The nonclassic secretion of thioredoxin is not sensitive to redox state

Marcel Tanudji, Sarah Hevi, and Steven L. Chuck


Thioredoxin (Trx) is a small, redox-active, cytosolic protein found in plants, bacteria, and eukaryotic organisms that has diverse cellular functions. In humans, a single gene for Trx codes for a protein of 105 amino acids with a molecular mass of 12 kDa that forms a compact spherical structure. Trx is an antioxidant that helps to maintain the cytosolic redox environment for proper protein folding and activates transcription factors such as necrosis factor κB (NF-κB) and activator protein-1 (AP-1). The active site of human Trx consists of Cys32-Gly-Pro-Cys35, and this motif is highly conserved among various species.

Trx can also alter the function of lymphocytes and neutrophils. Exogenous Trx protects lymphoid cells against TNF-α or hydrogen peroxide-mediated cytotoxicity and prevents apoptosis caused by depletion of glutathione and L-cysteine. In contrast to these beneficial effects, elevated plasma levels of Trx can block chemotaxis by neutrophils in response to lipopolysaccharide. An elevated level of plasma Trx was found to be associated with decreased survival in patients with acquired immune deficiency syndrome (AIDS) and correlated with reduced chemotaxis of neutrophils. Chemicals that are antioxidants or that increase glutathione could lower levels of Trx and thereby diminish its detrimental effects on neutrophils. N-acetylcysteine (NAC) is itself an antioxidant.
and also increases cellular glutathione. The administration of NAC increased survival in patients with AIDS (24).

Treatment with NAC was also reported to decrease the secretion of Trx by human T-cell leukemia virus I-transformed T cells (24). These findings raise the issue of whether NAC blocks the nonclassic secretory pathway or simply lowers cytosolic levels of Trx. We investigated this issue because no specific inhibitor of the nonclassic secretory pathway used by Trx is known. We also examined whether the secretion of Trx is sensitive to the redox status of the cell or Trx itself.

We investigated the heterologous secretion of human Trx from Chinese hamster ovary (CHO) cells. This approach enables the study of mutant forms of Trx without cross-reacting, endogenous human Trx. Similar to cancer cells that secrete endogenous Trx, transfected CHO cells secrete human Trx slowly but efficiently. This report is not inhibited by BFA, indicating that a nonclassic pathway is used. We characterize the secretion of Trx from CHO cells and demonstrate that its exportation does not depend on either the redox status of the cell or a functional active site in Trx.

**METHODS**

_Reagents_. Emmanuelle Wollman kindly provided the plasmid-encoding Trx (44), and David Silberstein generously provided polyclonal antibodies for Trx (3). Monoclonal antibodies against the myc epitope were purified by protein G affinity chromatography from media of MYC 1-9E10.2 cells (10). Lipofectamine 2,000, OPTI-MEM, Glutamax I, penicillin/streptomycin, fetal bovine serum, and protein A-agarose were purchased from Invitrogen. BFA was purchased from BioRad. Lipofectamine 2,000, OPTI-MEM, Glutamax I, penicillin/streptomycin, 1% nonessential amino acids, 1 mM L-glutamine. All cells were grown in 37°C medium (DMEM, 1% non-essential amino acids, 1 mM L-glutamine). For each 6-cm dish. Each of the components was resuspended to 500 µl of 5% SDS-methionine for 30 min at 37°C in 5% CO2 and labeled with 100 µCi/ml 35S-methionine for 30 min. At the end of the labeling period, the cells were washed once with chase medium (DMEM, 1% penicillin and streptomycin, 1% nonessential amino acids) and incubated for the indicated amount of time.

_Metabolic labeling with [35S]methionine_. CHO cells were incubated in methionine-free medium (DMEM minus methione and cysteine, 5% fetal bovine serum, 1% Glutamax I, 1% non-essential amino acids, 1x penicillin and streptomycin) for 30 min at 37°C in 5% CO2 and labeled with 100 µCi/ml 35S-methionine for 30 min. At the end of the labeling period, the cells were washed once with chase medium (DMEM, 1% penicillin and streptomycin, 1% nonessential amino acids) and incubated for the indicated amount of time.

_Metabolic labeling of MCF7 and HT-29 cells_ was performed similarly, except the cells were starved for 1 h and labeled for 1 h with 200 µCi/ml of [35S]methionine. Some cells were treated with 1 µg/ml BFA during the starvation, labeling, and chase periods.

**Immunoprecipitation, SDS-PAGE, and phosphor imaging.** For each dish of cells, the medium was collected and cells were lysed by the addition of 1 ml of 1X TXSBW (1% Triton X-100, 100 mM Tris-HCl, pH 8, 100 mM NaCl) in the presence of 1 mM PMSF. Both cell lysate and medium were centrifuged at 16,000 g for 15 min to remove debris, and the supernatants were transferred to fresh 1.5-ml tubes. Aliquots of cleared cell lysate and medium were saved for assays of lactate dehydrogenase (LDH) (see LDH assays). For immunoprecipitation, 200 µl of 5% TXSBW buffer (5% Triton X-100, 500 mM Tris-HCl, pH 8, 500 mM NaCl) and 1 mM PMSF were added to the cleared medium. Either 4 µl of anti-Trx antibodies or 6 µl of anti-myc antibodies prebound to protein G beads were added per 1 ml of cell lysate or medium. The samples were mixed by inversion and incubated at 4°C for 1 h before the addition of 10 µl of a suspension of protein A-agarose beads. Samples derived from U937 and IM9 cells were precleared of endogenous antibody by overnight rotation with 10 µl of a suspension of protein A-agarose beads before immunoprecipitation. The samples were rotated at 4°C overnight, washed twice in 1X TXSBW and twice in Tris-NaCl (100 mM Tris-HCl, pH 8, 100 mM NaCl), and resuspended in 1X SDS-PAGE buffer with 500 mM 1,4-dithiothreitol (DTT). The samples were incubated at 37°C for 30 min before boiling and loaded on a 15% polyacrylamide gel. At the completion of electrophoresis, the gels were destained for 30 min, soaked in 1 M sodium salicylate for 30 min, and dried. The gels were exposed to X-ray film for viewing or a phosphor-imaging screen (Molecular Dynamics) for quantitation.

**LDH assay.** Assays for LDH were carried out on 5-µl samples of cell lysate and medium after the chase period to...
Fig. 1. Thioredoxin (Trx) is secreted from human cells via a brefeldin A (BFA)-insensitive pathway. MCF-7, HT-29, U937, and IM9 cells were incubated in methionine-free medium for 1 h, labeled with [35S]methionine for 1 h, and chased in complete medium for 6 h (see METHODS for details). One dish of each cell line was treated with 1 μg/ml BFA, beginning with methionine starvation, until the end of the chase period [indicated by + over the lanes]. Cell lysates (C) and media (M) were collected and subjected to immunoprecipitation with anti-Trx antibodies. The washed immunoprecipitates were resolved by SDS-PAGE and fluorography. %Sec, percentage of Trx secreted as measured by a PhosphorImager.

Nonclassic secretion of endogenous Trx by human cells. Trx is secreted from lymphoblastoid cells, fibroblasts, airway epithelial cells, and cells derived from tumors of breast and colon cancers (4, 28, 30, 31, 37, 38, 40). Export of Trx via a nonclassic pathway has been demonstrated only for activated lymphocytes (28). We assayed the secretion of Trx from a variety of cells expressing endogenous Trx to verify that secretion is nonclassic and to obtain a reference with which to compare secretion from heterologous cells. From 14 to 44% of Trx is secreted from MCF-7 (breast cancer), HT-29 (colon carcinoma), U937 (histiocytic lymphoma), and IM9 (multiple myeloma) cells (Fig. 1). To demonstrate that Trx is secreted from these cells via a nonclassic pathway, pulse-chase assays were repeated in the presence of BFA to block ER-Golgi transport. BFA did not block the secretion of Trx from these cells. Assays for LDH indicated that <3% of total cellular LDH was present in the medium after 4 h (data not shown), verifying that the extracellular Trx did not arise from cell lysis. Thus Trx is actively secreted from these cells via a BFA-insensitive, nonclassic pathway.

Nonclassic secretion of human Trx in a heterologous cell system. To test the role of the active site or other residues on the secretion of Trx, we searched for a mammalian cell line that secretes Trx nonclassically but lacks cross-reacting, endogenous Trx. We found that CHO cells offer several advantages for these studies. First, CHO cells do not synthesize and therefore do not secrete endogenous Trx that could interfere with our assay for secretion. Pulse-chase analysis of untransfected CHO cells with [35S]methionine and subsequent immunoprecipitation with anti-Trx antibodies detected no cross-reactive protein bands corresponding to endogenous Trx in either the cell lysate or medium (Fig. 2A, lanes 1 and 2). Treatment of these cells with BFA did not alter this result (Fig. 2A, lanes 3 and 4). Second, CHO cells transiently transfected with a plasmid encoding human Trx readily express an immunoreactive 12-kDa protein in the cell (Fig. 2A, lane 5). Third, Trx is also secreted into the medium (Fig. 2A, lane 6) in proportions comparable to those of cells that secrete endogenous Trx (Fig. 1). Trx is secreted via a nonclassic pathway from CHO cells because BFA did not affect this secretion (Fig. 2A, lanes 7 and 8) in contrast to its complete inhibition of prolactin secretion via the ER-Golgi pathway (Fig. 2B, lanes 3 and 4). Assays for LDH showed that <5% cell lysis occurred during the 6-h chase period (data not shown). Thus Trx is actively exported through a nonclassic pathway in CHO cells. Overall, CHO cells are a model system to study the export of Trx because these cells do not contain cross-reactive 12-kDa proteins, synthesize and export human Trx nonclassically in amounts comparable to cells that secrete endogenous Trx, and are readily transfected in contrast to the tumor cells in Fig. 1.

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**%Sec.**
PPL

Fig. 2. Trx is secreted from transiently transfected Chinese hamster ovarian (CHO) cells through a nonclassic pathway. A: CHO cells were transfected with a plasmid encoding human Trx, incubated in methionine-free media for 30 min, labeled with [35S]methionine for 30 min, and chased in complete media for 6 h in the absence (lanes 1, 2, 5, and 6) or presence (lanes 3, 4, 7, and 8) of 1 μg/ml BFA as in Fig. 1. Cell lysates (C) and media (M) were collected and subjected to immunoprecipitation with anti-Trx antibodies. %Sec, percentage of Trx secreted into the media. B: CHO cells were transfected with a plasmid encoding preprolactin-myc (PPL), and the experiment in A was performed in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of BFA. The cell lysates and media were immunoprecipitated with anti-myc antibodies. %Sec, percentage of PPL secreted into the media.
Kinetics of Trx secretion by transiently transfected CHO cells. We next investigated the kinetics of Trx secretion. CHO cells transfected with Trx were labeled with [35S]methionine and chased at 37°C in medium lacking serum for 2, 4, 6, and 8 h. After separation by SDS-PAGE, the protein bands were quantitated by using a PhosphorImager, and the relative secretion for each time point was plotted (Fig. 3). The secretion of Trx is a slow but steady process with up to 50% being secreted into the medium after 8 h. The increase in secretion is not due to cell lysis because an assay for LDH showed that fewer than 5% of cells lysed even after the maximum chase time of 8 h (data not shown). Because an adequate proportion of Trx is secreted into the medium after 6 h of chase, we chose to use this time point for subsequent assays of Trx secretion.

Trx secretion is altered by temperature. Facilitated protein secretion is generally a temperature-dependent process. For example, secretion of proteins via the classic ER-Golgi secretory pathway is affected by alterations in temperature (33). On the other hand, passive diffusion through a pore is not affected by a change in temperature (21). We investigated the temperature sensitivity of Trx secretion to gain insight into whether it is a facilitated or passive process. CHO cells transfected with Trx were starved, labeled at 37°C, and then incubated in serum-free chase medium at 25, 37, and 42°C for 6 h (Fig. 4). As expected, about 40% of Trx synthesized in the cells is secreted after 6 h at 37°C. Lowering the chase temperature to 25°C drastically inhibited Trx secretion. On the other hand, the secretion of Trx increased by 15% when cells were incubated at 42°C during the chase period. However, assays of LDH indicated a 15% increase in cell lysis at this temperature that accounts for much of the increase in Trx in the medium (data not shown).

Trx secretion is affected by factors in serum. Some nonclassic secretory pathways are sensitive to factors in serum. For example, the secretion of HIV-tat and IL-1β is inversely proportional to the amount of serum in the medium (7, 29). However, the nonclassic secretion of other proteins, such as green fluorescent protein (GFP), is not affected by serum (39). We investigated whether factors present in fetal bovine serum also alter the secretion of Trx. CHO cells transfected with the Trx plasmid were starved, labeled with [35S]methionine, and chased in medium containing 0, 5, 10, and 20% fetal bovine serum (Fig. 5). The proportion of secreted Trx decreased as the concentration of serum increased. A similar inhibition of secretion by serum was observed with IM9 cells secreting endogenous Trx (data not shown). Because Trx is found in serum, it is possible that Trx in bovine serum could compete with radiolabeled human Trx for immunoprecipitation. Therefore, we mixed lysates and media of transfected CHO cells with 0, 5, 10, or 20% serum before immunoprecipitation, and we found that there was no effect on the amount of labeled human Trx recovered (data not shown). Thus factors in serum decrease the secretion of Trx by CHO cells.

Secreted Trx is not associated with externalized membrane vesicles. At least one nonclassically secreted protein, galectin-3, is secreted via blebs in the plasma membrane that become externalized vesicles (20). Such a mechanism could enable the posttranslational export of fully folded cytosolic proteins from the cell. We studied whether Trx, likewise, is secreted in exter-

![Fig. 3. Kinetics of Trx secretion by CHO cells. CHO cells transiently transfected with a plasmid encoding Trx were incubated in methionine-free media for 30 min, labeled with [35S]methionine for 30 min, and chased in complete media for 2, 4, 6, or 8 h. At each time point, cell lysate (C) and medium (M) were harvested and immunoprecipitated with anti-Trx antibodies. The graph shows the percentage of Trx secreted as assessed by a phosphor imager for each chase time.](Image)

![Fig. 5. The secretion of Trx is inhibited by factors in serum. CHO cells transiently transfected with a plasmid encoding Trx were starved for methionine for 30 min, labeled with [35S]methionine at 37°C for 30 min, and chased at 25, 37, or 42°C for 6 h. The cell lysate and medium were harvested and immunoprecipitated with anti-Trx antibodies as described in METHODS. %Sec, percentage of Trx secreted.](Image)
nalized membrane vesicles via a similar pathway. CHO cells transiently transfected with the Trx plasmid were labeled, and the medium was collected after 4 or 6 h. By examining the medium at two different times during the chase period, we would be better able to detect Trx if it is in membrane vesicles. The medium was clarified by low-speed centrifugation to remove debris and then subjected to high-speed centrifugation sufficient to pellet any vesicles (20). The supernatant and the pellet were subjected to immunoprecipitation with anti-Trx antibodies. All of the secreted Trx was in the supernatants; none of the secreted Trx was present in the pellet (Fig. 6). These data suggest that Trx is secreted freely into the medium and not in membrane vesicles from CHO cells.

N-acetyl cysteine reduces Trx secretion by downregulating Trx expression. No specific inhibitor of Trx secretion is known. Recently, it was shown that treatment with NAC resulted in the reduction of Trx secretion from ATL-2 cells (24). NAC acts as an antioxidant, and it also increases cellular glutathione levels. We investigated whether NAC actually blocks the secretory pathway used by Trx or whether the cellular level of Trx is simply decreased due to the increased availability of other antioxidants. When NAC is added during the chase period, no acute change in the secretion of Trx from CHO cells was detected (Fig. 7A). We also tested the effect of a longer treatment with NAC. CHO cells transiently transfected with human Trx were incubated with NAC for 16 h before assessing secretion. In these cells treated with NAC overnight, the absolute amount of Trx secreted is diminished compared with untreated cells (Fig. 7B). However, the level of Trx remaining in the cells treated with NAC after 6 h also is reduced so that the proportion of Trx secreted from cells treated with NAC is equivalent to that from untreated cells (Fig. 7B). Thus the reduction of Trx secretion by NAC is not due to a block in the Trx secretory pathway but rather from reduced cytosolic levels of Trx throughout the chase period.

Mutations in the active or regulatory homodimerization sites of human Trx do not impair secretion. Cysteine residues play key roles in the activity of Trx. The active site of human Trx consists of two cysteine residues at positions 32 and 35 relative to the NH$_2$ terminus. Changing either of these cysteine residues to serine renders the redox site inactive. In addition, human Trx contains cysteine at residues 62, 69, and 73. The formation of Trx homodimers that regulate the activity of the protein has been shown to depend on cysteine 73 (42).

We investigated whether the redox or regulatory status of Trx impacts its secretion. We mutated either

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Fig. 7. N-acetylcysteine (NAC) lowers cytosolic levels of Trx but does not block its nonclassic secretion. A: CHO cells transiently expressing Trx were left untreated (Un; lanes 1 and 2) or treated with 10 mM NAC just during the 6-h chase period (Chase; lanes 3 and 4). Cell lysates (C) and media (M) were subjected to immunoprecipitation with anti-Trx antibodies. %Sec, percentage of Trx secreted. B: CHO cells transiently expressing Trx were left untreated (Un; lanes 1 and 2) or treated with 10 mM NAC beginning 16 h before and continuing throughout the labeling and chase periods (O/N; lanes 3 and 4). Cell lysates (C) and media (M) were subjected to immunoprecipitation with anti-Trx antibodies. %Sec, percentage of Trx secreted.

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Fig. 8. Mutation of the cysteine residue at either the active site or the homodimerization site does not inhibit Trx secretion. CHO cells transiently expressing wild-type Trx (WT), a Trx-active site mutant (Trx C35S), or a Trx homodimerization site mutant (Trx C73S) were starved for methionine for 30 min, labeled with [$^{35}$S]methionine for 30 min, and chased for 6 h. At the end of the chase period, the cell lysate (C) and media (M) were collected and immunoprecipitated with anti-Trx antibodies. %Sec, percentage of each Trx protein secreted.
DISCUSSION

Several human cancer cells secrete endogenous Trx by a route other than the ER-Golgi pathway. Although secretion or surface expression of Trx from such tumor cell lines has been demonstrated previously, a nonclassic route of export has not been verified by using BFA for any cell type other than activated lymphocytes (28). Furthermore, this data establishes the proportion of Trx secreted in our assay as a standard for comparing the efficiency of secretion from heterologous CHO cells. Human Trx is secreted from CHO cells through a nonclassic pathway in amounts that compare favorably with tumor cells. The secretion of Trx is a slow but efficient process with up to 50% of the protein exported over 8 h. Secretion of Trx is reduced by low temperature and factors in serum. However, NAC does not affect secretion directly; rather, the antioxidant results in lower levels of Trx in the cytosol and medium. Using this heterologous system to study the secretion of Trx, we demonstrate that mutation of either the redox active site or the homodimerization site does not alter secretion of human Trx.

Trx is a cytosolic protein that is secreted selectively from several types of cells. Although the secretion of Trx from CHO cells is slow, it is specific and not simply due to diffusion or leakage out of the cell. First, low temperature reduces secretion of Trx. Second, we have expressed other cytosolic proteins by using transiently transfected CHO cells but did not observe any secretion into the medium (39). Therefore, the secretion of human Trx from these cells is not due to an artifact induced by liposome-mediated transfection. Third, assays of LDH show that cellular lysis is minimal and does not account for the significant proportion of Trx secreted. Thus human Trx is secreted actively via a nonclassic pathway from CHO cells.

It is interesting that the secretion of Trx from CHO cells is reduced with increasing serum concentration in the medium. Serum likewise inhibits the secretion of HIV tat protein (7). Recently, it has been shown that Trx is present in normal human serum (26). These observations raise the possibility that the nonclassic pathway by which Trx is secreted is inhibited by circulating Trx. Alternatively, other factors in serum block nonclassic secretion of Trx and other proteins such as HIV tat.

The redox status of the cytosol is maintained in part by the activity of Trx. Previously, it was demonstrated that the amount of Trx found in the media of cells treated with NAC is significantly decreased (24). These findings led to the suggestion that NAC blocks the secretion of Trx (24). Our investigations demonstrate that treatment with NAC does not have a direct effect on secretion. Instead, NAC decreases the level of Trx in the cytosol and thereby reduces the amount of Trx secreted. The proportion of Trx secreted, however, remains equivalent in the presence or absence of NAC. Furthermore, rendering the redox site of Trx inactive does not alter its secretion. Thus the redox status of the cell or Trx itself does not impact its secretion.

Proteins that are secreted generally contain a targeting signal to direct their export. In eukaryotic cells, signal sequences usually at the amino terminus of proteins mediate the targeting of proteins to the ER (5). Similarly, amino acid sequences direct secretion from bacteria from the sec-dependent or twin-arginine pathways. Therefore, it appears likely that a region of the Trx molecule functions as an export signal. We used the heterologous secretion of human Trx to study the effect of mutating the active site or a residue used in the formation of homodimers. Neither of these mutations significantly affected the secretion of Trx, suggesting that these biological activities are not involved in targeting. Little is known about targeting signals required for nonclassic protein secretion. The amino-terminal 96 amino acids of galectin-3 are necessary for its secretion (22), but the targeting signals for other nonclassically secreted proteins remain unknown. Our heterologous system of nonclassic secretion could facilitate studies of the export signal in human Trx.

Unlike other nonclassic pathways, the export route of Trx has not been identified. Several other proteins, such as IL-1β, acidic and basic fibroblast growth factor (FGF), galectins 1 and 3, and HIV-tat also appear to be secreted via nonclassic secretory pathways (6, 7, 12, 19, 20, 29). The secretion of these proteins is not attributable to cell lysis. On the basis of their response to inhibitors of secretion including serum, it appears that these proteins do not use one common pathway for nonclassic secretion. An ATP-binding cassette protein has been implicated in the secretion of IL-1β (16), a sodium-potassium ATPase has been linked to the secretion of bFGF (11), and membrane shedding results in the export of galectin-3 (20). However, the export pathway of Trx is unknown. The selective secretion of human Trx by CHO cells indicates that a nonclassic secretory pathway is present in these cells that is capable of recognizing human Trx as a substrate for export. The endogenous substrates secreted by this pathway remain to be identified.

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REFERENCES


