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Redox Control of Caspase-3 Activity by Thioredoxin and Other Reduced Proteins

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Caspases are cysteine proteinases that play a critical role in the execution phase of apoptosis. The active site cysteine residue must be reduced for caspase activity. Thioredoxins are redox proteins that catalyze the reduction of cysteine residues. We have examined the ability of various recombinant human thioredoxins to activate caspase-3. The EC_{50} for caspase-3 activation by reduced thioredoxin-1 was 2.5 μM, by reduced glutathione 1.0 mM and by dithiothreitol 3.5 mM. A catalytic site redox-inactive mutant thioredoxin-1 was almost as active as thioredoxin-1 in activating caspase-3. Caspase activation was shown to correlate with the number of reduced cysteine residues in the thioredoxins. Reduced insulin and serum albumin were as effective on a molar basis as thioredoxin-1 in activating caspase-3. Thus, caspase-3 activation is not a specific effect of thioredoxins but is a property shared by other reduced proteins.

Key Words: **thioredoxin; glutathione; caspase-3; apoptosis.**

Caspases are a family of aspartate-specific cysteine proteinases that play a critical role in the execution phase of apoptosis (1, 2). Caspases are normally present in the cell in the proenzyme form that requires limited proteolysis for enzymatic activity (3). The activated caspases require reduction of a catalytic site cysteine residue as well as other cysteine residues around the catalytic site for enzymatic activity (4, 5). Thiol alkylating agents and spontaneous thiol oxidation inhibit caspase activity (6, 7).

Thioredoxin-1 is a low molecular weight (12 kDa) redox protein (8) found at increased concentrations in a number of human cancers (9–15). Recombinant human thioredoxin-1 stimulates cell growth (13, 16–18) and protects cells from apoptosis (19). Cells stably transfected with human thioredoxin-1 show increased tumor growth and inhibited apoptosis (20, 21). Redox activity is necessary for the growth stimulating and anti-apoptotic effects of thioredoxin-1 (17, 20). The cysteine (Cys) residues at the conserved -Cys-Gly-Pro-Cys-Lys active site of thioredoxin-1 undergo reversible oxidation-reduction catalyzed by the NADPH-dependent flavoprotein thioredoxin reductase (22). Reduced thioredoxin-1 is able to exert redox control through thiol-disulfide exchange over proteins that have cysteine residues that are important for activity. One such group of thioredoxin regulated proteins that have been extensively studied are the redox-sensitive transcription factors. Reduced thioredoxin-1 increases the DNA binding and *trans*activates transcription factors such as NF-κB (23, 24), the glucocorticoid receptor (25, 26) and activator protein-2 (27). Thioredoxin-1 is much more potent in increasing the transcription factor DNA binding than small molecular weight thiols such as 2-mercaptoethanol, L-cysteine, N-acetyl cysteine or reduced glutathione (23, 25, 28). This has lead to the suggestion that thioredoxin-1 specifically increases redox-sensitive transcription factors DNA binding in cells (23, 28).

Because of its ability to reduce cysteine residues reduced thioredoxin-1 might reverse the oxidative inactivation of caspases. However, thioredoxin-1 is an inhibitor of apoptosis (20, 21) and it would be an anomalous finding if thioredoxin were to result in caspase activation which is the final step in apoptosis. We have examined the direct effects of reduced thioredoxins upon caspase activity. Thioredoxin-1 was found to be a more potent activator of caspase-3 activity than either glutathione or dithiothreitol (DTT). However, it was only marginally more active than a catalytically inactive mutant thioredoxin-1 and had similar activity to other reduced proteins in activating caspase-3. Thus, redox activation of caspase-3 *in vitro* is a general feature of reduced proteins and not a specific effect of thioredoxin.

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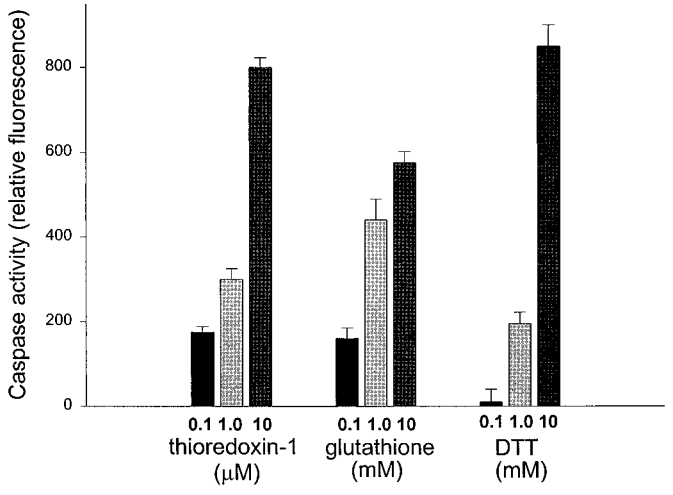

FIG. 1. Stimulation of caspase-3 activity by reduced recombinant wild-type thioredoxin-1, reduced glutathione and DTT. Purified active recombinant human caspase-3 was used for the assay with added thiols at the concentrations shown. Values are the mean of 3 determinations and bars are ±SE.

MATERIALS AND METHODS

Caspase assay. Purified human recombinant active caspase-3 was obtained from PharMingen International (San Diego, CA). Caspase activity was measured using a caspase-3 fluorescent assay kit (Contech Labs Inc., Palo Alto, CA). Assays were performed according to the manufacturer's instructions with modifications for a 96-well flat-bottom plate. Active caspase-3, 400 ng/ml, was used for the reaction and DTT was omitted from the reaction buffer. All reagents were added except for the substrate, mixed and then allowed to incubate for 10 min at 37°C before adding 5 μl of 1 mM fluorescence substrate Ac-DEVD-AFC to 50 μM final concentration. The mixture was incubated for 60 min at 37°C and fluorescence measured at excitation/emission wavelengths of 380/460 nm. Each assay was performed in triplicate.

Oxidized and reduced proteins. Recombinant human wild-type thioredoxin-1 (9), mutant $Cys^{73} \rightarrow Ser$ thioredoxin-1 (C73S) (29), redox inactive catalytic site mutant $Cys^{32} \rightarrow Ser/Cys^{35} \rightarrow Ser$ thioredoxin-1 (C32S/C35S) (17) and recombinant human truncated mitochondrial thioredoxin-2 (30) were expressed in *E. coli* and purified as previously described. Human bovine serum albumin and bovine insulin were obtained from Sigma Chemical Co. (St Louis, MO). Stock solution of the proteins at 100 μM in water were incubated with 10 mM DTT or 5 mM diamide for 2 h at room temperature and then passed twice through a Sephadex G-25 desalting column (Pharmacia, Piscataway, NJ) to remove DTT and diamide. All solutions were gassed with O_2-free He before use.

RESULTS

Caspase-3 activity was completely dependent upon the presence of a reduced thiol added to the assay. DTT, 10 mM, which is typically used in the assay of caspase activity gave maximum activity. Other thiols also activated caspase-3 (Fig. 1). The EC_{50}s for caspase-3 activation by reduced thioredoxin-1, reduced glutathione and DTT were 2.5 μM, 1000 μM and 3,500 μM, respectively. The addition of NADPH and thioredoxin reductase to 10 μM reduced thioredoxin-1 did not result in further increase in caspase-3 activity suggesting that there were sufficient reducing equivalents in the pre-reduced thioredoxin to activate all of the caspase-3 (results not shown).

When other reduced forms of thioredoxin were employed they were also effective at stimulating caspase-3 activity, including the redox-inactive catalytic site mutant thioredoxin-1 C32S/C35S (Fig. 2A). There was a significant negative correlation between the EC_{50}s for stimulation of caspase-3 activity by the different reduced thioredoxins and the number of cysteine residues in the proteins ($r = 0.98$, $P < 0.05$) (Fig. 2B).

Albumin and insulin were also tested for their ability to increase caspase-3 activity and were found to be as effective as effective as thioredoxin, with approximate EC_{50} values of 2.3, 1.0 and 4.1 μM for reduced thioredoxin-1, albumin and insulin, respectively. The oxidized forms of the proteins were considerably less active (Fig. 3).

DISCUSSION

The caspases have an active site cysteine nucleophile (4) and other cysteine residues located near the active site (5) that are essential for catalytic activity. Caspase proteinase activity is lost in the presence of the thiol-oxidizing agent diamide (31), thiol-alkylating agents such as iodoacetamide, *N*-ethylmaleimide (6) and thimerosal (5), oxidized glutathione (7) and dithiocarbamate disulfides (7). The assay for caspase activity is typically carried out in the presence of millimolar concentrations of DTT. We found that without added DTT there was very little caspase activity, presumably due to the spontaneous oxidation of the active site cysteine nucleophile. Other thiols such as reduced glutathione and reduced human thioredoxin could activate caspase activity but thioredoxin-1 was about 400-fold more potent on a molar basis than glutathione and 1400-fold more potent than DTT in activating caspase-3. What was surprising was that the reduced catalytic site redox inactive mutant thioredoxin-1, $Cys^{32} \rightarrow Ser/Cys^{35} \rightarrow Ser$, was almost as active as wild-type thioredoxin-1 in stimulating caspase-3 activity. Human thioredoxin-1 has 3 cysteine residues (Cys^{62}, Cys^{65} and Cys^{73}) in addition to the catalytic site cysteine residues and all cysteine residues appeared to be equally effective at transferring reducing equivalents to activate caspase-3. There was a correlation between the number of cysteine residues in the mutant thioredoxins, 4 in $Cys^{73} \rightarrow$ Ser thioredoxin-1, 3 in $Cys^{32} \rightarrow Ser/Cys^{35} \rightarrow$ Ser thioredoxin-1 and 2 in and truncated mitochondrial thioredoxin-2, and the decrease in caspase-3 stimulating activity. We then showed that other reduced proteins, bovine insulin and human serum albumin, which

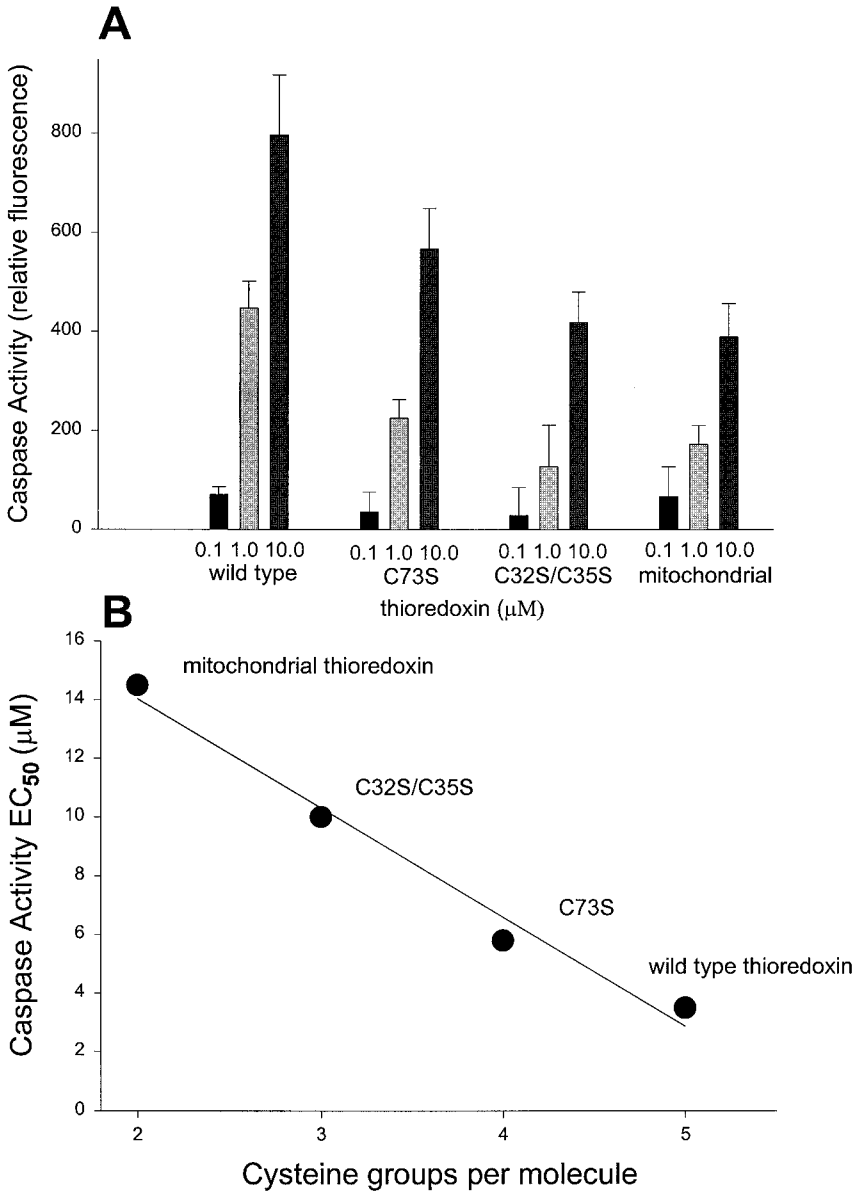

FIG. 2. Effect of different reduced thioredoxins on caspase-3 activity. Purified active recombinant human caspase-3 was used for the assay with reduced wild-type thioredoxin-1, $Cys^{73} \rightarrow Ser$ mutant thioredoxin-1 (C73S), $Cys^{32} \rightarrow Ser/Cys^{35} \rightarrow Ser$ mutant thioredoxin-1 (C32S/C35S) and mitochondrial thioredoxin-2. (A) Concentration response using the thioredoxin concentrations shown. Values are the mean of 3 determinations and bars are ±SE. (B) Relationship between cysteine content of the different reduced thioredoxins and EC_{50} for stimulation of caspase-3 activity. The line is a regression analysis of the data.

have reducible non-structural cysteine residues (32),[2] were equally effective on a molar basis as thioredoxin in activating caspase-3. It, thus, seems likely that in the reducing environment of the cell caspase activity will be maximal as long as there are reduced protein thiols present. Only when thiol buffering capacity is severely depleted as when cells are treated with the thiol depleting agent diamide (31) or NO which nitrosylates thiol groups (33), is caspase activity inhibited. Thioredoxin does not seem any more likely than any other reduced protein to activate caspase activity. Indeed, its concentration in the cell which is around 1 μM, makes it unlikely to play a major role in directly maintaining caspase activity. Indeed, thioredoxin-1 protects cells against apoptosis (19, 21) and would not be expected to increase caspase activity which is responsible for the final execution phase of apoptosis (1). Preliminary studies have shown that MCF-7 breast

[2] Swiss-Prot Accession No. PO2768.

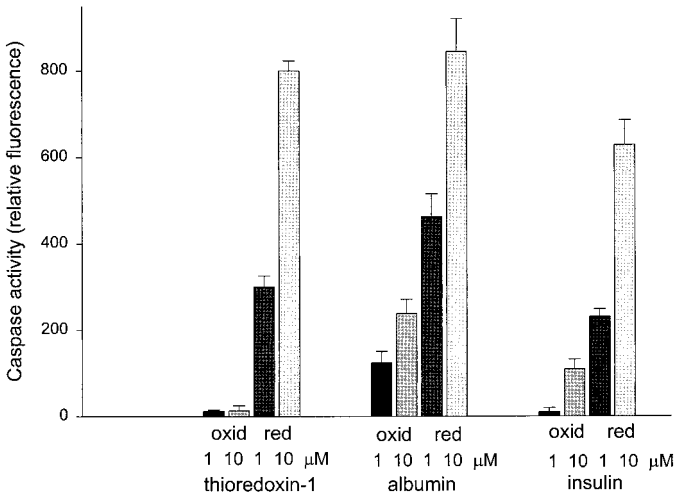

FIG. 3. Effect of reduced and oxidized proteins on caspase-3 activity. The proteins used were oxidized (oxid) and reduced (red) wild-type human thioredoxin-1, human serum albumin and bovine insulin at the concentrations shown. Values are the mean of 3 determinations and bars are ±SE.

cancer cells stably over-expressing thioredoxin have decreased caspase-3 activity upon exposure to apoptotic stimuli (unpublished observations). Thus, thioredoxin may reduce thiol groups on other proteins and indirectly negatively regulate caspase activity.

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