Gamma-Interferon-inducible Lysosomal Thiol Reductase (GILT)

MATURATION, ACTIVITY, AND MECHANISM OF ACTION*

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We recently identified a gamma-interferon-inducible lysosomal thiol reductase (GILT), constitutively expressed in antigen-presenting cells, that catalyzes disulfide bond reduction both in vitro and in vivo and is optimally active at acidic pH. GILT is synthesized as a 35-kDa precursor, and following delivery to major histocompatibility complex (MHC) class II-containing compartments (MIICs), is processed to the mature 30-kDa form via cleavage of N- and C-terminal propeptides. The generation of MHC class II epitopes requires both protein denaturation and reduction of intra- and interchain disulfide bonds prior to proteolysis. GILT may be important in disulfide bond reduction of proteins delivered to MIICs and consequently in antigen processing. In this report we show that, like its mature form, precursor GILT reduces disulfide bonds with an acidic pH optimum, suggesting that it may also be involved in disulfide bond reduction in the endocytic pathway. We also show that processing of precursor GILT can be mediated by multiple lysosomal proteases and provide evidence that the mechanism of action of GILT resembles that of other thiol oxidoreductases.

Antigen-presenting cells such as dendritic cells, macrophages, and activated B cells stimulate CD4+ T cells by presentation of MHC class II-peptide complexes. Generation of class II epitopes requires proteolysis of endocytosed antigens in MIICs (1). Several studies have shown that reduction of disulfide bonds in proteins is a critical step for antigen processing, mainly by facilitating unfolding of proteins and their subsequent cleavage by proteases (2–4). In an earlier study, Collins et al. (2) provided evidence that in the endocytic pathway reducing activity was present primarily in lysosomes while absent from early recycling endosomes. They proposed that disulfide bond reduction in lysosomes was largely catalyzed by a high concentration of cysteine, which is transported into the lumen by amino acid transporters. In a recent report, we suggested that along with cysteine, a γ-interferon-inducible lysosomal thiol reductase (GILT), originally described as IP30 (registered as IFI30, Human Gene Nomenclature Data Base), may contribute to the reducing activity of MIICs (5).

GILT is synthesized as a 35-kDa soluble glycoprotein (6). Following transport to MIICs via the mannose-6-phosphate receptor, it is processed to the mature form by proteolytic cleavage of its N- and C-terminal propeptides (5). At residues 46–49 there is a CXXC motif similar to the WCGH/PCK motif of proteins in the thioredoxin family (7–10). This class of enzymes catalyzes, at a neutral pH, dithiol oxidation, disulfide bond reduction, and disulfide bond isomerization (11). The reduction of disulfide bonds by the mature form of GILT in vitro proved to be optimal at acidic pH (5). We also showed that C46GCG49 constituted the GILT active site. Mutation of either cysteine to serine abolished in vitro thiol reductase activity (5).

The precursor form of GILT, via its N-terminal propeptide, binds specifically to certain HLA-DR alleles (12). Although the significance of this association has not been determined, the observation raised the question of whether precursor GILT is functionally distinct from its mature form. Another distinction between the two forms is that, following secretion, precursor GILT forms disulfide-linked dimers (6). In this study, we examine the thiol reductase properties of the precursor and its processing to mature GILT and also investigate its mechanism of action.

EXPERIMENTAL PROCEDURES

Cells and Antibodies—The human B lymphoblastoid line (B-LCL), Pala (13), has been described previously. COS-7 cells were from the American Type Culture Collection. The rabbit antisera R.GILT (5), R.IP30N (12), R.IP30C (12), and the mouse monoclonal antibodies MAP.IP30 (12), DA6.147 (14), and IG12 (15), have been previously described.

Enzymes and Inhibitors—The following proteases and inhibitors were purchased from Sigma: cathepsin B; chloroquine, 2,3,3′-trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (E64d), iodoacetamide (IAA), leupeptin, pepstatin A, Cathepsins D and L and N-(benzoyloxycarbonyl)-L-phenylalanine-vinylsulfone phenyl (LHVS) were purchased from Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT), and morpholinurea-leucine-homophenylalanine-vinylsulfone phenyl (LHVS) was generously provided by Dr. Hidde Ploegh, Harvard Medical School. N-Glycosidase F was purchased from Roche Molecular Biochemicals.

GILT Purification—Human GILT was affinity-purified from B-LCLs using the monoclonal antibody MAP.IP30 coupled to Bio-Gel A15m beads as described previously (5).

Polymerase Chain Reaction-based Mutagenesis—The generation of the cysteine to serine mutant GILT derivatives (C46S, C48S, C48S/C49S) and the cloning of wild-type and mutant GILT cDNAs into the expression vector pcDNA 3.1 puromycin (pcDNA 3.1 puromycin) was described previously (5).

Transient Transfections in COS-7 Cells—COS-7 cells were seeded overnight in T75 flasks, grown to 80% confluence, and transfected with 20 μg of GILT or mutant GILT cDNAs using CellFECTIN (Life Tech-
FIG. 1. Precursor GILT secreted from COS-7 cells is dimeric and exhibits variable glycosylation. COS-7 cells were transfected with cDNAs encoding wild-type or C46S/C49S mutant GILT, and precursor and mature forms of GILT were immunoprecipitated from the supernatants and extracts, respectively. In A, eluates were immunoblotted with antisera that recognize both the precursor and mature GILT (R.GILT), the N-terminal propeptide (R.IP30N), or the C-terminal propeptide (R.IP30C). Only the precursor forms of GILT were detected with R.IP30N and R.IP30C, yet multiple species were revealed. B shows that deglycosylation of precursor GILT with N-glycosidase F resulted in detection of only a single species following immunoblotting with R.GILT. In C, eluates were analyzed by nonreducing SDS-PAGE and immunoblotted with R.GILT. The majority of the secreted precursor GILT for both the wild-type and C46S/C49S were dimers, demonstrating that dimerization occurs independently of the active-site cysteines.
was only detected with R.GILT, while precursor GILT was detected by all three antisera. However, multiple species of both wild-type and mutant precursor GILT were revealed. GILT has three potential N-glycosylation sites at residues 37, 69, and 82 (5), suggesting that the multiple bands revealed in the immunoblots were likely to result from differences in glycosylation. To test this, the eluates were treated with N-glycosidase F, which removes N-linked glycans. Immunoblotting with R.GILT showed that deglycosylation converted all the precursor GILT to a single species (Fig. 1B). The data suggested that protein overexpression in COS-7 results in incomplete glycosylation of a fraction of secreted precursor GILT, perhaps by overwhelming the glycosylation machinery.

Luster et al. (6) previously showed that following secretion, precursor GILT forms disulfide-linked dimers. To confirm this, and to determine whether dimerization involved the active-site cysteines, eluates containing precursor wild-type and C46S/C49S GILT were separated by nonreducing SDS-PAGE and immunoblotted with R.GILT (Fig. 1C). For both wild-type and mutant, the majority of the GILT detected was dimeric, indicating that neither Cys-46 nor Cys-49 are required for dimerization.

**Thiol Reductase Activity of Precursor GILT**—To test for thiol reductase activity we employed the assay previously described which uses $^{125}$I-F(ab')$_2$ as a substrate (5). Eluates from MAP.IP30 beads were activated with 25 μM DTT. Wild-type and mutant forms of precursor and mature GILT were eluted in a total of 120 μl. Different volumes of the eluates (10, 20, and 40 μl) were incubated with denatured $^{125}$I-F(ab')$_2$ at 37 °C for 1 h at pH 4.5. Reduction of F(ab')$_2$ into Fab' and H' and L chains was analyzed by nonreducing SDS-PAGE followed by autoradiography, quantitating by image analysis. Fig. 2, A and C, show that even 10 μl of wild-type precursor GILT reduced F(ab')$_2$ approximately 50%, whereas C46S/C49S precursor GILT was inactive at all concentrations (Fig. 2, B and C). The activity paralleled that of mature GILT.

To determine whether precursor GILT has the same acidic pH optimum as the mature form, we assayed for activity in the pH range of 4.0–7.0. By N-glycosidase F digestion and Western blotting we confirmed that, although varying in glycosylation, only precursor GILT was immunonisolated from the supernatants of GILT-expressing COS-7 cells (Fig. 3A). Eluates containing mature GILT were also analyzed by immunoblotting to confirm that similar amounts were assayed at the various pH values (Fig. 3A). From preliminary titrations performed at pH 4.5 we determined the volumes of precursor and mature GILT eluates that exhibited similar thiol reductase activity (data not shown) and used this volume for assaying activity at the different pH values. Fig. 3B shows that, like mature GILT, precursor GILT reduced F(ab')$_2$ with an acidic pH optimum. Quantitation of reduction is shown in Fig. 3C. The slight apparent difference in pH optimum is within experimental error and is not reproducible.

**In Vitro Digestion of Precursor GILT**—From immunofluorescence and electron microscopy data described earlier, it appears that precursor GILT first enters the endocytic pathway in early endosomes and is processed to the mature form in multivesicular late endosomes (5). To determine whether processing is mediated by specific enzymes, we examined purified lysosomal cathepsins for their ability to cleave precursor GILT in vitro. Eluates were incubated with the various cathepsins either with or without inhibitors. The alkylating reagent, iodoacetamide, was used to inhibit cathepsin B activity. Pepstatin A inhibits aspartyl proteases, including cathepsin D, and Z-FY-CHO and LHVS inhibit cathepsin L and cathepsin S, respectively (19, 20). To simplify the immunoblotting pattern, the samples were deglycosylated prior to SDS-PAGE. Probing with antisera R.GILT, R.IP30N, and R.IP30C revealed that cathepsin B only cleaved the C-terminal propeptide, while cathepsin D only cleaved the N-terminal propeptide (Fig. 4A–C). Cathepsin L cleaved both propeptides well, and cathepsin S cleaved the N-terminal propeptide completely and the C-terminal propeptide partially (Fig. 4, A–C). Also, addition of the specified inhibitors blocked proteolysis by each of the cathepsins (Fig. 4, A–C). It was previously shown that 5 μM LHVS specifically inhibited cathepsin S in vitro (21). However, 0.8 μM recombinant cathepsin S was used in the in vitro digestion. Titrating LHVS in the range of 5 nM to 2 μM, we determined that 0.5 μM LHVS was necessary to block proteolysis by recombinant cathepsin S, used at 0.8 μM in vitro. The ability of LHVS to completely inhibit cathepsin S in less than a 1:1 molar ratio is presumably because a fraction of the cathepsin S is proteolytically inactive.

**Inhibition of GILT Maturation in Vivo**—Pala cells were metabolically labeled with $^{35}$S-methionine for 30 min and chased with 15-fold excess cold methionine/cysteine for up to 4 h in the presence of chloroquine (30 μM), or a mixture of leupeptin (1 mm), E64d (30 μM), and pepstatin A (30 μM). The
cells were detergent-extracted, GILT immunoprecipitated with MAP.IP30, and the samples analyzed by SDS-PAGE followed by autoradiography. By increasing lysosomal pH, chloroquine inhibits all acid-optimal proteases. Consequently, chloroquine prevented GILT maturation (Fig. 4D). However, addition of two cysteinyl protease inhibitors, leupeptin and E64d, and an aspartyl protease inhibitor, pepstatin A, failed to arrest maturation (Fig. 4D). Nor did any of these inhibitors affect GILT maturation when used individually (data not shown). This suggests that other proteases besides the cysteine proteases inhibited by leupeptin and E64d and aspartyl proteases inhibited by pepstatin A can cleave precursor GILT. Overall, the data argue that multiple lysosomal proteases contribute to GILT maturation.

Activation of GILT by L-Cysteine and Cysteinyl Glycine—Physiological reductants, which have been proposed to contribute to the reducing activity in lysosomes, include cysteine and cysteinyl glycine. Pisoni et al. (22) have demonstrated that cysteine is efficiently delivered to the lysosomes by a cysteine-specific transport system. The role of cysteinyl glycine, however, is more ambiguous. Cysteinyl glycine is a by-product of the extracellular cleavage of glutathione by the ectoenzyme, γ-glutamyl transpeptidase (23). It has been proposed that this dipeptide is then transported across the plasma membrane and into lysosomes. Although the evidence that cysteinyl glycine is a physiological lysosomal reductant is circumstantial, its potential role cannot be excluded.

The in vitro thiol reductase assay with iodinated F(ab′)2 was performed with purified GILT (1 μg) at pH 4.5 as described above, substituting 25 μM DTT with l-cysteine and cysteinyl glycine at 0.1, 0.5, and 1 mM (Fig. 5). Fig. 5A shows reduction of F(ab′)2 by the two reductants alone or with GILT. Inclusion of L-cysteine or cysteinyl glycine at 1 mM with GILT resulted in a significant increase in reduction of F(ab′)2 relative to reduction driven by the reductants alone. The concentration of cysteine or cysteinyl glycine required to activate GILT was 1 mM, 40-fold greater than for DTT (25 μM). The same concentration of both reductants was also able to activate precursor GILT immunoprecipitated from COS-7 cell supernatants (Fig. 5C). Estimates of the cysteine concentration in endosomes and lysosomes range from several hundred μM to several tens mM (24), suggesting that physiological cysteine concentrations can serve to mediate reduction by GILT. We are unaware of any quantitation of the level of cysteinyl glycine in lysosomes.

In addition to l-cysteine and cysteinyl glycine, we also examined glutathione for its ability to activate GILT in vitro. Inclusion of 1 mM glutathione in the assay with either form of
GILT did not increase reduction of F(ab’)2 relative to reduction by glutathione alone (data not shown).

In Vitro Trapping of F(ab’)2 Derivatives by C49S GILT — The reaction mechanism of the active-site motif (CXXC) in thioredoxin and protein disulfide isomerase has been well characterized (reviewed in Refs. 11 and 25). In the reaction, the N-terminal cysteine is believed to initiate nucleophilic attack on a disulfide bond, generating an enzyme-substrate mixed disulfide intermediate. Subsequent intramolecular nucleophilic attack by the second active-site cysteine stabilizes and traps both propeptides, while the cleavage of the C-terminal propeptide is weaker with cathepsin S. To examine in vitro proteolysis, PalA cells were starved, metabolically labeled with [35S]methionine, and chased for up to 4 h in the presence of chloroquine (30 μM) or a mixture of leupeptin (1 mM), E64d (30 μM), and pepstatin A (30 μM). GILT was immunoprecipitated from cell lysates with MAP.IP30, and samples were analyzed by 13% reducing SDS-PAGE and autoradiography (D). Chloroquine inhibited GILT maturation, while leupeptin, E64d, and pepstatin A together did not affect processing.

DISCUSSION

Following synthesis, lysosomal enzymes undergo posttranslational glycosylation, which occurs in the ER and Golgi, and limited proteolysis. The nature and site of intracellular proteolysis are variable, even within a family of lysosomal enzymes. For example, cleavage can be internal, or N- and C-terminal, and can occur in the lysosomes themselves or prior to delivery to lysosomes, in compartments such as the ER and post-Golgi organelles. Proteolysis can involve multiple steps and may be essential for enzyme activation. Lysosomal proteases such as cathepsins B, D, and L are synthesized as inactive zymogens (reviewed in Ref. 28). Following transport to lysosomes, the acidic pH induces autocatalytic proteolysis, generating the mature, active cathepsins.

Examples where lysosomal proteolysis does not appear to regulate enzymatic activity include β-galactosidase and asparagylglucosaminidase. β-Galactosidase is synthesized as a 86-kDa precursor and is processed to a 64-kDa mature form by removal of a 20-kDa C-terminal propeptide (29). The precursor form associates in the lysosome in a multienzyme complex, which also includes α-neuraminidase and cathepsin A (30–33). From expression in Chinese hamster ovary cells, it was shown...
that the secreted precursor β-galactosidase was functionally identical to the mature form, demonstrating that the C-terminal propeptide is not enzymatically inhibitory (34).

Aspartylglucosaminidase is also synthesized as a precursor and becomes enzymatically active in the ER when the 42-kDa form is internally cleaved into two subunits, a 27-kDa pro-α chain and a 17-kDa chain (35). After delivery to the lysosome, the pro-α chain is further trimmed at the C terminus to the mature 24-kDa form with no effect on the enzymatic activity. Our previous study (5) only examined the thiol reductase activity of mature GILT. Here we have shown that the precursor form of GILT can also reduce disulfide bonds, indicating that neither the N- nor C-terminal propeptides inhibit the reductase activity.

In vitro cleavage of either or both the N- and C-terminal propeptides of precursor GILT can be mediated by the lysosomal proteases, cathepsins B, D, L, and S (Fig. 4). Although GILT maturation was arrested by chloroquine in vivo, a mixture of the cysteinyl protease inhibitors, E64d and leupeptin, and the aspartyl protease inhibitor, pepstatin A, failed to prevent maturation (Fig. 4D). These findings suggest that GILT processing is not mediated by any specific enzyme and argue that the N- and C-terminal prosequences are readily accessible to multiple lysosomal proteases.

Precursor GILT was purified as a secreted product from overexpressing COS-7 cells. Fig. 1 shows that the isolated material contains four species of different apparent molecular weights. N-Glycosidase F treatment converted the upper three bands to the apparent Mr of the lower band (Fig. 1B). This indicates that the lower, fainter band corresponds to unglycosylated precursor, and the upper three correspond to precursor GILT containing three, two, and one N-linked glycans, respectively. Notably, mature GILT isolated from detergent extracts of the same cells does not show evidence of variable glycosylation. This may be because at least one N-linked glycan must be converted to the mannose-6-phosphate-bearing form for proper targeting to the endocytic pathway. A second reason, not necessarily exclusive of the first, is that the absence of one or more N-linked glycans renders GILT sensitive to proteolysis in the lysosome or MIIC.

We also showed that cysteine and cysteinyl glycine were able to activate precursor and mature GILT (Fig. 5C). Although the role of cysteinyl glycine as a physiological reductant is unclear, cysteine has clearly been implicated as a pertinent reductant. Pisoni et al. (22) first demonstrated that lysosomes in murine macrophages transported cysteine effectively from the cytosol into the lumen using a specific transport system. Gainey et al. (36) later reported that, although in a macrophage hybridoma cell line cysteine was only transported into lysosomes, in a murine B cell line cysteine was effectively transported into endosomes as well as lysosomes. They argued that the site of disulfide reduction of endocytosed proteins is not necessarily confined to lysosomes and is variable among the different types
of antigen-presenting cells. The finding that precursor GILT exhibits enzymatic activity raises the possibility that it may be involved in disulfide bond reduction early in the endocytic pathway. As described previously, precursor GILT colocalizes with intact invariant chain, presumably associated with MHC class II αβ dimers, largely in early endosomes/early MIICs. The pH of these organelles is believed to be 5.5–6.0. We have shown that precursor GILT, as well as mature GILT, can still effectively reduce disulfide bonds in this pH range (Fig. 3). The presence of cysteine in compartments preceding lysosomes (36) suggests that precursor GILT may be sufficiently active in early endosomes/early MIICs to initiate the reduction of internalized protein antigens prior to proteolysis. This may be important in preserving class II binding epitopes by allowing class II molecules to bind to unfolded proteins or fragments of proteins, prior to their complete degradation. Interestingly, we showed previously that precursor GILT itself binds in vivo to HLA-DRw52 molecules (12). This interaction was enhanced by chloroquine treatment, suggesting that invariant chain proteolysis and release of peptide-binding MHC class II αβ dimers may occur more readily in the endocytic pathway than proteolysis of other proteins. This again is consistent with unfolded proteins becoming available for MHC class II binding prior to significant proteolysis.

Recently, Gingras et al. (37) reported that GILT (referred to as IP30 in the report) could be purified from human placenta by affinity chromatography using an affinity column consisting of phenyl-leucine coupled to epoxy-activated agarose. Other proteins that were copurified included the carboxypeptidases cathepsin A and plasma glutamate carboxypeptidase and the endoprotease cathepsin D. The isolation of GILT could have resulted from either direct binding to the dipeptide Phe-Leu or binding to cathepsin A, cathepsin D, or plasma glutamate carboxypeptidase. Our own preliminary data suggest that GILT can bind Phe-Leu-agarose directly (data not shown). Because the other proteins copurified by Gingras et al. (37) were proteases, we tested whether GILT could perhaps also be a peptidase or a protease using a variety of peptide and protein substrates. However, we were unable to demonstrate that GILT possessed either activity (data not shown). Whether the affinity of GILT for the hydrophobic dipeptide Phe-Leu reflects an additional function is unknown, but an intriguing possibility is that it represents a binding site for hydrophobic regions of

![Fig. 6. Proposed mechanism of reduction by GILT.](http://www+jbc.org/)

![Fig. 7. Trapping of F(ab')2 derivatives by C49S GILT.](http://www+jbc.org/)
proteins and that GILT may play a role in protein unfolding in addition to its thiol reductase activity.

The demonstration that both the precursor and mature forms of GILT are enzymatically active begs the question of why GILT is synthesized as a precursor at all. The N- and C-terminal propeptides may simply be required for proper folding in the ER, or they may serve a different function. There are two cysteine residues in the C-terminal propeptide, one of which may play a role in dimerization of precursor GILT, although the role of dimerization is itself unclear. Conceivably these cysteine residues could regulate the activity of the enzyme in some way that is not readily apparent in the in vitro assay we have employed. The functions of the propeptides can be approached by mutagenesis experiments.

The data presented in Fig. 7 show that mutagenesis of the second cysteine in the active site generates a “trapping” phenotype like that of analogous mutants of other members of the thioredoxin family. Thus, the mechanism of action of GILT is likely to be similar. Redox reactions involving cysteine residues require de-protonation of a thiol group, in the active site of the enzyme in the case of reduction, as indicated schematically in Fig. 6. The acidic pH optimum of GILT argues that the region surrounding the active site must have evolved to facilitate de-protonation of Cys-46 for the initiation of the nucleophilic attack on the target disulfide bond. Precisely how this occurs may become evident when the three dimensional structure of GILT is uncovered.

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