Thioredoxin Peroxidase Is a Novel Inhibitor of Apoptosis with a Mechanism Distinct from That of Bcl-2*

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Thioredoxin peroxidase (TPx) is a member of a newly discovered family of proteins that are conserved from yeast to mammals and to which natural killer enhancing factor belongs. These proteins are antioxidants that function as peroxidases only when coupled to a sulfhydryl reducing system. The physiological function of TPx in cells is not yet known. Here we demonstrate that when the human TPx II, a member of this family, is stably overexpressed in Molt-4 leukemia cells, it protects from apoptosis induced by serum deprivation, ceramide, or etoposide. TPx II, like Bcl-2, is able to inhibit release of cytochrome c from mitochondria to cytosol, and it inhibits lipid peroxidation in cells. TPx II, unlike Bcl-2, could prevent hydrogen peroxide accumulation in cells, suggesting that it functions upstream of Bcl-2 in the protection from apoptosis and may be implicated as an endogenous regulator of apoptosis.

Thioredoxin peroxidase is a member of a family of proteins initially discovered from yeast (1) and rat (2) to be important in protecting glutamate synthetase oxidation by a metal ion catalyzed reaction, and this protection required the presence of a thiol (1). Later it was discovered that this protein indeed acts as a peroxidase but requires thioredoxin or a thiol-containing intermediate to carry on its peroxidase function (3). The gene encoding TPx II has been demonstrated to protect cells from different inducers of apoptosis, again suggesting a role for oxidation products in regulating cell death (15, 16). These considerations prompted us to investigate whether thioredoxin peroxidase could also function as an inhibitor of apoptosis.

EXPERIMENTAL PROCEDURES

Materials

Molt-4 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) at 37 °C in an environment containing 5% CO2 and 95% air. Monoclonal anti-cytochrome c antibody was purchased from Pharmagen. Polyclonal antibody against TPx II was prepared as described (3). 2,7-Dichloro-fluorescin diacetate was from Molecular Probes (Eugene, OR). Etoposide was obtained from Sigma.

Methods

Transfection of TPx II DNA—Molt-4 cells (3 × 10^6/ml) were electroporated with PCRTM 3.1-Uni vector (Invitrogen) and without full-length TPx II DNA (10 μg) (17). A heterogeneous population of positive clones was selected with G418 (Life Technologies, Inc.) and maintained in RPMI 1640 containing 10% FBS, 25 mm HEPES, pH 7.4, and 0.5 mg/ml of G418. To determine the expression of TPx II, whole cell extracts (10 μg/lane) were separated on a 12% SDS-polyacrylamide gel, Western blotted with the polyclonal anti-TPx II antibody, and visualized with the ECL reagents (Amersham) as described (3).

Cell Treatment—Cells (5 × 10^6) were washed twice with phosphate-buffered saline (PBS) and once with serum free RPMI 1640 containing 25 mm HEPES, pH 7.5. Washed cells were seeded at a density of 5 × 10^5/ml in serum free RPMI 1640 containing 25 mm HEPES, pH 7.5. To study the effects of ceramide, etoposide, or H2O2, cells were washed once and seeded (at a density of 5 × 10^5/ml) with RPMI 1640 containing 2% FBS and 25 mm HEPES, pH 7.5. Control cells were treated with appropriate amounts of vehicle (ethanol for ceramide and Me2SO for etoposide) diluted in the same culture medium as that for the drugs. At desired time intervals, aliquots of cells were removed for various assays. Cell viability was assessed by trypan blue exclusion.

PARP Cleavage—Cells (5 × 10^6) were pelleted by centrifugation, rinsed once with ice-cold PBS, resuspended in 100 μl of PBS containing 1 mm EDTA, 1 mm EGTA, and 1 mm phenylmethylsulfonyl fluoride, diluted with 5 × SDS sample buffer, and boiled for 10 min. Intact and cleaved PARP were detected by Western blot as described (18) using anti-PARP anti-serum from Enzyme System Products (Dublin, CA).

Lipid Peroxidation Assay—Cells (5 × 10^6) were washed twice with PBS and lysed by three cycles of repeated freezing and thaw in 300 μl of 200 mm Tris-HCl, pH 7.4. The amounts of representative lipid peroxide, malonaldehyde, were determined spectrophotometrically with a
TPx Inhibits Apoptosis Differently from Bcl-2

RESULTS

We stably transfected Molt-4 leukemia cells with vector control or cDNA encoding the mammalian thioredoxin peroxidase protein TPx II. After selection for stable transfectants, we demonstrated the overexpression of TPx II protein using Western blot analysis (Fig. 1). We next evaluated the cells for their sensitivity to inducers of apoptosis. The cells overexpressing TPx II were resistant to a variety of inducers of apoptosis. Fig. 2A demonstrates that vector-transfected cells were ~25 and ~40% dead by 24 and 48 h following serum deprivation, respectively, whereas cells overexpressing TPx II were significantly resistant to death in response to serum deprivation. Similarly, vector-transfected cells were easily induced to undergo cell death in response to other well known inducers of apoptosis such as ceramide treatment (Fig. 2B), whereas TPx II again protected cells from ceramide-induced cell death. We also tested the ability of TPx II to protect from apoptosis in response to the pharmacologic agent, etoposide, a chemotherapeutic agent that has been demonstrated to induce cell death by apoptosis (21). TPx II, but not vector control, protected cells from undergoing cell death in response to etoposide treatment (Fig. 2C). Bcl-2 has been well documented to protect cells from apoptosis (13). As Fig. 2D demonstrates, Bcl-2 was as effective as TPx II at protecting cells from ceramide-induced apoptosis. To confirm that cell death in response to these inducers was occurring by apoptosis, we evaluated the ability of these inducers to cleave the death substrate PARP (22). TPx II, but not control vector, inhibited PARP cleavage in response to up to 72 h of serum deprivation as well as in response to ceramide treatment (Fig. 2E). These studies demonstrate that TPx II inhibits apoptosis, and it appears to be as effective as Bcl-2.

Cytochrome c has recently been implicated in the apoptotic process, and it has been identified as a component required in a cell free system to induce activation of the protease cascade (23). Cytochrome c appears to be released from the mitochondria into the cytosol in response to several apoptotic stimuli. Bcl-2 has recently been demonstrated to block cytochrome c release and consequently rescues cells from activation of the caspases and cell death (19, 24). Up to this point of evaluation, TPx II appeared to act similar to Bcl-2 in protecting cells from apoptosis; however, it was unclear if TPx II functioned upstream, in parallel, or at the same site as Bcl-2 in the apoptotic pathway. Therefore, in an attempt to order thioredoxin peroxidase in the apoptotic pathway with respect to Bcl-2, we next evaluated its ability to modulate cytochrome c translocation. Cytochrome c release into the cytosol was evaluated in cells that overexpress Bcl-2 as well as cells that overexpress TPx II. We first evaluated if the cell-permeable ceramide analog C6-ceramide is able to induce cytochrome c release into the cytosol, because ceramide has been demonstrated to function upstream of Bcl-2 in the apoptotic pathway (25). The addition of C6-ceramide (20 μM) resulted in release of cytochrome c from mitochondria to cytosol seen as early as 2 h. Importantly, Bcl-2, protected cells from cytochrome c release in response to ceramide (Fig. 3A). Interestingly, TPx II also protected cells as effectively as Bcl-2 from cytochrome c release (Fig. 3B). We also assayed cytochrome c release in response to the chemotherapeutic agent etoposide, and the results show that TPx II (Fig. 3D) was as effective as Bcl-2 (Fig. 3C) in protecting cells from cytochrome c release. Because release of cytochrome c is considered an early event in the commitment to apoptosis, this shows that TPx II functions similar to Bcl-2 in this phase.

To further understand the mechanism by which TPx II inhibits apoptosis and to compare this with Bcl-2, we next evaluated the effects of TPx II on lipid peroxidation, given the biochemical function of TPx II as a peroxidase. Lipid peroxidation has been implicated as an intermediate in the apoptotic pathway that is thought to precede DNA fragmentation and morphological changes of apoptosis (20, 26), but it is not completely clear if it occurs prior to or after mitochondrial events. Fig. 4 shows that TPx II was able to potently inhibit lipid...
peroxidation in response to serum deprivation (Fig. 4A), C₆-ceramide (20 μM) (B), and etoposide (40 μM) (C). Treatments were for the indicated times. D, Bcl-2 also protected cells from lipid peroxidation in response to C₆-ceramide (20 μM) for 2 h. The results are the averages of duplicate determinations and are representative of three experiments.

lipid peroxidation is downstream of the site of action of Bcl-2. Importantly, these results indicated to us that even though TPx II may act as a peroxidase, it inhibited lipid peroxidation at least partly indirectly by inhibiting mitochondrial release of cytochrome c and was still indistinguishable from Bcl-2.

Bcl-2 has been demonstrated to allow cellular hydrogen peroxide accumulation during apoptosis and to prevent the effects of exogenous hydrogen peroxide on cell death (14), suggesting that Bcl-2 functions downstream of an initial oxidative stimulus. We, therefore, evaluated the effects of TPx II on the intracellular accumulation of hydrogen peroxide and the ability of TPx II to protect from hydrogen peroxide-mediated apoptosis. Fig. 5A shows that TPx II protected from hydrogen peroxide for the indicated concentrations and times. C, TPx II protected cells from hydrogen peroxide induced cytochrome c release. D, TPx II but not Bcl-2 protected cells from intracellular H₂O₂ accumulation in response to exogenous H₂O₂ (80 μM) treatment for the indicated time. The results in A and B are averages of duplicate determinations and are representative of three separate experiments. The cytochrome c results shown in C are representative of three experiments. The data in D are the means ± S.D. of duplicate determinations from three experiments.
DISCUSSION

In this study we demonstrate several interesting and novel points about regulation and ordering of the apoptotic pathway. First, we demonstrate that the protein thioredoxin peroxidase is a potent inhibitor of apoptosis. Second, we demonstrate that TPx II inhibits cell death by a mechanism distinct from Bcl-2 and probably upstream of the site of action of Bcl-2. We also demonstrate that hydrogen peroxide generation in cells is upstream of cytochrome c release. And finally, the results show that TPx II but not Bcl-2 is able to prevent hydrogen peroxide accumulation in cells. This allows us to order the peroxidase upstream of Bcl-2 and cytochrome c release in the apoptotic pathway as suggested in Scheme I.

These studies have several interesting and important implications. First, these data demonstrate that a protein with a hitherto unknown cellular function is involved in protecting cells from apoptosis in response to reactive oxygen species. This protection is at a point distinct from that of Bcl-2.

Second, this protein is functionally linked to thioredoxin and thioredoxin reductase in electron transfer (3). It has recently been demonstrated that the adult T-cell leukemia-derived factor (which was originally described as a factor that induces production of interleukin-2 receptor α by T-cells infected by the human T-cell lymphotropic virus I, and is thought to act like a growth factor) is the human homologue of thioredoxin (28).

Adult T-cell leukemia-derived factor has recently been demonstrated to inhibit tumor necrosis factor α- and anti-Fas antibody-induced apoptosis (29). It has also been implicated in imparting resistance to chemotherapeutic agents of ALL cell lines (30). These results, coupled with our data on the protection of apoptosis by thioredoxin peroxidase, place thioredoxin/TPx II in a crucial position in the regulation of apoptosis.

Inducer $\rightarrow$ hydrogen peroxide $\rightarrow$ cytochrome c $\rightarrow$ caspase $\rightarrow$ apoptosis generation $\cap$ release activation $\cap$ Bcl-2

Scheme I. A proposed scheme for ordering the apoptotic pathway with respect to TPx II and bcl-2.

Third, the identity of the gene encoding TPx II with that encoding NKEF, which has been shown to be induced by oxidative stress (5), implies a potential physiologic role for this factor in the protection of cells from death by oxidative damage. Oxidative stress and damage by free radicals have been implicated in several pathologic states including cancer (31, 32), several neurodegenerative diseases such as muscular dystrophy (33), Alzheimer’s (34, 35) and Parkinson’s diseases (36), amyotrophic lateral sclerosis (37), atherosclerosis/asemic injuries (31, 38), and aging (39, 40). Interestingly, chromosomal localization of the human TPx II gene demonstrated that it resides on chromosome 13q12 (4). Importantly, the breast cancer susceptibility gene BRCA2 is localized to chromosome 13q12-q13 (41), and a form of Duchenne-like muscular dystrophy is localized to 13q1 (27). It is possible, therefore, as proposed by Pahl et al. (4), that TPx II could be considered a candidate gene for some of these disorders.

In conclusion, our data that TPx II/NKEF is an effective inhibitor of apoptosis in response to several different insults, coupled with its role as an antioxidant, place it in a critical position for elucidating mechanisms of apoptosis. The results also suggest a possible role for TPx II in diseases involving apoptosis induced by oxidative damage.