Cutting Edge: Induction of the Antigen-Processing Enzyme IFN-γ-Inducible Lysosomal Thiol Reductase in Melanoma Cells Is STAT1-Dependent but CIITA-Independent

Patrick W. O'Donnell, Azizul Haque, Michael J. Klemsz, Mark H. Kaplan and Janice S. Blum

*J Immunol* 2004; 173:731-735; doi: 10.4049/jimmunol.173.2.731
http://www.jimmunol.org/content/173/2/731
Cutting Edge: Induction of the Antigen-Processing Enzyme IFN-γ-Inducible Lysosomal Thiol Reductase in Melanoma Cells Is STAT1-Dependent but CIITA-Independent

Patrick W. O’Donnell, Azizul Haque, Michael J. Klemsz, Mark H. Kaplan, and Janice S. Blum

Presentation and CD4+ T cell responses to Ag in the context of MHC class II molecules require processing of native proteins into short peptides for optimal MHC binding and TCR recognition (1, 2). Pathways for Ag processing have been best defined in professional APCs and involve Ag denaturation via unfolding coupled with proteolysis (3–5). Within this processing pathway, the reduction of disulfide bonds is a critical step (6–9). A 30-kDa IFN-γ-inducible thiol reductase, IFN-γ-inducible lysosomal thiol reductase (GILT),3 functions to catalyze thiol bond reduction, thus unfolding native protein Ag and facilitating further processing via cellular proteases. In contrast with professional APCs such as B cells, class II-positive human melanomas expressed relatively little to no GILT protein or mRNA. Tumor cell GILT expression was partially restored with IFN-γ treatment but unlike other genes required for class II Ag presentation, GILT was not regulated by CIITA. Rather, studies revealed STAT1 plays a direct role in IFN-γ-inducible GILT expression. These results define a molecular mechanism for the uncoupled regulation of MHC class II genes and the processing enzyme GILT in human melanomas. The Journal of Immunology, 2004, 173: 731–735.

GILT expression correlates with class II protein levels in professional APCs: constitutive in class II-positive B cells and inducible by IFN-γ in macrophages (12). In contrast, studies with class II-positive tumors such as melanomas suggest the expression of MHC class II and GILT may not always be linked. GILT expression is absent or expressed at low levels in a majority of class II-positive human melanomas (12). This observation suggests that Ag-processing reactions may differ in melanomas and professional APCs, thus influencing tumor escape from immune surveillance.

The signaling mechanisms which regulate IFN-γ-dependent gene transcription have been well characterized (16, 17). IFN-γ stimulates a cell through a specific receptor followed by activation of Jak 1 and 2, which phosphorylate tyrosine residues on the intracellular domain of the receptor. These phosphotyrosine residues serve as docking sites for STAT1 monomers which are subsequently phosphorylated by Jak 1 and 2. Phospho-STAT1 dimerizes and transits to the nucleus to activate the transcription of numerous target genes (18–20). IFN-γ treatment of macrophages and nonprofessional APCs including many tumors can induce class II Ag expression by activation of STAT1 and the induction of specific isoforms of the transcription factor CIITA. Yet, whether STAT1 or CIITA play a role in the expression of GILT has not been tested.

As melanomas represent the most lethal cancer of the skin, with resistance to many chemotherapeutic treatments, novel immunotherapeutic strategies to treat and prevent recurrence of these tumors are being explored (21, 22). CD4+ and CD8+ T cells specific for melanoma-derived Ag can be isolated from patients suggesting that immune defenses can be mobilized to recognize and attack these tumors (23). In addition, therapeutic delivery of large numbers of CD4+ and CD8+ T cells to patients correlates with tumor regression and disease remission (24). Thus, understanding the mechanisms which guide Ag processing and CD4+ T cell responses to Ag in the context of MHC class II molecules requires processing of native proteins into short peptides for optimal MHC binding and TCR recognition (1, 2). Pathways for Ag processing have been best defined in professional APCs and involve Ag denaturation via unfolding coupled with proteolysis (3–5). Within this processing pathway, the reduction of disulfide bonds is a critical step (6–9). A 30-kDa IFN-γ-inducible thiol reductase, IFN-γ-inducible lysosomal thiol reductase (GILT),3 functions to catalyze thiol bond reduction, thus unfolding native protein Ag and facilitating further processing via cellular proteases. In professional APCs such as B cells and macrophages, GILT is optimally active at low pH and has been demonstrated to play a key role in Ag processing and the display of immunodominant epitopes by class II molecules (8, 10–15).

3 Abbreviations used in this paper: GILT, IFN-γ-inducible lysosomal thiol reductase; c.m., conditioned medium.
processing reactions in melanomas and professional APCs may be important to enhancing these experimental therapies.

The goal here was to define at a molecular level the mechanisms that control GILT expression in melanomas. Studies confirmed very low or undetectable basal levels of GILT protein in human class II-positive melanomas. IFN-γ treatment of these tumors enhanced the amount of intracellular GILT protein and mRNA, although the level of GILT protein and message in these cytokine-treated tumors was still less than that found in human B lymphoblasts. Although GILT secretion has been observed in certain B cells and stimulated macrophages (10, 11), cellular release of GILT was not observed in resting or cytokine-activated tumors. Rather, GILT produced by tumors was retained in cells and correlated with mRNA levels. CIITA, a master switch promoting constitutive class II expression in B cells and IFN-γ induction in macrophages (25–27), was not responsible for basal or inducible GILT expression in melanomas. Studies using B cells lacking or expressing CIITA, as well as mutant B cells missing members of the RFX protein family (28, 29) demonstrated further that GILT was not regulated transcriptionally by those same factors that control MHC class II expression. Instead, studies indicated IFN-γ induction of GILT expression in tumors was dependent on activation of STAT1.

Materials and Methods

Cells, IFN-γ stimulation, and Western analysis

Cells were cultured as previously described (12, 30, 31), stimulated with human IFN-γ (100–500 U/ml; R&D Systems, Minneapolis, MN), and whole cell protein extracts were prepared with the addition of phosphatase inhibitor mixtures 1 and 2 (Sigma-Aldrich, St. Louis, MO). Western analysis using whole cell lysate (25 μg protein) was performed as previously described (8). The following Abs were used: actin (NeoMarkers, Fremont, CA), GILT (rabbit antiserum, Vishnu a gift from P. Cresswell, Yale University, New Haven, CT), total STAT1 (Upstate Biotechnology, Lake Placid, NY), and MHC class II α and β (DA6.147 and XD5.A11).

Biotin-labeled DNA oligonucleotide pull-down, RNA isolation, and Northern analysis

Whole cell lysate (100 μg protein) was incubated with a biotin-labeled dsDNA oligonucleotide as previously described (32) using the following 5’ biotin-linked oligonucleotides: no. 1: GCCACGTCGAAGCTCCTCCG GCTC, no. 2: CCCATGCGCCCTCCCTTGAAGGCCAGGTGC, and complementary nonbiotinylated DNA oligonucleotide. Oligonucleotide pull-down competition assays were performed as above with addition of 10-, 100-, 500-fold nonbiotinylated DNA oligonucleotide. Oligonucleotides with a p53 binding site: TCCGAACTTGCCTCCGGGACTAGT (33), or a STAT1 consensus binding site: TGAGCCTGATTTCCCGGAATGAGGC (34) and complementary oligonucleotides were used to generate double-stranded competitors. Total RNA was extracted from cells using Tri-Reagent (Molecular Research Center, Cincinnati, OH), and Northern analysis performed (35).

Results and Discussion

GILT expression in melanomas is controlled at the level of gene transcription and not reductase secretion

Despite measurable levels of MHC class II protein expression, a majority of the human melanomas express only low levels of a thiol reductase GILT, an enzyme linked to efficient Ag processing (12). Two such melanomas, 1359mel and J3, were tested to determine mechanisms controlling IFN-γ induction of GILT in these tumors. In the absence of IFN-γ treatment, very low or no GILT protein was detectable in 1359mel or J3 (Fig. 1A). Upon cytokine treatment, both melanomas displayed increased GILT protein levels. Melanoma responses to IFN-γ stimulation at multiple time points were also tested and GILT protein levels increased in stimulated tumors until at least 48 h (data not shown). Previous work has shown that reductase glycosylation can lead to changes in GILT protein migration during electrophoresis (12). Such differences were not noted here, suggesting that the intracellular GILT isoform detected was similar in each of the tumors and B-LCL lines tested. These studies demonstrate that in contrast to B cells, resting tumor cells display uncoupled expression of GILT and class II DR. However, the cytokine inducibility of GILT in DR-positive melanomas indicated that these tumors can synthesize low levels of full-length GILT mRNA and protein in response to IFN-γ.

Macrophages and some B cells are able to express increased GILT levels in response to IFN-γ, however, these monocyte and lymphoid B cell lines secrete measurable amounts of the reductase into their extracellular medium (10, 11). Melanomas have also been shown to secrete select lysosomal proteases, thus, protein secretion could account for reduced reductase levels observed in melanomas (36). Remarkably, neither resting nor cytokine-treated 1359mel secreted any detectable GILT protein (Fig. 1A). In addition, GILT secretion was not observed using J3 tumor cells including a subclone stably transfected to constitutively express GILT under the control of a viral promoter. In contrast, the human B cell line Pala secreted measurable levels of GILT in conditioned media (c.m.) (data not shown). Although either cytokine treatment or gene transfection could be used to increase GILT protein levels in tumors, GILT was not detectable in conditioned media from tumor cells. Therefore, the reduced expression of GILT in melanomas was not due to a defect in the sorting or retention of this lysosomal enzyme.

Differences in gene transcription were tested as a potential mechanism to explain the reduced GILT levels in melanomas.

![FIGURE 1.](http://www.jimmunol.org/Downloaded/from)
Northern analysis revealed an abundance of GILT mRNA in the B cell Frev and the GILT-transfected tumor J3.GILT (Fig. 1B). However, in multiple human melanoma lines: 1359mel, SLM2mel, J3, M21, 1102mel, and M1106, little to no basal GILT RNA was detected. A consistent increase in GILT RNA was seen when J3 cells were treated with IFN-γ. These data suggest GILT protein levels in melanomas are the result of the low levels of GILT mRNA and not altered protein metabolism.

Neither basal nor inducible GILT expression is controlled by the transactivator CIITA

The ability of IFN-γ to trigger both GILT and MHC class II protein synthesis in several human melanomas (Fig. 1A) raised the possibility that similar transcription factors may govern the inducible expression of these molecules in tumor cells. To further probe the mechanism(s) responsible for the low level of GILT expression in class II⁺ human melanomas, the relative importance of transcription factors known to influence MHC class II abundance in response to signaling via the IFN-γ receptor was examined (16, 17). One transactivator, CIITA, necessary for the expression of genes within the MHC class II loci, as well as the invariant chain, is synthesized by professional and nonprofessional APCs in response to IFN-γ (17, 25, 27). To directly test the requirements for CIITA, IFN-γ-inducible GILT expression was analyzed by immunoblot in a small subset of melanomas that lack basal CIITA expression. These melanoma lines (mel 624, mel 888, and mel 1861) were transduced to ectopically express CIITA under the control of a retroviral promoter. mel 624 (Fig. 2A), mel 888, and mel 1861 (data not shown) all expressed low basal levels of GILT but no class II protein. Upon cytokine treatment, GILT expression increased in each of the parental tumors, but to a level below that observed for the professional APC Frev. RT-PCR demonstrated that all three melanoma lines lacked basal CIITA mRNA expression (data not shown). Only mel 888 expressed class II in response to IFN-γ (data not shown). When CIITA was ectopically expressed in these three tumors, there was no significant increase in GILT protein compared with the parental lines. Ectopic expression of CIITA, even in the absence of IFN-γ, did result in measurable class II DR levels in these tumor lines.

GILT expression was also examined in a B cell line lacking CIITA, RJ2.2.5 (Fig. 2B), as well as B cell lines (SJL, BLS-1, 6.1.6) lacking discrete RFx family members (data not shown). The loss of CIITA or RFx proteins in these cells results in a class II null phenotype known as bare lymphocyte syndrome (28, 29). GILT was detectable in all cell lines tested, regardless of the absence of CIITA or RFx family members. Furthermore, 1315 nos. 4 and 32, RJ2.2.5-derived subclones in which CIITA has been stably transfected to restore class II protein expression, showed no change in GILT levels. Thus, CIITA is neither sufficient nor required for basal or cytokine inducible expression of GILT in multiple cell types.

STAT1 is required for IFN-γ-inducible, but not basal, GILT expression

IFN-γ induction of CIITA is a downstream event, mediated by the STAT1 transcription factor which is activated by Jaks in response to cytokine engagement of the IFN-γ receptor (16, 25, 27, 37). To determine whether GILT gene expression might be linked to early events in IFN-γ signaling, the time course of GILT mRNA production was monitored in IFN-γ-treated tumors. GILT mRNA was detectable between 2 and 4 h of IFN-γ treatment and increased to a maximum level at 16 h poststimulation (Fig. 3A).

As activated Stat1 was observed in the melanomas following IFN-γ stimulation, two approaches were undertaken to determine whether STAT1 molecules function in regulating GILT gene expression. A panel of human fibrosarcoma tumors that expressed or lacked expression of STAT1 were tested for basal and IFN-γ-inducible GILT expression (Fig. 3B). No fibroblast-like (human fibrosarcoma cell line) had a low level of basal GILT expression that increased upon IFN-γ stimulation. However, in the STAT1 null U3A (2ftgh-derived), treatment with IFN-γ had no effect on the basal level of GILT expression. When STAT1 was reconstituted in this cell line yielding the U3ASTAT1 tumor, the IFN-γ induction of GILT expression was restored. Therefore, STAT1 is required for IFN-γ-inducible, but not basal, GILT gene expression in these human tumors.

To extend our results from the fibrosarcoma STAT1 null cell lines, a STAT1 dominant-negative expression plasmid was transfected into the melanoma line 1359mel (Fig. 3C). The dominant-negative STAT1β is a naturally occurring splice variant of the STAT1 gene which lacks the transactivation domain and has been well characterized in interfering with transactivation by the full-length STAT1 (18, 38). GILT was detectable in the cytokine-treated parental 1359 and 1359.pc (plasmid control) transfected lines, but GILT protein induction was reduced in 1359.STAT1β treated with IFN-γ. A decrease in MHC class II expression was observed in 1359.STAT1β cells treated with...
IFN-γ (data not shown), as expected due to the requirement for STAT1 activation for the induction of CIITA. This further supports a requirement for STAT1 in IFN-γ-inducible GILT expression in tumors.

Analysis of the human GILT promoter revealed at least two sequences which conform to minimal recognition sites for minimal promoter showing potential STAT binding site and sequences. A, STAT1 binding to two biotin-labeled STAT consensus sequence oligonucleotides from the GILT promoter. Biotin-labeled DNA oligonucleotides from the GILT promoter, immunoprecipitated with streptavidin agarose beads, electrophoresed and immunoblotted for STAT1. Specificity was established by adding increasing amount of unlabeled oligonucleotides from a p53 binding site or a STAT consensus oligonucleotide. Representative of three independent experiments.

FIGURE 4. STAT1 binds to biotin-labeled STAT consensus sequence oligonucleotides from the GILT promoter. A, Representation of the GILT promoter showing potential STAT binding site and sequences. B, STAT1 binding to two biotin-labeled STAT consensus sequence oligonucleotides from the GILT promoter. 1359 melanoma cells were stimulated with 500 U/ml IFN-γ for 4 h, cell lysates were incubated with biotin-labeled DNA oligonucleotides from the GILT promoter, immunoprecipitated with streptavidin agarose beads, electrophoresed and immunoblotted for STAT1. Specificity was established by adding increasing amount of unlabeled oligonucleotides from a p53 binding site or a STAT consensus oligonucleotide. Representative of three independent experiments.

Binding is competed with a STAT1 consensus sequence but not a p53 binding site oligonucleotide, lacking a consensus sequence. These results support a model wherein STAT1 binds sequences in the GILT promoter to regulate gene expression.

The ordered expression of subsets of genes involved in Ag processing and presentation may be a consequence of the temporal requirement for their function. During the generation of a pathogen-specific immune response, Ag reduction and proteolysis typically precede peptide loading and class II presentation. Therefore, in response to IFN-γ, the rapid induction of GILT via STAT1 activation would be most logical. Furthermore, induction of class II gene expression via IFN-γ is mediated by STAT1 activation through its induction of specific isoforms of the CIITA transcription factor. In the majority of melanomas, class II proteins are continuously synthesized and displayed on the cell surface due to the constitutive expression of CIITA via an enhancer element located in promoter III (39). Subverting class II function and tumor Ag presentation in these tumors may therefore be most easily accomplished by altering Ag processing and GILT expression.

Acknowledgments
We thank Drs. C. H. Chang, P. Robbins, N. Restifo, G. Stark, P. Cresswell, S. Topalian, W. Storkus for provision of cells and reagents. We also thank N. Schmidt, D. Zhou, P. Li, and S. Jackson for their helpful discussions and technical assistance.

References


