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Redox-Sensing Release of Human Thioredoxin from T Lymphocytes with Negative Feedback Loops[1]

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Thioredoxin (TRX) is released from various types of mammalian cells despite no typical secretory signal sequence. We show here that a redox-active site in TRX is essential for its release from T lymphocytes in response to H_2O_2 and extracellular TRX regulates its own H_2O_2-induced release. Human T cell leukemia virus type I-transformed T lymphocytes constitutively release a large amount of TRX. The level of TRX release is augmented upon the addition of H_2O_2, but suppressed upon the addition of *N*-acetylcysteine. In the culture supernatant of a Jurkat transfectant expressing the tagged TRX-wild type (WT), the tagged TRX protein is rapidly released at 1 h and kept at a constant level until 6 h after the addition of H_2O_2. In contrast, another type of transfectant expressing the tagged TRX mutant (C32S/C35S; CS) fails to release the protein. H_2O_2-induced release of TRX from the transfectant is inhibited by the presence of rTRX-WT in a dose-dependent manner. Preincubation of the transfectant with rTRX-WT for 1 h at 37°C, but not 0°C, results in a significant suppression of the TRX release, reactive oxygen species, and caspase-3 activity induced by H_2O_2, respectively. Confocal microscopy and Western blot analysis show that extracellular rTRX-WT added to the culture does not obviously enter T lymphocytes until 24 h. These results collectively suggest that the oxidative stress-induced TRX release from T lymphocytes depends on a redox-sensitive event and may be regulated by negative feedback loops using reactive oxygen species-mediated signal transductions. ***The Journal of Immunology*, 2004, 172: 442–448.**

Thioredoxin (TRX)[3] is a small (12 kDa) ubiquitous protein containing a conserved active site, -Cys-Gly-Pro-Cys-, and plays a variety of redox-related roles in organisms ranging from *Escherichia coli* to humans (1, 2). Human TRX was originally cloned as a soluble factor produced by human T cell leukemia virus type I (HTLV-I)-transformed ATL2 cells (3, 4) or as an autocrine growth factor produced by EBV-transformed B cells (5). Subsequent studies have shown that TRX is released from several cell types through a leaderless pathway in response to various stimulations (6, 7), and that APCs release TRX to provide the reducing environment for the activation of T lymphocytes (8). Expression of TRX in mammalian cells is enhanced by a variety of stresses such as H_2O_2, x-ray and UV exposure, or anticancer drug treatment (4, 9–12). In HIV- or hepatitis C virus-infected patients, reactive oxygen species (ROS), including H_2O_2, are spontaneously produced by neutrophils and monocytes and the concentration of TRX protein in plasma is elevated (13–19). Excessive ROS production leads to oxidative stress and may participate in the apoptosis of T lymphocytes, followed by mitochondrial oxidation, and effect the microsomal cytochrome P450 system and plasma membrane NADPH oxidases (20). Thus, ATL2 or Jurkat cells transfected with human *TRX* genes were used for the analysis of TRX release and apoptosis by H_2O_2. We report here that the TRX-wild type (WT), but not a TRX mutant (C32S/C35S; CS) is released from the transfectants in the presence of H_2O_2 and the released TRX limits H_2O_2-induced TRX release and apoptotic response by inhibiting further release of ROS.

Materials and Methods

Reagents and cells

ATL2 cells (HTLV-I-transformed T cell line) and Jurkat cells (human T cell lymphoma cell line) were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37°C. The stable transfectant cells, expressing *TRX* genes, were cultured in RPMI 1640 medium containing 400 μg/ml neomycin (G418; Nacalai Tesque, Kyoto, Japan) and the same compounds as described above. For Western blotting analysis, anti-FLAG mAb, M2 (anti-FLAG mAb), and anti-6x histidine tag mAb (anti-His mAb) were purchased from Sigma-Aldrich and Qiagen (Valencia, CA), respectively. Anti-human TRX mAb (anti-TRX mAb, mouse IgG1) was used as previously described (21).

Plasmid

Standard methods were used for DNA and protein manipulations. The expression vectors for TRX-WT and TRX-CS were amplified by the standard PCR method. The expression vector encoding the *TRX* gene was used as a template as previously described (3). The following oligonucleotides were prepared as primers for TRX-WT or TRX-CS constructs for the pQE80L vector: 5′-GGA TCC GTG AAG CAG ATC GAG AGC AAG-3′ (primer 1, 5′*Bam*HI) and 5′-GTC GAC TTA GAC TAA TTC ATT AAT GGT GGC-3′ (primer 2, 3′*Sal*I). Amplified DNA was subcloned into the vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and sequenced. After digestion with *Bam*HI and *Sal*I, the DNA fragment was inserted into the *Bam*HI-*Sal*I sites of the bacterial expression vector pQE80L (Qiagen). Each plasmid was transformed into competent *E. coli* (XL-1 Blue) cells, and the transformants were cultured for 4 h (A_{650}; 0.6) in terrific broth (Life Technologies, Grand Island, NY). Cells were harvested at 2 h after the addition of

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[3] Abbreviations used in this paper: TRX, thioredoxin; HTLV-I, human T cell leukemia virus type-I; NAC, *N*-acetylcysteine; WT, wild type; ROS, reactive oxygen species; LDH, lactate dehydrogenase; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; PI, propidium iodide.

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a final concentration of 1 mM isopropyl β-D-thiogalactopyranoside (Nacalai Tesque). In the case of the TRX-WT or TRX-CS/p3xFLAG-CMV-10 vector (Sigma-Aldrich), DNA fragments were amplified by PCR using two kinds of primers, a forward primer (primer 1′, 5′*Not*I) and a reverse primer (primer 2′, 3′*Bam*HI) and were inserted.

Purification of rTRX-WT or TRX-CS

6x Histidine-tagged rTRXs were purified from *E. coli*, which was transformed with pQE80L vectors containing *TRX-WT* and *TRX-CS* genes by Ni^{+}-chelating column chromatography according to the instruction manual of the QIAexpress system (Qiagen). Approximately 2.7 mg of purified rTRXs was obtained per milligram (wet) of cells after DTT treatment at 37ºC for 30 min. After PD-10 column chromatography (Amersham Bioscience, Piscataway, NJ), the purity of the rTRXs was confirmed by SDS-PAGE. The purity of the acquired rTRXs was over 98%. Alexa fluorescence-labeled (Alexa-) rTRX-WT or rTRX-CS was prepared using an Alexa Fluor 488 Protein Labeling kit (Molecular Probes, Eugene, OR) according to the instruction manual provided by the manufacturer. Briefly, 2 mg/ml of purified rTRXs was mixed and incubated in the reaction buffer including the fluorescent compounds for 1 h. The labeled rTRXs were subjected to gel filtration chromatography on a PD-10 column. The purity of the acquired Alexa-rTRXs was confirmed using SDS-PAGE and a Typhoon 9410 Variable Mode Imager (Molecular Dynamics, Sunnyvale, CA).

Stable transfectants for TRXs

For stable expression of the *TRX* genes and proteins, Jurkat cells were transfected with 10 μg of plasmid DNA: TRX-WT and TRX-CS/p3xFLAG-CMV-10 using a Gene Pulser electroporation system (Bio-Rad, Hercules, CA) with a capacitance of 960 μF and an electrical field of 320 V. The transfectants were cultured in RPMI 1640 medium containing 400 μg/ml G418 to screen stable transfectants. The drug-resistant bulk population of the cells was cloned by limiting dilution. Expression of TRX protein in the cells was confirmed by Western blot analysis. To avoid variation among clones, three randomly selected clones were tested.

Specific sandwich ELISA for TRX

The amount of TRX in the culture supernatant of cells was measured with a TRX ELISA kit (Redox Bioscience, Kyoto, Japan) as previously described with minor modification (22). 3,3′,5,5′-tetramethylbenzidine was used as a substrate for peroxidase and 30 min later stopping solution (2 N H_2SO_4) was added. The absorption at A_{450} was measured with an ELISA plate reader (Molecular Devices, Sunnyvale, CA).

Immunoprecipitation assay

Briefly, transfectant cells were exposed to 50 μM H_2O_2 and the supernatant of these cells was collected. The released FLAG-tagged TRXs in these supernatants were immunoprecipitated with 4 μg of monoclonal anti-human TRX mAb (anti-TRX mAb) and protein G-Sepharose beads (Amersham Biosciences) overnight at 4ºC.

Lactate dehydrogenase (LDH) activity assay

The LDH activity in culture supernatants was measured, as previously described (23), after the cells were treated with 50 μM H_2O_2 for 1, 3, or 6 h.

Assay for apoptosis

To detect the percent DNA contents of the sub-G_1 population, the cells were fixed in 70% ethanol on ice overnight after the stimulations. Then the cells were treated with 50 μg/ml RNase for 1 h at 37ºC and then incubated with propidium iodide (PI; Calbiochem, La Jolla, CA) staining solution for 1 h (24). The stained cells were analyzed by a flow cytometer (FACSCalibur; BD Biosciences, Mountain View, CA) using CellQuest software.

Measurement of caspase-3 activity

After the cells were stimulated with H_2O_2 for 24 h, the cell lysate was prepared with lysis buffer containing 0.5% Nonidet P-40, 50 mM Tris-HCl (pH 7.5), and protease inhibitors (Roche, Basel, Switzerland) from treated cells. The caspase-3 activity in this lysate was measured using a fluorometer (Spectra Fluor) as previously described (25).

Measurement of intracellular ROS

To measure the amount of intracellular ROS generated by the cells, 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes) was used. This compound is a nonfluorescent, cell-diffusible dye. Intracellular esterases cleave the acetyl groups from the molecule to produce nonfluorescent DCFH. This is trapped inside the cell and in the presence of ROS, DCFH is subsequently modified to fluorescent DCFH, which can be detected by flow cytometry. After the cells were incubated with 10 μg/ml rTRX-WT for 1 h, they were preloaded with 5 μM DCFH-DA at 37°C for 30 min. Each sample was analyzed using a FACSCalibur. The data are expressed as the mean fluorescence intensity.

Preparation of cytosolic fraction

The cytosolic fraction was prepared by cavitation and centrifugation (26). The treated cells were collected and suspended with hypotonic buffer after washing out with excess PBS buffer. Next, cavitation was performed using nitrogen gas at 1500 pounds per square inch on ice. The suspension was subjected to a series of sequential centrifugations: 1,500 and 50,000 rpm. The supernatant acquired from 50,000 rpm spin was used as the cytosolic fraction.

Confocal imaging

Imaging analysis was performed with a Bio-Rad RTS 2000 confocal laser microscope, a modified version of the Nikon RCM 8000 video-rate confocal instrument (Nikon, Melville, NY) (27). This system uses a krypton/argon laser of the American Laser Cooperation (Salt Lake City, UT). Data after capturing images were transferred directly from a real-time image-capturing subsystem to a hard disk developed specifically for this system by Nikon. These operations were all managed under Windows NT. For Fig. 4, images were taken at 2.1 s $frame^{-1}$ after 0, 24, 48, or 72 h. The culture medium was replaced with HEPES-buffered Krebs-Ringer solution before capturing the images by confocal laser.

Results

TRX release from HTLV-I-infected T lymphocytes is regulated by redox state

Since viral infections induce the production of ROS such as H_2O_2 in various cell types, we considered that oxidative stress may induce the release of TRX from HTLV-I-transformed T lymphocytes, ATL2 cells. The cells were cultured for 24 h with 10 or 50 μM H_2O_2, and TRX in the culture supernatants and the cell lysates was measured using the ELISA system. As shown in Table I, the addition of H_2O_2 increased the amount of TRX protein in both culture supernatant and cell lysate in a dose-dependent manner. Moreover, a reducing reagent, *N*-acetylcysteine (NAC) decreased the amount of TRX in the supernatant but not that in cell lysate (Table I). These results suggest that the release of TRX from HTLV-I-transformed T lymphocytes is regulated by redox status.

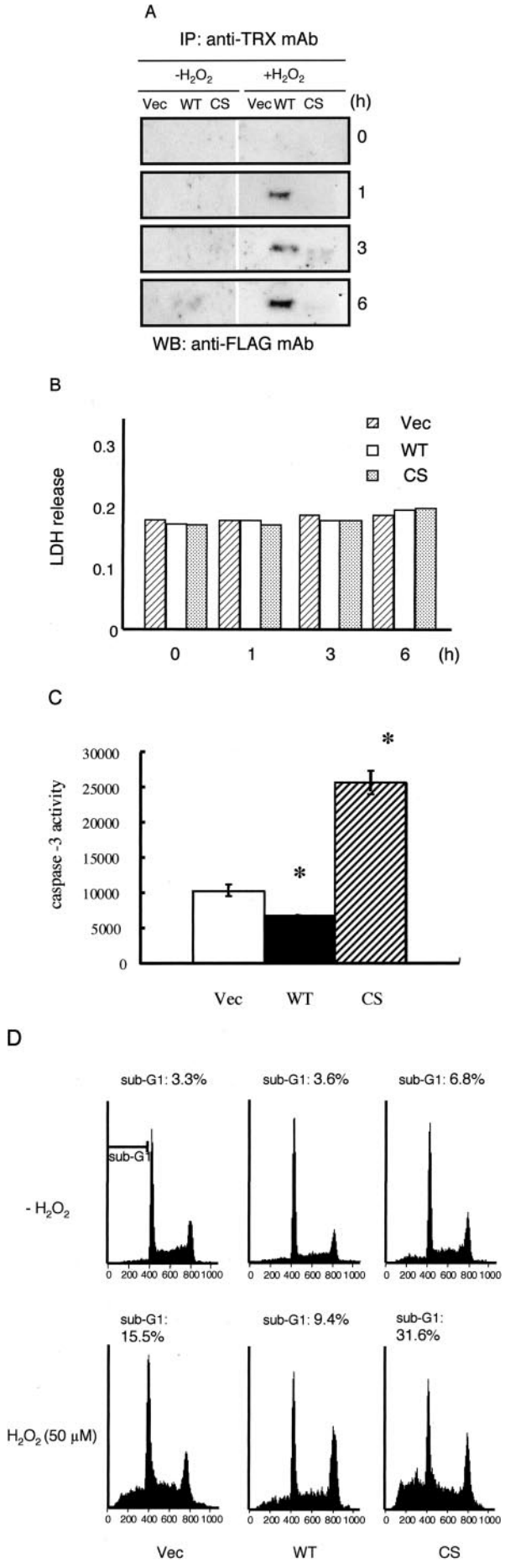
Redox-active site in TRX is indispensable for its release by H_2O_2

To test whether the redox-active site of TRX is involved in the release of TRX in response to H_2O_2, we generated stable transfectants of Jurkat cells transfected with vector DNA, the FLAG-tagged *TRX-WT* gene or the FLAG-tagged *TRX-CS* gene in which both cysteine residues of the active site of TRX were converted into serine. In the absence of H_2O_2, FLAG-tagged TRX was not detected in the culture supernatants of any transfectants by Western blot analysis using anti-FLAG mAb after the immunoprecipitation by anti-TRX mAb (Fig. 1*A*, *left panel*). In contrast, the

Table I. *Release of TRX in response to H_2O_2 and NAC*[a]

	H_2O_2			NAC	
	None	10 μM	50 μM	5 mM	10 mM
Supernatant	635	784	918	621	364
Cell lysate	1210	1930	2090	1280	1370

[a] A total of 1.2×10^6 cells of ATL2 cells was cultured in the absence or presence of H_2O_2 or NAC for 24 h, and the total weight (nanograms) of TRX in the culture supernatants or cell lysates was measured by a TRX ELISA system.



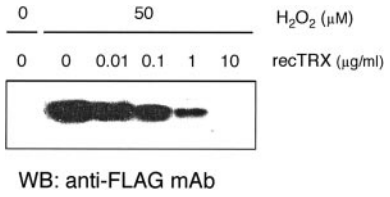


FIGURE 2. Inhibition of TRX release by the addition of extracellular TRX in a dose-dependent manner. Both 50 μM H_2O_2 and a concentration of 0, 0.01, 0.1, 1, or 10 μg/ml rTRX-WT were simultaneously added to the culture medium of the transfectant expressing TRX-WT. Each culture supernatant was recovered 3 h after the addition of H_2O_2 and analyzed by the same method used in Fig. 1*A*. rec, Recombinant.

tagged TRX was rapidly released from the Jurkat transfectant expressing TRX-WT at 1 h after the addition of 50 μM H_2O_2 to the culture, but the amount of tagged TRX in the culture supernatant did not significantly increase for the next 5 h (Fig. 1*A*, *right panel*). However, the tagged TRX was not detectable in the culture supernatants of the transfectant expressing TRX-CS within 6 h after the addition of H_2O_2 (Fig. 1*A*, *right panel*). To exclude the possibility that H_2O_2 might have induced necrosis of the transfectant expressing the tagged TRX-WT, the LDH activity in the culture supernatant was determined until 6 h after the addition of 50 μM H_2O_2 in the culture. As shown in Fig. 1*B*, LDH activity in the culture supernatant of all transfectants at 1, 3, or 6 h after the start of culture was comparable to that at 0 h, indicating that necrosis had not begun within 6 h. Then, the transfectants were assessed for oxidative stress-induced apoptosis. As a preliminary experiment to test whether the concentration of H_2O_2 (50 μM) used in this report induces apoptosis of the cells transfected with the control vector, *TRX-WT* or *TRX-CS*, caspase-3 activity in the tranfectants was measured after a 24-h culture in the presence of H_2O_2 (Fig. 1*C*). The caspase-3 activity of the transfectant expressing TRX-WT was significantly lower than that of the control vector. In contrast, that of the transfectant expressing TRX-CS was significantly higher than that of the control. Furthermore, the rate of apoptosis was determined as percent DNA content of sub-G_1 using flow cytometric analysis with PI staining. In the absence of H_2O_2, the percent DNA content of sub-G_1 was 3.3% in cells transfected with control vector, 3.6% in cells with *TRX-WT*, and 6.8% in cells with *TRX-CS*. After the addition of H_2O_2, the cells transfected with *TRX-WT* and control vector showed 9.4 and 15.5% apoptosis, respectively. In contrast, 31.6% of TRX-CS transfectants underwent apoptosis in the presence of H_2O_2. The results clearly show that the caspase-3 activity of the transfectant expressing TRX-WT was weaker than that of the control, while conversely, the activity of caspase-3 in the transfectant expressing TRX-CS was much stronger than that of the control. These results indicate that the expression of TRX-WT in Jurkat cells protects against apoptosis, whereas the expression of the mutant TRX-CS enhances apoptosis. Thus, it is suggested that the intact sequence of a redox-active site (-CPGC-) in

FIGURE 1. Release of tagged TRX from Jurkat transfectants in response to H_2O_2. *A*, FLAG-tagged TRX-WT or FLAG-tagged TRX-CS was immunoprecipitated by using ADF11 mAb from the culture supernatants of each Jurkat transfectant in the absence ($-H_2O_2$) or presence of 50 μM H_2O_2 ($+H_2O_2$) for 0, 1, 3, or 6 h. *B*, LDH activity in each culture supernatant of the transfectant exposed to 50 μM H_2O_2 for 1, 3, or 6 h. Vec, Cells transfected with vector. *C*, Caspase-3 activities of transfectants were measured after stimulation with 50 μM H_2O_2 for 24 h. *, $p < 0.01$, when compared with control. *D*, The percent DNA content of sub-G_1 transfectants was measured after stimulation with 50 μM H_2O_2 for 48 h.

TRX protein is indispensable for its active release from T lymphocytes by H_2O_2 and that TRX is rapidly released within 1 h, irrespective of the cellular sensitivity to H_2O_2-induced apoptosis.

TRX release is regulated by extracellular TRX

Since the amount of tagged TRX in the culture supernatant of the Jurkat transfectants was constant for 6 h, it is possible that the TRX released from the transfectants might regulate further its own release. To test this possibility, various doses of rTRX-WT (0.01–10 μg/ml) were added to the culture of the Jurkat transfectant expressing FLAG-tagged TRX-WT along with 50 μM H_2O_2. After 3 h, the tagged TRX in the culture supernatants was immunoprecipitated using anti-TRX mAb (anti-TRX mAb) and protein G-Sepharose beads. The samples were analyzed by SDS-PAGE, followed by Western blotting using anti-FLAG mAb. As shown in Fig. 2, the tagged TRX in the supernatants decreased in inverse proportion to the amount of rTRX-WT added to the culture and was undetectable at 10 μg/ml (0.8 μM) rTRX-WT. The expression level of the intracellular tagged TRX was not affected by the concentration of rTRX-WT added (data not shown). These results

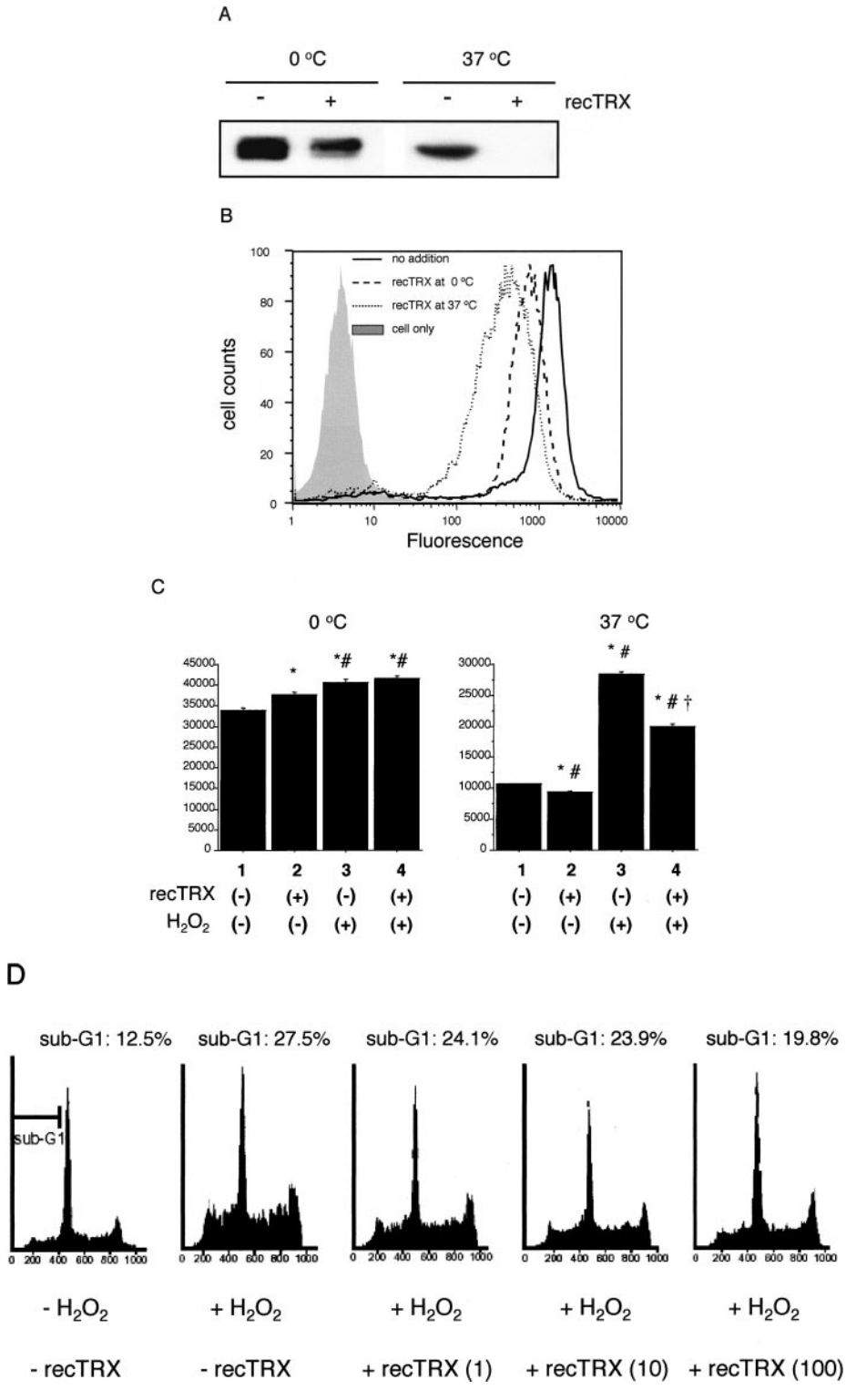


FIGURE 3. Suppression of H_2O_2-induced cell events by extracellular rTRX. *A*, After the pretreatment of the transfectant expressing TRX-WT with 10 μg/ml rTRX-WT for 1 h at 0 or 37°C, 50 μM H_2O_2 was added to the culture. Each culture supernatant was recovered 3 h after the addition of H_2O_2 and analyzed by the same method used in Fig. 1*A*. *B*, The transfectant expressing TRX-WT was preincubated with 10 μg/ml rTRX-WT at 0 or 37°C for 1 h. After being washed with medium, the cells were incubated with DCFH-DA for 30 min, and these treated cells were analyzed by flow cytometer. *C*, The transfectant expressing TRX-CS was cultured with 50 μM H_2O_2 for 24 h after treatment with rTRX for 1 h at 0 or 37°C. Caspase-3 activity in the supernatant of these treated cells was measured. *, $p < 0.01$ vs 1; #, $p < 0.01$ vs 2; †, $p < 0.01$ vs 3. Values of p were calculated using one-way ANOVA followed by Sheffe's post hoc tests. *D*, The transfectant expressing TRX-CS was cultured with 50 μM H_2O_2 in the absence or presence of rTRX in a dose-dependent manner. After the 48-h stimulation, the cells were stained by PI and measured by the FACS system (micrograms per milliliter). rec, Recombinant.

indicate that H_2O_2-induced TRX release from the cells is inhibited by a suitable dose of TRX-WT in the culture supernatant.

Pretreatment with rTRX-WT suppresses H_2O_2-induced release of TRX and apoptosis

To clarify the roles of exogenous TRX-WT in the regulatory mechanism of TRX release, the transfectant expressing TRX-WT was stimulated by the addition of H_2O_2 after pretreatment with rTRX-WT. The cells were preincubated with 10 μg/ml (0.8 μM) rTRX-WT for 1 h at 0 or 37°C. After a wash with the culture medium, the cells were subsequently cultured in the presence of 50 μM H_2O_2 for 3 h. The release of tagged TRX was not fully inhibited by preincubation with rTRX-WT at 0°C (Fig. 3*A*). However, the preincubation at 37°C remarkably suppressed the release of tagged TRX from the cells (Fig. 3*A*). It appeared that the interaction of rTRX-WT with the transfectant expressing the tagged TRX resulted in prevention of the H_2O_2-induced release of TRX from the cells via the cellular active process. To investigate the cellular active processes involved in the regulation of TRX release, the intracellular ROS production after the treatment of the transfectant with rTRX-WT was analyzed using a flow cytometer. The Jurkat transfectant expressing the tagged TRX-WT was incubated with 10 μg/ml rTRX-WT for 1h at 0 or 37°C, followed by 5 μM DCFH-DA for 30 min. Fig. 3*B* shows that ROS production after the incubation with rTRX-WT at 0 and 37°C was reduced 36 and 48% compared with that without rTRX-WT, respectively. As possible signal transduction mediated by ROS, apoptotic signaling was subsequently analyzed. It has been shown that H_2O_2 induces apoptosis of Jurkat cells (28). Since the caspase-3 activity of the transfectant expressing TRX-CS was significantly higher than that of the control (Fig. 1*C*), the effect of the preincubation with rTRX-WT was examined using the transfectant. Fig. 3*C* clearly showed that the caspase-3 activity after the addition of H_2O_2 in the culture preincubated with rTRX-WT was significantly inhibited at 37°C, but not at 0°C. Moreover, after the pretreatment of rTRX-WT at concentrations of 0, 1, 10, and 100 μg/ml, the percent DNA content of sub-G_1 cells was 27.5, 24.1, 23.9, and 19.8%, indicating a dose-dependent suppression of apoptosis by rTRX-WT (Fig. 3*D*). Hence, the results collectively suggest that extracellular rTRX-WT regulates ROS production and its mediated independent signal transductions responsible for the H_2O_2-induced release of TRX and apoptosis.

Extracellular TRX enters cells

As a mechanism for extracellular TRX to regulate cellular signal transduction, the possibility that the extracellular TRX enters the cells was examined. Alexa fluorescence-labeled (Alexa-) rTRX-WT or TRX-CS was prepared and added at a concentration of 100 ng/ml into culture medium of ATL2 cells. Confocal microscopy revealed that some cells cultured with Alexa-rTRX-WT emitted fluorescence within 24 h and almost all did after 72 h (Fig. 4*A*). In contrast, the cells cultured with Alexa-rTRX-CS failed to emit fluorescence after 72 h (data not shown). To confirm the cellular localization of Alexa-rTRX-WT, each subcellular fraction of the cells cultured with the His-tagged rTRX-WT for 0, 24, 48, or 72 h was prepared and analyzed by Western blotting using anti-His mAb. Fig. 4*B* clearly shows that the tagged TRX-WT accumulated in the cytosolic fraction in a time-dependent manner. Almost the same result was observed in Western blot analysis of particulate fractions containing plasma membranes (data not

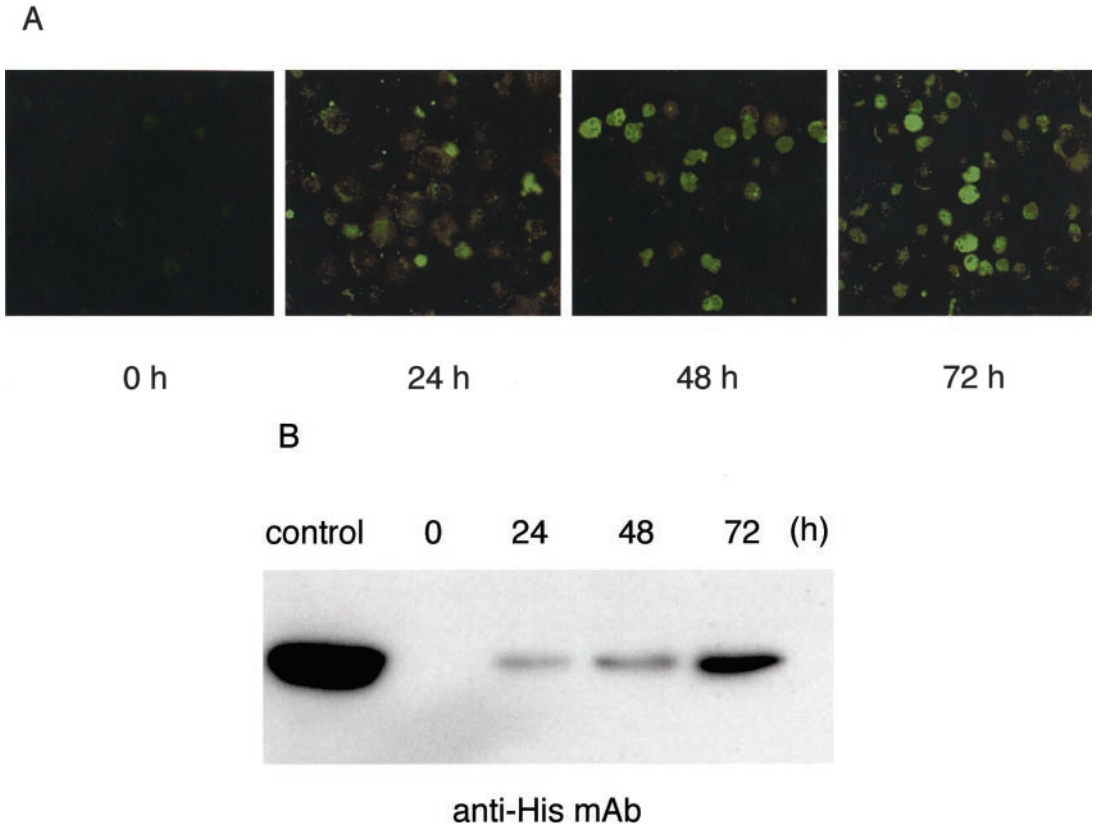


FIGURE 4. Entry of rTRX-WT into the cells. *A*, ATL2 cells were incubated with 100 ng/ml Alexa-rTRX-WT for 24, 48, or 72 h. After the incubation, the cells were washed with an excess amount of HEPES-buffered Krebs-Ringer solution and observed by confocal microscopy. *B*, Jurkat cells were incubated with 100 μg/ml His-tagged rTRX-WT for 0, 24, 48, or 72 h. The cytosolic fractions from each cell were prepared by the described method and analyzed by Western blotting using anti-His mAb. Control, Recombinant TRX.

shown). The data clearly indicate that extracellular TRX-WT enters cultured cells on a regular basis within 24 h.

Discussion

It has been reported that HTLV-I-transformed T lymphocytes secrete a large amount of TRX protein in the culture supernatant (3, 29). Although the data of Table I and Fig. 1*A* indicate that two cysteines at the redox-active site of TRX might regulate the oxidative stress-induced release of TRX, it remains unclear whether the reducing enzymatic activity of TRX is required for its release. A recent report showed that the release of TRX-C35S containing a serine instead of a cysteine at residue 35 is not altered by the redox status of the cells (30). We obtained the same result in that TRX-C35S could be released from Jurkat cells under oxidative stress (data not shown). Hence, it is suggested that Cys^{32} at the redox-active site of TRX is indispensable for the oxidative stress-induced TRX release.

Another question regarding the release of TRX is related to the nature of the mechanism by which the redox-sensitive release occurs. In an *E. coli* system, Inaba and Ito (31) have discovered a thiol-mediated protein folding system on the periplasmic membrane of many secreted proteins that have a disulfide bond. This system includes the TRX family of proteins. It is thus an intriguing possibility that a similar thiol-mediated redox-sensitive release mechanism is present in mammalian cell systems. Such a mechanism may be used in the release of leaderless proteins in various cell types in the immune and endocrine systems.

In terms of the kinetics of release after the addition of H_2O_2, TRX-WT was rapidly released from the transfectant within 1 h and its level in the culture supernatant was almost unchanged for 6 h (Fig. 1*A*). Although the concentration of intracellular TRX was ~10-fold higher than that of extracellular TRX as a result of Western blot analysis using anti-FLAG mAb (data not shown), the concentration of the TRX released did not increase any further after 1 h. We speculate that the release is regulated by a sensing mechanism, which may monitor the concentration of extracellular TRX. This hypothesis may be supported by the result that the H_2O_2-induced TRX release from the Jurkat transfectant was reduced in the presence of rTRX-WT whose concentration in the culture was comparable to that of endogenous TRX released from ATL2 cells (Fig. 2 and Table I.). It is likely that the TRX transmits an inhibitory signal to shut off further release via an autocrine and/or paracrine feedback loop because the H_2O_2-induced TRX release was completely suppressed by preincubation with rTRX at 37°C, but not fully at 0°C (Fig. 3*A*). Therefore, it seems that Jurkat cells or HTLV-I-transformed cells are equipped with TRX-sensing molecules on the cell surface, which may specifically recognize TRX protein, transmit signals into the cell, and regulate the release of TRX from the cells under physiological conditions.

It is quite natural that the extracellular rTRX can function to quench hydroxyl radicals in the culture using a TRX-dependent system. In fact, the level of ROS production after the preincubation with rTRX was significantly reduced (Fig. 3*B*). However, we expect that the amount of extracellular rTRX was not enough to quench both 50 μM H_2O_2 and subsequent ROS production since the concentration of rTRX-WT added to the culture, 10 μg/ml (~0.8 μM), was much lower than that of H_2O_2 (32). It is also reported that an isoform of TRX-dependent peroxidase, peroxiredoxin IV, which can scavenge hydroxyl radicals in culture, exists on the cell surface. This system may be partly involved in the suppression of TRX release. Thus, it appears that the signal transduction pathways may regulate H_2O_2-induced TRX release. In support of this hypothesis, it is reported that LPS-induced pro-IL-1 production and IL-1 secretion corresponded to the production of ROS and activation of c-Jun N-terminal kinase, p38, and extracellular signal-regulated kinase (33). Further study is required to elucidate the signal transduction pathways involved in the regulation of the H_2O_2-induced release of TRX.

As another mechanism accompanying a negative feedback loop, we speculate that the intracellular TRX that accumulated with the entry of extracellular TRX regulates the release. In fact, we demonstrated that extracellular rTRX-WT could enter ATL2 cells or Jurkat cells within 24 h by the confocal microscopic observation and Western blot analysis (Fig. 4). However, we failed to visualize the entry of extracellular rTRX-WT at a concentration of 100 μg/ml after the preincubation at 37°C for 1 h (data not shown). In this experiment, the concentration of rTRX-WT used in Fig. 3 was not appropriate for confocal microscopy because of the high fluorescence intensity of the culture medium containing Alexa-labeled rTRX-WT. Although it is possible that an undetectable amount of rTRX-WT enters the cells for 1 h, it is unlikely that this entry mechanism of TRX mainly functions to inhibit the TRX release by the preincubation with rTRX.

In summary, we suggest that the release of TRX from T lymphocytes is regulated by a negative feedback loop sensing the concentration of TRX in and/or out of cells for the purpose of maintaining the physiological condition of the cells. We are now proceeding to identify the target molecules on the plasma membrane for extracellular TRX, which might be involved in the signal transduction and the entry into the cells.

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