to the same volume as the supernatants using ice-cold double distilled H_2O containing 2 μM cytochalasin D and incubated on ice for 60 min. Equal amounts of the samples (2 μg of the G-actin fractions and the corresponding amount of the F-actin fractions) were loaded to each lane and analyzed by Western blotting with anti-actin antibody. Actin in each fraction was analyzed using a chemiluminescence documentation system (Bio-Rad, Hercules, CA) and the ratio of F-actin to G-actin was quantified by the Image J software.

Co-immunoprecipitation assay

Cells were lysed with 1% NP-40 in PBS (pH 7.5) containing 1 m*M* Na_3VO_4, 25 m*M* NaF, and protease inhibitors. The cell lysates were incubated with the anti-actin mAb overnight. The samples were then mixed with Protein A/G plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA). After incubation for 4 h, the beads were washed three times with lysis buffer and the bound proteins were released from the beads by boiling in 100 μl of SDS-PAGE sample buffer for 15 min. The samples (20 μl/lane) were analyzed by Western blotting with anti-Trx1 antibody.

Expression and purification of His-tagged Trx1

Overnight cultures of *E.* coli strain BL21 (DE3) transformed with pET24a (+)-Trx1 were diluted 1:100 in Luria-Bertani medium containing kanamycin (50 ng/ml) and incubated at 37°C with shaking to an OD_{600} of 0.6. Isopropyl-h-d-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.5 m*M*. After a further 4 h of growth, cells were pelleted at 5000 *g* for 10 min at 4°C and then resuspended in 10 ml sonication buffer (50 m*M* sodium phosphate pH 8.0, 300 m*M* NaCl, 1 m*M* PMSF). The cells were then sonicated and centrifuged at 20,000 *g* for 20 min at 4°C. The cleared lysate was incubated in a batch with 2 ml 50% Ni-NTA (Clontech, Mountain View, CA) resin for 1 h at 4°C. The resin was loaded into a column, washed with 2 ml sonication buffer and then with 20 ml 50 m*M* sodium phosphate pH 8.0, 300 m*M* NaCl, 20 m*M* imidazole. The bound protein was eluted with 50 m*M* sodium phosphate pH 8.0, 300 m*M* NaCl, 100 m*M* imidazole.

His-Fusion protein pull-down assays

The expression of His-tagged Trx1 was induced by 0.05 m*M* IPTG and was purified by His purification modules.

Cell lysates were added to the His Purification Modules that had been immobilized with His and His-Trx1 respectively, incubated for 3 h at 4°C and washed with washing buffer to remove nonspecifically-bound protein. Bound protein was eluted with 800 m*M* imidazole, resolved on SDS-PAGE, and detected by actin antibody.

Determination of the oxidation state of actin

Cells in 6-well plates were incubated for 30 min at 37°C in the presence of different concentrations of H_2O_2. To prevent postlysis disulfide exchange, cells were precipitated directly in 5% trichloro-aceticacid (TCA). Precipitates were washed in 70% acetone and resuspended in 40 μl of reaction buffer (50 m*M* Tris-HCl, pH 6.8, 1% SDS) supplemented with a cocktail of protease inhibitors (Roche, Basel, Switzerland) with 10 m*M* freshly prepared AMS. Samples were incubated for 30 min at room temperature and for 10 min at 37°C. A sample (20 μl) of nonreducing loading buffer was added, and samples were resolved by SDS-PAGE.

MTT assay

Cells were seeded in 96-well plates at a density of 50,000 cells per well and allowed to attach overnight at 37°C. H_2O_2 was used to induce apoptosis at the doses indicated. Following insult, MTT was added to the wells at a final concentration of 0.5 mg/ml and the plates were incubated at 37°C for 4 h. Supernatants were then aspirated off and formazan crystals were dissolved with 200 μl of anhydrous DMSO. After a 5-min incubation at room temperature, the absorbance at 595 nm was determined in the microtiter plate reader.

Detection of cell apoptosis

Cells were seeded in 6-well plates and allowed to attach overnight at 37°C, then treated with 400 μM H_2O_2 for 30 min. To determine the proportion of apoptotic cells, cells were stained with AnnexinV-FITC and PI. The cells were washed with PBS, resuspended in 100 μl binding buffer, incubated for 15 min with 5 μl AnnexinV-FITC and 50 μg/ml PI in the dark, then subjected to flow cytometric analysis.

Measurement of cellular T-AOC levels

The total antioxidant capacity of cells (T-AOC) was measured by reduction of Fe^{3+}. Briefly, 300 μl freshly prepared FRAP reagent (25 ml acetate, 2.5 ml TPTZ, 2.5 ml $FeCl_3$ mixture) was warmed to 37°C and a reagent blank reading was taken (M1) at 593 nm; 10 μl of sample was then added, along with 30 μl H_2O; Absorbance (A) readings were taken after 4 s. The change in absorbance (ΔA_{593nm}) between the final reading selected and the M1 reading was calculated for each sample and related to ΔA_{593nm} of a Fe^{2+} standard solution tested in parallel.

Measurement of cellular ROS levels

ROS levels were measured with the use of the fluorescent signal H_2DCF-DA, which is a redox-sensitive nonfluorescent compound. After the dye enters into cells, it is converted into fluorescent DCF under the oxidation of cellular ROS. After cells transfected with Trx1 or its mutants were stimulated with H_2O_2, they were collected and incubated with 10 μM DCFH-DA (dissolved in DMSO) for 30 min at 37°C, they were washed three times with PBS (pH 7.4), and the relative levels of fluorescence were quantified (485 nm excitation filter and 535 nm emission filter). The measured fluorescence values were expressed as a percentage of the fluorescence in control cells.

Results

Trx1 is involved in the redox regulation in SH-SY5Y cells by its redox active cysteine residues

It has been shown that SH-SY5Y cells are highly sensitive to oxidative stress and responsive to both extracellularly administered and pre-conditioning induced Trx1. To test the ability of Trx1 to protect cells from oxidative stress and the role of its redox active cysteine, SH-SY5Y cells were transiently transfected with pcDNA3-Trx1, pcDNA3-Trx1 (C32/35S), or empty vector. Cellular total antioxidative capability (T-AOC) was measured after stimulation with different concentrations of H_2O_2 for 30 min. The levels of cellular T-AOC were decreased by H_2O_2 in a dose-dependent manner. Without any stimulation, the levels of T-AOC in cells transfected with pcDNA3-Trx1 were more than two times the control and empty vector ($P < 0.05$, $n = 6$). Under stimulation with 800 μM H_2O_2, the T-AOC levels in cells transfected with pcDNA3-Trx1 and pcDNA3-Trx1 (C32/35S) were still higher

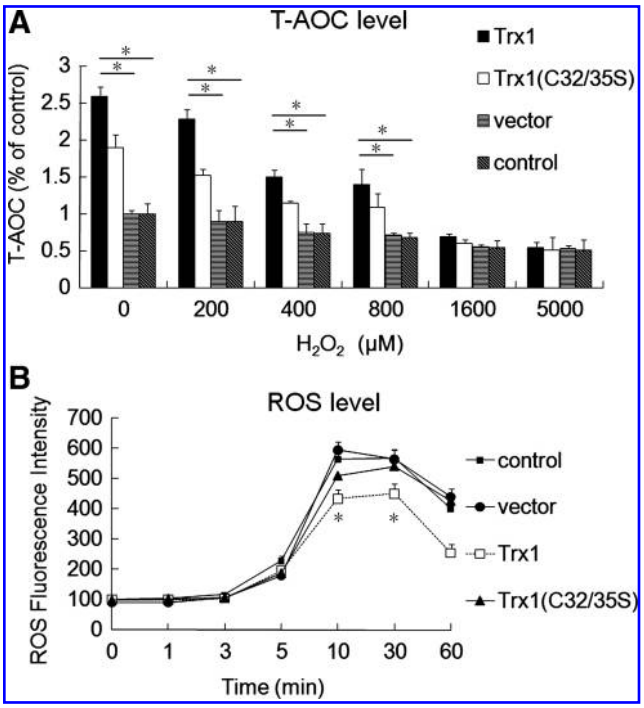

FIG. 1. Trx1 is involved in the redox regulation in SH-SY5Y cells by its redox active cysteine residues. (**A**) SH-SY5Y cells were transfected with Trx1, Trx1(C32/35S), or empty vector for 18 h, then stimulated with increasing concentrations of H_2O_2 for 30 min, and the cellular total antioxidative capability (T-AOC) was assayed. (**B**) SH-SY5Y cells were transfected with Trx1, Trx1 (C32/35S), and empty vector for 18 h, then stimulated with 200 μM H_2O_2, and the level of intracellular ROS was measured at different time points.

than cells transfected with the empty vector (Fig. 1A, $P < 0.05$, $n = 6$). Although cells transfected with pcDNA3-Trx1 (C32/35S) demonstrated a lower T-AOC level than cells transfected with pcDNA3-Trx1, the T-AOC level was much higher than that of cells transfected with empty vector. This suggests that besides redox active sites, other cysteines in Trx1 are also involved in the cellular redox regulation. We also measured the cellular ROS levels after H_2O_2 stimulation. After 200 μM H_2O_2 stimulation, intracellular ROS levels increased to the highest value in 10 to 30 min, then decreased quickly. This observation means that the cells have the ability to scavenge cellular ROS. At 10 min, the ROS levels in Trx1 and Trx1 (C32/35S) transfected cells were obviously lower than that in empty vector transfected cells (Fig. 1B, $P < 0.05$, n = 3). At 30 min, the ROS levels in empty vector transfected cells were 1.5 times higher than in Trx1 transfected cells ($P < 0.05$, $n = 3$). These results suggested that Trx1 plays an important role in cellular redox regulation in SH-SY5Y cells. Redox active cysteine residues are indispensable for this effect, however, other cysteine are also involved in this process.

Trx1 protects the actin cytoskeleton from oxidative insults

Protein thiols are targets of oxidative stress. Many studies on oxidative stress have shown that Cys374 of actin is highly reactive to oxidizing agents and that oxidative damage elicits changes in actin cytoskeleton organization (12). To study the effects of oxidative stress on cytoskeleton structure and whether Trx1 can protect the actin cytoskeleton from oxidative stress, the structure of the actin cytoskeleton was assessed by the immunofluorescence staining and the ratio of F-actin incorporated into the cytoskeleton versus G-actin was measured by the G-actin/F-actin *in vivo* assay kit. In SH-SY5Y cells, 100 μM H_2O_2 stimulation for 30 min impeded actin polymerization. For Trx1 transfected SH-SY5Y cells, the actin cytoskeleton was more resistant to H_2O_2 induced oxidative stress (Fig. 2A). H_2O_2 significantly decreased the ratio of F-actin to G-actin in a dose dependent manner both in control cells and in cells overexpressing Trx1 (Fig. 2B). By comparison, we found that the ratio was much higher in Trx1 over-

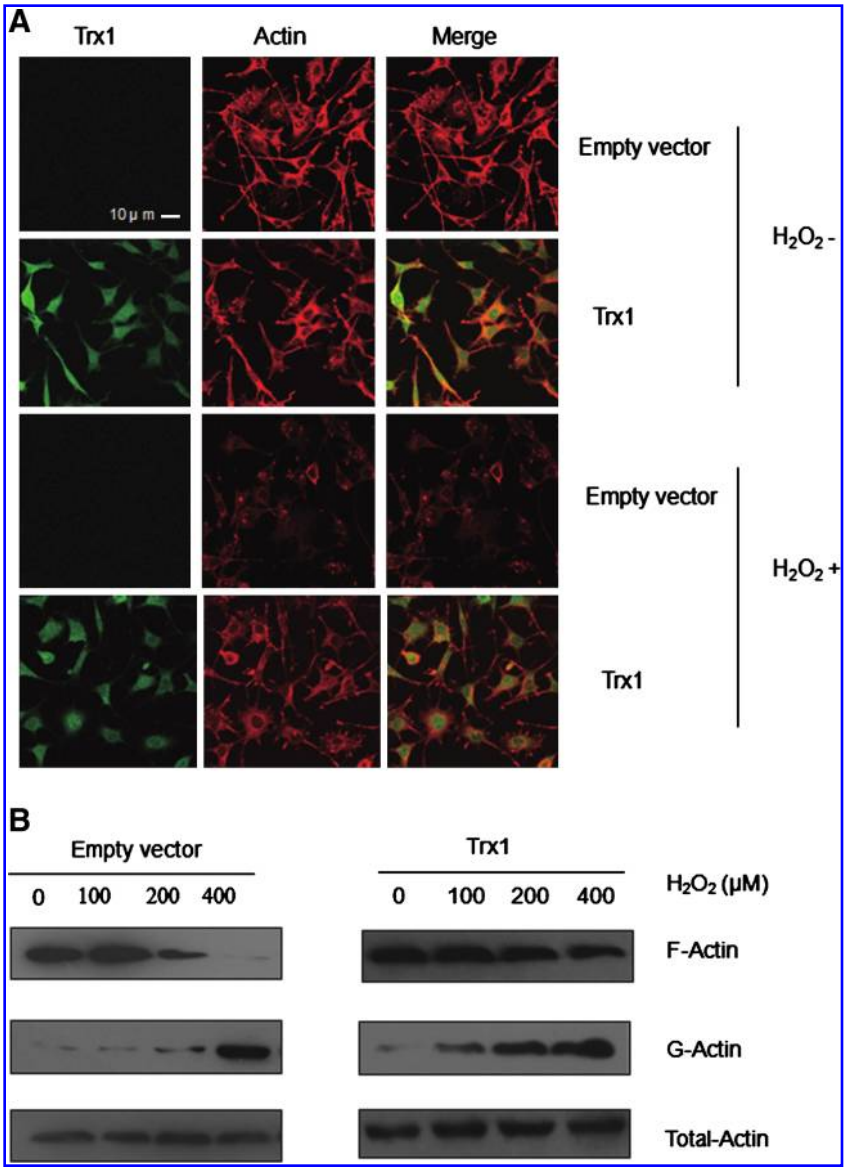

FIG. 2. Trx1 protects the actin cytoskeleton from oxidative stress. (**A**) SH-SY5Y cells stably transfected with empty vector or with Trx1 were treated with 100 μM H_2O_2 for 30 min, and then fixed with 4% paraformaldehyde. Immunofluorescene staining was carried out with FITC-labeled antibody for Trx1 and TRITC-phalloidin for F-actin. (**B**) Cells were treated with various concentrations of H_2O_2 for 30 min, and then lysed with F-actin stabilization buffer. The supernatant (G-actin) was separated from the pellets (F-actin). Equal volumes of the samples were analyzed by Western blotting with anti-actin antibody. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

expressing SH-SY5Y cells than that in control cells under H_2O_2 stimulation and normal condition (Supplemental Fig. 1; see www.liebertonline.com/ars). This suggests that Trx1 could protect actin from disorganization.

The regulation of Trx1 on the redox state of actin

Actin contains reactive sulfhydryl groups, which are the moieties generally most reactive towards H_2O_2. To evaluate the effect of Trx1 on the actin redox state, SH-SY5Y cells were transfected with either pcDNA3-Trx1 or pcDNA3 empty vector, and then treated with increasing concentration of H_2O_2. Reduced and oxidized forms of actin could be distinguished by AMS modification, since a free cysteine increases the molecular mass of the protein by ~500 Da. Without any treatment, the reduced band was identified clearly either in Trx1 transfected cells or control cells. In control cells, the reduced band got fainter with the increasing concentration of H_2O_2 stimulation, it disappeared when H_2O_2 concentration increased to 200 μM. However, in Trx1 transfected cells, there is still much amount of reduced actin in cells under 400 μM H_2O_2 stimulation (Fig. 3, Supplemental Fig. 2; see www.liebertonline.com/ars).

Trx1 is involved in the redox regulation of actin by their direct interaction

Because Trx1 has been shown to directly associate with target proteins in exerting its reducing action (4), we postulated that Trx1 might physically associate with actin. First, a pull-down assay was used to test the direct interaction between actin and Trx1. For this purpose, a cDNA encoding Trx1 was subcloned into pET24a (+) in frame, resulting in His-Trx1. The His-Trx1 protein was immobilized on agarose beads and incubated with the cell lysate. After extensive washing, bound proteins were analyzed by Western blotting using actin antibody. The results demonstrated that actin specifically bound to His-Trx1. In control experiment, there was no actin bound to the agarose. If DTT and β-ME were added to the incubation buffer at a high concentration, actin couldn't bind to Trx1 any more (Fig. 4A). This interaction between actin and Trx1 was further confirmed by co-immunoprecipitation experiment, which was performed in SH-SY5Y cells transfected with pcDNA3-Trx1 (Fig. 4B). The cell lysate was immunoprecipitated by actin antibody, then detected by Trx1 antibody. Because of the endogeneous expression of Trx1, the Trx1 band in the precipitation could also be detected in cells without Trx1 transfection. Furthermore, we performed Co-IP experiment in cells overexpressed thioredoxin reductase (TrxR), we found the interaction between Trx and actin was reduced significantly in these cells (Supplemental Fig. 3; see www.liebertonline.com/ars). These results implicate the existence of a direct interaction between Trx1 and actin and indicate that the interaction is disulfide-dependent.

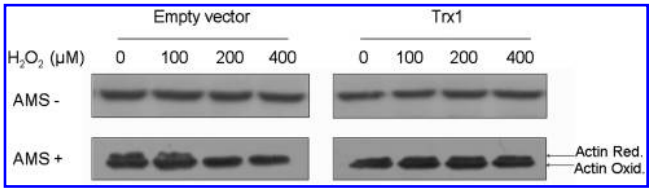

FIG. 3. Effect of Trx1 on the redox state of actin. SH-SY5Y cells stably transfected with the Trx1 plasmid or empty vector were incubated with increasing concentration of H_2O_2 for 30 min, and then precipitated in 5% TCA. The precipitate was resuspended in reaction buffer containing 10 mM AMS. Samples were resolved by nonreducing SDS-PAGE.

The interaction between Trx1 and actin decreases with increasing concentration of H_2O_2

It has been shown that addition of increasing concentration of diamide to cells results in oxidation of the active site and oxidation of the nonactive site at higher concentration. With the increasing of ROS levels, free thiols in Trx1 can be oxidized by the formation of intramolecular disulfide. Cys62–Cys69 disulfide could provide a means to transiently inhibit Trx1 activity under conditions of oxidative stress (21). When the concentration of H_2O_2 was increased to 400 μM, the interaction between Trx1 and actin was attenuated significantly (Fig. 5). The amount of Trx1 immunoprecitated by actin is shown in Supplemental Fig. 4 (see www.liebertonline.com/ars). The results suggest that the interaction between Trx1 and actin is redox-dependent.

Cysteine 62 in Trx1 is the key site that interacts with actin

In addition to active site of cysteine, there are still three cysteines at positions of 62, 69, and 73 in Trx1. To determine

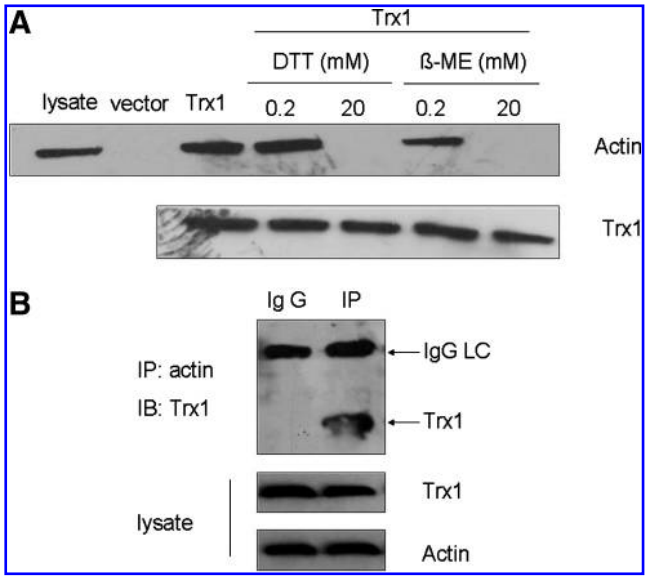

FIG. 4. Interaction between Trx1 and actin *in vitro* and *in vivo*. (**A**) His-fusion protein pull-down assay. SH-SY5Y cell lysate was added to the His purification modules that had been immobilized with His and His-Trx1, incubated for 3 h, then washed with washing buffer to remove protein bound nonspecifically. In the incubation buffer, different concentrations of DTT and β-ME were added to test their interaction under reducing conditions. (**B**) SH-SY5Y cells were transfected with pcDNA3-Trx1. The cell lysate was incubated with the anti-actin mAb for 4 h. The samples were then mixed with Protein A/G plus agarose. After incubation for 4 h, the beads were washed and the bound proteins were released from the beads by boiling in SDS-PAGE buffer. The samples were analyzed by Western blotting with anti-Trx1 antibody.

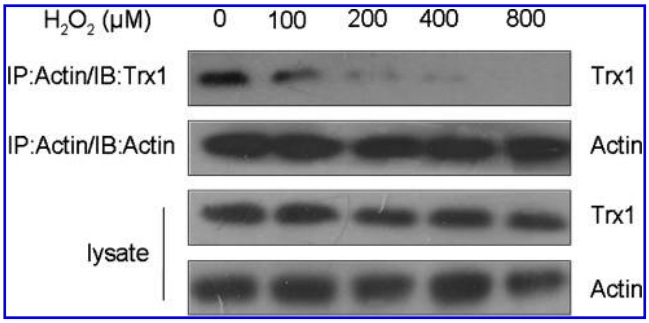

FIG. 5. Interaction between Trx1 and actin is affected by the cellular redox state. SH-SY5Y cells transfected with the Trx1 plasmid were incubated with increasing concentrations of H_2O_2 for 30 min. Cell lysate was immuoprecipitated by actin antibody, then detected with Trx1 antibody. The expression of Trx1 and actin in cells was analyzed by Western blotting.

which cysteine is the key site in Trx1 to interact with actin, we first mutated the redox active cysteine residues. By co-immunoprecipitation assay, the interaction between Trx1 (C32/35S) mutant and actin is the same as that of wild-type Trx1 (Fig. 6A and Supplemental Fig. 5; see www.liebertonline.com/ars). Then we made mutants of Trx1 bearing Cys–Ser at the sites of 62, 69, and 73. The binding capability of wild-type Trx1 and its mutants was measured by immunoprecipitation (Fig. 6B). Co-IP experiment between Trx1 (C62S) and actin was also performed in the presence or absence of DTT or β-ME. The result demonstrated that there exists very weak

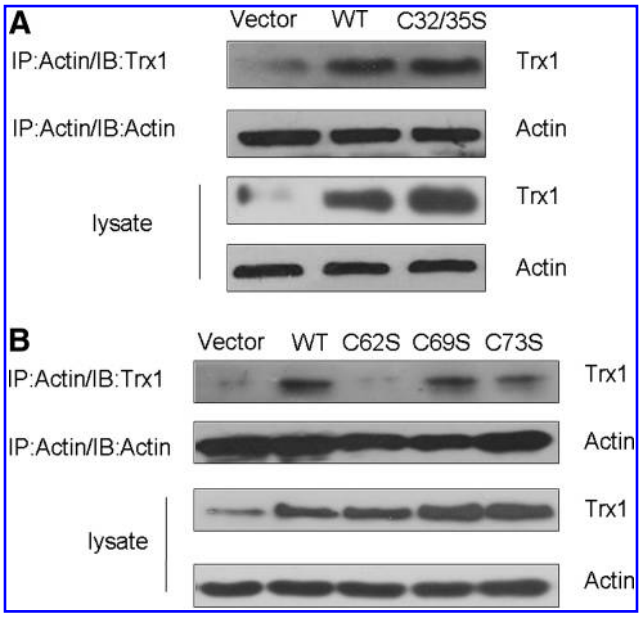

FIG. 6. Cysteine 62 plays an important role in the anti-apoptotic function of Trx1 in SH-SY5Y cells. (**A**) SH-SY5Y cells were transfected with pcDNA3-Trx1, pcDNA3-Trx1 (C32/35S), and pcDNA3 empty vector. Lysates were immunoprecipitated using actin antibody and probed with Trx1 antibody (*upper lane*). The cell lysates were also probed directly with Trx1 and actin antibody (*second and third lane*). (**B**) pcDNA3, pcDNA3-Trx1 and pcDNA3-Trx1(C62S, C69S, C73S) were overexpressed in SH-SY5Y cells. Cell lysates were used for immunoprecipitation.

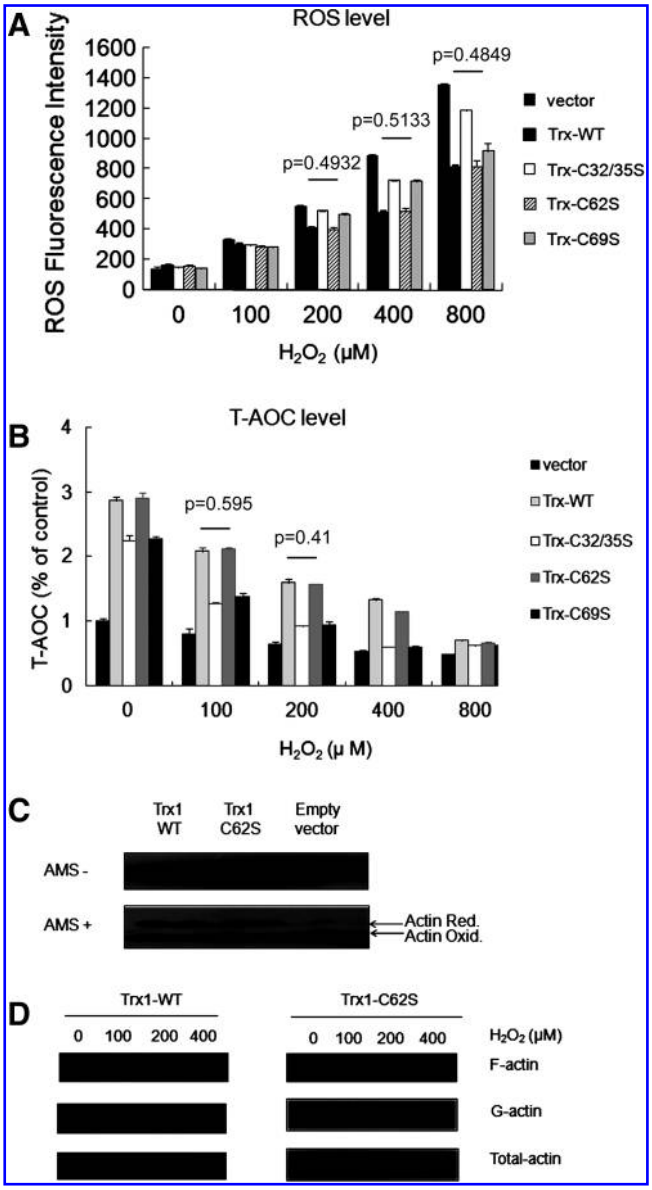

FIG. 7. Cysteine 62 in Trx1 is not the crucial site for cellular redox regulation, but mediates the regulation of actin polymerization under H_2O_2 stimulation. (**A**), (**B**) Trx1-C62S has the same capacity as Trx1 to reduce the ROS levels and increase T-AOC. SH-SY5Y cells were transfected with pcDNA3-Trx1, pcDNA3-Trx1 (C62S), pcDNA3-Trx1 (C69S), pcDNA3-Trx1 (C32/35S), or empty vector for 18 h, then stimulated with increasing concentrations of H_2O_2 for 30 min, the levels of ROS and T-AOC were analyzed as before. (**C**) Cysteine 62 is crucial to keep actin in reduced status. SH-SY5Y cells were transfected with pcDNA3-Trx1, pcDNA3-Trx1 (C62S), or empty vector, and then stimulated with 400 μM H_2O_2. Reduced actin was modified by AMS and resolved by nonreducing SDS-PAGE. (**D**) The ability of Trx1 to protect actin from depolymerization under oxidative stress was reduced when Cysteine 62 was mutated. Cells were transfected with pcDNA3-Trx1 or pcDNA3-Trx1 (C62S), and then stimulated by increasing concentrations of H_2O_2. F-actin and G-actin were isolated as before and analyzed by Western blotting.

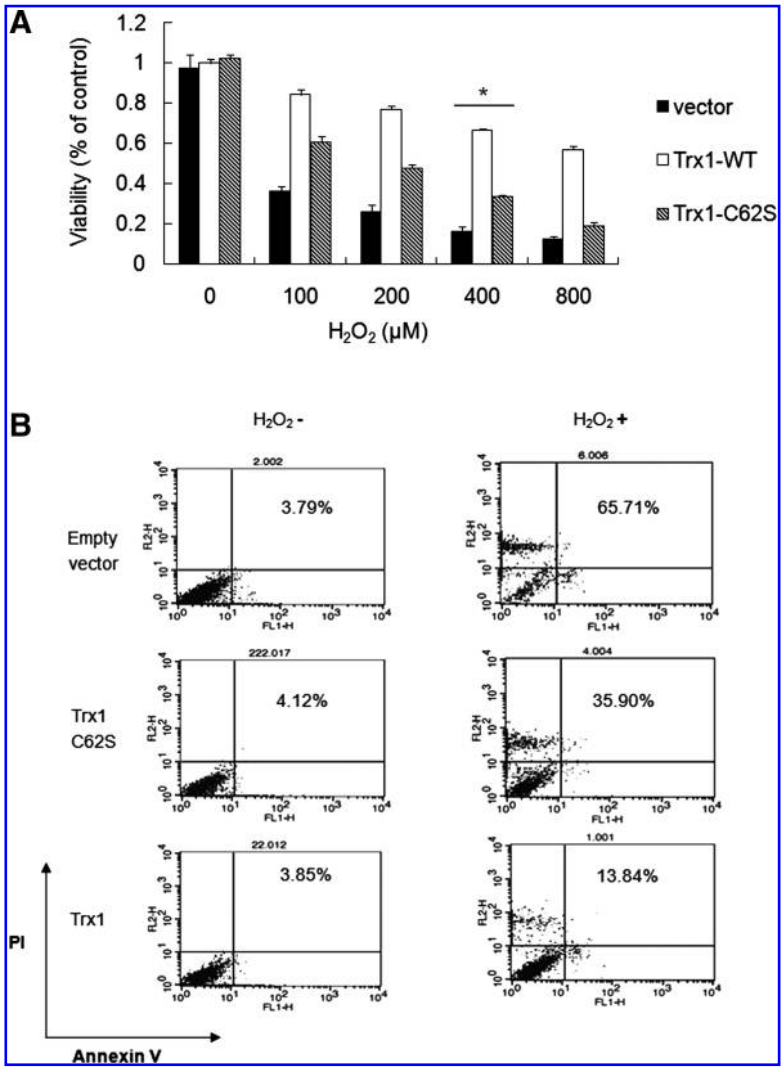

FIG. 8. Cysteine 62 plays an important role in the anti-apoptotic function of Trx1 in SH-SY5Y cells. (**A**) Effects of Trx1 and Trx1 (C62S) on cell viability. SH-SY5Y cells were transfected with pcDNA3-Trx1, pcDNA3-Trx1 (C62S), or empty vector for 18 h, and then stimulated with increasing concentrations of H_2O_2. Cell viability was analyzed by the MTT assay. (**B**) Protection of Trx1 and Trx1 (C62S) from oxidative stress-induced apoptosis. SH-SY5Y cells were transfected with pcDNA3-Trx1, pcDNA3-Trx1 (C62S), or empty vector for 18 h, and stimulated with 400 μM H_2O_2 for 30 min. Apoptosis was identified by Annexin V and PI staining, and measured by FACS. The percentage of apoptotic cells is given in the figures.

interaction between Trx1 (C62S) and actin. In the presence of DTT or β-ME, this interaction disappeared (Supplemental Fig. 6; see www.liebertonline.com/ars). The results demonstrated that Cysteine 62, and not Cysteine 69 or Cysteine 73, plays an important role in mediating the interaction between Trx1 and actin. When this site was mutated, the interaction between Trx1 and actin was reduced significantly.

Cysteine 62 in Trx1 is not the crucial site for cellular redox regulation, but mediates the regulation of actin polymerization under H_2O_2 stimulation

SH-SY5Y cells were transfected with pcDNA3-Trx1 or pcDNA3-Trx1 (C62S, C69S, C32/35S), respectively, stimulated by H_2O_2, and the levels of ROS and T-AOC were measured. Stimulating with increasing concentration of H_2O_2, the cellular ROS levels in cells transfected with pcDNA3 empty vector and pcDNA3-Trx1 (C69S, C32/35S) were much higher than in cells transfected with pcDNA3-Trx1 and pcDNA3-Trx1 (C62S), especially under higher H_2O_2 concentrations. The effect of Trx1 on cellular ROS level, however, was almost the same as that of Trx1 (C62S) (Fig. 7A, $P = 0.4932$, $n = 6$). The effect of Trx1 (C62S) on the regulation of cellular redox state was also validated by the T-AOC assay (Fig. 7B). Under 400 μM H_2O_2 stimulation, the amount of reduced actin in pcDNA3-Trx1 transfected cells was much higher than that in pcDNA3-Trx1(C62S) and pcDNA3 empty vector transfected cells, indicating that cysteine 62 in Trx1 is especially involved in the regulation of actin redox status (Fig. 7C, Supplemental Fig. 7B; see www.liebertonline.com/ars). The amounts of F-actin and G-actin in the presence of different concentration of H_2O_2 were quantified by Western blotting after cells were transfected with pcDNA3-Trx1 or pcDNA3-Trx1 (C62S) (Fig. 7D, Supplemental Fig. 7A; see www.liebertonline.com/ars). The ratio decreased both in pcDNA3-Trx1 and pcDNA3-Trx1 (C62S) transfected cells with the increasing concentration of H_2O_2 stimulation. However, the value was much higher in pcDNA3-Trx1 transfected cells. These results suggest that Cysteine 62 is an important site that keeps actin in a reduced state and maintains its organization, although it is not directly involved in the regulation of the cellular redox state.

Cysteine 62 plays an important role in the anti-apoptotic function of Trx1 in SH-SY5Y cells

It has been reported that Trx1 has anti-apoptotic functions. Trx1 can bind to ASK1 through Cys 32 and Cys 35 and inhibit stress-induced apoptosis (19). *S*-Nitrosylation of Cys 69 also

contributes to inhibition of apoptosis (8). Increasing evidence indicates that apoptosis can be transmitted through the actin cytoskeleton (6, 7). The function of Cysteine 62 in actin regulation suggests that Cysteine 62 is also required for the anti-apoptotic function of Trx1 in SH-SY5Y cells. To elucidate the function of Cysteine 62 in anti-apoptosis, SH-SY5Y cells were transfected with empty vector, wild-type Trx1 or Trx1 (C62S), and then stimulated with 400 μM H_2O_2. Cell viability and apoptosis were then analyzed. With the increasing concentrations of H_2O_2, cell viability decreased in empty vector, Trx1 and Trx1 (C62S) transfected cells. Overexpression of wild-type Trx1 significantly increased cell viability. Mutation of Cysteine 62, in which the redox regulatory domain was intact, can also improve cell viability, but its capacity to increase cellular viability was reduced by 20% as compared with over expression of wild-type Trx1 (Fig. 8A, $P < 0.05$, $n = 9$).The results showed that in empty vector transfected cells, 65.71% of the cells underwent apoptosis under 400 μM H_2O_2 stimulation, Trx1 and Trx1 (C62S) reduced the number of apoptotic cells to 13.84% and 35.90%, respectively (Fig. 8B). These data suggest that the mechanism of Cysteine 62 in preventing oxidative stress-induced apoptosis is different from that of other cysteines.

Discussion

A growing body of evidence indicates that the actin system is one of the most sensitive constituents of the cytoskeleton to the oxidant attack. Recent redox proteomics studies detected actin as the most prominent protein oxidized in response to exposure of cells to oxidants (5, 7, 10, 12). We reasoned that a direct redox regulation of actin *in vivo* could be one of the most important processes regulating the dynamics of the microfilament system. In our study, Trx1 was identified as interacting with actin and protecting the actin cytoskeleton from oxidative stress. Moreover, actin can be kept at reduced status, even at a higher concentration of H_2O_2 stimulation under the protection of Trx1.

Trx1 is expressed ubiquitously in mammalian cells and contains a conserved Cys-Gly-Pro-Cys active site (Cys 32 and Cys 35) that is essential for the redox regulatory function (1, 16). In addition to the conserved cysteine residues in the active site, three additional structural cysteine residues (Cys 62, Cys 69, and Cys 73) are present in the structure of the human Trx1 (16). Trx1 is *S*-nitrosylated on Cys 69, this is required for scavenging ROS and for preserving the redox regulatory activity, and contributes to the protein's anti-apoptotic functions (8). It has been identified that the Cys 73 residue is involved in dimerization of Trx1 *via* an intermolecular disulfide bond formation between Cys 73 of each monomer in the oxidized state. The biological function of the Cys 62 and Cys 69 residues in the nonactive domain remains to be fully elucidated. Some studies suggested that the formation of a disulfide bond between Cys 62 and Cys 69 gave a way to transiently inhibit Trx1 activity for redox signaling under oxidative stress (21). Our results demonstrated a new role for Cys 62, although it is not a key site that is involved in cellular redox regulation, which plays an important role in mediating its interaction with actin. This interaction disappeared with the increasing concentration of H_2O_2 stimulation. One possible reason is that the intramolecular disulfide bond formation inhibits the activity of Trx1.

Different H_2O_2 concentrations have different oxidative effects of functional relevance, leading to dimer formation, glutathionylation, and depolymerization of the actin system, depending on the location of the actin molecules, the source of the oxidant, and the availability of surrounding reducing systems (12). Many studies on oxidative stress have shown that both Cys 374 and Cys 272 of β-actin are highly reactive to oxidizing agents. Chemical modification of Cys 374 affects polymerizability and profilin binding (3, 15). The intracellular thiol homeostasis is maintained by the thioredoxin and glutaredoxin systems, that utilize NADPH as reducing equivalents to reduce proteins (9, 18). Thus, oxidative modification may be restored by these redoxins and glutaredoxins. *In vivo*, the direct redox control of actin by Trx1 could be one of the most important processes regulating the dynamics of the microfilament system.

It has been demonstrated that Trx1 could protect cells from apoptosis by the thiol oxidoreductase activity (4, 8, 17). Moreover, reduced Trx1 forms a complex with the apoptosis signaling regulating kinase-1 (ASK1), and protects cells from apoptosis by inhibiting ASK1 (19). In this study, we showed that Cys 62 in Trx1 plays an important role in protecting cells from apoptosis, independently of its role in the enzyme active site. Our results suggested that Trx1, by binding to actin and regulating its dynamics, could protect cells from apoptosis. Collectively, the results presented here indicate that the previously described massive production of ROS and the striking effect of Trx1 on cell viability and apoptosis under oxidative stress are largely transmitted through the actin cytoskeleton.

The results of oxidative stress on protein thiols and disulphides in *Mytilus edulis* revealed by proteomics suggest that actin and protein disulphide isomerase are redox targets (14). Actin was also identified by affinity chromatography assay to be a Trx1 target in eukaryotic unicellular green alga (13). Both actin and Trx1 are evolutionarily conserved proteins. Thus, we speculate that the protection of actin from oxidative insult by Trx system could be a universal regulatory mechanism.

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Author Disclosure Statement

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Abbreviations Used

AMS = 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid
ASK1 = apoptosis-signal-regulated kinase 1
β-ME = β-mercaptoethanol
DTT = dithiothreitol
FACS = fluorescence-activated cell sorting
H_2O_2 = hydrogen peroxide
MTT = methylthiazolyldiphenyl-tetrazolium bromide
PI = propidium iodide
ROS = reactive oxygen species
T-AOC = total antioxidative capability
Trx1 = thioredoxin-1

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