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Functional Requirements for the Lysosomal Thiol Reductase GILT in MHC Class II-Restricted Antigen Processing

K. Taraszka Hastings, Rebecca L. Lackman, and Peter Cresswell

Ag processing and presentation via MHC class II is essential for activation of CD4+ T lymphocytes. γ-IFN-inducible lysosomal thiol reductase (GILT) is present in the MHC class II loading compartment and has been shown to facilitate class II Ag processing and recall responses to Ags containing disulfide bonds such as hen egg lysozyme (HEL). Reduction of proteins within the MHC class II loading compartment is hypothesized to expose residues for class II binding and protease trimming. In vitro analysis has shown that the active site of GILT involves Cys46 and Cys49, present in a CXXC motif that shares similarity with the thioredoxin family. To define the functional requirements for GILT in MHC class II Ag processing, a GILT-deficient murine B cell lymphoma line was generated and stably transduced with wild-type and cysteine mutants of GILT. Intracellular flow cytometry, immunoblotting, and immunofluorescence analyses demonstrated that wild-type and mutant GILT were expressed and maintained lysosomal localization. Transduction with wild-type GILT reconstituted MHC class II processing of a GILT-dependent HEL epitope. Mutation of either Cys46 or Cys49 abrogated MHC class II processing of a GILT-dependent HEL epitope. In addition, biochemical analysis of these mutants suggested that the active site facilitates processing of precursor GILT to the mature form. Precursor forms of GILT-bearing mutations in Cys200 or Cys211, previously found to display thiol reductase activity in vitro, could not mediate Ag processing. These studies demonstrate that the thiol reductase activity of GILT is its essential function in MHC class II-restricted Ag processing.

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The MHC class II Ag processing pathway generates cell surface MHC class II peptide complexes essential for the activation of CD4+ T lymphocytes (reviewed in Ref. 1). MHC class II α- and β-chains are generated and form heterodimers in the endoplasmic reticulum, where they associate with invariant chain (Ii). The N-terminal cytoplasmic domain of Ii targets the class II-II complex to the trans-Golgi network and endocytic pathway. Ii is sequentially cleaved leaving a C-terminal portion of Ii, class II-associated Ii peptide (CLIP), which protects the class II peptide binding groove from binding peptides outside of the class II loading compartment. In the acidic environment of the lysosomes, cathepsins are generally activated by autotaxic cleavage of a propeptide, which blocks the active site in the precursor form. Cathepsins are responsible for the proteolysis of endocytosed exogenous proteins and endogenous proteins localized to this compartment, for the generation of class II binding peptides. In the lysosomal compartment, the class II-related molecule H2-M (HLA-DM in humans) interacts with class II-CLIP and facilitates the exchange of CLIP for peptides generated in the endocytic compartment. MHC class II peptide complexes are then directed to the cell surface.

Reduction of Ags is important for MHC class II processing and presentation (2–5). γ-IFN-inducible lysosomal thiol reductase (GILT) is a reductase that is localized to MHC class II loading compartments and has maximal reductase activity at the acidic pH found in these compartments (6). GILT is constitutively expressed in APCs and is up-regulated by IFN-γ in other cell types (6, 7). GILT is synthesized as a 35-kDa precursor and targeted to the endocytic pathway via the mannose-6-phosphate receptor (6, 8). In the endocytic pathway, N- and C-terminal propeptides are cleaved to generate a 28-kDa mature form by multiple cathepsins (9). The mature form is found in multivesicular late endosomes and multilamellar lysosomes (6, 8). Additionally, a small portion of precursor GILT is secreted as a disulfide-linked dimer and has reductase activity (9). A thioredoxin-like CXXC motif involving Cys46 and Cys49 constitutes GILT's reductase active site in a cell-free assay using 125I-F(ab’2)2 as a substrate (6). Similar to thioredoxin, the N-terminal Cys46 initiates nucleophilic attack on a disulfide bond. There is formation of a mixed disulfide GILT substrate intermediate with subsequent intramolecular attack by Cys49 resulting in the release of the reduced substrate (9).

GILT has been shown to facilitate MHC class II-restricted Ag processing (8, 10). Hen egg lysozyme (HEL) is an excellent model Ag for evaluating the role of protein structure in Ag processing because it has four intrachain disulfide bonds (11) and is resistant to proteolytic cleavage without prior reduction (12). Intracellular processing of HEL to generate the I-Aβ-restricted HEL peptide involving residues 74–88 (HEL74–88) is entirely dependent on the presence of GILT (8). HEL74–88 contains two cysteines at positions 76 and 80 that are each involved in a disulfide bond (11), and previous studies support the need for reduction of disulfide bonds for presentation of this epitope (12). In contrast, processing of the I-Aβ-restricted HEL epitope involving residues 20–35 (HEL20–35),

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4 Abbreviations used in this paper: Ii, invariant chain; CLIP, class II-associated Ii peptide; GILT, γ-IFN-inducible lysosomal thiol; HEL, hen egg lysozyme; MSCV, murine stem cell virus.
containing one cysteine involved in a disulfide bond, is not affected by the absence of GILT, possibly because the topology of these residues renders them accessible to proteolysis without reduction by GILT or acidic pH alone is sufficient to denature this region for MHC class II binding (8). Despite the similar processing of some HEL epitopes in vitro, the recall response to HEL in GILT knockout mice was about one-tenth of that seen in the wild-type mice (8). Similar reductions in recall responses were seen with immunization with other proteins containing disulfide bonds such as bovine RNase A and human IgG (8). Only a slight difference was seen after immunization with bovine α-casein, an Ag that does not contain disulfide bonds (8). Therefore, GILT is critical in the processing and presentation of some MHC class II peptide complexes and is important in the development of immune responses to protein Ags that contain disulfide bonds.

In this study, we have generated B cell lymphoma lines from GILT knockout and wild-type mice. To evaluate the molecular requirements for the function of GILT in intracellular MHC class II-restricted Ag processing, we stably transduced the GILT-deficient B cell line with mutants of the reductase active site and mutants that disrupt the processing of GILT. Mutation of Cys46 or Cys49, either singly or together, resulted in a loss of processing of the GILT-dependent HEL74–88 epitope, indicating that GILT’s reductase activity is its essential function in MHC class II-restricted Ag processing. Furthermore, we showed by mutation of Cys46 and Cys49 that the reductase active site facilitates the processing of precursor GILT to the mature form and that, in mutants in which Cys200 or Cys211 are mutated, the preserved precursor form of GILT is not sufficient to function in Ag processing.

Materials and Methods

Animals and cell lines

GILT knockout mice were generated as previously described and backcrossed 10 times onto the C57BL/6 background (8). Eμ-myc transgenic mice were generated by Adams et al. (14), and Eμ-myc transgenic mice on the C57BL/6 background were provided by Dr. R. Medzhitov (Yale University, New Haven, CT). Mice with the Eμ-myc transgene (c-myc oncogene coupled to the Ig μ enhancer) were backcrossed onto GILT knockout mice. Mice were housed in a pathogen-free facility. These studies were approved by the institutional review committee.

Wild-type and GILT-deficient B cell lymphoma lines were generated by in vitro transfection with the Eμ-myc transgene as previously described (14). Briefly, the lymph nodes and spleens were harvested from Eμ-myc transgenic mice and wild-type and GILT knockout backgrounds after they developed spontaneous lymphoma. Cells were adapted to tissue culture in RPMI 1640 medium supplemented with 10% FBS, HEPES, and 2-ME (50 μM). The B cell lymphoma line derived from Eμ-myc transgenic mice on the C57BL/6 background was termed BμMyc.1, and the cell line derived from Eμ-myc transgenic mice on the GILT knockout background was named BμMyc.GKO.1. The following other cell lines were used: 293T (human renal epithelial cell line expressing SV40 large T Ag), Priess (human B cell line), B04 (I-AK-restricted murine T cell hybridoma recognizing HEL74–88) (15), and Hb1.9 (I-AK-restricted murine T cell hybridoma recognizing HEL20–33) (15).

Generation of wild-type and mutant GILT retroviral vectors

The PCR-based generation of cysteine to serine point mutations at residues 46, 49, 46/49, 200, and 211 of human GILT was previously described (6, 14). The PCR pairs were amplified with PCR primers containing restriction sites for EcoRI and BamHI. The PCR fragments were cloned into the retroviral expression vector pCLeco encoding gag, pol, and env cDNAs using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen Life Technologies). Supernatants containing retrovirus were collected from the transfected 293T cells after 36 and 60 h of culture at 32°C. At these time periods, BμMyc.GKO.1 cells were resuspended in a 1:2 dilution of supernatants containing retrovirus with medium supplemented with polybrene (8 μg/ml final) followed by centrifugation for 90 min at 1258 g at 32°C. Transduced BμMyc.GKO.1 cells were cultured overnight at 32°C and then maintained at 37°C.

Flow cytometry and immunofluorescence

For flow cytometric analysis of cell surface expression, the FcyRII/RII was blocked by preincubating cells with rat anti-mouse CD16/CD32 mAb (1 μg per million cells, Mouse BD Fc Block; BD Biosciences) in PBS with 1% BSA and 0.05% sodium azide for 5 min on ice. Cells were stained with FITC or PE-conjugated mAbs against murine CD45R (B220), I-AK, CD43, IgM, IgD, CD24 (heat-stable-Ag HSA), CD23, CD21 and corresponding isotype controls (BD Biosciences) and were fixed with 1% paraformaldehyde in PBS. Cell-associated fluorescence was measured using a FACS Calibur flow cytometer (BD Biosciences) and analysis performed using FlowJo software (Tree Star). For intracellular flow cytometric analysis, cells were fixed with 3.7% formaldehyde, permeabilized with 0.05% saponin, and stained with FITC-conjugated anti-human GILT mAb (MaP.IP30; BD Biosciences) or FITC-conjugated mouse IgG1 isotype control (BD Biosciences) with or without 4 h pretreatment with 100 μg/ml brefeldin A.

For the immunofluorescence studies, BμMyc.GKO.1 cells stably expressing wild-type or mutant GILT were attached to Alcan blue-coated coverslips, fixed with 3.7% formaldehyde, and permeabilized with 0.05% saponin. Cells were stained using the rabbit anti-human GILT serum followed by Alexa Fluor 488-conjugated Fab’2 goat anti-rabbit IgG (Molecular Probes) and rat anti-mouse class II mAb (TIB120), which was a gift from Dr. R. Medzhitov, with Alexa Fluor 546-conjugated Fab’2 goat anti-rat IgG (Molecular Probes). Images were collected using a Leica TCS SP2 confocal microscope.

Immunoblotting

Immunoblotting was performed as previously described (18). Briefly, cells (4 × 106 cells per gel lane) were lysed in TBS with 1% Triton X-100 for 30 min on ice. Samples were separated by nonreducing SDS-PAGE (12% w/v acrylamide) and electrophoretically transferred to immonobilon-P membrane (Millipore). The membrane was blocked in PBS with 0.2% Tween 20 and 5% dehydrated milk and probed with rabbit anti-human GILT serum (1/10,000) (6) or rat anti-GRP94 mAb (1/5000; StressGen Biotechnologies) as a loading control. The membranes were then washed, incubated with HRP-conjugated goat anti-rabbit or anti-rat IgG (1/5000; Jackson ImmunoResearch Laboratories) and ECL substrate (SuperSignal West Pico; Pierce), and exposed to film.

Metabolic radiolabeling and immunoprecipitation

BμMyc.GKO.1 cells transduced with wild-type, CA465 or CA9S GILT (2 × 106 cells/sample) were incubated in medium without methionine or cysteine for 1 h at 37°C, labeled for 1 h with 1 mCi of [35S]methionine/cysteine labeling mix (PerkinElmer), and chased in an excess of unlabelled methionine and cysteine for the indicated time periods. Cells were lysed in TBS with 1% Triton X-100 for 30 min on ice. The postnuclear supernatants were precleared with normal mouse serum (Sigma-Aldrich) and protein G-Sepharose (GE Healthcare) and immunoprecipitated with mouse anti-human GILT mAb (MaP.IP30) (19) or normal mouse serum and protein G-Sepharose. Samples were separated by reducing SDS-PAGE (12%) and imaged by autoradiography. Samples were quantitated using a Molecular Dynamics Storm PhosphorImager system.

Class II Ag processing assay

MHC class II expression was maintained by FACS sorting of cells stained with anti-I-AK-FITC (BD Biosciences) using a FACS Vantage SE flow cytometer (BD Biosciences) followed by iFN-γ treatment with 100 U/ml for 24 h. The cells were then treated with (Calbiochem) B04 or Hb1.9 T cell hybridoma clone in the presence of 105 cell equivalents per gel lane were incubated in medium without methionine or cysteine for 1 h at 37°C, labeled for 1 h with 1 mCi of [35S]methionine/cysteine labeling mix (PerkinElmer), and chased in an excess of unlabelled methionine and cysteine for the indicated time periods. Cells were lysed in TBS with 1% Triton X-100 for 30 min on ice. The postnuclear supernatants were precleared with normal mouse serum (Sigma-Aldrich) and protein G-Sepharose (GE Healthcare) and immunoprecipitated with mouse anti-human GILT mAb (MaP.IP30) (19) or normal mouse serum and protein G-Sepharose. Samples were separated by reducing SDS-PAGE (12%) and imaged by autoradiography. Samples were quantitated using a Molecular Dynamics Storm PhosphorImager system.

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Results

Generation and characterization of GILT-deficient and GILT-expressing B cell lymphoma lines

To evaluate the molecular requirements for the function of GILT in Ag processing, we first needed to generate a GILT-deficient APC that could be stably transduced with wild-type and mutant GILT. Murine B cell lymphoma lines were generated by in vivo transformation with the Eμ-H9262-myc transgene as previously described (14). The 90% of Eμ/H9262-myc transgenic mice with the c-myc onco-gene coupled to the IgH/H9262 enhancer developed spontaneous lymphoma in vivo, manifested by diffuse lymphadenopathy and splenomegaly 2–5 mo after birth. Eμ/H9262-myc transgenic mice were backcrossed with the GILT knockout mice. Lymph node cells and splenocytes from tumors were harvested from the Eμ/H9262-myc transgenic mice on the wild-type and GILT knockout backgrounds. Seventy percent of the cell lines started were successfully adapted to culture. The B cell lymphoma line derived from the GILT knockout background was named Bμ/H9262Myc.GKO.1.

Stages of B cell development are defined by differential expression of cell surface markers (20). For example, pro-B cells are CD45Rlow (B220) and CD43high (20). Pre-B and immature B cells are CD45Rint and CD43low, and immature B cells are distinguished from pre-B cells by expression of IgM. Expression of CD24 (HSA) increases with B cell development (20). Mature B cells are CD45Rhigh and do not express CD43, and mature B cells can be further distinguished from immature B cells by higher expression of IgD (20). Markers of peripheral stages of mature B cells CD21 and CD23 can be used to identify follicular mature B cells (CD21+CD23−) and marginal zone B cells (CD21−CD23+) (20). As shown in Fig. 1A, BμMyc.GKO.1 cells displayed an immature B cell phenotype. BμMyc.GKO.1 cells expressed wild-type and mutant human GILT by intracellular FACS. BμMyc.GKO.1 cells transduced with vector alone (negative control), wild-type, or mutant C46S, C49S, C46SC49S, C200S, or C211S GILT, and the Priess B cell line (positive control) were fixed with formaldehyde, permeabilized with saponin, and then stained with FITC-conjugated anti-GILT mAb (MaP.IP30) (solid line histogram) or FITC-conjugated isotype control mAb (dashed line histogram). Transduced GILT was homogeneously expressed in the BμMyc.GKO.1 cells. Wild-type GILT and the mutants C46S, C49S, and C46SC49S were expressed at equivalent levels. The mutants C200S and C211S, which have impaired GILT processing, were expressed at slightly lower levels.

Expression and subcellular localization of wild-type and cysteine mutants of GILT

We hypothesized that GILT’s reductase activity is its essential function in MHC class II-restricted Ag processing. To test this hypothesis, wild-type human GILT and cysteine mutants of the reductase active site (C46S, C49S, and C46SC49S) were stably expressed in the GILT-deficient B cell lymphoma line BμMyc.GKO.1. Additionally, we were interested in the possible role of Cys211 in Ag processing. Remarkably, although Cys211 is present in the C-terminal propeptide and presumably nonessential to the function of the mature form, C211S GILT and a mutant in its proposed disulfide partner...
Cys200, C200S GILT, are impaired in processing to the mature form, although the mutant precursors remain active (16). Human and murine GILT share ~70% sequence identity, and the cysteines in the mature form and C-terminal propeptide are entirely conserved (8). We elected to use human GILT in these studies because retrovirally transduced wild-type or mutated GILT were stained with anti-GILT serum and anti-MHC class II mAb and analyzed by confocal microscopy. As shown in green in Fig. 2, staining with anti-GILT serum revealed a punctate pattern consistent with staining of late endosomes and lysosomes. Staining with anti-MHC class II mAb (Fig. 2, red) or anti-lysosome-associated membrane protein-1 mAb (data not shown) revealed a similar pattern. The merged images demonstrated that GILT localized with MHC class II (Fig. 2, yellow) and lysosome-associated membrane protein-1 (data not shown). No staining was observed with the secondary Ab alone, and no staining for GILT was observed in B2Myc.GKO.1 cells transduced with vector alone (data not shown). These data demonstrate that retrovirally transduced wild-type GILT or mutant C46S, C49S, C46SC49S, C200S, and C211S GILT maintain the correct subcellular localization within the B2Myc.GKO.1 cells.

**Effect of cysteine mutations on GILT maturation**

To further evaluate the expression and processing of the wild-type and mutant forms of human GILT in the B2Myc.GKO.1 cells, cell lysates were analyzed by nonreducing SDS-PAGE and immunoblotting with rabbit anti-GILT serum. This antiserum recognizes both precursor and mature forms. GRP94 served as a loading control. Wild-type GILT and the active site mutants C46S, C49S, and C46SC49S were detected in both precursor and mature forms. C200S and C211S GILT exhibited minimal or impaired processing.

![Figure 2](image2.png) Figure 2. Colocalization of wild-type and cysteine mutants of human GILT with MHC class II. B2Myc.GKO.1 cells transduced with wild-type (WT) or mutated GILT were fixed with formaldehyde, permeabilized with saponin, stained with anti-human GILT mAb or isotype control mAb and examined by flow cytometry (Fig. 1B). There was homogenous expression of the transduced GILT in the B2Myc.GKO.1 cells. Wild-type GILT and the active site mutants (C46S, C49S, and C46SC49S) were expressed at equivalent levels. The mutants that exhibit impaired GILT processing (C200S and C211S) appeared to be expressed at slightly lower levels.

Immunofluorescence staining was performed to determine the subcellular localization of wild-type and mutant forms of GILT. B2Myc.GKO.1 cells expressing wild-type or mutated GILT were fixed with paraformaldehyde, permeabilized with goat anti-rabbit IgG (green, left column) and anti-MHC class II mAb (TIB120) with Alexa Fluor 546-conjugated F(ab’)2 of goat anti-rat IgG (red, middle column), and analyzed by confocal microscopy. Merged images (yellow, right column) are also shown.
GILT was detected at steady state (Fig. 3, lanes 6 and 7). As previously observed when they were overexpressed in COS-7 cells (16), C200S and C211S GILT exhibited impaired processing to the mature form. For wild-type and mutant GILT, the precursor form was detected as a doublet (Fig. 3). Multiple molecular weight species of precursor GILT are also observed upon overexpression in COS-7 cells and are due to variable N-linked glycosylation (9).

The immunoblotting analysis, which represents the steady state, suggested that there was slightly more precursor form relative to mature GILT.

**FIGURE 4.** Effect of mutation of active site cysteines on GILT maturation. B2Myc.GKO1 cells transduced with wild-type (WT) (A and D), C46S (B), or C49S (E) human GILT were starved, metabolically labeled with [35S]methionine and cysteine, and chased for the time periods indicated. Postnuclear supernatants of detergent cell lysates were immunoprecipitated with anti-GILT mAb (MaP.IP30) or normal mouse serum as a control (C). Samples were analyzed by reducing SDS-PAGE (12%) and imaged by autoradiography. C and F, The amount of precursor and mature GILT was quantitated for each time point using a PhosphorImager. The mature form of wild-type, C46S, and C49S GILT was graphed as a percentage of total intracellular GILT for each time point (mature GILT divided by the sum of precursor plus mature GILT).

**FIGURE 5.** Cell surface expression of MHC class II in B2M-myc transformed B cell lymphoma cells. B2Myc.GKO.1 cells stably transduced with vector alone, wild-type (WT) GILT, or mutant GILT (C46S, C49S, C46SC49S, C200S, or C211S) and treated with IFN-γ for 24 h. Cells were stained with anti-I-Ab mAb directly conjugated with FITC (solid line histogram) or a negative isotype control mAb directly conjugated with FITC (dashed line histogram), and then analyzed by flow cytometry. I-Ab expression in B2Myc.1 cells, which endogenously express GILT, is shown for comparison. B2Myc.GKO.1 cells, transduced with wild-type or mutated GILT, and B2Myc.1 cells had equivalent cell surface expression of MHC class II.
mature form for the single and double cysteine mutants involving Cys46 compared with wild-type GILT (Fig. 3). This suggestion led us to evaluate the kinetics of GILT maturation in B/H9262Myc.GKO.1 cells expressing wild-type, C46S and C49S GILT to determine whether the active site cysteines are involved in the processing of precursor to mature GILT. Pulse chase analysis was performed by metabolically labeling cells with [35S]methionine and cysteine for 1 h and chasing with excess cold methionine and cysteine for up to 9 h. At intervals, the cultures were harvested, and cells were extracted with detergent. GILT was immunoprecipitated with mouse anti-GILT mAb (MaP.IP30), the samples were analyzed by reducing SDS-PAGE followed by autoradiography (Fig. 4, A, B, D, and E), and quantitated using a PhosphorImager (Fig. 4, C and F). For wild-type, C46S, and C49S GILT, the precursor form was long-lived (Fig. 4). This result is similar to that observed in murine primary B cells (21), but is quite different from results in human B lymphoblastoid lines, in which little precursor GILT remains after 3 h of chase (19). The immunoblotting analysis in Fig. 3 shows that the mature form predominates for both wild-type and mutant GILT species, suggesting that mature GILT accumulates slowly over time and is stable. A more mature form was present in wild-type GILT compared with C46S or C49S GILT (Fig. 4 A and D compared with Fig. 4 B and E). Quantitation showed that processing from the precursor to mature form of GILT was reduced in both active site cysteine mutants. Less mature form was generated for C46S compared with wild-type GILT (25% of total intracellular GILT at 3 h compared with 40% of total intracellular GILT at 6 h) (Fig. 4C) and for C49S compared with wild-type GILT (40% compared with 55% of total intracellular GILT at 3 h) (Fig. 4F). This difference does not appear to be due to differences in secretion of precursor GILT because immunoprecipitation of GILT from the culture supernatants revealed that ~35% of GILT was secreted in both the wild-type and C46S forms (data not shown).

Effect of cysteine mutants on Ag processing

To evaluate the effect of cysteine mutants on MHC class II-restricted Ag processing, we first needed to demonstrate equivalent MHC class II expression by the B/H9262Myc.GKO.1 cells transduced with wild-type or mutated GILT and that wild-type human GILT could reconstitute the Ag processing activity in murine B cells. Cell surface expression of MHC class II decreased over time in both B/H9262Myc.1 and B/H9262Myc.GKO.1 cells (data not shown). To

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GILT-dependent HEL74–88 epitope. B2M-Myc.GKO.1 cells transduced with vector alone, WT, C46S, C49S, C46SC49S, C200S, or C211S GILT were pretreated with IFN-γ for 24 h to increase MHC class II expression. The B cell lymphoma cells were then cocultured with B04 T cell hybridoma cells and 1 mg/ml HEL, 10 μg/ml HEL74–88 peptide, or 1 mg/ml BSA. C, Quantitative comparison of the generation of the GILT-dependent HEL74–88 epitope by wild-type and mutant GILT species. B2M-Myc.GKO.1 cells transduced with vector alone, WT, C46S, C49S, C200S, or C211S GILT and FACS-sorted for expression of I-Ab were cocultured with B04 T cell hybridoma cells and varying concentrations of HEL. D, MHC class II-restricted Ag processing of GILT-independent HEL20–35 HEL epitope. As in B, except used HEL20–35 peptide and Hb1.9 T cell hybridoma cells. In each case, the IL–2 concentration in the culture supernatants was determined by ELISA in at least two experiments with each condition performed in triplicate. Data represent the mean ± 1 SD of the IL–2 concentration for a representative experiment. Coculture with peptide, which is exchanged on the cell surface and does not require processing, served as a positive control. The IL–2 concentration with coculture with BSA was below the limit of detection and served as the negative control.
maintain the levels of MHC class II expression required for Ag processing assays, high MHC class II-expressing cells were selected by FACS and then treated with IFN-γ (100 U/ml) for 24 h as necessary. B4Myc.GKO.1 cells transduced with vector alone, wild-type GILT, or mutated human GILT and B4Myc.1 cells endogenously expressing murine GILT had equivalent cell surface expression of MHC class II (Fig. 5). The addition of wild-type or mutated GILT did not alter cell surface levels of MHC class II (Fig. 5). MHC class II Ag processing assays were performed by coculturing T cell hybridomas specific for HEL epitopes in the context of I-Aβ along with APCs and intact HEL, HEL peptide, or BSA as a control. IL-2 production by the T cell hybridoma was measured by ELISA. B4Myc.GKO.1 cells transduced with vector alone were unable to process the GILT-dependent class II-restricted HEL74–88 epitope recognized by the B04 T cell hybridoma (Fig. 6A, left). Transduction with wild-type human GILT reconstituted the processing of the GILT-dependent HEL74–88 epitope and stimulation of the B04 T cell hybridoma (Fig. 6A, middle) to the level seen in B4Myc.1 cells, which endogenously express murine GILT (Fig. 6A, right). For all APCs, coculture with the specific HEL74–88 peptide, which is exchanged on the cell surface and does not require intracellular processing, resulted in equivalent IL-2 production by the B04 hybridoma (Fig. 6A). IL-2 production with coculture with BSA was below the limit of detection (Fig. 6A).

B4Myc.GKO.1 cells transduced with GILT mutants of the reductase active site, C46S or C49S singly or together, were unable to efficiently process the GILT-dependent HEL74–88 epitope (Fig. 6B). Because these mutants were equivalently expressed, localized, and processed, this result demonstrated that GILT reductase activity is necessary for its function in intracellular MHC class II processing. Additionally, B4Myc.GKO.1 cells transduced with C200S and C211S GILT, which substantially impair GILT maturation, were unable to efficiently process the GILT-dependent HEL epitope (Fig. 6B). To further explore the activity of the mutated forms of GILT, B4Myc.GKO.1 cells transduced with vector alone, wild-type GILT, and mutant C46S, C49S, C200S, and C211S GILT were cocultured with a range of concentrations of HEL and the B04 T cell hybridoma. B4Myc.GKO.1 cells transduced with C49S, C200S, and C211S GILT were able to process HEL and stimulate the B04 T cell hybridoma approximately one-tenth as efficiently as wild-type GILT (Fig. 6C). Transduction with vector alone or C46S GILT did not stimulate IL-2 production even at high concentrations of HEL (Fig. 6C). The defect in the MHC class II processing of the GILT-dependent HEL epitope was not due to a global defect in MHC class II processing. B4Myc.GKO.1 B cell lines stably transduced with vector alone, wild-type GILT, or cysteine mutants of GILT demonstrated equivalent processing of the GILT-independent HEL20–35 epitope recognized by the Hb1.9 T cell hybridoma (Fig. 6D).

**Discussion**

B cell lymphoma lines B4Myc.1 and B4Myc.GKO.1 were generated from wild-type C57BL/6 and GILT knockout mice, respectively, using in vivo transformation with the Eμ-myc transgene. These cells displayed an immature B cell phenotype as demonstrated by cell surface expression of CD45R, low expression of CD43, expression of IgM, and little to no expression of IgD (Fig. 1A). It is unclear whether the unstable MHC class II (I-Aβ) expression on B4Myc.1 and B4Myc.GKO.1 cells is due to the B cell developmental stage, type of CIITA expression, a specific effect of c-myc, or transformation in general. Expression of class II increases with B cell development, with maximal expression in mature B cells, and with decreased expression upon maturation into plasma cells (22, 23). I-A expression in murine B cells appears in development on a portion of pre-B cells and all immature B cells from the adult bone marrow; however, IgM+ pre-B and IgM+ immature B cells derived from the fetal developmental pathway, occurring in neonatal lymphoid organs and extending up to 1 mo of age, lack class II expression (23, 24). The frequency of IgM+ B cells expressing class II decreases over time in stromal cell cultures of bone-marrow derived progenitors; it is uncertain whether this is due to loss of class II expression or expansion of class II cells (23). B4Myc.GKO.1 cells up-regulated MHC class II expression in response to IFN-γ, which could be due to expression of type IV CIITA. B cells constitutively express large amounts of type III CIITA and small amounts of type IV and type I CIITA (25). IFN-γ has been shown to increase the activity of CIITA promoter IV in B cells and may contribute to up-regulation of MHC class II expression (26). It is possible that overexpression of c-myc may down-regulate MHC class II via L-myc and N-myc overexpression decreases the transcriptional activity of CIITA promoter IV through binding to the E-box transcription factor-binding site in small cell lung cancer and neuroblastoma cell lines (27). Alternatively, loss of MHC class II may be due to the general instability of transformed cell lines.

The GILT-deficient B cell lymphoma line B4Myc.GKO.1 was stably transduced with wild-type human GILT, mutants of the reductase active site (C46S, C49S, C46SC49S) or mutants that disrupt processing of GILT (C200S, C211S). Based on intracellular flow cytometric analysis, immunofluorescence studies, and immunoblotting analysis, wild-type and mutant GILT species were homogenously and equivalently expressed and maintained the late endosomal/lysosomal localization found for endogenous GILT (Figs. 1B, 2, and 3). These cell lines provide a useful tool for biochemical and cellular analysis of GILT.

In this study, we showed that GILT reductase activity is an essential function in MHC class II Ag processing (Fig. 6B). Mutation of Cys66 or Cys69 of the CXCC reductase active site, either singly or together, eliminated efficient intracellular processing of the GILT-dependent HEL74–88 epitope and the production of cell surface peptide-MHC class II complexes for T cell stimulation. An ~10-fold lower amount of T cell stimulation was reproducibly observed when B4Myc.GKO.1 cells transduced with C49S GILT were cocultured with intact HEL (Fig. 6C). However, no intracellular processing was observed with C46S GILT even at high concentrations of HEL (Fig. 6C). This result further supports the hypothesis that Cys66 is the active site cysteine that initiates nucleophilic attack on the substrate disulfide bond. In C49S GILT, therefore, the N-terminal Cys66 could still generate mixed disulfide GILT-substrate intermediates. A small amount of reduction and substrate release may be mediated by a separate reducing agent, lysosomal cysteine, for example. Some members of the thio-redoxin superfamily, such as glutaredoxin, are able to catalyze efficient substrate oxidation with only the N-terminal active site cysteine (28).

As previously shown, C200S and C211S GILT had defects in maturation and were expressed predominantly as the precursor form at steady state (Fig. 3) (16). Although the wild-type precursor form has reductase activity in vitro (9), the precursor forms of C200S and C211S GILT were ~10-fold less efficient in Ag processing compared with wild-type GILT (Fig. 6C). We were interested in the function of the likely disulfide pair Cys200 and Cys211 because Cys211 is located in the C-terminal propeptide and dispensable to the function of the mature form, yet is able to affect the processing and reductase activity. Precursor C200S and C211S GILT species immunosisolated from COS-7 cells have weak activity at acidic pH and better activity at neutral pH in a cell-free
assay (16). The markedly reduced Ag processing activity of C200S and C211S GILT could have a number of explanations. Although loss of the Cys^{200} and Cys^{211} disulfide pair apparently allowed for sufficient folding to exit the endoplasmic reticulum and localize to the lysosomes (Fig. 2), it may result in decreased protection from lysosomal proteolysis or instability at acidic pH compared with wild-type precursor. C200S and C211S GILT may therefore have reduced activity in Ag processing due to diminished amounts. Alternatively, but perhaps less likely, Cys^{200} and Cys^{211} could be essential for Ag processing activity by precursor GILT, or the precursor form of GILT in general may be less active in intracellular Ag processing.

In addition, we have identified a novel role for the reductase active site. Based upon an analysis of the rate of maturation, mutation of Cys^{46} or Cys^{19} in the reductase active site reduced the processing of the precursor to mature form of GILT (Fig. 4). This defect in C46S and C49S GILT was not previously observed in COS-7 transfectants (6) or J3 melanoma cell transfectants at steady state (data not shown). The effect may be specific for professional APCs or perhaps more readily observed in B{\textsc{b}}Myc:GKO.1 cells, which inherently have a lower level of processing of precursor to mature GILT. GILT reductase active site could autocatalyze the reduction of the precursor form to expose the dibasic cleavage sites flanking the propeptide sequences to lysosomal cathepsins. For example, the reductase active site could reduce the disulfide bond predicted between Cys^{200} in the mature form and Cys^{211} in the C-terminal propeptide, and thus, aid cleavage of the propeptide. However, this possibility seems unlikely because eliminating this disulfide bond by mutating either Cys^{200} or Cys^{211} to serine resulted in impaired processing of GILT. Alternatively, GILT’s reductase active site could play a role in maintaining the activity of lysosomal cysteine proteases that are responsible for cleavage of GILT’s N- and C-terminal propeptides.

The pulse-chase analysis in Fig. 4 showed that even wild-type human GILT is incompletely processed. Even after 24 h, ~50% remained in the precursor form, and the rate of processing of the remaining pool appeared to be very slow or nonexistent (data not shown). Incomplete processing was also evident at steady state, shown in Fig. 3 by immunoblotting. This processing was previously observed for murine GILT in the A20 B cell line (8) and in primary B cells (21), but has not been observed in either human EBV-transformed B cells or in COS-7 cells (9, 19). Staining of the B{\textsc{b}}Myc:GKO.1 transductant with an Ab to the N-terminal propeptide indicated that the precursor form does not colocalize with MHC class II molecules (data not shown), but we have not yet identified the compartment where it resides. We are currently working to resolve this potentially interesting phenomenon.

Reduction of Ags is an important step in MHC class II processing and presentation. Destabilizing protein structure by acidification and reduction can allow MHC class II binding of the full-length protein or a protein fragment (3, 5). In addition, some epitopes from HEL and other proteins must be reduced for efficient stimulation of T cells (4, 13). Reduction facilitates lysosomal proteolytic digestion of Ags and generation of antigenic peptides bound to MHC class II for T cell stimulation (2). However, reduction is not favored at the acidic pH found in the lysosomal compartment. In fact, lysosomes of adenocarcinoma lines were found to be oxidizing rather than reducing (29). The constitutive expression of GILT in APCs is likely to account for the enhanced reduction of proteins in the lysosomal compartment. Lysosomal proteases have the ability to both generate and destroy antigenic epitopes (30), and MHC class II binding can protect the bound epitope from proteolysis (31, 32). We propose that reduction by GILT facilitates MHC class II Ag processing by exposing constrained epitopes for MHC class II binding, thus, protecting them from protease digestion.

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Disclosures
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References


