Acute Cadmium Exposure Inactivates Thioltransferase (Glutaredoxin), Inhibits Intracellular Reduction of Protein-glutathionyl-mixed Disulfides, and Initiates Apoptosis*

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Oxidative stress broadly impacts cells, initiating regulatory pathways as well as apoptosis and necrosis. A key molecular event is protein S-glutathionylation, and thioltransferase (glutaredoxin) is a specific and efficient catalyst of protein-SSG reduction. In this study 30-min exposure of I93 and Jurkats to cadmium inhibited intracellular protein-SSG reduction, and this correlated with inhibition of the thioltransferase system, consistent with thioltransferase being the primary intracellular catalyst of deglutathionylation. The thioltransferase system contributed very little to total deglutathionylase activity. Thioltransferase and GSSG reductase in situ displayed similar dose-response curves (50% inhibition near 10 μM cadmium in extracellular buffer). Acute cadmium exposure also initiated apoptosis, with I93 cells being more sensitive than Jurkats. Moreover, transfection with antisense thioltransferase cDNA was incompatible with cell survival. Collectively, these data suggest that thioltransferase has a vital role in sulfhydryl homeostasis and cell survival. In separate experiments, cadmium inhibited the isolated component enzymes of the thioltransferase and thioredoxin systems, consistent with the vicinal dithiol nature of their active sites: thioltransferase (IC50 ~ 1 μM), GSSG reductase (IC50 ~ 1 μM), thioredoxin (IC50 ~ 8 μM), thioredoxin reductase (IC50 ~ 0.2 μM). Disruption of the vicinal dithiol on thioltransferase (via oxidation to C22-SS-C25; or C25S mutation) protected against cadmium, consistent with a dithiol chelation mechanism of inactivation.

Thioltransferase (glutaredoxin) is a member of the TDOR1 enzyme family, which also includes thioredoxin and their corresponding reductase enzymes GSSG reductase and thioredoxin reductase, respectively. Thioltransferase is a 12-kDa cytosolic protein that has been characterized in vitro as a specific catalyst for the reduction of protein-glutathionyl-mixed disulfides (protein-SSG) (1–5). The reaction catalyzed by thioltransferase is also selective for GSH as the reducing substrate (3). This thiol-disulfide interchange reaction is likely crucial for maintaining intracellular thiol status (2, 6). Under normal conditions the intracellular milieu is predominately reducing (typical GSH/GSSG ratio ~ 100), but physiological and pathophysiological processes like aging, cardiovascular and neurodegenerative diseases, AIDS, and cancer chemotherapy can shift the redox balance toward an oxidizing milieu. A crucial component of the cellular redox balance is modulation of the thiol-disulfide status of critical cysteine residues on proteins, and a prevalent modification of cysteine residues is reversible S-glutathionylation of proteins. Accumulation of protein-SSG has been reported in different cell types under a variety of oxidative conditions (7–11). With many sulfhydryl proteins, glutathionylation is inactivating. Examples include the transacting factor NF-1 (12), protein tyrosine phosphatase 1B (13), and phosphofructokinase (14, 15). With other proteins glutathionylation can be an activation event. Examples include HIV-1 protease (16, 17) and microsomal glutathione S-transferase (18). Contrasting effects of reversible glutathionylation for different proteins studied in vitro suggest that protein-SSG formation is a mechanism of regulation and/or intracellular signal transduction (2, 6, 11, 13, 17, 19) and implicate thioltransferase as a key player in these cellular processes.

Cysteine residues can be modified reversibly in other ways that also can affect the activities of proteins. Examples include sulfenic acids and intra- or intermolecular disulfides. Thioltransferase does not catalyze reduction of these oxidized forms unless they react first with GSH to form protein-SSG (2). However, thioredoxin has been reported to catalyze reduction of these sulfhydryl modifications (4, 15, 20–22), displaying preferential reduction of intramolecular disulfides and protein-sulfenic acids compared with intermolecular disulfides and protein-SSG (15, 20). In contrast to thioltransferase, thioredoxin is neither an efficient nor selective catalyst for the reduction of protein-SSG (1, 4, 15). The different substrate selectivities of thioltransferase and thioredoxin suggest that the two TDOR enzyme systems may contribute synergistically to sulfhydryl homeostasis.

Cadmium is a heavy metal that exhibits both acute and chronic toxicity. In cases of severe acute exposure, death can result in a few days and long term exposure to lesser amounts can result in damage to kidney, lung, and bone tissue (23). Cadmium also triggers stress-like responses in various signaling cascades (24), including modulation of the regulation of the...
tumor suppressor gene p53, various cytokines, and proteins like metallothionein and glutathione synthetase. Cadmium has no known beneficial effects in the human body, but it is used extensively in the production of human consumables like batteries, plastics, various pigments, and metal coatings. Although Cd\(^{2+}\) has been implicated as a pro-oxidant, it differs from other pro-oxidant metal ions like Cu\(^{2+}\) and Fe\(^{2+}\), because it does not participate in redox cycling (23). This implicates direct binding of cadmium to critical cellular components as the mechanism of toxicity, and proteins with vicinal disulfides are expected to be particularly sensitive to inactivation by cadmium (25).

Recently it was reported (26) that short term incubation of HT4 neuronal cells with cadmium led to an increase in the level of cellular protein-SSG. Because cadmium is not a direct oxidant, we hypothesized that protein-SSG accumulation was likely due to inhibition of thioltransferase, the expected catalyst of protein-SSG deglutathionylation in cells (2, 4, 15, 19). To test this hypothesis we examined the effects of cadmium exposure on H9 and Jurkat cells, two T-cell-derived lines that have been extensively studied for their responses to oxidative stress.

Here we report that cadmium treatment of cells is associated with specific coordination to the TDOR proteins, all of which contain vicinal cysteine residues. This coordination leads to potent inhibition of the thioltransferase system in situ, and this effect corresponds to a concomitant decrease in the rate of cellular protein-SSG dethiolation. Although the enzymes of the thioredoxin system are also sensitive to cadmium inhibition in vitro, the thioredoxin system does not contribute significantly to cellular protein-SSG reduction. Together these data indicate that the thioltransferase system is the primary mechanism for homeostatic deglutathionylation of protein-SSG in cells. In addition, acute treatment of cells with cadmium in the concentration range that inhibits thioltransferase leads to apoptosis, and cells transfected with the antisense cDNA of thioltransferase is necessary for cell survival and that it plays a role in initiation of apoptosis.

**Experimental Procedures**

**Materials—GSSG reductase (bovine and yeast) was purchased from Sigma. Thioredoxin reductase (bovine and Escherichia coli) was purchased from Sigma and American Diagnostics (Greenwich, CT). E. coli thioredoxin was obtained from Calbiochem. NADPH was purchased from Roche Molecular Biochemicals. L-cysteinyl-glutathione disulfide was purchased from Toronto Research Chemicals, Inc. Lipoamide disulfide (0.6-8-thioctic acid amide) was obtained from Sigma. RPMI 1640 medium, fetal bovine serum, penicillin-streptomycin mix, glutamine, trypan blue, Lipofectin, and Lipofectamine were from Life Technologies, Inc. All other chemicals were reagent grade from standard sources. Dr. David Davis (National Institutes of Health) generously donated the Jurkat cells (clone E6–1), and the H9 cells and K562 cells were obtained from the American Type Culture Collection. pRep4 and pRep10 expression vectors were from Invitrogen. Sephadex G-75 gel filtration resin was purchased from Pharmacia. Cadmium, Thioltransferase, Protein-SSG Reduction, and Apoptosis 26557

**Preparation of BSA-SSG\(^{35S}\)—BSA-SSG\(^{35S}\) was prepared as described previously (5), with the following modifications. After the reaction of Cys-carboxymethyl-BSA with N-[(carboxymethyl)pyridyl bis(3,3-dithiopropionate) was quenched with glycine, BSA was separated from small molecules by dialysis against 100 mM sodium phosphate, pH 7.0, overnight with two changes of buffer. The modified BSA was then treated with 4 mM \(^{35S}\)GSH for 1 h at room temperature. The resulting BSA-SSG\(^{35S}\) product was separated from \(^{35S}\)GSH as described previously, and it typically had a 98% SS-equivalency with BSA.

**Cell Maintenance—**H9, Jurkat, and K562 cells were grown in RPMI 1640 medium supplemented with 15% fetal bovine serum (10% for K562 cells), penicillin and streptomycin (50 units/ml and 50 μg/ml, respectively), and 2 mM glutamine. The cells were maintained between 1 × 10\(^5\) and 1 × 10\(^6\) cells/ml in a humidified 37 °C incubator containing 5% CO\(_2\) for no more than 20 days. The cells were passed every 2–3 days.

**Cell Exposure to Cadmium—**Cells in log phase growth (6 × 10\(^5\) to 1 × 10\(^6\), within 48 h of passage) were centrifuged and resuspended in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM sodium phosphate, 1.4 mM potassium phosphate, pH 7.4) to a density of 7 × 10\(^6\) cells/ml. These cells were treated by adding 100-μl aliquots of concentrated cadmium solutions in deionized water to 5 ml of the cells so that final cadmium concentrations were between 0 and 100 μM. Controls (100 μl of H\(_2\)O only) were performed in parallel. The cells were incubated at 37 °C at 5% CO\(_2\) for 30 min. Cadmium chloride and cadmium acetate gave equivalent results.

**Cell Lysates—**Cells (which settled during treatment) were resuspended in a minimal amount (usually 1 ml) of one of the various treatment solutions and centrifuged, and the resulting supernatant was removed by suction. Cell pellets were resuspended in lysis buffer, pH 7.4 (adapted from Ref. 29) (50 mM Tris-Cl, 2 mM sodium phosphate, 0.7 mM potassium phosphate, 0.75% Triton X-100 (v/v), 369 mM NaCl, 1.35 mM KCl, 2 μl/ml dimethyl sulfoxide, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 25 μg p-nitrophenyl, p'-guanidinobenzoate), in the absence or presence of 50 mM NEM. NEM was added to alkylate remaining free sulfhydryls when the extracts were being analyzed for protein-SSG. Protein content was assessed with the BCA assay using BSA as standard.

**Assay of the Thiol-disulfide Oxidoreductase Activities in Cell Lysates—**Lysates of control cells and cadmium-treated cells were analyzed for thioltransferase activity with the standard radiolabel assay (3), which monitors time-dependent release of radioactivity from BSA-SSG\(^{35S}\). Lysates of yeast and PGN buffer with or without GSSG reductase (2 units/ml, final) were warmed separately to 30 °C, mixed, and then an aliquot of BSA-SSG\(^{35S}\) (0.1 mM, final) was added to initiate the reaction (total volume 0.5 ml). Aliquots of the resultant reaction mixtures (30 °C) were precipitated with ice-cold trichloroacetic acid (10%, final) at 0.5, 1, 2, and 3 min. After centrifugation, the supernatants were analyzed for \(^{35S}\)GSH for no more than 20 days. The cells were passed every 2–3 days.

**In Vitro Inhibition of the Thiol-disulfide Oxidoreductase Enzymes by Cadmium—**Cadmium-mediated inhibition of the TDOR enzymes was monitored for 5 min at 340 nm, and thioredoxin reductase activity was followed for 8 min at 405 nm in separate assays. The rates were presented as nanomoles of product/min/mg of cellular protein. Although typical procedures include EDTA in the assay mixtures, we purposely omitted EDTA to avoid competitive chelation of cadmium. Instead buffers were routinely pretreated with Chelex (0.1 g/ml) that had been washed thoroughly with deionized water (18-Megohm resistance).

**In Vitro Inhibition of the Thiol-disulfide Oxidoreductase Enzymes by Cadmium—**Cadmium-mediated inhibition of the TDOR enzymes was
tested in PGN buffer treated with Chelex (as above). GSH was included in all of the inhibition reactions, because it is naturally present in the intracellular environment. Because 5,5'-dithiobis(nitrobenzolic acid) reacts with GSH immediately, thioredoxin reducectase activity was assayed with a lipoamide, a known substrate for thioredoxin reductase (31) that is not reduced by GSH.

All four TDOR enzymes were tested similarly for inhibition by cadmium. Concentrated PGN buffer, containing the respective enzymes, and stock solutions of cadmium in water (or water alone) were pre-warmed (5 min) separately to 30 °C. Then 30 μl of the cadmium solution (or water) was added to the enzyme solutions to achieve final cadmium concentrations of 0–200 μM. The reactions were immediately initiated with the addition of substrate (final volume 200 μl). Each enzyme was tested with seven concentrations of cadmium (0.1–200 μM) encompassing the full range of inhibition for each enzyme.

Activity of thioredoxin transferase (wild type or mutant) in the absence or presence of cadmium was assayed using both spectrophotometric and radiolabel assays (3). The spectrophotometric assay was adapted for use with a 96-well plate reader (Thermomax, Molecular Devices), and yeast GSSG reductase was omitted as a control. The spectrophotometric assay is similar to yeast GSSG reductase, so that the final concentrations of potential cadmium chelation by DTTO. To estimate the magnitude of this DTTO effect, inhibition of thioredoxin reductase by cadmium was tested (as above) in the absence and presence of 0.5 mM DTTO.

Cadmium-mediated Inactivation of Oxidized Thioredoxin—Thioredoxin transferase (wild type) was pretreated with or without cysteine-SG (50 mM) in 100 mM potassium phosphate buffer (pH 7.5) for 10 min at 30 °C. Thioredoxin transferase samples so treated were tested for sensitivity to IAA and cadmium (as above) in the presence of medium until they were passed into larger flasks. A protein adduct formed under conditions of oxidative stress. In all of the inhibition reactions, because it is naturally present in the intracellular environment. Because 5,5'-dithiobis(nitrobenzolic acid) reacts with GSH immediately, thioredoxin reductase activity was assayed with a lipoamide, a known substrate for thioredoxin reductase (31) that is not reduced by GSH.

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Table I

Rates of BSA-SSG([35S]) deglutathionylation by cell lysates

| Enzyme System | k_{cat} (min^{-1}) | K_M (mM) | k_{cat}/K_M (min^{-1} mM^{-1}) |
|---------------|--------------------|----------|-------------------------------|
| Thioltransferase | 2200 | 0.04 | 55000 |
| Thioredoxin | 8 | 0.7 | 11 |

The two enzymes were assayed in K phosphate buffer pH = 7.5 with NADPH and GSH and 2 units/ml GSSG reductase (human thioltransferase), or with NADPH and 90 nM thioredoxin reductase (E. Coli thioredoxin). The activities were measured with various concentrations of cysteine-SSG (2–1000 μM), and the non-enzymatic rates were subtracted. The substrate concentration dependence of the initial rates were plotted as velocity vs. [cysteine-SSG], and the data were fit to a rectangular hyperbolic relationship by non-linear least squares analysis to obtain the respective values for k_{cat} and K_M. At least three determinations of initial rates were measured for each concentration of cysteine-SSG.

Inactivation of Cellular Thioltransferase by Cadmium—To test whether cellular thioltransferase was inactivated, H9 and Jurkat cells were treated with cadmium (0–100 μM) for 30 min in PBS. Cell-derived thioltransferase activity was lost in a dose-dependent manner as reflected by the diminution in the rate of reduction of BSA-SSG[35S] as a function of cadmium concentration (Fig. 1A). This inactivation was irreversible, because removal of cadmium from the cell lysates by overnight dialysis at 4 °C did not change the percentage activity remaining in the cadmium-treated samples relative to control samples dialyzed in parallel (data not shown). Cellular GSSG reductase was also inhibited by cadmium over the same concentration range as thioltransferase (Fig. 1B). The lack of observable cellular thioredoxin-mediated reduction of protein-SSG (Table I) indicates that the effect of cadmium on deglutathionylation can be ascribed exclusively to the thioltransferase system.

To investigate the effect of cadmium on thioltransferase-mediated catalysis of reduction of endogenous protein-SSG in situ, cells were pretreated with 0.5 mM H_2O_2 in PBS to maximize intracellular protein-SSG formation. After replacement of the H_2O_2-containing PBS with complete medium, the initial rates (=25% substrate depletion) of deglutathionylation of endogenous protein-SSG in control (0–0.5 min) and cadmium-treated cells (0–2 min) were found to be 0.47/min and 0.12/min, respectively (Fig. 2). This difference corresponds to a 75% net loss of protein-SSG deglutathionylation capacity after exposure of cells to cadmium plus H_2O_2 compared with H_2O_2 alone, however, the control rate may be an underestimate, because it is defined only by the earliest point that could be measured.

Inactivation of Cellular GSSG Reductase by Cadmium—GSH-independent reactions, respectively).

Inactivation of Cellular Thioltransferase by Cadmium—To investigate the effect of cadmium on thioltransferase activity by cadmium treatment: H9 cells (dotted bars) and Jurkat cells (solid bars) in PBS were treated with various concentrations of cadmium for 30 min. Lysates were prepared and assayed for protein content and thioltransferase activity as described under "Experimental Procedures." Each experiment was repeated at least seven times, and the activities are presented as nanomoles/min/mg (mean ± S.E.).

The loss of in situ protein-SSG deglutathionylation activity (Fig. 2) is consistent with cadmium-mediated inactivation of cellular thioltransferase activity measured in vitro (Fig. 1A). The 75% inactivation (intact cells) was somewhat different from the 90% inactivation (measured in vitro), but this apparent discrepancy can be reconciled (see "Discussion").

Cadmium Affects the Accumulation of Protein-SSG—We tested whether cadmium treatment of H9 and Jurkat cells would result in accumulation of cellular protein-SSG as observed in mouse neuronal cells (HT4) (26, 37). Cadmium treatment alone did not lead to a substantial change in detectable protein-SSG in H9 or Jurkat cells (data not shown), suggesting less basal oxidative stress in H9 and Jurkat cells compared with HT4 cells. Nevertheless, cadmium treatment did inacti-
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Fig. 2. Time courses of intracellular deglutathionylation of protein-SSG[35S] after treatment with H2O2 (●) or cadmium and H2O2 (○). The cellular pool of GSH was radiolabeled as described under “Experimental Procedures,” and the cells were treated with cadmium in PBS; 20 min into the cadmium treatment the control cells and cadmium-treated cells were further treated with 0.5 mM H2O2 for 10 min at 37 °C to produce intracellular protein-SSG[35S]. The cells were resuspended (an aliquot was taken as the t = 0) and centrifuged, and the cell pellets were resuspended in replete medium at 37 °C. Then 1-ml aliquots were taken at the time points indicated and centrifuged, and the cell pellets were lyzed in the presence of 50 mM NEM. These lysates were dialyzed to remove free radiolabel and then analyzed for DTT-releasable radioactivity ([35S]GSH). The amount of [35S]GSH released by DTT at t = 0 corresponded to 50 ± 2.5% and 50 ± 2.3% of total protein-associated radioactivity for control and cadmium-treated cells, respectively. The data are presented as fraction of total protein-associated [35S]GSH (mean ± S.E., n = 3) compared with what was released at t = 0 (maximum release). The initial rates were calculated from the data for the 0- to 0.5-min time period for control cells and the 0- to 2-min time period for cadmium-treated cells (i.e. where substrate depletion was not greater than 25% in either case).

We then tested whether cadmium pretreatment altered the pattern of protein-SSG formation induced by H2O2. In fact, cells treated with cadmium and H2O2 gave a very similar array of protein-SSG[35S] bands on SDS-polyacrylamide gel electrophoresis as did the cells treated with H2O2 alone (Fig. 3B). This result indicates that cadmium was not an effective competitor for the majority of glutathionylation sites on thiol-containing proteins. This observation was confirmed by densitometric measurements of the entire lanes (see Fig. 3 legend), and it is consistent with the affinity of cadmium for vicinal thiols versus isolated thiols that serve as glutathionylation sites (see below). The apparent molecular masses of the predominant glutathionylated proteins in H9 cells after maximal H2O2 treatment (± cadmium) were 97, 64, and 45 kDa, and a 23-kDa doublet.

Relative Sensitivity of the Thiol-disulfide Oxidoreductase Enzymes to Cadmium Inhibition in Vitro—Because all of the TDOR enzymes contain vicinal thiols at their active sites, we determined their relative sensitivities to inhibition by cadmium in vitro. GSH was included in all of the reaction mixtures to mimic intracellular thiol status. Among the four TDOR enzymes tested, thioredoxin reductase was the most sensitive (Fig. 4, IC50 ~ 0.2 μM). Thioltransferase and GSSG reductase both displayed IC50 values near 1 μM. The apparent IC50 value for thioredoxin was 40 μM in the presence of 0.5 mM DTT (which is necessary for turnover of thioredoxin in the absence of thioredoxin reductase). To test the potential effect of DTT on cadmium inhibitory potency, we retested cadmium inhibition of thioredoxin reductase in the presence of 0.5 mM DTT, and found the cadmium concentration-response relationship shifted to the right by about a factor of 5 (data not shown).

Accordingly, the IC50 for inhibition of thioredoxin in the absence of DTT can be estimated at 8 μM, as depicted by the solid line in Fig. 4.

To test further the supposed vicinal thiol chelation mechanism of cadmium inactivation/inhibition, the cadmium sensitivity of unaltered thioltransferase was compared with two altered forms; namely, (a) oxidized thioltransferase, where the active site vicinal thiols are converted to an intramolecular disulfide (C22-SS-C25), and (b) mutant thioltransferase (C7S, C25S, C78S, C82S) where the active site vicinal thiols are now a thiol (Cys22) and a hydroxyl (Ser25). Pretreatment of wild type thioltransferase with disulfides converts the active site to C22-SS-C25 and protects the enzyme from alkylation inactivation by iodoacetic acid (1, 38). Therefore, thioltransferase was preincubated in the absence or presence of cysteine-SSG, followed by treatment with no agent (control), IAA, or cadmium. The oxidized thioltransferase retained full activity, whereas the reduced (dithiol) thioltransferase was inactivated by IAA and cadmium consistent with the mechanism of vicinal thiol chelation for cadmium-mediated inactivation/inhibition (Table III). The mutant thioltransferase was 10-fold less sensitive to cadmium-mediated inactivation (IC50 ~ 10 μM) compared with wild type (1 μM). The residual inhibitory effect of cadmium on the mutant thioltransferase is likely due to the conservative substitution of cysteine with serine, which retains some ability to coordinate with cadmium to form a chelate complex. In fact, this inhibition was fully reversed upon dilution, in contrast to the wild type enzyme (Table III), i.e. the mutant without vicinal thiols was insensitive to irreversible inactivation.

Effects of Cadmium Treatment on Cell Viability—Cellular toxicity, apoptosis, and necrosis have previously been reported as consequences of cadmium treatment, but these responses have usually been determined after exposures much longer than those used in the present study. Therefore, we measured the effect of acute cadmium exposure on cell viability. A 30-min cadmium treatment followed by replacement of the medium resulted in inhibition of cell growth followed by cell death as measured by trypan blue exclusion (Fig. 5). Death of cadmium-treated cells compared with control cells was evident at 12 h for both cell lines. However, H9 cells (Fig. 5, left panels) were more sensitive to cadmium than were Jurkat cells (Fig. 5, right panels).

Whether apoptosis accounted for cadmium-mediated cell death was determined using three different methods. Nuclear staining with acridine orange and ethidium bromide showed characteristic apoptotic morphology in both cell types, but H9 cells were more sensitive than Jurkat cells. H9 cells also showed clear DNA laddering, whereas Jurkat cells showed none (data not shown). The apoptotic response to cadmium was quantified by flow cytometry of propidium iodide-stained cells (Table IV). By all measures H9 cells were more sensitive to cadmium-mediated apoptosis and cell death than were the Jurkat cells.

Transfection of Cells with Antisense Thioltransferase cDNA Impacts Cell Survival—To manipulate the level of thioltransferase directly, cells were transfected with sense or antisense thioltransferase cDNA (Fig. 6). It was expected that derivative cell lines with various amounts of thioltransferase would be established by manipulating K562 cells (relatively low endogenous thioltransferase) and Jurkat cells (relatively high endogenous thioltransferase). Remarkably, in three separate experiments with both K562 and Jurkat cells, transfection with antisense thioltransferase cDNA was not compatible with cell survival. Cells transfected with antisense cDNA for thioltransferase did not survive selection (Fig. 6B), whereas cells transfected with vector alone (Fig. 6A) or cells transfected with sense...
**FIG. 3.** 
H$_2$O$_2$-induced protein-SSG accumulation in H9 cells ± cadmium pretreatment. A, [35S]GSH-radiolabeled cells were treated with 100 μM cadmium in PBS (dotted bars) or PBS alone (solid bars), as described under “Experimental Procedures.” The cells were then centrifuged and resuspended in replete medium, and 1-ml aliquots were treated with the indicated concentrations of H$_2$O$_2$ for 10 min, and then centrifuged. The cell pellets were analyzed as described under Fig. 2. The data are presented as percentage of total protein radiolabeled by DTT (mean ± S.E., n = 3). B, lysates of radiolabeled H9 cells treated with 0.5 mM H$_2$O$_2$ ± cadmium, were combined and treated ± DTT at equivalent protein concentrations. Equal amounts of protein (1.7 mg) were run on a 10–20% SDS-polyacrylamide gel electrophoresis gel, transferred to a polyvinylidene difluoride membrane, exposed to film, and finally treated with Ponceau S. Right panel, densitometric scan of membrane after staining with Ponceau S. 

**FIG. 4.** 
Inhibition of the thiol-disulfide oxidoreductase enzymes by cadmium. Purified thioredoxin ( ), thioredoxin reductase ( ), and thioredoxin reductase ( ) were assayed for activity in the presence of GSH and cadmium at 30 °C as described under “Experimental Procedures.” The solid line represents the theoretical results for thioredoxin in the absence of DTT (see text). Results are presented as percentage activity remaining ± S.E. The control activities for each enzyme were as follows: Thioredoxin reductase (0.33 units ml$^{-1}$), thioredoxin (1.3 units ml$^{-1}$), GSSG reductase (2.0 units ml$^{-1}$), thioredoxin with 0.5 mM DTT (ΔA$_{340}$ nm min$^{-1}$ ml$^{-1}$ lag time = 18 A$_{340}$ nm min$^{-1}$ ml$^{-1}$).

cDNA for thioredoxin all survived selection (Fig. 6C and data not shown). K562 cells transfected with sense cDNA for thioredoxin displayed expected increases in deglutathionylase activity (Fig. 6C), whereas Jurkat cells transfected with sense cDNA did not display the expected increase (data not shown). The collective data of Figs. 5 and 6 suggest that thioredoxin is required for cell viability (see “Discussion”).

**DISCUSSION**

Thioredoxin and thioredoxin are relatively abundant cytosolic enzymes distributed ubiquitously in nature and considered to play important roles in sulfhydryl homeostasis. The two enzymes are distinguished by different cofactors and by different substrate specificities. Thioredoxin specifically catalyzes the reduction of glutathione-containing mixed disulfides, in particular protein-SSG. Although thioredoxin can catalyze reduction of protein-SSG substrates, it is far less efficient than thioredoxin (Table II) (1, 4, 15, 21) and would not be expected to contribute to physiological deglutathionylation. Reversal of oxidative stress-induced protein-SSG formation has been observed in numerous studies (8–11, 19, 39, 40), but the enzymatic basis for the deglutathionylation was not addressed. In this report the thioredoxin-mediated (GSH-dependent) rate of BSA-SSG$^{[35S]}$ reduction by cell lysates is at least 18 times greater than the thioredoxin-mediated (GSH-independent) rate of BSA-SSG$^{[35S]}$ reduction. Moreover, the time frame of thioredoxin-mediated reduction of BSA-SSG$^{[35S]}$ by cell lysates (3 min) correlates with the time frame of intracellular deglutathionylation observed for control cells shown in Fig. 2. Thus, cellular protein-SSG dethiolase activity is essentially

**TABLE III**

Effects of IAA and cadmium pretreatment on various forms of thioredoxin.

|           | Control | IAA | Cadmium |
|-----------|---------|-----|---------|
| Reduced   | 35.8 ± 3.4 | 5.2 ± 0.5$^a$ | 10.7 ± 1.3$^a$ |
| Oxidized  | 29.1 ± 3.4 | 29.8 ± 1.2 | 22.4 ± 1.5 |
| Mutant    | 18.6 ± 5.8 | 0.3 ± 0.1$^b$ | 22.5 ± 4.7 |

$^a$ Significantly different from control (p < 0.005).

$^b$ Significantly different from control (p < 0.1).
entirely attributable to cellular thioltransferase activity (Table I). Previously we found that thioltransferase accounted for all of the hemoglobin-SSG deglutathionylase activity of human hemolysates (2, 41), and Jung and Thomas (4) ascribed the major deglutathionylase activity of rat hepatocytes to thiol-transferase. Thus, it seems appropriate to generalize that thioltransferase is the primary intracellular catalyst of protein-SSG deglutathionylation.

The Effect of Cadmium on the Thiol-disulfide Oxidoreductase Enzymes—In the current study, cadmium treatment of cells in a range shown previously to alter cellular functions (24) inactivated intracellular thioltransferase and its coupling enzyme GSSG reductase (Fig. 1), consistent with in vitro effects of cadmium on these two enzymes (Fig. 4). Our results for mammalian GSSG reductase agree with those of Muller (42) but differ from another study that reported no inhibition (43). However, the latter study included EDTA in the assay of GSSG reductase, likely abolishing the cadmium effect. Mammalian thioredoxin reductase is quite sensitive to cadmium according to our in vitro data (IC50 '0.2 m). This result compares well with cadmium inhibition of E. coli thioredoxin reductase (25), and it is consistent with the reported protective effect of EDTA on mammalian thioredoxin reductase (44). Our in vitro results suggest that the thioredoxin system would be inhibited at lower concentrations of cadmium in situ than those that inhibit the thioltransferase system and intracellular protein-SSG de-thiolation. This analysis further supports the conclusion that only the thioltransferase system is pertinent to intracellular protein-SSG dethiolation.

The Mechanism of Cadmium-Enzyme Interaction—As suggested previously (25), the likely mechanism of cadmium-mediated inactivation/inhibition of the TDOR enzymes is coordination with the vicinal thiol groups contained in all four of these enzymes. Our current data support this interpretation in several ways: 1) oxidized thioltransferase (C22-SS-C25) was insensitive to cadmium-mediated inactivation (Table III); 2)

![Fig. 5. Inhibition of cell growth and cell death after cadmium treatment.](image-url)

Cells were treated with cadmium for 30 min in PBS, then this solution was removed and replaced with replete medium for the indicated times (see “Experimental Procedures”). Cell aliquots were incubated with trypan blue for 5 min, and then all cells were counted and analyzed for trypan blue exclusion. The results are expressed as cells/ml (△, H9; ○, Jurkat) and percentage of live cells (■). Left panels, H9 cells; right panels, Jurkat cells.

| Cadmium (µM) | H9 Cells % Apoptosis | Jurkat Cells % Apoptosis |
|--------------|-----------------------|--------------------------|
| 0            | 1 ± 0                 | 1 ± 1                    |
| 25           | 14 ± 8                | 2 ± 1                    |
| 100          | 74 ± 7                | 17 ± 5                   |

This table shows cadmium-induced apoptosis in H9 and Jurkat cells. Cells were treated with cadmium for 30 min, then the treatment solution was removed and replaced with fresh medium, as described under “Experimental Procedures”. The cells were returned to the 37 °C incubator for 23.5 hrs, at which time approximately 1.5 × 10⁶ cells were stained with propidium iodide (see “Experimental Procedures”), and analyzed by flow cytometry. The values represent the mean from 4 experiments ± SE.

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Cadmium, Thioltransferase, Protein-SSG Reduction, and Apoptosis

The Involvement of Cellular Cofactors in Thiol-disulfide Homeostasis—Cellular thioltransferase activity can be affected both directly (inactivation/inhibition) and indirectly (depletion of the second substrate, GSH). Others have reported that GSH is a necessary factor for cellular reduction of protein-SSG, and these results are consistent with thioltransferase being responsible for enzymatic deglutathionylation. In particular, regeneration of GSH by NADPH and GSSG reductase was reported to be the rate-limiting step in erythrocyte reduction of protein-SSG, and this was linked to erythrocyte glucose 6-phosphate dehydrogenase activity. Glucose 6-phosphate dehydrogenase was reported to be inhibited partially by cadmium in hepatocytes, but it was less sensitive than GSSG reductase (42). In another context we found that, in genetically altered CHO cells where glucose-6-phosphate dehydrogenase was knocked out and NADPH generation was compromised, endogenous reduction of disulfides was also impaired (45). These altered CHO cells appeared to be devoid of reduction activity attributable to the thioredoxin system; however, activity attributable to the thioltransferase system (GSH-dependent) was diminished but not abolished (45). This distinction is likely due to the direct dependence of the thioredoxin system on NADPH for turnover, whereas the thioltransferase system can operate without NADPH so long as the supply of GSH is sufficient (both for reduction of thioltransferase and formation of glutathionyl-disulfide substrates). Two mechanisms, de novo synthesis and NADPH-dependent turnover of GSSG, maintain cellular GSH. In the current study H2O2-treated cells replenished with medium (Fig. 2) reduced accumulated protein-SSG rapidly (>0.47/min, initial rate), whereas cells maintained in PBS displayed very little reduction of protein-SSG (<0.005/min, i.e. <1% of the rate in replete medium), indicative of GSH deprivation. These results reflect the rate-determining step of the thioltransferase catalytic cycle where regeneration of thioredoxin-S– from the thioredoxin-SSG intermediate requires GSH (3). Thus, cellular protein-SSG status could be altered by several different perturbations that impact on the activity of the thioltransferase system, including changes in GSH concentration, concentration of NADPH, and thioltransferase activity.

Cadmium and Cell Survival—Cadmium has been reported to cause apoptosis and necrosis depending upon the concentration and period of exposure (usually 8 h or more) (46–48). Cadmium treatment (4–50 μM) of CEM-C12 cells, a T-cell line, in medium with 2% serum for 18 h was associated with apoptosis (46). In another report (47) T-cells, monocytes, and B-cells were all shown to undergo cell death in response to cadmium treatment (0.1 μM to 10 mM) but only after 7 h or more of incubation with cadmium. The one exception was T-lymphocytes treated for only 1 h but with 10 mM cadmium, a very high concentration (47). Only 20% of the T-lymphocytes were viable 24 h later; however, apoptosis was not addressed as a mechanism of cell death. In another study CCRF-CEM, Raji, and

**Fig. 6. Viability of cells transfected with plasmids ± thioltransferase cDNA inserted in the antisense orientation.** Jurkat cells (A1) and K562 cells (A2) transfected with pRep containing no insert were viable 6 weeks after selection with hygromycin. Jurkat cells (B1) and K562 cells (B2) transfected with pRep containing the thioltransferase insert in the antisense orientation did not survive selection with hygromycin. C, K562 cells transfected in the sense direction (pRep10) survived selection and displayed increased thioltransferase activity, assayed as described under "Experimental Procedures" using equivalent amounts of cell lysates. Details of selection are described under "Experimental Procedures." Results are representative of three separate experiments.

mutant thioltransferase (vicinal cysteine replaced by serine, C25S) was fully protected against irreversible inactivation by cadmium (Table III), and it was 10-fold less sensitive to inactivation/inhibition by cadmium compared with wild type enzyme; and 3) cadmium treatment did not alter the profile of proteins that are glutathionylated in response to H2O2 treatment (Fig. 3B), consistent with a monothiol nature of these sites. Because cadmium pretreatment did not alter the profile of protein-SSGs induced by H2O2, this means that the endogenous substrates for thioltransferase were the same in control and cadmium pretreated cells. Hence the diminution of protein-SSG reduction by cadmium can be attributed unambiguously to inhibition of the thioltransferase enzyme system rather than to the alteration of substrate.

As hypothesized, the cadmium-mediated inhibition of thioltransferase activity in situ and in vitro (Figs. 1 and 4) correlated with cadmium-mediated inhibition of intracellular protein-SSG dethiolation (Fig. 2). When cells in PBS were treated with 100 μM cadmium, cellular thioltransferase activity measured in vitro was decreased by 90%. When cells in PBS were treated with 100 μM cadmium and 0.5 mM H2O2 and then resuspended in medium, the initial rate of intracellular deglutathionylation measured in situ was decreased by 75%. This apparent difference in inhibitory effect can reasonably be attributed to an underestimate of the initial rate for the untreated cells and/or to differences in conditions of the two experiments. In particular, addition of replete medium can recruit a portion of the endogenous thioltransferase protected from cadmium inactivation by formation of the intramolecular disulfide (C22-S-S-C25) in response to the oxidative H2O2 treatment, a modification readily reversed by GSH. In addition, a small portion of the net deglutathionylation in situ may be due to direct action of GSH that is not inhibited by cadmium and cannot be assessed separately as it can be in the in vitro measurements. Thus the in situ experiment (Fig. 2) probably reflects an underestimate of the actual sensitivity of thioltransferase to inactivation by cadmium.

In the current study H2O2-treated cells replenished with medium (Fig. 2) reduced accumulated protein-SSG rapidly (>0.47/min, initial rate), whereas cells maintained in PBS displayed very little reduction of protein-SSG (<0.005/min, i.e. <1% of the rate in replete medium), indicative of GSH deprivation. These results reflect the rate-determining step of the thioltransferase catalytic cycle where regeneration of thioredoxin-S– from the thioredoxin-SSG intermediate requires GSH (3). Thus, cellular protein-SSG status could be altered by several different perturbations that impact on the activity of the thioltransferase system, including changes in GSH concentration, concentration of NADPH, and thioltransferase activity.
Molt-3 cells were treated with cadmium in media containing 5% serum for 18 h, and all three cell lines underwent apoptosis at low concentrations of cadmium but underwent necrosis at higher concentrations (above 50 µM) (48).

In contrast, we examined whether a more limited cadmium exposure (30 min), which inactivates cellular thioltransferase activity (Fig. 1A), would lead to cell death or apoptosis. In H9 and Jurkat cells, 30-min cadmium treatment followed by incubation in fresh medium for 12 or more additional hours resulted in decreased cell viability according to trypan blue exclusion (Fig. 5). Cadmium treatment also caused growth inhibition and inflicted irreversible damage that led to death by apoptosis in a concentration-dependent manner (Figs. 5 and 6 and Table IV). Thus, loss of thioltransferase correlates with initiation of cellular apoptosis. However, Jurkat cells were less sensitive than H9 cells to cadmium-mediated apoptosis and cell death. This difference cannot be ascribed solely to loss of thioltransferase activity, because Jurkat and H9 cells displayed similar cadmium-concentration dependent losses (Fig. 1A). The basis for different extents of apoptotic cell death in these two cell types is unknown, possibly reflecting differences in regulation of the cellular apoptotic machinery among different cell types. A report (49) published after completion of the current study also showed relatively short term (2 h) exposure to cadmium (200 µM) led to apoptosis in U-937 cells. The authors suggested p38 mitogen-activated protein kinase activation as a specific event in initiation of apoptosis. Because p38 mitogen-activated protein kinase is activated by oxidative stress (50), and cadmium inhibition of thioltransferase exacerbates oxidative stress via interference with sulphydryl homeostasis, the two studies are complementary and identify mechanisms of cadmium action that may be related sequentially and/or synergistically.

The concept of initiation of apoptosis by modulation of thiol status has been addressed previously (51–55). Increasing the level of GSH is associated with inhibition of apoptosis mediated by a variety of known stimulators (56–59). Loss of GSH has also been shown to sensitize cells to apoptotic induction, but loss of GSH was not sufficient in itself to initiate apoptosis (52). These findings suggest that GSH acts to protect against the modification of sensitive protein-SH groups and further that a sulphydryl modification is necessary to initiate apoptosis by this pathway. Caspases, cysteine proteinases implicated as mediators of apoptosis, were shown to be regulated by changes in redox status (i.e. changes in GSH or thioredoxin) (54, 60), and elevated levels of cellular thioredoxin have been correlated with inhibition of apoptosis (61). We hypothesize that cellular apoptosis could be initiated by addition of sensitive sulphydryl groups on one or more specific proteins and that when the repair mechanisms for these proteins (thioltransferase and thioredoxin systems) are overwhelmed an apoptotic response occurs.

**Thioltransferase and Cell Survival**—To enable studies of the cellular function(s) of thioltransferase more directly we set out to alter the levels of thioltransferase by transfecting cells with sense and antisense thioltransferase cDNA. K562 cells transfected in the sense direction survived selection and showed increased levels of thioltransferase activity (Fig. 6C) and corresponding increases in thioltransferase protein content (data not shown). Clones derived from such transfected K562 cells maintained high levels of thioltransferase. However, after we froze the cells for storage and re-thawed them for analysis, they no longer retained high thioltransferase activity, and this led us to explore other cell types. Because the H9 and Jurkat lines had higher levels of endogenous thioltransferase, we attempted to diminish its content by transfection with antisense cDNA. Jurkat cells (and K562 cells) transfected with antisense cDNA for thioltransferase did not survive selection, whereas the cells transfected with empty vector did survive (Fig. 6, A and B). This suggests that loss of cellular thioltransferase was incompatible with cell survival.

In a study by another research group (62) the levels of thioltransferase were increased by transfection in some MCF-7 cells, but further increases were not observed in a cell line that already had elevated amounts of endogenous thioltransferase through chemical induction. They offered the interpretation that high levels of thioltransferase might be cytotoxic. In our K562 cells increased thioltransferase activity was not harmful (Fig. 6C), and Jurkat cells transfected with thioltransferase cDNA in the sense direction survived selection but did not show increased amounts of thioltransferase (data not shown). Thus, our results parallel the findings of Meyer and Wells (62). Although high levels of thioltransferase might be toxic in some situations, an alternative explanation is that cells may have mechanisms of feedback regulation at either the protein or mRNA level that limit the steady-state amount of thioltransferase. MCF-7 cells with higher levels of thioltransferase (62, 63) displayed resistance to Adriamycin, a mediator of oxidative stress. We interpret the correlation between increased thioltransferase and resistance to Adriamycin-induced oxidative stress as indicative of prevention of protein-SSG accumulation that could trigger apoptosis.

The process of reversible S-glutathionylation of key proteins has many potential cellular functions (2, 15, 18, 40, 64). The transmission of cellular signals is crucial to many cellular processes, including differentiation, proliferation, and apoptosis. The implication that various types of modification of sensitive sulphydryl groups are involved in these processes suggests that both thioltransferase and thioredoxin contribute to cellular regulation. Because they have different substrate preferences, they are predicted to have different but synergistic roles. Thus, loss of both TDOR systems (as in cadmium treatment) would make a cell especially prone to apoptosis.

**Conclusions**—Cadmium is a toxic agent that inhibits all of the enzymes of the thioltransferase and thioredoxin systems via interaction with vicinal thiols. This allowed us to show that thioltransferase is the preponderant mechanism of protein-SSG reduction in H9 and Jurkat cells. We also showed that acute treatment of cells with cadmium initiates cell death by apoptosis. Loss of thioltransferase activity is a common factor in cadmium- and antisense-mediated cell death, suggesting that loss of thioltransferase is an event that may initiate apoptosis. Together, these results reinforce the concept that thioltransferase is a key intracellular player in the enzymatic regulation of redox-sensitive proteins.

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