Redox amplification of apoptosis by caspase-dependent cleavage of glutaredoxin 1 and S-glutathionylation of Fas

Vikas Anathy,1 Scott W. Aesif,1 Amy S. Guala,1 Marije Havermans,1 Niki L. Reynaert,4 Ye-Shih Ho,2 Ralph C. Budd,2 and Yvonne M.W. Janssen-Heininger1

1Department of Pathology and 2Department of Medicine, University of Vermont, Burlington, VT 05405
3Institute of Environmental Health Sciences, Wayne State University, Detroit, MI 48202
4Department of Respiratory Medicine, Maastricht University Medical Center, Maastricht, Netherlands 6229ER

R eactive oxygen species (ROS) increase ligation of Fas (CD95), a receptor important for regulation of programmed cell death. Glutathionylation of reactive cysteines represents an oxidative modification that can be reversed by glutaredoxins (Grxs). The goal of this study was to determine whether Fas is redox regulated under physiological conditions. In this study, we demonstrate that stimulation with Fas ligand (FasL) induces S-glutathionylation of Fas at cysteine 294 independently of nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase–induced ROS. Instead, Fas is S-glutathionylated after caspase-dependent degradation of Grx1, increasing subsequent caspase activation and apoptosis. Conversely, overexpression of Grx1 attenuates S-glutathionylation of Fas and partially protects against FasL-induced apoptosis. Redox-mediated Fas modification promotes its aggregation and recruitment into lipid rafts and enhances binding of FasL. As a result, death-inducing signaling complex formation is also increased, and subsequent activation of caspase-8 and -3 is augmented. These results define a novel redox-based mechanism to propagate Fas-dependent apoptosis.

Introduction

Fas (CD95; Apo-1) is a member of the tumor necrosis factor receptor superfamily of death receptors that shares a conserved 80 amino acid death domain (DD) in their cytoplasmic tail critical in apoptosis signaling (Peter et al., 2007). Upon ligation of Fas, the sequential association of Fas-associated DD (FADD), pro forms of caspase-8 and -10, and cellular FADD-like IL-1β–converting enzyme inhibitory protein occurs, leading to the formation of the death-inducing signaling complex (DISC) with resulting oligomerization, processing, and activation of caspase-8 and execution of apoptosis via direct or indirect programs (Wajant, 2002). Fas is constitutively expressed in tissues, and although its role in apoptosis is well established, additional regulatory roles of Fas that include immune cell activation and proliferation have recently been suggested (Tibbetts et al., 2003).

The production of reactive oxygen species (ROS) has traditionally been associated with cellular and tissue injury as a result of the high reactivity of some oxidant species. Compelling data now exist to demonstrate that oxidants are used under physiological settings as signaling molecules that control processes such as cell division, migration, and mediator production (Lambeth, 2004; Janssen-Heininger et al., 2008). Amino acids that are targets for reversible oxidations are cysteines with a low pKa sulfhydryl group, and numerous classes of proteins contain conserved reactive cysteine groups. These cysteines can be reversibly oxidized to sulfenic acids, S-nitrosylated cysteines, or disulfides, or be irreversibly oxidized to sulfenic or sulfonic acids (Hess et al., 2005; Janssen-Heininger et al., 2008; for review see Forman et al., 2004). S-glutathionylation reflects the formation of a disulfide between the cysteine of glutathione and the cysteine moiety of a protein (also known as protein-mixed disulfide or PSSG [protein S-glutathionylation]) and has...
emerged as an important mechanism to regulate reversible cysteine oxidations as it occurs in the cellular environment where glutathione concentrations are in the millimolar range (Fernandes and Holmgren, 2004). Under physiological conditions, the thiol transferases glutaredoxin 1 (Grx1) and 2 in mammalian cells specifically catalyze reduction of PSSG, restoring the protein cysteine to the sulfhydryl state (Fernandes and Holmgren, 2004).

Various studies exist to support a role of redox regulation of the Fas death pathway. Caspases contain a reactive cysteine critical for enzymatic activity, and a role for nitric oxide in preventing caspase activation has been established based upon findings demonstrating that caspase-3 and -9 are S-nitrosylated under basal conditions to prevent activation (Mannick et al., 1999, 2001; Benhar et al., 2008). In response to a proapoptotic stimulus, such as Fas ligand (FasL), thioredoxin-2 (Trx2)–mediated denitrosylation of caspase-3 occurs, which is a process required for caspase-3 activation and subsequent execution of the apoptotic pathway (Mannick et al., 1999, 2001; Benhar et al., 2008). Fas-mediated apoptosome formation was also shown to involve ROS derived from mitochondrial permeability transition (Sato et al., 2004). Furthermore, Fas-dependent cell death in response to highly reactive oxidants has been reported in association with clustering of Fas (Huang et al., 2003; Shrivastava et al., 2004), whereas conversely antioxidant compounds attenuate Fas-dependent cell death (Huang et al., 2003). Based on those collective observations, we sought to establish the physiological relevance of redox-based regulation of Fas. In this study, we describe a novel mechanism whereby Fas-dependent cell death is regulated. This pathway is initiated via caspase-dependent degradation of Grx1, subsequent increases in S-glutathionylation of cysteine 294 of Fas (which promotes binding of FasL and enhances recruitment into lipid rafts), formation of SDS-resistant high molecular weight (MW) Fas complexes, and DISC, and subsequently further augments activation of caspases, thereby amplifying cell death.

**Results**

**Increases in PSSG by FasL occur independently of generation of ROS but instead are associated with degradation of Grx1**

S-glutathionylation represents a redox-based modification of cysteines, which is a regulatory switch that affects cell signaling. Therefore, we addressed whether levels of PSSG were increased after ligation of Fas in lung epithelial cells using nonreducing SDS-PAGE and immunoblot analysis with an antibody directed against glutathione. Results shown in Fig. 1A demonstrate a marked increase in PSSG that occurred as early as 1 h after Fas ligation. Increases in PSSG occurred with significant specificity based on the appearance of two bands of an apparent MW between 40 and 50 kD (Fig. 1A and C) that co-migrated with Fas (not depicted).

The current paradigm of redox-based signaling after stimulation of growth factor receptors is the production of ROS by the activation of NADPH oxidases, which causes inhibition of tyrosine phosphatases (Rhee et al., 2000). Overoxidation of the antioxidant molecule peroxiredoxin (Prx) is believed to be critical in promoting signaling by H$_2$O$_2$ (Wood et al., 2003). Results shown in Fig. 1B demonstrate that oxidized Prx (Prx-SO$_2$H) appeared between 2 and 3 h after stimulation with FasL, which are time points protracted relative to the accumulation of PSSG, which occurred as early as 1 h (Fig. 1A). Incubation of cells with diphenyliodonium (DPI), an inhibitor of NADPH oxidases, failed to attenuate the increases in cellular PSSG content, whereas overoxidation of Prx was completely inhibited by DPI (Fig. 1C). These findings suggest that FasL-induced increases in PSSG and Prx-SO$_2$H occur through independent mechanisms and demonstrate that increases in PSSG occur in an NADPH oxidase–independent manner.

Mammalian cells contain Grxs, which under physiological conditions act to reverse S-glutathionylated cysteines, restoring the protein cysteine to the sulfhydryl group. Levels of cytosolic Grx1 were markedly reduced by 2 and 4 h after ligation of Fas (Fig. 1D) with corresponding decreases in enzymatic activity (Fig. 1E). Consistent with the lack of effect of PSSG, DPI also failed to restore Grx1 levels in cells stimulated with FasL (Fig. 1C). In contrast to decreases in Grx1, expression of the related disulfide reductase Trx1 remained unchanged in response to ligation of Fas (Fig. 1D), demonstrating that the redox changes caused by ligation of Fas showed some specificity toward Grx1.

**Activation of caspases is required for degradation of Grx1 and S-glutathionylation of Fas**

Engagement of Fas causes a rapid activation of caspase-8 and -3 (Hengartner, 2000). Sequence analysis of murine Grx1 suggested that amino acids 43–46 (EFVD) and 56–59 (AIQD) may be putative cleavage sites of caspase-8 and -3, both of which have predicted affinity toward glutamic and aspartic acid residues (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200807019/DC1; Earnshaw et al., 1999). This raised the possibility that upon ligation of Fas, Grx1 was degraded in a caspase-dependent fashion. Indeed, pretreatment of cells with a generic caspase inhibitor, ZVAD-FMK, effectively blocked FasL-induced cleavage of caspase-8 and -3 and completely prevented FasL-induced degradation of Grx1 (Fig. 2A). Immuno-precipitation (IP) of Grx1 followed by immunoblot analysis of cleaved caspase-8 and -3 demonstrated an association between active caspases and Grx1 in cells after ligation of Fas, whereas in control cells, these associations were not observed (Fig. 2B). Incubation of recombinant Grx1 with active caspase-8 or -3 in vitro led to the formation of a fragment of ~8 kD, which was more apparent in response to caspase-3 as compared with caspase-8 (Fig. 2C). Consistent with the protection against Grx1 degradation (Fig. 2A), pretreatment of cells with ZVAD-FMK prevented the formation of detectable levels of PSSG after FasL stimulation (Fig. 2D). Based on our observations that proteins that were S-glutathionylated upon stimulation of cells with FasL comigrated with Fas, we speculated that Fas itself could be a target for S-glutathionylation. Lysates from FasL-treated cells were immunoprecipitated using an antiguithione antibody followed by detection of Fas by immunoblotting. After FasL stimulation, Fas-SSG (S-glutathionylated Fas) was detectable as
efficient to cause the formation of Fas-SSG. In aggregate, these findings demonstrate that after stimulation of cells with FasL, caspase-dependent degradation of Grx1 occurs in association with increases in Fas-SSG. Our data also suggest that caspase activation is necessary but may not be sufficient for degradation of Grx1.

Increased S-glutathionylation of Fas results in enhanced apoptosis in cells lacking Grx1

We next examined the impact of modulation of Grx1 on Fas-SSG and the subsequent sensitivity of cells to undergo apoptosis. Transfection of lung epithelial cells with a Grx1-specific siRNA caused a marked decrease in the cellular content of Grx1 (Fig. 3 A). Two independent approaches were used to assess Fas-SSG that encompassed IP with a specific antibody directed against overoxidized Prx (Prx-SO$_2$H). The bottom panel shows total Prx1 content. (C) Lack of requirement of NADPH oxidase activity in FasL-induced increases in PSSG. C10 cells were treated with FasL in the presence or absence of the inhibitor of 10 μM NADPH oxidase DPI. Lysates were prepared at the indicated times for assessment of PSSG as described in A. Total levels of Prx1, Prx-SO$_2$H, or Grx1 were assessed by immunoblotting. (D) Evaluation of total Grx1 and Trx1 content by immunoblotting in cells exposed to FasL + M2 as described in A. The bottom panel shows β-actin content as a loading control. (E) Evaluation of enzymatic activity of Grx1 in C10 cells exposed to 200 ng/ml FasL + 500 ng/ml M2 for 1–4 h. Results represent triplicate values from two independent experiments. The time-dependent decrease in Grx1 activity was significant at the level of P < 0.05 by Student’s t test. Error bars represent SEM.
Figure 2. FasL induces caspase-dependent cleavage of Grx1 and increases PSSG as well as S-glutathionylation of Fas. (A) Immunoblot analysis of cleaved caspase-8 (p18) and -3 fragments (p17 and p19) in C10 cells treated with FasL + M2 as described in Fig. 1 in the presence or absence of 10 μM ZVAD-FMK. The bottom panel shows total cellular content of Grx1. Note that expression of the pro form of caspase-8 remains unchanged during the course of the experiment. (B) Evaluation of the interaction between Grx1 and caspase-8 or -3 in cells. C10 cells were exposed to FasL + M2 as described in Fig. 1 A, and Grx1 was immunoprecipitated (IP) at the indicated times for the evaluation of association with active caspase-8 or -3 fragments via Western blotting. The bottom panel represents total content of proteins in whole cell lysates (WCL) that were used as the input for IP. Note that expression of the pro form of caspase-8 remains unchanged during the course of the experiment. (C) In vitro assessment of cleavage of Grx1 by caspase-8 or -3. 200 ng recombinant hGrx1 was incubated with 200 U active caspase-8 or -3. At the indicated times, samples were prepared for immunoblot analysis of hGrx1. Fragmented hGrx1 product is ~8 kD in size. Incubation of heat-inactivated caspase-8 and -3 with hGrx1 for 4 h largely prevented the formation of cleaved fragment (0 h). (D) Increases in overall PSSG are a response to ligation of Fas and are caspase dependent. Cells were incubated as described in A. ZVAD-FMK or vehicle was added to cells 2 h before ligation of Fas as well as 2 h after ligation. Lysates were resolved by nonreducing SDS-PAGE. Antiglutathione antibody was used to detect PSSG on immunoblots. The bottom panel shows total Fas content. (E) Caspase-dependent S-glutathionylation of Fas. C10 cells were incubated with FasL + M2 for 0.5, 1, or 2 h in the presence or absence of ZVAD-FMK. Cell lysates were subjected to nonreducing IP (+DTT) using antiglutathione antibody to IP S-glutathionylated proteins (IP: PSSG) before detection of Fas via Western blotting. As a reagent control to reduce S-glutathionylated proteins before IP, samples were incubated with 50 mM DTT (+DTT). The bottom panel shows total content of Fas, procaspase-8, cleaved caspase-8, and Grx1 in whole cell lysates. (F) S-glutathionylation of Fas requires the presence of caspase-8. C10 cells were transfected with control (Ctr) siRNA or caspase-8 (C8)-specific siRNA and 48 h later were incubated with FasL + M2 for 2 or 4 h. The top lane shows assessment of S-glutathionylation of Fas via IP of S-glutathionylated proteins using antiglutathione antibody (IP: PSSG) under nonreducing conditions (−DTT) before detection of Fas via Western blotting. As a reagent control to reduce S-glutathionylated proteins before IP, samples were incubated with 50 mM DTT (+DTT). The bottom panel shows total content of Fas, procaspase-8, cleaved caspase-8, and Grx1 in whole cell lysates. (G) Assessment of caspase-dependent degradation of Grx1 and S-glutathionylation of Fas in NIH 3T3 cells after ligation of Fas. Cells were treated with 500 ng/ml FasL + 1 μg/ml M2 for 1, 2, or 4 h in the presence or absence of ZVAD-FMK. S-glutathionylated proteins were immunoprecipitated as described in E before detection of Fas via Western blotting. The bottom panel represents Fas content, cleaved caspase-3, and Grx1 content in whole cell lysates.
Grx1 only after labeling of cells with biotinylated glutathione (Fig. 3 B), presumably because of the enhanced sensitivity of detection of PSSG using the latter approach as compared with the antiglutathione antibody. In addition to increasing Fas-SSG, FasL-stimulated activation of caspase-8 and -3 was also enhanced in cells subjected to Grx1 siRNA knockdown compared with controls (Fig. 3 C). Cells with lowered Grx1 content were less viable compared with controls, and Grx1 siRNA–treated cells were more sensitive to FasL-induced death than controls (Fig. 3 D). Similarly, in comparison to C57BL6 (wild type [WT]) controls, primary cultures of lung fibroblasts (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200807019/DC1) or proliferating CD4+ T lymphocytes derived from Glrx1−/− mice (Ho et al., 2007) demonstrated a higher extent of Fas-SSG upon receptor ligation (Fig. 3, E and F). Although we were unable to detect S-glutathionylation of Fas in the absence of FasL in lung fibroblasts (Fig. 3 E), possibly as a result of the aforementioned detection limits of our assay, baseline Fas-SSG was detected in Glrx1−/− fibroblasts (Fig. 3 F). Enhanced FasL-induced activation of caspase-8 and -3 was readily apparent in Glrx1−/− fibroblasts, which were also less viable before, or in response to Fas ligation compared with WT counterparts (Fig. 3, G and H).

Overexpression of Grx1 prevents increases in S-glutathionylation of Fas and attenuates caspase activation and apoptosis in response to receptor ligation

Because Grx1 deficiency caused an increase in FasL-induced caspase activation, Fas-SSG, and enhanced cell death, we speculated that overexpression of Grx1 would reduce levels of Fas-SSG and afford protection from FasL-induced apoptosis. Cells transfected with Flag-Grx1 were stimulated with FasL and examined for the S-glutathionylation status of Fas (Fas-SSG) by IP with antiglutathione antibody. Compared with pcDNA3 vector–transfected cells, overexpression of Grx1 (Fig. 4 A) markedly reduced Fas-SSG (Fig. 4 B) and markedly lowered the activities of caspase-8 and -3 (Fig. 4 C) induced in response to FasL. Overexpression of Grx1 also markedly enhanced survival (Fig. 4 D) in response to ligation of Fas as compared with controls.

S-glutathionylation of Fas promotes binding of FasL, recruitment to lipid rafts, formation of SDS-stable high MW complexes, and enhances formation of the death-inducing signaling complex

The localization of Fas in lipid rafts is essential for binding of FasL, assembly of DISC, and subsequently the induction of apoptosis (Hueber et al., 2002; Muppidi and Siegel, 2004). Therefore, we determined whether the extent of Fas-SSG affected these parameters. As expected, after stimulation of cells with FasL, an increase in the amount of Fas localized to the lipid rafts occurred based on its colocalization with raft marker caveolin1 (Fig. 5 A and Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200807019/DC1). IP of S-glutathionylated proteins revealed that in response to FasL ligation, Fas-SSG was present within the lipid raft fractions as well as soluble fractions (Fig. 5 A), whereas Grx1 was absent in the lipid raft fraction (S3 A). In cells overexpressing Grx1, the overall content of Fas and its S-glutathionylated state were decreased in lipid rafts compared with mock-transfected cells stimulated with FasL (Fig. 5 A and Fig. S3 A). Assessment of FasL binding demonstrated dose-dependent increases in WT cells. However, in cells overexpressing Grx1, no clear increases in FasL binding occurred at a range of concentrations (Fig. 5 B and Fig. S3 B). Furthermore, IP of the DISC revealed clear associations between FasL, Fas, FADD, and procaspase-8, which were diminished in cells overexpressing Grx1 (Fig. 5 C). Grx1 was absent in DISC in all conditions evaluated (Fig. 5 C and not depicted), which may sustain the presence of Fas-SSG in these signaling platforms. IP of FasL resulted in high MW SDS-stable PSSG complexes that were attenuated in cells overexpressing Grx1 and absent in samples treated with DTT (Fig. 5 D). Evaluation of Fas in these samples confirmed its presence in the high MW complex (~190 kD) after IP with FasL. This high MW Fas complex was sensitive to decomposition by DTT and markedly decreased in Grx1-overexpressing cells (Fig. 5 D), demonstrating that PSSG contributes to the formation of high MW Fas complexes that are known to be required for the induction of apoptosis (Feig et al., 2007). In cells lacking Grx1, a marked increase in binding of FasL occurred (Fig. 5 B and Fig. S3 C) in association with more IP of Fas (Fig. 5 E). Collectively, these observations demonstrate that the status of S-glutathionylation of Fas regulates binding of FasL, the ability of Fas to move into lipid rafts, formation of high MW Fas complexes, and assembly of DISC.

DD cysteine 294 is essential for FasL-induced S-glutathionylation of Fas, FasL binding, cleavage of caspase-8 and -3, and cell death

Murine Fas contains a total of 24 cysteines, out of which 20 are present in the ectodomain, and four are located in the DD (GenBank accession no. AB124113). The ectodomain cysteines are known to form intramolecular disulfide bonds and therefore do not represent likely targets for S-glutathionylation. This prompted us to search for potential cysteine targets for S-glutathionylation within the DD of Fas through the generation of constructs in which cysteines were mutated to alamines. Fas mutants, C194A, C271A, C294A, or WT Fas was transfected into fibroblasts derived from lpr mice that lack Fas (Drappa et al., 1993). After stimulation with FasL, Fas-SSG was apparent in cells transfected with WT, C194A, or C271A mutant constructs. In contrast, Fas-SSG was not apparent in cells expressing C294A mutant Fas (Fig. 6 A). In lpr fibroblasts or lung epithelial cells, expression of WT, C194A, or C271A Fas constructs resulted in enhanced formation of active caspase-8 and -3 fragments after ligation of Fas, which was not apparent in cells expressing comparable levels of C294A mutant Fas (Fig. 6 B and not depicted). Cell death in response to FasL was largely abrogated in lpr fibroblasts expressing C294A mutant Fas in comparison with cells expressing WT, C194A, or C271A mutant versions of Fas (Fig. 6 C and Fig. S4 A, available at http://www.jcb.org/cgi/content/full/jcb.200807019/DC1). Surface expression
Figure 3. Increased S-glutathionylation of Fas, caspase-8 activity, and cell death in cells lacking Grx1. (A) Assessment of S-glutathionylation of Fas after knockdown of Grx1. C10 cells were transfected with Grx1 siRNA or control (Ctr) siRNA and treated with FasL + M2 for the indicated times. S-glutathionylated proteins were immunoprecipitated using antiglutathione antibody (IP: PSSG). Samples treated with 50 mM DTT to reduce S-glutathionylated proteins (+DTT) were used as reagent controls. The content of Fas, Grx1, and actin in whole cell lysates (WCL) used as the input for IP are shown in the bottom panels.
Discussion

The regulation of biological processes via redox-based signaling events is becoming increasingly apparent, and a role for reversible and dynamic cysteine oxidations that encompass PSSG therein is also emerging (Janssen-Heininger et al., 2008). However, the relevance of these events and the precise molecular targets in apoptotic signaling via death receptors remains unclear. The findings from this study define a new redox-based regulatory system that controls apoptosis after engagement of Fas. We demonstrate in this study that initial activation of caspase-8 and/or -3 causes degradation of Grx1, resulting in S-glutathionylation of Fas at cysteine 294, which subsequently enhances binding of FasL, aggregation of Fas, accumulation of Fas in lipid rafts, DISC assembly, and further activation of caspases, causing a propagation of apoptotic cell death (Fig. 6 G).

Activation of signaling after stimulation of growth factor receptors requires the reversible cysteine oxidation of protein tyrosine phosphatases, which occurs after the activation of NADPH oxidases and resultant increases in levels of hydrogen peroxide (Rhee et al., 2000). Our findings illuminate a new paradigm in oxidant-dependent signal transduction, as we demonstrate that redox-dependent apoptotic signaling can be initiated in an NADPH oxidase–independent manner. Instead, we have identified a caspase-initiated mechanism of oxidative signaling through direct or indirect degradation of the thiol repair enzyme Grx1. It is of relevance to note that FasL–induced caspase activity was recently shown to cleave and inactivate the mitochondrially localized antioxidant enzyme, manganese superoxide dismutase (Pardo et al., 2006), creating a redox imbalance in mitochondria associated with enhancement of apoptosis, which is in line with our current observations.

Although numerous studies exist demonstrating that steady-state levels of PSSG are increased in cells after exposure to H₂O₂ (for review see Forman et al., 2004), our current observations demonstrate that FasL effectuated marked increases in levels of PSSG with notable specificity based on the observed restricted patterns of S-glutathionylation (Fig. 1 A). These findings support the concept that redox-based signaling has a high degree of specificity, is compartmentalized to limited targets, and therefore has the ability to modulate selective pathways (Janssen-Heininger et al., 2008). However, it is important to highlight that increases in PSSG are not the only redox changes that occur after ligation of Fas. Denitrosylation of active-site cysteines of caspases has been reported after ligation of Fas in association with enhancement of their activity and apoptosis (Mannick et al., 1999, 2001), and recently, a role of Trx2 has been suggested herein (Benhar et al., 2008). Moreover, we demonstrate for the first time that over-oxidation of Prx1 also occurred after ligation of Fas, likely as a result of activation of NADPH oxidases (Fig. 1 C). Importantly, over-oxidation of Prx1 occurred with delayed kinetics relative to S-glutathionylation. The relative interplay between thioredoxin-catalyzed caspase denitrosylation, Fas-S-glutathionylation, and Prx1 overoxidation is unclear at this time, but it is tempting to speculate that endogenous S-nitrosylation is important in homeostatic
was detected in a variety of primary cell types and cell lines and was demonstrated to be functionally significant based on our observations, demonstrating that S-glutathionylation of Fas promotes FasL binding and enhances trafficking of Fas into lipid rafts and assembly of DISC, thereby amplifying the strength of the apoptotic signal. Cysteine 294 is located in the carboxyterminal end of DD of mouse Fas and is conserved in the DDs of rat and human Fas (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200807019/DC1). Analogous to conditions favoring S-nitrosylation (Hess et al., 2005), the flanking of cysteine 294 of Fas by acidic and basic amino acids, its localization within the carboxyterminal tail, and the hydrophobic compartment formed by lipid rafts are plausible factors that favor its susceptibility for S-glutathionylation or sustain this cysteine oxidation. This scenario highlights the possibility that other DD-containing receptors may also be regulated through S-glutathionylation, although this remains to be formally tested.

These findings, demonstrating that modulation of Grx1 greatly impacts Fas-dependent proapoptotic signaling, identify Grx1 as a survival factor that protects cells against apoptosis. Indeed, a role of Grx1 as a survival factor is supported by findings that demonstrate its ability to enhance the activation of nuclear factor κB in association with deglutathionylation of cysteine 179 of IKK-β (Reynaert et al., 2006). Furthermore, a role for Grx2 in the protection against dopamine-induced
apoptosis has been reported via its ability to induce activation of nuclear factor κB (Daily et al., 2001). Antiapoptotic effects of Grx have also been linked to its regulation of the redox state of Akt (Murata et al., 2003) and activation of Ras-phosphoinositide 3-kinase and c-Jun N-terminal kinase pathways (Daily et al., 2001). In contrast, a proapoptotic role for Grx1 has been identified in endothelial cells stimulated with tumor necrosis factor α. In the latter study, Grx activity increased in response to tumor necrosis factor α, and Grx1 associated with S-glutathionylated caspase-3, caused its deglutathionylation, and enhanced its

Figure 5.  **Assessment of FasL binding, presence of Fas in lipid rafts, and DISC formation after manipulation of Grx1.**  
(A) Evaluation of S-glutathionylation of Fas in lipid rafts in cells stimulated with FasL + M2 and the impact of overexpression of Grx1. Cells were transfected with pcDNA3 or Flag-Grx1 and stimulated with FasL + M2 for 20 min. Lipid raft fractions (3 and 4) and soluble fraction (12) were subjected to IP with antiguathione antibody and analyzed by immunoblotting for Fas. PSSG was decomposed with 50 mM DTT as a reagent control before IP. The middle and bottom panels reflect immunoblot assays of Fas and the raft marker caveolin1 present in the input samples. Complete fractionation is shown in Fig. S3 A (available at http://www.jcb.org/cgi/content/full/jcb.200807019/DC1).  
(B) Assessment of FasL binding to cells after manipulation of Grx1. Cells were subjected to control (Cr) and Grx1 siRNA transfection. In separate experiments, cells were transfected with pcDNA3 or Grx1 plasmids. After 48 h, cells were trypsinized and incubated with ascending doses of FasL + M2 for 20 min. Binding of FasL to cells was evaluated after incubation with FITC-conjugated anti–mouse antibody and evaluation of 10,000 events via flow cytometry. Binding of FasL to cells is reflected as mean fluorescence intensity (MFI), and absolute values are plotted on the y-axis. The x-axis depicts ascending concentrations of FasL. Note that differences absolute fluorescence intensities between pcDNA3 and control siRNA–transfected cells may be a result of the different transfection procedures. Confirmation of Grx1 overexpression and knockdown is shown in Fig. S3 B and Fig. S3 C, respectively.  
(C) Assessment of FasL-interacting proteins in cells overexpressing Grx1. pcDNA3 or Grx1-transfected C10 cells were treated with M2 alone or FasL + M2. Cells were lysed, and 700 μg of protein was subjected to IP using protein G agarose beads to isolate DISC proteins. After SDS-PAGE, samples were analyzed by immunoblotting for Fas, FADD, procaspase 8, cleaved caspase-8, and Grx1.  
(D) Evaluation of PSSG and Fas content in high MW complexes after IP of FasL + M2 or M2 alone via nonreducing SDS PAGE. As a control, samples were treated with DTT before electrophoresis.  
(E) Assessment of interaction between FasL and Fas in WT primary tracheal epithelial cells or cells lacking Glrx1. Cells were exposed to M2 alone or FasL + M2 for 30 min, lysed, and 700 μg of protein was subjected to IP using protein G agarose beads. After SDS-PAGE, samples were analyzed by immunoblotting for Fas. WCL, whole cell lysate.
In summary, our study reveals a new dimension in regulation of the Fas signaling pathway that is redox based in nature. Caspase-initiated degradation of Grx1 and subsequent S-glutathionylation of Fas represents a feed-forward amplification enzymatic activity (Pan and Berk, 2007). These conflicting data demonstrate that the outcome of S-glutathionylation is clearly controlled by ligand-dependent modulation of Grx activity and the molecular targets of Grx1.

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loop to enhance apoptosis (Fig. 6 G). This study identifies Grx1 as an attractive target to modulate death receptor–induced apoptosis.

**Materials and methods**

**Reagents and antibodies**

The following antibodies were used: Fas (Millipore), FADD (MBL International), caspase-8 (Enzo Biochem, Inc.), caspase-3 (Cell Signaling Technology), glutathione (Virogen), bixin (Jackson Immunoresearch Laboratories), Prx1 and Prx-SO2H (Lab Frontier), Grx1 (American Diagnostica Inc.), Trx1 (Santa Cruz Biotechnology, Inc.), caveolin1 and J02 (BD), and β-actin and Flag (M2; Sigma-Aldrich). Secondary antibodies were obtained from GE Healthcare, Jackson Immunoresearch Laboratories, or Invitrogen.

**Cell culture**

A line of murine alveolar type II epithelial cells (C10), NIH 3T3 cells (provided by A. Howe, University of Vermont, Burlington, VT), primary lung fibroblasts, tracheal epithelial cells, CD4+ T lymphocytes from WT and Grx1−/− mice, or primary lung fibroblasts derived from lpr mice were used. Cells were isolated and propagated as described previously (Shrivastava et al., 2004; Reynaert et al., 2006; Hinshaw-Makepeace et al., 2008). NIH 3T3 cells were grown in Dulbecco’s minimum essential medium containing 10% FBS, 100 U/ml penicillin-streptomycin, 2.5 mg/ml glucose, and 10 μg/ml pyruvate. Before treatment with FasL, cells were starved in serum-free medium for 2 h.

**FasL treatment and assessment of cell death**

C10 cells were treated with 200 ng/ml FasL-Flag (Enzo Biochem, Inc.) + 0.5 μg/ml anti-Flag cross-linking antibody. Fibroblasts or CD4+ T lymphocytes were treated with 500 ng/ml FasL + 1 μg/ml M2. As reagent controls, cells were treated with M2 alone. Cell death was assessed using the MIT assay (Promega). Activation of caspase-8 and -3 was measured using reagents (Caspase-Glo 8 and Caspase-Glo 3/7; Promega).

**Grx1 activity assay**

Cells were lysed in 137 mM Tris-HCl, pH 8.0, 130 mM NaCl, and 1% NP-40. Lysates were cleared by centrifugation, equalized for protein content, and resuspended in 137 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% CHAPS, and 20 mM N-ethylmaleimide with protease inhibitor cocktail (Santa Cruz Biotechnology, Inc.), caveolin1 and JO2 (BD), and glutathione disulfide reductase [Roche], 0.35 mM NADPH, 1.2 U glutathione reductase [Roche], 1.2 U glutathione peroxidase [Sigma-Aldrich], and 0.1% CHAPS, and 20 mM cysteine-SO2H at 30 °C. Consumption of NAPDH was followed spectrophotometrically at 340 nm. Data are expressed in units, in which 1 U equals the oxidation of 1 μmol NADPH/min/mg protein.

**Cleavage of human Grx1 (hGrx1) by caspases**

hGrx1 (American Diagnostica Inc.) was resuspended in 50 mM Hepes, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, and 400 μM DTT. Recombinant human caspase-3 or -8 (EMD) was incubated with hGrx1 for the indicated time period at 37°C. Reaction mixtures were analyzed by immunoblot analysis for Grx1.

**IP of Grx1 and interacting caspases**

C10 cells were treated with Fasl. Lysates were prepared (20 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, and 0.5% NP-40 with protease inhibitor cocktail), and Grx1 was immunoprecipitated from 500 μg of protein using 1 μg/ml anti-Grx1 antibody using protein G agarose beads. The samples were analyzed via SDS-PAGE using antibodies that detect cleaved p18 and pro p55 forms of caspase-8 and p17 and p19 fragments of caspase-3, respectively. Immunoprecipitated Grx1 was detected using anti-Grx1 antibody. As a control, lysates were incubated with isotype control IgG.

**IP of S-glutathionylated proteins**

Cells were exposed to Fasl and M2 as indicated. Lysates were prepared (50 mM Tris, pH 7.4, 150 mM NaCl, 0.25% SDS, 1% NP-40, 0.5% CHAPS, and 20 mM N-methylmaleimide with protease inhibitor cocktail [Sigma-Aldrich]), and protein content was equalized. 2 μg/ml anti-Grx1 antibody was added to immunoprecipitate glutathionylated proteins using protein G agarose beads. Samples were analyzed by immunoblotting using anti-Grx antibody. Fractions from sucrose gradients were treated with 0.25% SDS and 10 mM N-methylmaleimide for 1 h before IP. As a control, a portion of the lysate was treated with 50 mM DTT to remove glutathionylated proteins, and these samples were purified through columns (MicroBioSpin; Bio-Rad Laboratories) to remove DTT before subsequent IP.

Alternatively, cells were pretreated with biotinylated glutathione ethyl ester as described previously (Reynaert et al., 2004), and lysates were immunoprecipitated using anti-Fas antibody. The samples were subsequently analyzed by immunoblotting using anti-Fas antibody.

**Lipid raft preparation**

C10 cells were starved for 2 h and treated with 1 μg/ml Fasl + M2 2 μg/ml for 20 min at 37°C. Subsequent steps were performed on ice. Cells were washed twice with cold PBS and lysed (Muppidi and Siegel, 2004). Cells were scraped into a grinder (Wheaton) and gently homogenized. Homogenates were placed on the bottom of a centrifuge tube (SW41; Beckman Coulter), mixed with 85% sucrose, and overlaid with 35% and 5% sucrose. The gradient was allowed to settle for 30 min on ice before centrifugation for 16 h at 200,000 g. 1-ml fractions were collected and analyzed by immunoblotting (Muppidi and Siegel, 2004).

**DISC isolation and analysis**

DISC isolation was performed according to Holler et al. (2003). In brief, C10 cells (n = 1 × 107 cells/60-mm dish; after transfection, n = 1 × 107 cells/60-mm dish) were starved for 2 h and treated with 1 μg/ml Fasl plus cross-linking antibody and 2 μg/ml M2 for 20 min at 37°C. Subsequent steps were performed on ice. Cells were washed once with PBS, lysed for 10 min, and processed as described previously (Holler et al., 2003).

**Site-directed mutagenesis**

Site-directed mutagenesis of WT mouse Fas was performed using the following primers with a site-directed mutagenesis kit (QuickChange II XL; Agilent Technologies): mfasC194A, (forward) 5′-GTACCGGAAAAGAAAAGGCTCGAGGAAAAGACGAGG-3′ and (reverse) 5′-CCCTGTCTCTTTTCCAGGCTTCTTCTCTCTCTGACGACAGCTGGGACCTCC-3′; mfasC271A, (forward) 5′-GAAAAAGTCCAGCTTCTTGCTGGCTGTAACCAACTCTAGG-3′ and (reverse) 5′-CCATACAGAATGGTGACCGAGCCAGGACGCTGGACTTT-3′; and mfasC294A, (forward) 5′-GGTTCTACAAAAAGCGCAAGCCCGCAGGAAA CTTTAGG-3′ and (reverse) 5′-CTAGAGGTTCTGGCGGTCGCTGCTTGTGGTACCA-3′. Mutated constructs were verified by sequence analysis.

**Evaluation of binding of FasL and surface expression of Fas**

After transfection, cells were trypsinized, scraped into capillary tubes, centrifuged briefly, and incubated on ice with 1% FBS containing PBS with anti-Fas antibody (J02; 1 μg/ml) or with isotype control antibody. After 20 min, cells were washed and incubated with 1 μg/ml FITC-conjugated secondary antibody for 20 min. Cells were fixed, and 10,000 events were analyzed by flow cytometry (BD). To assess binding of FasL, cells and M2 (1.5 μg/ml) for 20 min, washed, and incubated with 1 μg/ml FITC-conjugated anti–mouse antibody before fixation and subsequent assessment of 10,000 events via flow cytometry. To evaluate whether intrinsic differences in binding of FasL to WT or C294A Fas occurred in the absence of FSGS, lpr fibroblasts were transfected with WT or C294A mutant Fas, lysed (Holler et al., 2003), and 200 μg lysate was incubated with 100, 300, or 1,000 ng/ml Fasl + 2 μg/ml M2 at 4°C for 12–16 h. Samples were subjected to IP and evaluation of Fas content via Western blot analysis.

**Image processing and statistical analysis**

Digital images were acquired by scanning x-ray film on a photo scanner (perfection 2450; EPSON), Photoshop (Adobe) and Illustrator (CS3; Adobe) were used to create and assemble figures. Images were obtained from samples run on the same gel. In some cases, lanes were reassembled for consistency, as is indicated by a vertical dividing line. In Fig. S3 A, the vertical black lines demarcate different gels. When required, contrast and brightness were adjusted equally in all lanes. No other manipulations were done. All experiments were performed three times. Data were analyzed by one-way analysis of variance (ANOVA) using the Tukey test to adjust for multiple comparisons or the Student’s t test where appropriate (Excel; Microsoft). Data from multiple experiments were averaged and expressed as mean values ± SEM.

**Online supplemental material**

Fig. S1 shows the sequence of mouse Grx1 and putative caspase cleavage sites (A) and the lack of Fas-SSG in cells exposed to staurosporine (B). Fig. S2 shows confirmation of the lack of Grx1 in fibroblasts derived from Grx1−/− mice. Fig. S3 shows Fas trafficking into lipid rafts in response to Fas ligation in cells transfected with control or Grx1 plasmid (A) and confirmation of increases and decreases in content Grx1 after overexpression and knockdown, respectively (B and C). Fig. S4 shows...
assessments of expression levels of WT and mutant Fas proteins. Fig. 5S shows alignment of primary sequences of mouse, rat, and human Fas proteins. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200807019/DC1.

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References


Figure S1. **Putative caspase cleavage sites in Grx1 and lack of Fas-SSG formation in response to other agonists.** (A) Putative caspase cleavage sites in mouse Grx1 protein, which was predicted based on the consensus sequences. (B) Lack of Fas-SSG in response to staurosporine (STS). C10 were exposed to 1 µM staurosporine or FasL + M2 for the indicated times. Cell lysates were subjected to nonreducing IP (-DTT) using antiglutathione antibody to immunoprecipitate S-glutathionylated proteins (IP:PSSG) before detection of Fas via Western blotting. As a reagent control to reduce S-glutathionylated proteins before IP, samples were incubated with 50 mM DTT (+DTT). Bottom panels represent the content of Fas, cleaved caspase-3, Grx1, and actin in whole cell lysates (WCL). Note that all samples were run on the same gel. Black lines indicate that intervening lanes have been spliced out.

Mouse Glutaredoxin 1 (Grx1)

MAQEFVNCKI QSGKVVFIK PTCPYCRKTQ EILSQLPFKQ GLLEFVDITA 50
TNNTSALIQDV LQQLTGARTV PRVFIGKDCI GCCSDLISMQ QTGEILMTRK 100
Q1GALQL 107

**EFVD, AIQD - Putative Caspase cleavage sites**
Figure S2. **Confirmation of lack of Grx1 in primary lung fibroblasts derived from Glrx1<sup>−/−</sup> mice.** An immunoblot for Grx1 (top) and Fas as a control (bottom) is shown.

Figure S3. **Trafficking of Fas into lipid rafts, knockdown, and overexpression of Grx1 in C10 cells.** (A) Assessment of Fas trafficking into lipid rafts in cells transfected with pcDNA3 or Grx1 in response to administration of FasL + M2 or M2 alone. Cells were fractionated via sucrose gradient centrifugation, and fractions 1–12 were assessed for lipid raft markers caveolin 1 and flotillin 1, Fas, and Grx1. Lipid rafts fractions (LRF) 3 and 4 were identified based on the colocalization of caveolin 1 and flotillin 1. (B and C) Confirmation of enhanced Grx1 expression in cells transfected with Grx1 plasmid (B) or loss of Grx1 after siRNA knockdown (C). Grx1 expression was evaluated by immunoblot analysis. Fas expression is shown as a loading control (Ctr). This figure accompanies experiments shown in Fig. 5 (B and C). In all cases, lanes 1–12 indicate samples that were run on one gel. Black lines indicate that intervening lanes have been spliced out.
Figure S4. **Confirmation of expression levels of WT Fas and Fas mutant constructs.** (A–C) Fas was evaluated via immunoblot analysis. Actin is shown as a loading control. A, B, and C accompany experiments shown in Fig. 6 (C, D and E, and F, respectively).

Figure S5. **Alignment of primary sequences of mouse, rat, and human Fas.** Cysteine 294 of mouse Fas is shown in bold, and putative target cysteines in rat and human Fas are also bolded. The boxed areas highlight surrounding basic and acidic amino acids that may favor its susceptibility toward S-glutathionylation.