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Glutathione Peroxidase-1 Protects from CD95-induced Apoptosis*

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Through the induction of apoptosis, CD95 plays a crucial role in the immune response and the elimination of cancer cells. Ligation of CD95 receptor activates a complex signaling network that appears to implicate the generation of reactive oxygen species (ROS). This study investigated the place of ROS production in CD95-mediated apoptosis and the role of the antioxidant enzyme glutathione peroxidase-1 (GPx1). Anti-CD95 antibodies triggered an early generation of ROS in human breast cancer T47D cells that was blocked by overexpression of GPx1 and inhibition of initiator caspase activation. Enforced expression of GPx1 also resulted in inhibition of CD95-induced effector caspase activation, DNA fragmentation, and apoptotic cell death. Resistance to CD95-mediated apoptosis was not due to an increased expression of anti-apoptotic molecules and could be reversed by glutathione-depleting agents. In addition, whereas the anti-apoptotic protein Bcl-xL prevented CD95-induced apoptosis in MCF-7 cells, it did not inhibit the early ROS production. Moreover, Bcl-xL but not GPx1 overexpression could suppress the staurosporine-induced late generation of ROS and subsequent cell death. Altogether, these findings suggest that GPx1 functions upstream of the mitochondrial events to inhibit the early ROS production and apoptosis induced by CD95 ligation. Finally, transgenic mice overexpressing GPx1 were partially protected from the lethal effect of anti-CD95, underlying the importance of peroxide formation (and GPx1) in CD95-triggered apoptosis.

The CD95 (CD95/APO-1) receptor/CD95 ligand (CD95L)[1] system is a key signal pathway involved in the regulation of apoptosis in various human cells including myeloid, T-lymphoid, fibroblast, and malignant glioma cells (1, 2). CD95 is 36-kDa transmembrane type I receptor, which belongs to the nerve growth factor/tumor necrosis factor receptor superfamily (3). Upon binding of CD95L or agonistic anti-CD95 antibodies, an adapter molecule, called FADD, is instantly recruited to the so-called Death Domain of CD95. This results in the binding and activation of procaspase-8, leading to its processing to generate an active molecule capable of cleaving and activating downstream effector caspases, ultimately resulting in cell dismantling (4).

Numerous studies (5, 6) have implicated oxidative stress in apoptosis and most particularly in CD95-induced cell death. Indeed, various antioxidants such as thioredoxin (7), catalase (8), Cu,Zn-dependent superoxide dismutase (9), *N*-acetylcysteine (NAC) (10–12), GSH (13–15), and *N*-*t*-butyl-phenylnitrone (11) can prevent CD95-mediated apoptosis in several cell types. Reports showing that CD95 ligation results in ROS generation in human B-lymphoma (16), T-leukemia (10, 11, 17), or glioma (9) cells, and monocytes (13) provide additional evidence that ROS are key molecules in CD95-induced cell death. Conversely, oxidative stress has also been shown to promote CD95 or CD95L expression in different cell types. Indeed, addition of pro-oxidants such as H_2O_2 (18, 19), paraquat (20), or hypoxia and subsequent reoxygenation (20) have been reported to be potent inducers of CD95 and/or CD95L.

Further support that oxidative stress and CD95 are intimately associated in apoptosis came from the observation that intracellular GSH levels modulate CD95-induced cell death. Depletion of GSH levels in Jurkat cells through overexpression of transaldolase rendered these cells highly susceptible to anti-CD95 antibodies (10). Reciprocally, CD95-resistant variants of T-leukemia cells exhibit higher GSH content than original cells (17, 21) and become sensitive to CD95 when incubated with L-buthionine-(*S,R*)-sulfoximine (BSO) or in GSH-free/cysteine-free medium to deplete GSH (21). Similarly, increasing the GSH concentration in activated human peripheral T-cells resulted in total protection against CD95-induced apoptosis (22). Moreover, CD95 ligation has been shown to decrease intracellular GSH content in Jurkat T-lymphocytes by stimulating the efflux of GSH (23). However, acute GSH depletion has also been reported to prevent CD95-induced apoptosis in some instances (see Ref. 24 and references therein), whereas chronic depletion enhances apoptosis (25). Thus, how GSH is connected with CD95-induced cell death signaling pathways still remains to be clarified.

The relationship between oxidative stress, GSH levels, and CD95-induced apoptosis prompted us to examine the role of

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[1] The abbreviations used are: CD95L, CD95 ligand; BSO, L-buthionine-(*S,R*)-sulfoximine; NAC, *N*-acetylcysteine; GPx, glutathione peroxidase; GSH, glutathione; ROS, reactive oxygen species; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DAPI, 4′,6-diamidino-2-phenylindole; DEVD, benzyloxycarbonyl-Asp-Glu-Val-Asp-chloromethyl ketone; Ac-DEVD-AMC, Ac-Asp-Glu-Val-Asp-aminomethylcoumarin; ZVAD, benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethyl ketone; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; ERK, extracellular signal-regulated protein kinase; DCFH-DA, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester).

glutathione peroxidase (GPx), one of the major enzymes responsible for ROS detoxification in mammalian cells, in CD95 apoptotic signaling. Four forms of human GPx have been described, including the classical cytosolic/mitochondrial selenium-dependent GPx1, the gastrointestinal GPx2, the plasma enzyme GPx3, and the phospholipid hydroperoxide GPx4 (26). Nomura and co-workers (27) have reported that the latter GPx could suppress apoptosis induced by several stresses including etoposide and UV irradiation through the inhibition of cytochrome *c* release and caspase-3 activation. Regarding GPx1, we recently reported that overexpression of this GPx can abolish doxorubicin-induced sphingolipid signaling, a phenomenon accompanied by inhibition of ROS formation and partial protection against doxorubicin-induced apoptosis (28).

To assess the yet undetermined role of GPx1 in CD95-induced cell death, we used human breast carcinoma T47D cells, which were stably transfected with a cDNA encoding human GPx1 (29). Generally, breast cancer cell lines are known to resist CD95-mediated apoptosis, but T47D cells may represent an exception inasmuch as they express high levels of CD95 (30). The susceptibility of GPx1-overexpressing cells to CD95, in terms of toxicity and cell signaling, was compared with that of parental cells, which are characterized by low endogenous GPx activity. Here we show that not only could overexpression of GPx1 strongly inhibit CD95-induced ROS formation, caspase activation, and apoptosis but also partially protect mice from the lethal effect of anti-CD95. These findings suggest that GPx plays a critical role in CD95 signaling by regulating effector caspase activation.

EXPERIMENTAL PROCEDURES

Reagents—*N*-Acetylcysteine, L-buthionine-(*S,R*)-sulfoximine, 4′,6-diamidino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and sodium selenite were supplied from Sigma. Z-Val-Ala-DL-Asp-fluoromethyl ketone, Z-Asp-Glu-Val-Asp-chloromethyl ketone (DEVD), and Ac-Asp-Glu-Val-Asp-aminomethylcoumarin (Ac-DEVD-AMC) were from Bachem (Voisins-Le-Bretonneux, France). 6-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (DCFH-DA) was from Molecular Probes (Leiden, The Netherlands). Anti-CD95 (CH-11 antibody) was from Beckman Coulter (Marseille, France). RPMI 1640 Glutamax, hygromycin, G418, and antibiotics were from Invitrogen; fetal calf serum was from BioMedia (Boussens, France).

Cell Culture—The human breast cancer T47D cell line, a differentiated epithelial substrain of ductal carcinoma origin, was transfected with pML-Hygro or pML-Hygro-HCMV-GPx plasmids, which contains part of a cDNA clone encoding human GPx1 (29). Empty vector and HCMV-GPx-transfected cells are designated T47D/H3 and T47D/GPx, respectively. These cells were grown in a humidified 5% CO_2 atmosphere at 37 °C in RPMI 1640 medium containing Glutamax (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), hygromycin B (150 μg/ml), sodium selenite (1 μM), and heat-inactivated fetal calf serum (10%).

The breast carcinoma MCF-7 sublines, stably transfected to express the CD95 receptor (denoted MCF-7/CD95) or both CD95 and Bcl-xL (denoted MCF-7/CD95/Bcl-xL), were kindly provided by Dr. V. Dixit (Genentech Inc., San Francisco, CA). They were grown in RPMI medium supplemented with G418 (200 μg/ml) and hygromycin (150 μg/ml).

The wild-type (A3) and caspase-8 mutant (I9–2) Jurkat cells were kindly provided by Dr. J. Blenis (Harvard Medical School, Boston), and cultured in RPMI 1640 medium (31).

Determination of Cytotoxicity—Cell viability was evaluated by using the tetrazolium-based MTT assay (32).

Flow Cytometry Analyses—Adherent cells were detached by incubation for 10 min with EDTA (5 mM) in phosphate-buffered saline (PBS). After washing with PBS, cells were incubated for 30 min at 4 °C in the presence of phycoerythrin-labeled anti-CD95 antibody (20 μl, Beckman Instruments), washed again, and analyzed on a FACScan (BD Biosciences) cytometer.

DNA Fragmentation Assay—After treatment with anti-CD95, T47D cells were washed twice in PBS, lysed for 20 min at 4 °C in 0.5 ml of lysis buffer (0.5% Triton X-100 v/v, 20 mM EDTA, and 5 mM Tris-HCl, pH 8.0), and then centrifuged for 20 min at 27,000 × *g* in order to separate the DNA fragments from the chromatin pellet. The DNA content of pellet (resuspended in 1 ml of 1 mM EDTA in 10 mM Tris-HCl, pH 8.0 buffer) and supernatant was determined by the fluorometric DAPI procedure (33).

Determination of ROS—ROS production was assessed using DCFH-DA. This probe is an uncharged cell-permeant molecule, which is cleaved by nonspecific esterases once inside the cell, and releases carboxydichlorofluorescein that is oxidized in the presence of ROS. Exponentially growing cells were labeled with 10 μM DCFH-DA for 30 min at 37 °C before the reaction was stopped. Cells were washed three times with PBS. Cell pellets were suspended in 1 ml of distilled water and sonicated at 4 °C. The cell-associated fluorescence was recorded at excitation and emission wavelengths of 495 and 525 nm, respectively, using a Jobin-Yvon 3D fluorometer.

Fluorogenic DEVD Cleavage Enzyme Assay—After incubation with anti-CD95, cells were sedimented. Cell pellets were homogenized in 10 mM HEPES, pH 7.4, 42 mM KCl, 5 mM $MgCl_2$, 0.5% CHAPS, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin. Reaction mixtures contained 100 μl of cell lysates and 100 μl of 40 μM Ac-DEVD-AMC. After 30 min of incubation at room temperature, the amount of the released fluorescent product aminomethylcoumarin was determined at 351 and 430 nm for the excitation and emission wavelengths, respectively.

Western Blot Analyses—Analysis of caspase cleavage was assessed by Western blot using the cell lysates prepared for DEVD cleavage enzyme assay. Samples were loaded onto a 15% SDS-polyacrylamide gel, electrophoresed, and transferred to a nitrocellulose membrane. Caspase-3 and its cleaved fragments were detected by using a rabbit polyclonal antiserum (a kind gift of Dr. D. Nicholson, Merck-Frosst, Pointe-Claire, Quebec, Canada); caspase-7 was detected with a rabbit polyclonal antiserum (Oncogene, France Biochem, Meudon, France); caspase-8 was detected using a rabbit polyclonal antiserum (a kind gift of Dr. G. Cohen, Leicester, UK) (34) and a goat anti-rabbit secondary antibody (Bio-Rad). Western blot was also used to analyze the expression of Bcl-2 (using a monoclonal antibody, Dako, Trappes, France), Bcl-xL, and Mcl-1 (using antibodies from Pharmingen), Bid (using a rabbit antibody kindly provided by Dr. X Wang, Dallas, TX), ERK (using a rabbit polyclonal antibody, Santa Cruz Biotechnology), phospho-ERK (using a monoclonal antibody, Cell Signaling Technology, Beverly, MA), and usurpin/FLIP (using a rabbit polyclonal antibody kindly provided by Dr. D. Nicholson). An anti-β-actin (Sigma) was used as a control for protein loading.

Preparation of Mitochondria and Western Blot Analysis of Cytochrome c—Mitochondrial preparations were carried out as described previously (35). Briefly, cell samples were washed once with ice-cold PBS and resuspended with ice-cold buffer A (20 mM HEPES, pH 7.5, 0.1% bovine serum albumin, 1 mM sodium EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin A) containing 250 mM sucrose. After swelling on ice for 5 min, the cells were homogenized with 15–20 strokes of a Kontes Dounce homogenizer with the B pestle (Kontes Glass Company, Vineland, NJ), and the homogenates were centrifuged at 750 × *g* for 5 min at 4 °C. The supernatants were then pelleted at 10,000 × *g* for 15 min at 4 °C. Resulting pellets containing mitochondria were resuspended in cold buffer A. The resulting supernatants ("cytosolic" extracts) were further cleared at 20,000 × *g* for 30 min at 4 °C. For Western blot analysis, equal amounts of mitochondrial and cytosolic proteins were separated on 15% SDS-PAGE and then transblotted to nitrocellulose. Monoclonal antibodies used against cytochrome *c* and cytochrome oxidase subunit II were from Pharmingen and Molecular Probes, respectively.

In Vivo Studies—Age-matched female GPx6 and GPx13 transgenic mice (36) and control mice (B6C3F1, obtained from Charles River Laboratories) of 10–16 weeks of age were intraperitoneally injected with 0.36 μg Jo2 antibody (Pharmingen) per g of body weight in a total volume of 200 μl of sterile saline. Mice were checked for mortality every 30 min.

Statistical Analyses—For *in vitro* assays, Student's *t* test was used for statistical analysis. For animal studies, Kaplan-Meier survival rates were compared using the Logrank test.

RESULTS

GPx1 Inhibits CD95-induced Apoptotic Cell Death—As CD95-mediated apoptosis has been shown to implicate the production of ROS, we investigated the effect of GPx1 overexpression on cell death induced by an agonistic anti-CD95 anti-

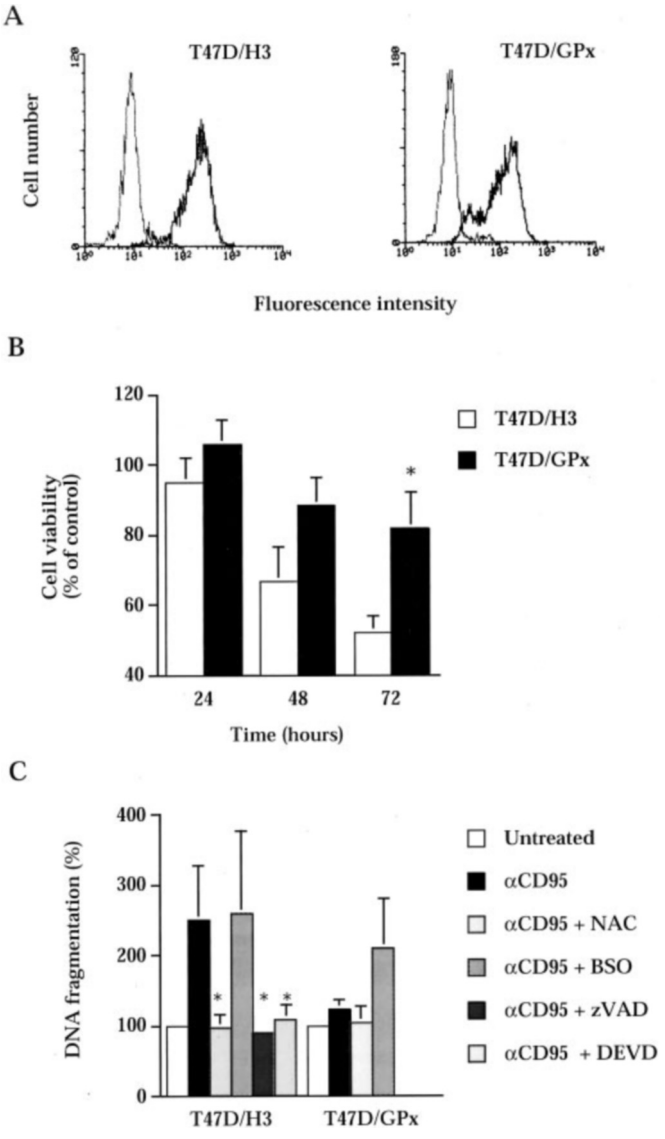

FIG. 1. **Effect of GPx1 overexpression on CD95-induced cytotoxicity and DNA fragmentation in T47D cells.** *A*, expression of CD95 receptor in T47D/H3 and T47D/GPx cells as determined by flow cytometry. The *thin* and *thick lines* depict the isotype-matched negative control and anti-CD95 antibody, respectively. *B*, T47D/H3 and T47D/GPx cells were incubated under serum-free conditions for the indicated times in the presence of 250 ng/ml anti-CD95. Cell viability was assessed by MTT assay, by comparing untreated and anti-CD95-treated cells at each time. Results are means ± S.E. of 3–8 independent experiments. Note that prolonged incubation of T47D cells in serum-free medium did not cause loss of cell viability. *C*, T47D/H3 and T47D/GPx cells were incubated under serum-free conditions with 250 ng/ml anti-CD95 for 48 h in the absence or presence of NAC (10 mM), BSO (250 μM), ZVAD (25 μM), or DEVD (25 μM) (inhibitors were added to the cell culture medium 2 h prior to addition of anti-CD95). Quantitative DNA fragmentation was determined by the spectrofluorometric DAPI procedure. Results are means ± S.E. of 2–5 independent determinations. The *asterisk* denotes $p < 0.05$.

body. This was examined in cells overexpressing GPx (T47D/GPx) as compared with cells transfected with an empty vector (T47D/H3), which express similar levels of CD95 antigen (Fig. 1*A*). As illustrated in Fig. 1*B*, the CD95-induced time-dependent cytotoxic effect was considerably reduced in GPx1-expressing cells (after 72 h of exposure to the anti-CD95, the viability of T47D/GPx cells exceeded 80%, whereas that of control cells approximated 50%). The resistance of T47D/GPx cells was further confirmed by examining the rate of DNA fragmentation following 48 h of treatment with anti-CD95. As shown in Fig. 1*C*, only a small (1.2 times) increase in DNA fragmentation was observed in the GPx1-overexpressing cells (as compared with a 2.5-fold elevation in control cells).

To substantiate the role of the antioxidant enzyme GPx1 in CD95-induced cell death, control experiments were carried out by manipulating the intracellular levels of GSH. Whereas the GSH precursor and ROS scavenger NAC completely protected T47D/H3 cells from CD95-induced cell death, the GSH-depleting agent BSO sensitized both control and GPx1-expressing cells to anti-CD95 (Fig. 1*C*). These compounds have been shown previously (28) to increase or decrease, respectively, the GSH levels in T47D cells, suggesting that the observed variations in cell viability are indeed related to GSH levels.

The resistance of T47D/GPx1 cells to the lethal effect of anti-CD95 was linked to a protection from apoptosis, as indicated by the suppression of DNA fragmentation in T47D/H3 conferred by the rather non-selective caspase inhibitors ZVAD and DEVD (37) (Fig. 1*C*). Consistent with these results, activation of effector and initiator caspase processing was found in T47D cells upon CD95 ligation (Fig. 2). Caspase activity, as measured by the cleavage of the fluorogenic tetrapeptide substrate Ac-DEVD-AMC, increased in T47D/H3 cells within 6 h, peaking at 24 h post-treatment (Fig. 2*A*). Only a very moderate, progressive increase (less than 1.5-fold) was noted in GPx1-overexpressing cells. Moreover, Western blot analysis confirmed that executioner caspase-3 (Fig. 2*B*) and caspase-7 (not shown) processing was reduced in T47D/GPx cells. Processing of the initiator caspase-8, which was detected as early as 15–30 min (data not shown), appeared essentially unaffected by GPx1 overexpression (Fig. 2*B*). Finally, CD95-induced cytochrome *c* release from mitochondria was diminished in GPx1-expressing cells (Fig. 2*C*).

CD95-induced Early ROS Generation Is Suppressed by GPx1 Overexpression and Caspase Inhibitors—Because CD95-mediated apoptosis has been described to be accompanied by the production of ROS, we next investigated the effect of GPx1 overexpression on intracellular ROS levels measured in anti-CD95-treated T47D cells. Fig. 3*A* shows that CD95 ligation induced a rapid increase in ROS (probably mostly peroxide) levels as measured by dichlorofluorescein fluorescence. This phenomenon was detectable within the first 15 min of incubation and peaked around 60 min; no increase in ROS levels was observed at later points, *e.g.* at 24 h (data not shown). In contrast, anti-CD95 failed to produce any detectable ROS increase in T47D/GPx cells, suggesting that in these cells GPx1 overexpression strongly reduced DCF-detected ROS accumulation. Of interest is the finding that caspase inhibitors suppressed the CD95-induced ROS increase in control cells (Fig. 3*B*), indicating a protease-dependent mechanism in ROS generation.

CD95-induced ROS Production Is Caspase-8-dependent—To explore further the molecular events that lead to ROS production after CD95 ligation, and because this production was found to be dependent on caspase activation (Fig. 3*B*), we investigated the role of initiator caspases. To this end, ROS production was monitored in human leukemia Jurkat cells expressing or not expressing caspase-8 (31); the caspase-8 mutant cell line expresses very low levels of caspase-8 (see Fig. 4*C*) and is resistant to anti-CD95-triggered cell death (data not shown). Although wild-type and caspase-8 mutant Jurkat cells expressed comparable levels of CD95 (Fig. 4*A*), CD95 ligation was not accompanied by early ROS production in the caspase-8 mutant cell line (Fig. 4*B*). In the control cell line, generation of ROS was detectable at 60 min, that is concomitantly to the first measurable cleavage of caspase-8 (Fig. 4*C*). Caspase-3 processing occurred no earlier than 60 min. These data indicate that

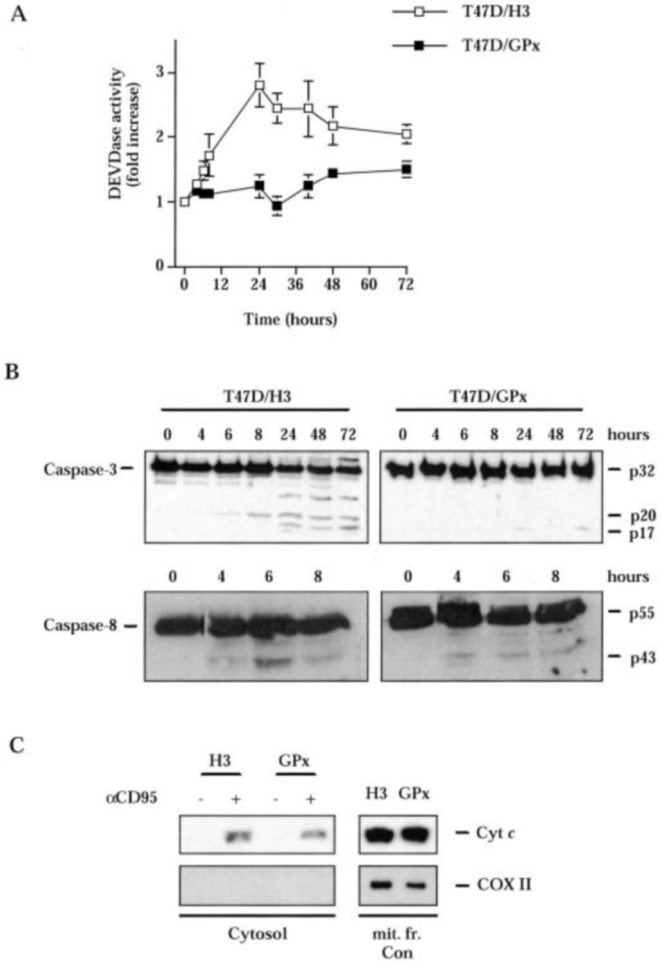

FIG. 2. **Effect of GPx1 overexpression on CD95-induced caspase activation and cytochrome *c* release in T47D cells.** T47D/H3 and T47D/GPx cells were incubated under serum-free conditions with 250 ng/ml anti-CD95 for the indicated times. Cells were then harvested, and DEVDase activity was determined as described under "Experimental Procedures" (*A*). Data are means ± S.E. of 3–6 independent experiments. *B,* extracts from cells treated as above were subjected to 15% SDS-PAGE and immunoblotted with anti-caspase-3 or caspase-8. *C,* alternatively, mitochondrial and cytosolic extracts were prepared from cells incubated for 24 h with or without anti-CD95, separated by SDS-PAGE, and immunoblotted using anti-cytochrome *c* and anti-cytochrome oxidase subunit II (which serves as a marker for mitochondrial contamination of cytosolic extracts) antibodies. A mitochondrial extract from nontreated cells (*mit. fr. Con*) was used as a positive control for cytochrome *c* (*Cyt c*) and cytochrome oxidase subunit II (COX II).

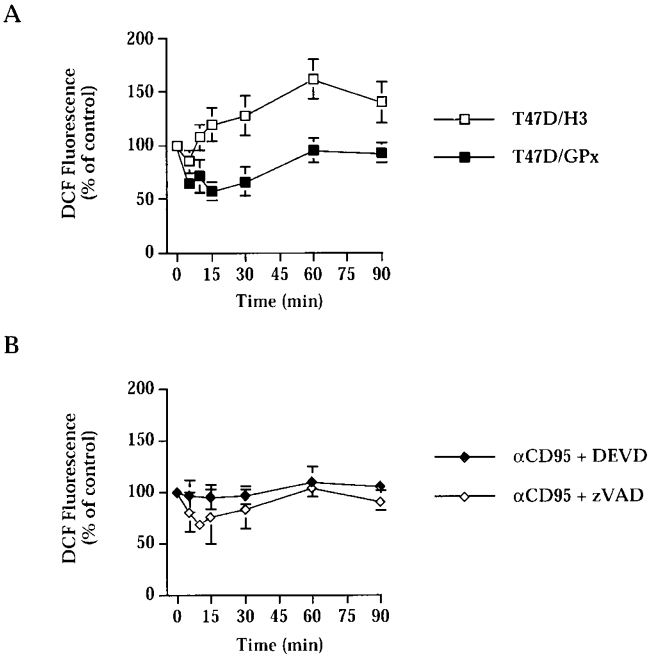

FIG. 3. **Effect of GPx1 and caspase inhibitors on CD95-induced ROS production in T47D cells.** *A*, T47D/H3 and T47D/GPx cells were incubated for the indicated times with 250 ng/ml anti-CD95 under serum-free conditions. *B*, T47D/H3 cells were treated with anti-CD95 in the presence of 25 μM of ZVAD or DEVD. The DCFH-DA probe was added to the cells 30 min before each reaction was stopped. Cells were washed and cell fluorescence was quantified as described under "Experimental Procedures." Data are means ± S.E. of 2–8 independent experiments.

CD95-induced ROS production immediately follows activation of the initiator caspase-8. Complementary studies further showed that caspase-8 cleavage precedes ROS generation. Indeed, incubation of Jurkat cells with antioxidants (NAC, pyrrolidinedithiocarbamate, or butylated hydroxyanisole) did not affect CD95-induced proteolytic processing of caspase-8 (Fig. 4*D*).

Resistance of GPx1-expressing Cells to Anti-CD95 Is Not Linked to Up-regulation of Anti-apoptotic Proteins—Because different proteins, including FLIP/usurpin (38), members of the Bcl-2 family (39, 40), and ERK (41, 42), are known to counteract the proapoptotic action of CD95 ligation, we examined whether the expression level of these proteins was affected by GPx1 overexpression. As shown in Fig. 5, similar levels of FLIP/usurpin, Bcl-xL, Mcl-1, Bid, and ERK (either the total or active, phosphorylated forms) were noted in T47D/H3 and T47D/GPx cells, excepted for Bcl-2 that was less expressed in GPx1-overexpressing cells. These data indicate that the resistance of T47D/GPx cells is not due to an increased expression of these proteins. Interestingly, the lower level of Bcl-2 in GPx1-expressing cells is reminiscent of a previous observation reporting a decreased amount of Bcl-xL in murine fibrosarcoma cells overexpressing another antioxidant enzyme, Mn-superoxide dismutase (43).

Bcl-xL Overexpression Does Not Affect the CD95-induced Early Production of ROS—Because CD95-mediated apoptosis is known to involve mitochondria, and because expression of members of the Bcl-2 family are able to suppress the mitochondrial events, leading to protection from apoptosis in some cell types (see "Discussion"), we investigated whether overexpression of these proteins could alter the above detected production of ROS. To this end, we employed MCF-7 cells, another cell line derived from a human breast carcinoma, that overexpress CD95 and the anti-apoptotic molecule Bcl-xL (44). As published previously (35, 44, 45), Bcl-xL overexpression (Fig. 6*A*) resulted in protection of MCF-7 cells from anti-CD95-induced cell death (Fig. 6*B*) and caspase-7 proteolytic cleavage (Fig. 6*C*). As the MCF-7 cell line is devoid of caspase-3 because of the functional deletion of the *CASP-3* gene (46), caspase-7 appears to be the only executioner caspase activated in these cells. However, the early ROS production triggered by anti-CD95 remained unaffected by Bcl-xL overexpression (Fig. 6*D*). These data suggest that the early CD95-induced generation of ROS is upstream of the mitochondria.

Bcl-xL but Not GPx1 Overexpression Prevents Staurosporine-induced ROS Production and Cell Death—The protein kinase inhibitor staurosporine is a well established apoptosis inducer that acts through mitochondrial dysfunction (47). As such, the apoptotic program launched by this compound can be blocked by overexpression of Bcl-2 members (48, 49). To explore further the site of action of GPx1 in the apoptotic cascade, we next investigated whether GPx1 overexpression, in comparison with

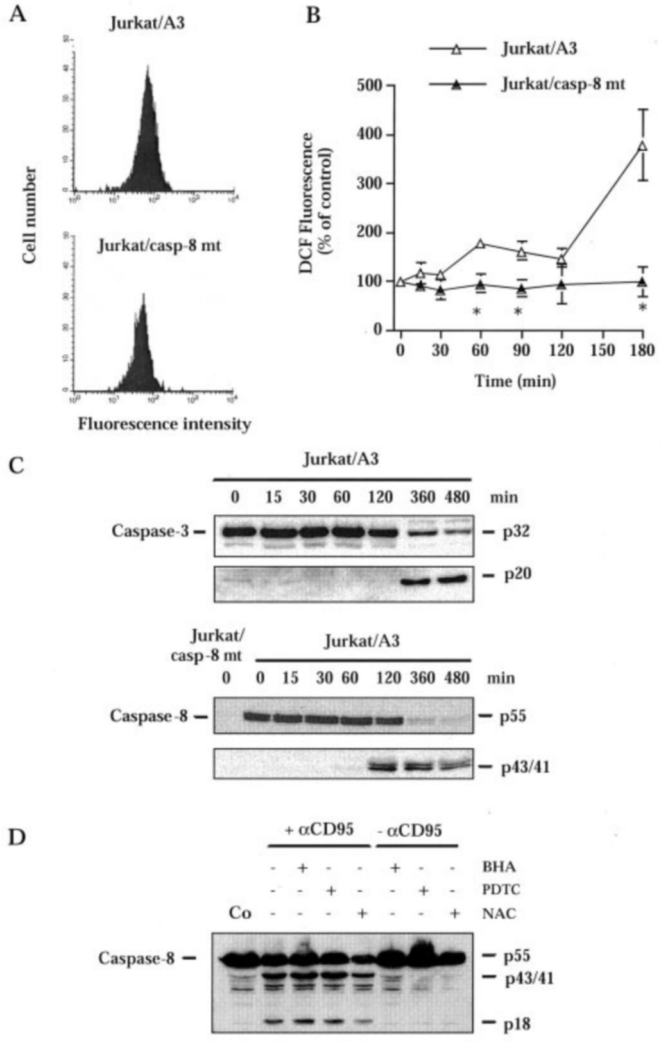

FIG. 4. **Caspase-8 activation is required for CD95-induced ROS production in Jurkat cells.** Control (*Jurkat/A3*) and caspase-8-deficient (Jurkat/casp-8 mt) Jurkat cells were analyzed for CD95 expression by flow cytometry (*A*). They were incubated with 100 ng/ml anti-CD95 in the absence of serum. At the indicated times, ROS production was determined using the DCFH-DA probe as described under "Experimental Procedures" (*B*). Results are means of 2–4 independent experiments; the *asterisk* indicates $p < 0.02$. Alternatively, caspase-3 and caspase-8 processing was monitored by Western blot analysis (*C*). *D*, Jurkat cells were incubated for 360 min in the presence or absence of 50 ng/ml anti-CD95 and NAC (20 mM), pyrrolidinedithiocarbamate (*PDTC*, 50 μM), or butylated hydroxyanisole (*BHA*, 200 μM), and caspase-8 processing was examined. Antioxidants were added to the cells 60 min before treatment with anti-CD95. *Co*, untreated control cells. Similar data were obtained in three separate experiments.

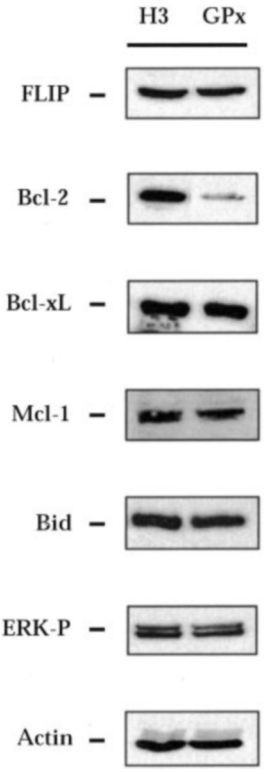

FIG. 5. **Expression of anti-apoptotic molecules in T47D/H3 and T47D/GPx cells.** T47D/H3 and T47D/GPx cell extracts were subjected to 15% SDS-PAGE and immunoblotted with antibodies against FLIP, Bcl-2, Bcl-xL, Bid, Mcl-1, phosphorylated ERK (*ERK-P*), and β-actin.

Bcl-xL, could prevent the staurosporine-induced effects. Fig. 7 shows that, although Bcl-xL overexpression suppressed the late ROS increase elicited by staurosporine (Fig. 7*B*), overexpression of GPx1 did not block this generation of ROS (Fig. 7*A*). As a consequence, Bcl-xL but not GPx1 overexpression protected breast cancer cells from staurosporine-induced cell death (Fig. 7, *C* and *D*). Altogether, these data indicate that the site of action of GPx1 differs from that of Bcl-xL and is upstream of the mitochondria.

GPx1 Overexpression Partially Protects Mice from C95-induced Mortality—In order to evaluate further the role of GPx1 in CD95-triggered cell death, we injected the Jo2 anti-CD95 antibody into normal and GPx1 transgenic mice. This antibody has been shown previously (50) to lead to the rapid death of mice due to severe liver damage by apoptosis. Two lines of mice carrying multiple copies of the GPx1 transgene (36) were tested. As illustrated in Fig. 8, these transgenic mice exhibited some (but significant) resistance to anti-CD95 antibody. Whereas control animals were all dead 5 h post-injection, GPx1 transgenic mice survived longer (some were even alive 24 h later). These observations further support the notion that oxidative stress and GPx1 play important functions in CD95-induced apoptosis.

DISCUSSION

CD95-induced apoptosis occurs through a complex signaling network involving the recruitment of the adaptor protein FADD and subsequent activation of procaspase-8, which then triggers the proteolytic processing of effector caspases (caspase-3 and -7), ultimately leading to cell dismantling through cleavage of a variety of vital substrates (4). Mitochondria are involved in this process through the release of pro-apoptotic molecules such as cytochrome *c*, which activate effector caspases. However, whereas some cell types (so-called type I) require activation of caspase-8 and are independent of the apoptogenic activity of mitochondria, others (type II) depend on the release of mitochondrial factors that results in the activation of caspase-3 and -8 (51). Hence, overexpression of Bcl-2 or Bcl-xL blocks caspase activation and apoptosis only in type II cells.

In addition to these established pathways, the production of ROS appears as an important signaling component of CD95-mediated apoptosis. Indeed, not only is CD95 engagement followed by ROS generation in different cell types (9, 10, 13, 16, 17, 52, 53), but also various enzymatic and non-enzymatic antioxidants can protect cells against CD95-induced cell death. In accordance with these observations, the present study demonstrates that anti-CD95 antibodies trigger the generation of ROS in human breast carcinoma and leukemia cells, and that overexpression of the antioxidant enzyme GPx1 confers resist-

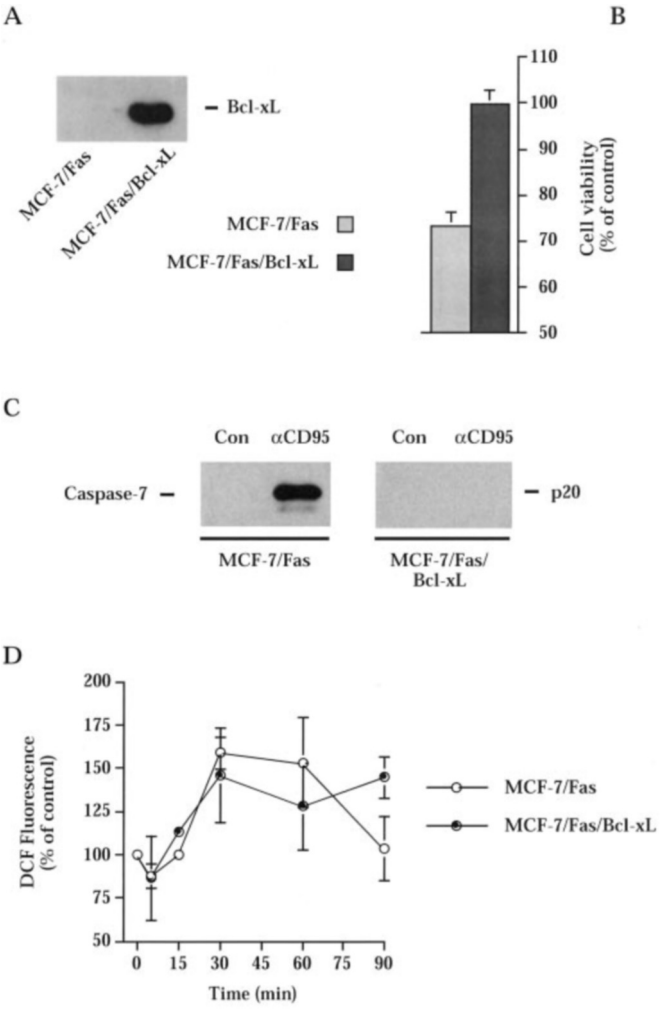

FIG. 6. **Effect of Bcl-xL overexpression on CD95-induced ROS production, caspase activation, and cell viability in MCF-7 cells.** Expression of Bcl-xL in MCF-7/CD95 and MCF-7/CD95/Bcl-xL cells was determined by Western blot (*A*). MCF-7/CD95 and MCF-7/CD95/Bcl-xL cells were treated with 100 ng/ml anti-CD95. After 48 h of incubation, cell viability was assessed by MTT assay (*B*), and caspase-7 processing was examined by Western blot (*C*). Alternatively, ROS production was evaluated after incubation for the indicated times with 100 ng/ml anti-CD95 (*D*). The DCFH-DA probe was added to the cells 30 min before each reaction was stopped. Cells were washed, and cell fluorescence was quantified as described under "Experimental Procedures." Data are means ± S.E. of 2–5 independent experiments.

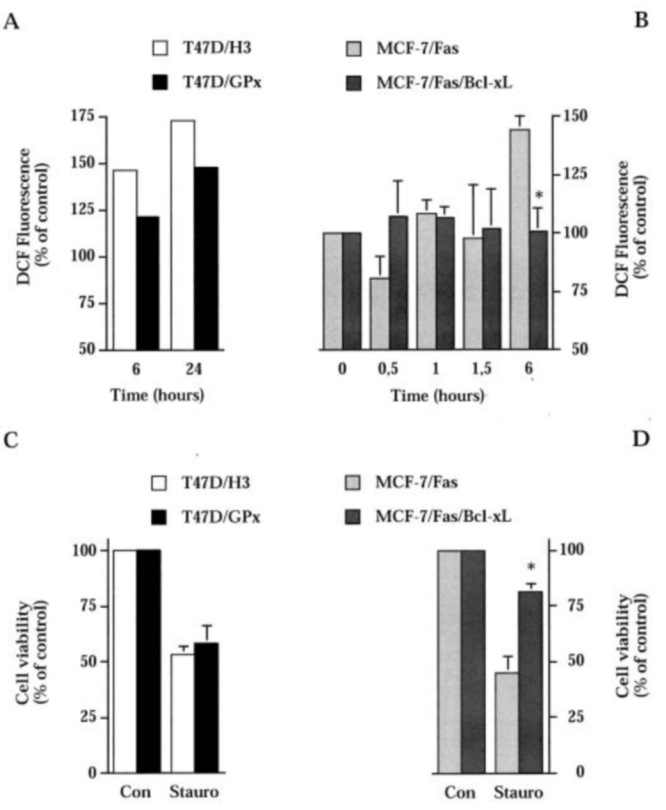

FIG. 7. **Effect of GPx1 and Bcl-xL overexpression on staurosporine-induced ROS production and viability of T47D and MCF-7 cells.** T47D/H3 and T47D/GPx cells (*A*) and MCF-7/CD95 and MCF-7/CD95/Bcl-xL cells (*B*) were incubated with staurosporine (250 nM). At the indicated times, ROS production was determined using the DCFH-DA probe as described under "Experimental Procedures." Alternatively, T47D and MCF-7 cell viability was assessed by MTT assay (*C* and *D*, respectively). Results are means of at least two independent experiments. The *asterisk* indicates $p < 0.001$.

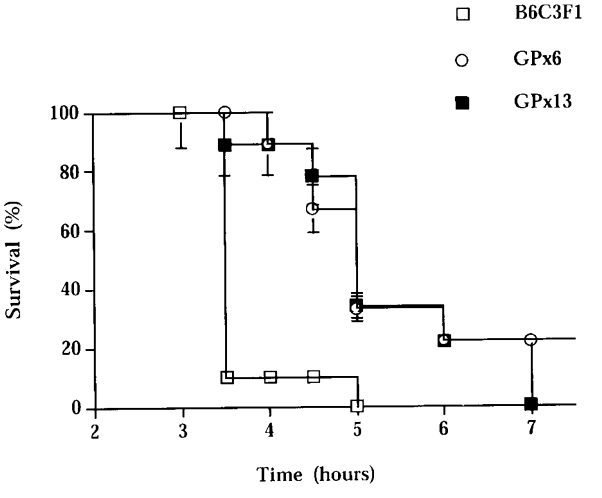

FIG. 8. **Differential resistance of GPx1 transgenic mice to anti-CD95 lethality.** Control (B6C3F1, $n = 10$) and GPx1 transgenic mice (line GPx-6, $n = 9$, and line GPx-13, $n = 9$) were injected with anti-CD95 (Jo2) antibody, and mortality was monitored every 30 min. Results are presented as a Kaplan-Meier survival curve. A significant difference ($p < 0.001$ and 0.0025) between control and GPx1 transgenic mice survival was observed both at 3.5 and 4.5 h, respectively.

ance to CD95-induced apoptosis. These findings are in line with reports showing that overexpression of the cytosolic Cu,Zn-superoxide dismutase and catalase (9), use of superoxide dismutase mimetics (54), or elevation of intracellular GSH levels (by interfering with the pentose phosphate pathway and the levels of NADPH) (10) all attenuate CD95-induced apoptotic cell death. Addition of catalase to neutrophils also prevented CD95-induced apoptosis (8). Moreover, it is well established that the apoptotic action of CD95 is modulated by reduced GSH levels, as indicated by the following: (i) the protective effect of exogenous GSH and the GSH precursor NAC (10, 13, 14, 22, 55); (ii) the deleterious effect of GSH-depleting agents such as BSO (10, 14, 21, 22); and (iii) the GSH efflux that occurs during CD95-mediated apoptosis (23). Of interest, however, is the finding that, in contrast to what is observed with GPx1, enforced expression of the mitochondrial GPx4, which acts on lipid peroxides, fails to suppress CD95-mediated apoptosis of leukemic cells, whereas it protects from other insults such as etoposide, staurosporine, UV radiations, or 2-deoxyglucose (27). Therefore, a specific subset of antioxidants including GPx1 appear to play a critical role in the apoptotic program initiated by CD95 ligation.

Although the above and present observations strengthen the notion that ROS production is an important component of

CD95-induced cell death signaling, they raise two main questions. The first one is the cellular source of ROS. Mitochondria are notorious for ROS production, but other subcellular sites also trigger oxidative stress (6). With regard to CD95-induced cell death, other sources have been proposed in addition to the mitochondrial production that accompanies disruption of the transmembrane potential ($\Delta\Psi_m$), GSH depletion, and opening of the permeability transition pore (52, 56, 57). A very rapid production of ROS has been observed in human lymphoma cell lines after CD95 ligation that was attributed to stimulation of NADPH oxidase (16). Consistent with this, neutrophils having a genetic defect in the NADPH components are partially resistant to CD95-induced cell death (8). Also, the contribution of lipoxygenase and NO synthase to ROS production cannot be excluded because a protective effect of potential inhibitors of these enzymes (nordihydroguaiaretic acid and diphenyleneiodonium, respectively) has been reported (10, 16).[2] It is thus likely that CD95 ligation promotes two types of ROS generation, an early (*i.e.* during the first 2 h) increase by a yet uncharacterized mechanism and a late (*i.e.* 6–24 h post-treatment) production from mitochondria.

This scenario seems compatible with the data of the present study. On the one hand, CD95 ligation triggers a rapid ROS production in human breast carcinoma cells, which is abolished by GPx1 overexpression, blocked by caspase inhibitors, but not affected by overexpression of the anti-apoptotic protein Bcl-xL. Moreover, in leukemic cells the early CD95-induced ROS production requires a functional caspase-8. These findings suggest that this early generation of ROS, possibly due to NADPH oxidase, occurs downstream of initiator caspases and upstream of the mitochondrial dysfunction. On the other hand, the ROS production (and subsequent cell death) elicited by staurosporine is a quite late event, which is not suppressed by GPx1, but is abrogated by Bcl-xL overexpression. In addition, overexpression of mitochondrial GPx4 can inhibit staurosporine- but not CD95-induced cytochrome *c* release and cytotoxicity (27). Thus, CD95 ligation triggers an early formation of ROS which precedes mitochondrial changes and is sensitive to GPx1.

The second question raised by this and previous studies is the role of the early ROS production in CD95-mediated apoptosis. This ROS increase may stimulate opening of the permeability transition pore and cause a disruption of mitochondrial membrane potential (6, 53), leading to executioner caspase activation. This is in accordance with the observed time course of caspase-3 and -7 proteolytic processing in T47D/H3 cells, which occurred hours after the initial ROS increase. In addition to these mechanisms, ROS could also regulate caspase activity, in particular that of the initiator caspase-8 (53). Although the death signal triggered by CD95 ligation is known to propagate essentially through a protease cascade (4), the participation of ROS in this signaling pathway becomes more obvious. Not only could ROS amplify the death signals, but also they may serve as an important "starter" of the mitochondrial events as shown by the inhibitory role of GPx1 (and other antioxidants as well) on cell death induction.

In summary, the present study demonstrates that overexpression of GPx1 in breast carcinoma cells abrogates the CD95-induced early production of ROS, leading to protection against caspase activation and apoptotic cell death. Further evidence for a protective function of GPx1 against CD95-mediated apoptosis is provided by the relative resistance of GPx1 transgenic animals to the liver injury induced by an anti-CD95. In addition, GPx1 functions upstream of the mitochondrial pathway, via a mechanism distinct from that of Bcl-xL. Another instance of protection from stress-induced apoptosis that occurs upstream of the site of action of Bcl-2 is the expression of the antioxidant thioredoxin peroxidase (58). Of note, a thioredoxin homologue, the adult T-cell leukemia-derived factor, is able to inhibit anti-CD95-induced apoptosis (7). Altogether, these results place oxidative damage as an important component of the CD95-triggered apoptotic cell death machinery and open new perspectives for manipulation of apoptosis.

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[2] In addition, preliminary data from this laboratory indicated that CD95-induced ROS production can be prevented by preincubating T47D cells with diphenyleneiodonium, a compound known to inhibit flavoproteins such as NADPH oxidase and NO synthase.

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