Glutathione Dependence of Caspase-8 Activation at the Death-inducing Signaling Complex*

Hannes Hentze‡§¶, Ingo Schmitz§**, Markus Latta‡, Andreas Krueger‡, Peter H. Krammer‡, and Albrecht Wendel‡

From ‡Biochemical Pharmacology, Department of Biology, University of Konstanz, D-78457 Konstanz, Germany and the §German Cancer Research Center, D-69120 Heidelberg, Germany

Apoptosis triggered by the death receptor CD95 (APO-1 or Fas) is pivotal for the homeostasis of the immune system. We investigated differential effects of glutathione depletion on CD95-triggered apoptosis in T and B cell lines as well as the glutathione dependence of caspase-8 activation. In B lymphoblastoid SKW6.4 cells, CD95-mediated apoptosis was prevented upstream of caspase-8 activation and caspase-3-like activity after acute glutathione depletion by diethyl maleate or cis-chloro-dinitrobenzene. Immunoprecipitation of the death-inducing signaling complex (DISC) revealed that the DISC was still formed in the glutathione-depleted state. The first cleavage step of procaspase-8 activation at the DISC, however, was inhibited. Accordingly, under cell-free conditions, radiolabeled procaspase-8 was processed at the immunoprecipitated DISC only after the addition of exogenous dithiothreitol or reduced glutathione. We also observed suppression of CD95-mediated apoptosis in glutathione-depleted CEM and H9 cells. Notably, Jurkat cells still died upon CD95 engagement under this condition, displaying incomplete nuclear fragmentation and a partial switch to necrosis; this may be explained by reduced cytochrome c/dATP-mediated caspase activation observed in cytosol from glutathione-depleted Jurkat cytosol. Our data indicate that the activation of caspase-8 at the DISC and hence CD95-mediated apoptosis induction shows a cell-specific requirement for intracellular glutathione.

Elimination of functionally impaired or potentially dangerous cells by apoptosis is of paramount importance for the organism. Apoptosis mediated by the death receptor CD95 (also called APO-1 and Fas) has previously been investigated intensively, especially in the immune system and the liver. Triggering of CD95 by agonistic antibodies (αCD95) or the naturally occurring CD95 ligand (CD95L) initiates trimerization of CD95 and recruitment of the adapter molecule Fas-associated death domain (FADD)† and procaspase-8 to form the death-inducing signaling complex (DISC) (1).

Caspase-8 is expressed as two isoforms, i.e. caspase-8/a and caspase-8/b (2). In the DISC, both forms of procaspase-8 are autoproteolytically cleaved to intermediates of 43 and 41 kDa, respectively. Subsequently, a heterotrimer of two small (10 kDa) and two large (18 kDa) subunits is formed that constitutes the active enzyme (3, 4). Then caspase-8 can directly cleave caspase-3 and thereby propagate apoptosis (5). Alternatively, caspase-8 can cleave Bid, and truncated Bid then translocates to the mitochondria and induces the release of apoptogenic factors such as cytochrome c (cyt c) (6–8). In the cytoplasm, cytochrome c binds to Apaf-1 forming an ~700-kDa apoptosome complex to which procaspase-9 is recruited. At the apoptosome, caspase-9 is activated (9–11), in turn cleaving other caspses such as caspase-8, -3, and -7 (12). In different cell types, apoptosis is initiated either by activation of large amounts of caspase-8 at the DISC followed by rapid cleavage of caspase-3 (type I cells, e.g. SKW6.4, H9), or the mitochondrial apoptosis pathway is the preferred route as a result of reduced DISC formation in those cells (type II cells, e.g. Jurkat, CEM) (13).

Reduced glutathione (GSH) is the major antioxidant of the cell and affects numerous central cellular functions such as metabolism, cell growth, transcription, and apoptosis (14, 15). GSH has a dual role in cell death; on the one hand, depletion of GSH triggered neuronal death (16), potentiated endothelial cell death induced by polychlorinated biphenyls (17) or apoptosis of leukemic cells induced by o xo-cholesterol (18), and also augmented toxicity of cisplatin in mice (19). These findings might be explained by an indirect enhancement of reactive oxygen species (ROS) production (20). As effectors of ROS that activate or potentiate apoptosis pathways, generation of ceramide and kinase or p53 activation have been proposed (21).

On the other hand, pro-oxidative conditions including direct depletion of GSH can be cytoprotective in many systems; oxidants such as dithiocarbamates or superoxide attenuated CD95-mediated apoptosis (22–25) as well as tumor necrosis factor receptor-1-mediated hepatocyte apoptosis (26). The depletion of intracellular GSH prevented NO-induced apoptosis of macrophages (27) as well as tumor necrosis factor receptor-1 and CD95-mediated hepatocyte apoptosis in vivo (28–30). Vice versa, antioxidants such as piperazinediethanesulfonic acid (CAPS), 3-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfonic acid (PIPES), 1,4-piperazinediethanesulfonic acid (CAPS), 3-(2-cyclohexylamino)-1-propanesulfonic acid (GSH-E), glutathione monoethylster.

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§ These authors contributed equally to this work.
¶ Present address: Inst. of Molecular and Cell Biology, 30 Medical Dr., 117609 Singapore.
** Present address: Lab. of Immunobiology, Dana-Farber Cancer Inst. and Dept. of Medicine, Harvard Medical School, Boston, MA 02115.
† To whom correspondence should be addressed: Inst. of Molecular and Cell Biology, Caspases and Cell Death Group, 30 Medical Dr., 117609 Singapore. Tel.: 65-874-3019; Fax: 65-874-7791117; E-mail: hentze@imcb.nus.edu.sg.

** The abbreviations used are: FADD, Fas-associated death domain; aF, 7-aminot-4-trifluoromethylcoumarin; BSO, buthionine-sulfoximine; CDNB, 1,4-chloro-2,4-dinitrobenzene; cyt c, cytochrome c; DEM, diethyl maleate; DISC, death-inducing signaling complex; DTT, dithiothreitol; FLIP, FLICE-inhibitory protein; GSH, reduced glutathione; GSSG, glutathione disulfide; ROS, reactive oxygen species; mAb, monoclonal antibody; PBS, phosphate-buffered saline; CHAPS, 3-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; CAPS, 3-(2-cyclohexylamino)-1-propanesulfonic acid; GSH-E, glutathione monoethyl ester.
versa, antioxidants were reported to enhance or facilitate apoptosis, e.g., the antioxidant α-lipoic acid enhanced CD95-mediated apoptosis in Jurkat cells (31), and the antioxidant protein thioredoxin was necessary for apoptosis induction in MCF-7 cells (32, 33). As a proposed mechanism, it has been demonstrated in many studies that under cell-free conditions, caspase activity or caspase activation requires a reducing environment: (i) oxidants such as NO or hydrogen peroxide blocked caspase-3 activity (23, 34); (ii) the reductants GSH or DTT were necessary to ensure full caspase-3 activity (28); and (iii) the thiol oxidant disulfiram and oxidized glutathione (GSSG) directly inhibited apoptosome-mediated caspase-3 activation (22). Respective studies for the glutathione dependence of caspase-8 activation, however, are lacking.

Sufficient GSH appears to be a prerequisite for death receptor-mediated apoptosis in some systems (see above). This might be due to the redox sensitivity of either caspases or the DISC, because FADD and procaspase-8 contain a number of reactive cysteine residues. This study therefore addressed whether acute depletion of GSH influences DISC formation, DISC activity, and the ensuing apoptosis in the cell lines SKW6.4, Jurkat, CEM, and H9. We further investigated whether caspase-8 activation at the immunoprecipitated DISC is redox-dependent under cell-free conditions.

**EXPERIMENTAL PROCEDURES**

**Materials**—Glutathione monohydrate was obtained from Calbiochem (La Jolla, CA), 1-cis-chloro-2,4-dinitrobenzene (CDNB) was from Fluka (Buchs, Switzerland), N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl-coumarin (DEVD-afc) and Pefablock® were from Biomol (Hamburg, Germany), and protein A-Sepharose was from Sigma. The dyes Hoechst-33342 and SYTOX were bought from Molecular Probes (Hamburg, Germany), and protein A-Sepharose was from Sigma. The dyes Hoechst-33342 and SYTOX were bought from Molecular Probes (Eugene, OR). All other reagents not further specified were purchased from Sigma. Activating αCD95 antibody (αCD95, clone CH11) and polyclonal IgG3 horseradish peroxidase-coupled secondary antibody (goat anti-mouse) were purchased from Pharmingen (San Diego, CA).

For DISC analysis, isotype-specific secondary antibodies from Southern Biotechnology Associates (Birmingham, AL) were used. The αFADD mAb was purchased from Transduction Laboratories (Lexington, KY), and the polyclonal αCD95 antibody was from Santa Cruz (sc-715, Santa Cruz, CA). The caspase-8 mAb C15 (mouse IgG2b) (2), the α-FLIP mAb NF6 (mouse IgGl) (35), and the αAPO-1 mAb (agonistic αCD95, IgG3x, used for stimulation of the DISC and CD95 immunoprecipitation) (36) were generated as described. Other recombiant enzymes not further specified were purchased from Roche Molecular Biochemicals or Sigma. Cell culture plates and other plastic materials were purchased from Greiner (Frickenhausen, Germany). Culture media were from Biochrom (Berlin, Germany), and penicillin, streptomycin, and fetal calf serum were bought from Invitrogen.

**Cell Culture**—The human B lymphoblastoid cell line SKW6.4 (ATCC, Manassas, VA) and the T leukemia cell lines Jurkat (clone E6-1), H9, and CEM (all ATCC) were grown in suspension in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum at 37 °C in 5% CO₂ and were passaged routinely every 2–3 days in a ratio of 1:10 or 1:5 depending on cell density. For experiments, the cells in passages 5–10 were centrifuged (460 × g, 4 °C, 5 min), resuspended in medium, plated in 96-well plates (100 µl of cell suspension, 10³ cells/well), and incubated with the compounds indicated (given in 100 µl/well; final volume, 200 µl/well) under the same conditions described for cell maintenance. To determine the amount of necrosis or apoptosis in lymphoid cells, the cultures were stained with a mixture of the membrane-permeable dye Hoechst-33342 (500 ng/ml) and the membrane-impermeable dye SYTOX (500 nm) for 5 min at 37°C. The amount of normal, necrotic (damaged/SYTOX-permeable membrane, normal nuclei) and apoptotic (impermeable membrane, condensed/fragmented nuclei) cells were scored with a fluorescence microscope.

**Determination of Glutathione**—The amount of GSH was quantified according to the enzymatic cycling method originally described by Tietze (37). Briefly, the cells were washed three times in ice-cold PBS, subsequently frozen at −80 °C in 1% sulfosalicylic acid, thawed, and the supernatants were separated from precipitated proteins by centrifugation (5 min, 14,000 × g, 4 °C). The supernatants and standards (0–5 µM GSH) were diluted in 0.1 M HCl containing 10 mM EDTA, and total glutathione was quantified with an ACP 5040 analyzer (Eppendorf, Hamburg, Germany).

**Measurement of Caspase-3-like Activity**—The activity of caspase-3-like proteases was measured on microtiter plates according to Thornberry (38). The cells were lysed (freeze-thaw in lysis buffer: 25 mM HEPES, pH 7.5, 5 mM MgCl₂, 1 mM EGTA, 1 mM Pefablock®, and 1 µg/ml each pepstatin, leupeptin, and aprotinin, 0.1% Triton X-100), the lysate was subsequently centrifuged (15 min, 14,000 × g, 4°C), and the clear supernatant was stored at −80 °C. The samples were diluted 1:10 with substrate buffer (60 µM fluorogenic substrate DEVD-afc in 50 mM HEPES, pH 7.4, 1% sucrose, 0.1% CHAPS, 10 mM DTT or as indicated) at a final volume of 100 µl, and blanks contained 10 µl of extraction buffer plus 90 µl of substrate buffer. Generation of free aef at 37 °C was kinetically determined by fluorescence measurement (excitation, 385 nm; emission, 505 nm) using the fluorometer plate reader Victor² (Wallac Instruments, Turku, Finland). Protein concentrations of the corresponding samples were estimated with the Pierce assay, and the activity was calculated using serially diluted standards (0–5 µM aef).

**Cytochrome c/dATP-induced Caspase Activation**—Jurkat cells were maintained at 2 × 10⁶ cells/sample in 50-µl Falcon tubes, treated as indicated, and subsequently centrifuged (5 min, 460 × g, 4°C). The pellet was lysed by repeated freeze-thawing in 50 µl of hypotonic extraction buffer (50 mM PIPES, pH 7.4, 50 mM KCl, 2 mM MgCl₂, 5 mM EGTA, 1 mM Pefablock®, and 1 µg/ml each pepstatin, leupeptin and
Glutathione and DISC Activity

**Statistics**—All data are given as the means ± S.D. The statistical differences were determined using the software GraphPad Instat® by one-way analysis of variance followed by the Tukey multiple comparison test of the control versus other groups. P < 0.01 was considered significant.

**RESULTS**

**Glutathione Depletion and Repletion in SKW6.4 Cells**—Initially, various compounds were tested for their capacity to lower GSH in SKW6.4 cells. The GSH synthesis inhibitor buthionine-sulfoximine (BSO) induced a sustained decrease in intracellular GSH to about half of the initial concentration (Fig. LA). Either of the two GSH S-transferase substrates diethylmaleate (DEM) and 1-cis-chloro-2,4-dinitrobenzene (CDNB) given in combination with BSO depleted intracellular GSH to below 10% of the initial concentration in 1 h. This depletion occurred without measurable toxicity within 4 h as determined by SYTOX/Hoechst staining. Further experiments showed that DEM was toxic at concentrations >2 mM and that CDNB was toxic at concentrations >20 μM. Notably, SKW6.4 cells in later passages were generally prone to higher basal toxicity and hence tolerated GSH depletion less well; therefore only cells in early passages were used for following experiments.

In the CD95 DISC, we investigated whether CD95-induced apoptosis is arrested in glutathione-depleted SKW6.4 cells. Next, we investigated whether CD95-mediated apoptosis is arrested in glutathione-depleted SKW6.4 cells.
mediated apoptosis in SKW6.4 cells is affected by GSH variations. As shown in Fig. 2A, exposure of cells to a CD95-activating antibody (CH-11) for 4 h resulted in about 70% apoptotic cells as determined by Hoechst/SYTOX exclusion staining. When cells were depleted of GSH by DEM or CDNB and then subjected to CD95 activation by CH-11, only <10% apoptotic cells were found after 4 h (Fig. 2, A and B).

Upon death receptor stimulation, apoptosis is initiated by caspase-8 and executed by caspase-3-related caspases (43, 44). All known caspases are redox-sensitive proteases (22, 23, 45). Therefore, caspase-3-like proteolytic activity was first examined upon CD95 stimulation. 4 h after CD95 engagement, a high DEVD cleaving activity was determined (Fig. 2C, filled squares). In the presence of DEM alone or DEM plus BSO, caspase activity was suppressed at this time to 42% or <20%, respectively. Because the DEVD-afc cleavage assay was carried out in the presence of 10 mM of the thiol reductant DTT, a condition sufficient to reactivate oxidized caspasas (34, 46, 47), we concluded that the observed decrease in caspase-3-like activity represents a reduction of executioner caspase activation rather than a reduction in activity itself. Western blot analysis of caspase-8 activation after CD95 triggering showed that the active caspase-8 p18 fragment was not detectable in cytosolic extracts upon GSH depletion by DEM (Fig. 2D), indicating that CD95 signal transduction was blocked upstream of caspase-8 cleavage to its active form. Therefore, CD95 DISC formation and activity was eventually analyzed in SKW6.4 cells (see below).

Repletion of Intracellular Glutathione Restores Activation of Executioner Caspases—Caspases can form mixed disulfides with agents such as dithiocarbamates, resulting in their inactivation (22). Therefore, a possible direct interaction of DEM or CDNB with caspases was addressed by two experimental approaches. First, the influence of GSH repletion on CD95-induced caspase activation was examined. Caspase-3-like activity 4 h after CD95 engagement was inhibited by CDNB in a concentration-dependent manner (Fig. 3A; data for DEM not shown). In SKW6.4 cells depleted of GSH by 5 μM CDNB, 2 mM GSH ester was sufficient to significantly restore activation of executioner caspases (Fig. 3B). In addition, CDNB (up to 20 μM) or DEM (up to 4 mM) did not affect caspase-3-like activity when directly added in vitro to the cleavage assay in the absence of DTT (data not shown). These data imply that reagents that deplete GSH enzymatically do not directly interfere with the enzymatic activity of caspases but rather disable CD95-mediated apoptotic signaling via secondary effects following depletion of GSH.

DISC Formation but Not Caspase-8 Processing Is Intact Following GSH Depletion in SKW6.4 Cells—The active caspase-8 p18 fragment was not detectable in cytosolic extracts of SKW6.4 cells after GSH depletion by DEM and CD95 ligation (Fig. 2D). Therefore, CD95 DISC formation as a prerequisite for caspase-8 activation was analyzed (Fig. 4A). The DISC contains several proteins including FADD, the long and short form of c-FLIP (FLICE-inhibitory protein), and caspase-8. These proteins all contain several cysteine residues, and we therefore investigated whether the proper assembly of the DISC is affected by GSH.

After maintenance of SKW6.4 cells for 1 h in medium containing BSO plus 0.25 to 1 mM DEM, the DISC was stimulated and immunoprecipitated with the antibody αAPO1 as described (1, 40). Western blots of the precipitated proteins revealed that even in the presence of up to 1 mM DEM, both forms of c-FLIP (short and long c-FLIP), FADD and both caspase-8 splice variants (caspase-8/a and caspase-8/b) were still recruited to CD95 (Fig. 4A). Also, the amount of precipitated CD95 itself did also not change under different GSH levels. These data show that DISC formation also took place under GSH-depleted conditions.

Activation and turnover of procaspase-8 at the DISC is mediated by two subsequent cleavage steps resulting in the release of the active subunits p18 and p10 into the cytoplasm (2, 41). Initial processing leads to the generation of p43/p41 cleavage intermediates. Notably, this first step in caspase-8 maturation was suppressed in a concentration-dependent fashion upon GSH depletion (Fig. 4A). At a concentration of 1 mM DEM, which entirely suppressed apoptosis and activation of executioner caspases (Fig. 2), caspase-8 cleavage was not detectable at the DISC level. This is in line with the absence of active p18 caspase-8 in the cytoplasm after exposure of the cells to DEM (Fig. 2C). Notably, c-FLIP long was still cleaved to the 43-kDa intermediate. This may be due to the low enzymatic activity of procaspase-8 or to residual enzymatic active caspase-8 under GSH-depleted conditions. Taken together, these data show that in SKW6.4 cells, a depletion of intracellular GSH by GSH S-transferase substrates arrests the execution of CD95-mediated apoptosis at the level of caspase-8 proenzyme processing at the DISC.

FIG. 3. Restoration of CD95-triggered caspase activation by GSH repletion in SKW6.4 cells. A, cells were GSH-depleted by the indicated concentrations of CDNB; and 10 mM GSH-E was added 1 h later. 2 h after the addition of CDNB, apoptosis was induced by αCD95 (100 ng/ml). The caspase-3-like activity was determined in cytosolic extracts after 4 h. B, in a similar experiment, the cells were GSH-depleted with 0.2 mM BSO/5 μM CDNB, and GSH was repleted by the indicated concentrations of GSH-E. A p value < 0.01 based on analysis of variance followed by the Tukey multiple comparison test is indicated (** versus untreated control). The values are depicted as the means ± S.D. from three wells. con, control.
Caspase-8 Activation under Cell-free Conditions Requires a Strong Reductant—In view of the complex redox regulation in intact cells, we additionally investigated whether activation of caspase-8 at the DISC depends on GSH also under cell-free conditions. We precipitated CD95 from nonstimulated and stimulated SKW6.4 cells to perform an in vitro caspase-8 cleavage assay (40, 41). After immunoprecipitation, DISC samples were washed to eliminate cellular GSH and cellular components and resuspended in buffer with or without DTT or GSH, respectively. Then the samples were incubated with 35S-labeled procaspase-8/a. Procaspase-8/a was processed to the cleavage products p43, p26, p18, and p10 only when reducing agents such as DTT or GSH were present (Fig. 4B). In the absence of reducing agents, no cleavage of procaspase-8/a was detectable. Our experiments indicate that full activation of caspase-8 at the DISC depends on the presence of a strong reductant, which may be DTT in vitro or its physiological equivalent GSH in living cells.

GSH Depletion Differently Affects CD95-mediated Apoptosis in CEM, H9, and Jurkat Cells—The role of GSH depletion in CD95-mediated apoptosis was further studied in the cell lines CEM, H9, and Jurkat. GSH depletion was achieved with the same nontoxic concentrations of BSO and DEM as described for SKW6.4 cells (Fig. 1A), with the exception that CEM cells tolerated only up to 0.5 mM DEM. In both CEM and H9 cells, apoptosis elicited by the CD95-activating antibody CH-11 was suppressed when cells were depleted of glutathione (Fig. 5, A and B) according to what we have found in SKW6.4 cells (Fig. 2). In contrast, lowering GSH only slightly suppressed apoptosis of Jurkat cells (Fig. 5C). Here, however, nuclear fragmentation was incomplete and atypical compared with cells treated only with αCD95, despite the nuclei appeared to be condensed (Fig. 5E, blue cells). We also noticed that a higher proportion of the cells now underwent necrosis instead of apoptosis (Fig. 5, C and E, green cells). Thus, only the type of cell death upon CD95 engagement changed in GSH-depleted Jurkat cells with a partial switch from apoptosis to necrosis, indicating that apoptosis signaling downstream of mitochondria might have been affected by low GSH.

Therefore, we eventually investigated whether apoptosome-triggered caspase activation might also depend on the presence of GSH. We used a cell-free system based on cytosolic lysates and cyt c/dATP in which caspase-3 activation triggers caspase-3 activation (9, 12, 39). Jurkat cells were depleted of GSH by DEM or CDN, and then cytosolic samples were prepared, and cyt c/dATP was added. After 30 min at 37 °C, about 400 microunits/mg DEVD cleaving activity as a readout for caspase-3 activation was detected in control cytosol (Fig. 5D). This activity was suppressed by 70% in GSH-depleted cytosol. Notably, caspase-3 activity was not restored after the addition of DTT. Suppression of executioner caspase activity after GSH depletion was also seen in SKW6.4 and Jurkat cells when apoptosis was induced by the unspecific kinase inhibitor staurosporine, a stimulus that does not include CD95 activation (5 μM staurosporine, 4 h of incubation time; SKW6.4 cells; STS, 810 ± 30 microunits/mg; STS + BSO/DEM: 190 ± 20 microunits/mg; Jurkat cells: STS, 670 ± 20 microunits/mg; STS + BSO/DEM: 50 ± 15 microunits/mg). Thus, the cellular SH-redundent GSH also appears to be important for caspase activation via the apoptosome pathway.

Fig. 4. GSH dependence of caspase-8 activation at the DISC in SKW6.4 cells and in a cell-free system. A, cells (10⁶/sample) were treated with BSO (0.2 mM) plus DEM (0.25, 0.5, and 1 mM, respectively) as indicated, and CD95 was immunoprecipitated. DISC preparation was performed as described under “Experimental Procedures.” Lane 1 (–) shows the negative control with αAPO1 bound to protein A-Sepharose. Detection of c-FLIP₈, p43-c-FLIP₂, and c-FLIP₆ proteins was performed using the NF6 α-FLIP antibody. The same blot was stripped and subsequently developed with C15 α-caspase-8 mAb, which detects both caspase-8 isoforms (zymogens: p55/p53) and the cleavage intermediates p43/p41. The secondary antibody also detected IgG3 of αAPO1 used for CD95 immunoprecipitation. FADD was detected using an αFADD mAb, and CD95 was immunodetected by an αCD95 polyclonal antibody serving as a control for precipitation efficiency and protein loading. B, CD95 was immunoprecipitated from 2 × 10⁶ either untreated (– anti-APO1) or αCD95-treated (+ anti-APO1) SKW6.4 cells. Immunoprecipitates were washed and incubated with in vitro translated 35S-labeled procaspase-8/a in the absence or presence of 10 mM DTT, 10 mM GSH, and 10 μM NADPH + 0.2 unit/ml GSSG reductase as indicated. After 24 h, the samples were analyzed on a 15% SDS-polyacrylamide gel. The positions of procaspase-8/a and its fragments p43, p26, p18, and p10 are indicated. The upper part of the gel was exposed for 16 h, and the lower parts were exposed for 5 days.
DISCUSSION

In this study, we report the in vitro GSH dependence of the most upstream caspase in CD95 signaling, caspase-8. We also showed that CD95-triggered apoptosis can be inhibited by GSH depletion in SKW6.4, CEM, and H9 cells. These observations are consistent with previous in vivo studies demonstrating that GSH is a prerequisite for death receptor-mediated hepatic apoptosis in mice (28–30). Besides by GSH depletion, the initiation of CD95-triggered apoptosis can also be inhibited by ROS (25), an event that inevitably produces GSSG by the activity of glutathione peroxidase and thus indirectly depletes GSH. Yet a difference exists between an acute depletion of GSH, which does not necessarily result in an induction of redox stress, and primary redox stress, which in turn may lead to changes in the GSH/GSSG ratio and eventually to GSH depletion. Because ROS are known to inhibit active caspases (23), it is important to note that the following findings exclude an inhibition of caspase activation and cell death by ROS in our experiments:

(i) activation of caspase-9 and caspase-8 in cell-free systems was dependent on the presence of a reductant (Figs. 4B and 5D), but a production of ROS does not occur under these conditions;

(ii) incubation of SKW6.4 cells with various antioxidants that are known to lower ROS in the cell culture (e.g. catalase, superoxide dismutase) failed to reverse apoptosis inhibition exerted by BSO/CDNB (not shown); likewise, a broad array of enzymatic and nonenzymatic antioxidants failed to restore sensitivity toward CD95-triggered hepatocyte apoptosis after phorone-induced GSH depletion (28), a condition that also lacks ROS production (48); and

(iii) oxidative stress was shown to inhibit CD95-mediated apoptosis of Jurkat T cells indirectly by lowering intracellular ATP (49). We therefore checked the concentration of intracellular ATP under GSH-depleted condi-
tions in our experiments and found no differences from control cells within the time frames studied here (not shown). As we also ruled out a direct interference of the GSH depletors used with caspases (Fig. 3), the reduced intracellular GSH concentration as such is responsible for halting apoptosis initiation at the level of caspase-8.

The question arises as to why GSH depletion did not block cell death in Jurkat cells despite the fact that caspase-3-like activity was suppressed. Previously, it has been demonstrated that oxidant-mediated caspase inactivation leads to necrosis in HepG2 cells, and low GSH also switched the mode of cell death from apoptosis to necrosis in leukemic cell lines treated with cytotoxic drugs (50, 51). With regard to Jurkat cells, an inhibition of caspase activation downstream of mitochondria by either peptide inhibitors or ATP depletion lead to necrotic rather then apoptotic cell death (52–54). Thus, first, one possible explanation for our data is that in Jurkat cells, residual low amounts of GSH after enzymatic depletion were still sufficient to allow a partial activation of caspase-8 at the DISC that was not detected by the methods used. Therefore, truncated Bid may still have been generated by low caspase-8 activity and subsequently induce loss of mitochondrial transmembrane potential in Jurkat cells, even though activation of executioner caspasess downstream of the mitochondria is now impaired (Fig. 5D). This condition would eventually lead to aberrant apoptosis and necrotic cell death as seen in our experiments (Fig. 5E).

Second, two groups identified a necrotic, caspase-independent pathway of CD95 in caspase-8-deficient Jurkat cells, which is originating from FADD (55, 56). Although a physiological relevance for this type of caspase-8-independent CD95-mediated necrosis remains to be shown, this pathway may provide an alternative explanation for our findings. Active caspase-3 is strictly required for certain nuclear apoptotic hallmarks such as chromatin condensation and DNA fragmentation (57). Thus, third, the observation that nuclear condensation still occurred under low GSH in Jurkat cells, whereas nuclear fragmentation appeared incomplete (Fig. 5E), may be explained by caspase-independent, apoptosis-inducing factor-triggered nuclear changes (58). Collectively, these findings show that cell death, even though not of pure apoptotic nature, can still occur in this cell line when downstream caspase-driven mechanisms are impeded.

The data of our study suggest that under GSH depletion, apoptosis was arrested in SKW6.4 cells because of an inhibition of the first cleavage step of caspase-8 activation rather than by an impairment of caspase activity. We also revealed that the activation of executioner caspases was suppressed in the absence of GSH when the apoptosis/caspase-9 pathway was triggered in a cell-free system (Fig. 5D), and also a suppression of staurosporine-triggered caspase activation in SKW6.4 and Jurkat cells. Thus, our data are consistent with a recent report that caspase-3 activity does not interfere with the activity of caspase-8 under intracellular conditions but rather can halt caspase-3 activation at an upstream step, possibly at the level of caspase-9 (59). Furthermore, the group of S. Orrenius reported previously that the balance between GSH and oxidized GSSG also influences cyt c/dATP-mediated caspase activation under cell-free conditions, which also shows that the apoptosis-mediated pathway is redox-dependent (22). As to the mechanism that renders the procaspases inactivatable by changes in the GSH/GSSG system, direct disulfide formation of the active site reactive cysteine or other cysteines within the enzyme is likely to occur (22), since similar redox mechanisms have been found to control lymphocyte activation by modulating NFκB binding activity (14). Collectively, these data argue that the activation of both upstream caspases, caspase-8 and caspase-9, rather than caspase activity itself is dependent on the thiol-disulfide status.

Whereas prevention of CD95-triggered apoptosis in hepatocytes in vivo (28–30) and in three leukemic cell lines in vitro (this study) was very pronounced, it did not occur in HepG2 or Hepa1–6 cells and was less prominent in primary cultured murine hepatocytes.2 Obviously, the inability of cells depleted of GSH to initiate CD95-triggered cell death appears to be restricted to distinct experimental systems, indicating that additional factors might determine whether apoptosis induction depends on sufficient availability of GSH in a given cell type. A proposal with broader perspective for further investigation is that the redox-active protein thioredoxin, capable of reactivating oxidized caspases (60), might be a second crucial regulator of caspase activation and present in different amounts in different cell types. In fact, two recent studies showed that caspase-8-driven apoptosis in MCF-7 cells is promoted by overexpression of thioredoxin plus thioredoxin reductase, and, moreover, that thioredoxin anti-sense confers resistance against apoptosis induction (32, 33). One can conclude from these and other studies that the delicate balance between physiological antioxidants (GSH and thioredoxin) and endogenously produced or exogenous oxidants (ROS and nitric oxide) finally determines whether caspase-initiated apoptosis can precede or is halted. Further studies on the redox regulation of caspase activation are likely to reveal additional regulatory factors and mechanisms controlling cell death.

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2 H. Hentze, M. Latta, and A. Wendel, unpublished observations.
