AN UNEXPECTED FUNCTIONAL LINK BETWEEN LYPOSOMAL THIOL REDUCTASE AND MITOCHONDRIAL MANGANESE SUPEROXIDE DISMUTASE

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Running title: Gamma interferon inducible lysosomal thiol reductase (GILT) regulates cellular proliferation

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Gamma interferon Inducible Thiol reductase (GILT) is an enzyme involved in initial steps of antigen processing and presentation. Recently we have shown that GILT is also expressed in mouse T cells where it exerts an inhibitory role on T cell activation. In this study, we identified mitochondrial manganese superoxide dismutase (SOD2) as one of the key intermediaries affected by GILT expression in fibroblasts. Expression and activity of SOD2 is reduced in the absence of GILT, due to reduced SOD2 protein stability. Forced increase in SOD2 expression in the absence of GILT restores fibroblast proliferation to wild type levels. Thus, GILT appears to have a fundamental role in cellular proliferation mediated through its influence on SOD2 protein activity and expression.

Enzymes of the thiol reductase family carry out reduction, oxidation, and isomerization of protein disulfide bonds in cytosol (for example-thioredoxin) [1, 2], mitochondria [3], endoplasmic reticulum (protein disulfide isomerase) [2] and lysosomes (GILT). The majority of these enzymes are functional at neutral or slightly alkaline conditions [4], they have similar three-dimensional structure and all feature a conservative active-site loop containing two cysteines in the sequence -C-G-P-C [5]. GILT is a unique and unusual member of the thiol reductase family because its optimal enzymatic activity is at a low pH (4.5–5.5) [6], [7], [8], and has an atypical active site (-C-G-A-C-).

GILT is synthesized as a 35KD soluble glycoprotein precursor and is transported to endosomal compartment via the Mannose-6-P-receptor pathway [9]. It is processed into the mature form (30kDa) by proteolytic removal of N- and C-terminal peptides. The protein has an approximate molecular mass of 30 kDa and was therefore initially named IP-30 [6]. In addition to endosomal/lysosomal localization, GILT is secreted in the tissue culture medium of the GILT-expressing cell lines [7], [10], and is present in mouse sera (M. Marić, unpublished observations). GILT is constitutively expressed in professional antigen presenting cells (APCs) but it is also inducible by pro-inflammatory cytokines such as IFNγ, TNFα, and IL1β [9].

Using GILT-/- mice as a model, we have shown that GILT catalyzes initial unfolding of antigenic protein (protein becomes more accessible for further processing by cathepsins) and therefore facilitates protein/peptide binding to MHC class II molecules [10]. By changing the redox state of exogenous antigenic proteins with disulfide bonds, GILT initiates the adaptive immune response. However, we have shown that GILT is constitutively expressed in T cells and has a role in the regulation of T cell activation. This is so far the only known GILT function not related to MHC class II processing [11]. GILT-/- T cells show increased proliferation and cytotoxic T cell activity in response to anti-CD3 stimulation. This observation suggests that GILT has a more fundamental role in cellular processes than just reduction of antigens in antigen processing pathway.
We hypothesized that the effect of GILT observed in T cells, is not unique and significant only to T cells but that fundamentally affects cellular proliferation in other cell types. In support of this hypothesis, we show that GILT-/- mouse fibroblasts also have increased levels of proliferation.

Furthermore, we show in this study that a possible mechanism for the regulatory role of GILT in cellular proliferation involves affecting steady state levels of a mitochondrial enzyme SOD2, involved in scavenging of reactive oxygen species (ROS) [12],[13]. Our data indicate that the expression, stability and function of mitochondrial manganese SOD (SOD2) is significantly decreased in GILT-/- mouse fibroblasts. These data suggest an unexpected functional link between lysosomal and mitochondrial enzymes involved in oxidative-reductive processes.

EXPERIMENTAL PROCEDURES

Mice, primary cells and cell lines. C57BL/6 mice were purchased from Jackson (Bar Harbor, ME). GILT -/- mice [10] were bred and maintained in Georgetown University pathogen-free animal facility. All mice were used at 6-12 week of age.

Primary fibroblasts were isolated from GILT -/- and wild type (WT) mouse spleens and/or ears as previously described [14]. GILT-/- and WT SV40 large T antigen immortalized mouse fibroblast cell line was generated from GILT-/- and WT mice in Dr. Peter Creswell’s laboratory at Yale University. Stable GILT -/- transfectants with mouse GILT and/or hSOD2 (kind gift from Dr. M. Williams, U. of Maryland) were made using Lipofectamine 2000 (Invitrogen) standard protocol. Both constructs were subcloned into pcDNA3.1 vector with zeocin resistance.

Cell proliferation. GILT-/- and WT mouse fibroblast cell lines were plated in triplicates at density of 1x10^6 cells/well (unless indicated otherwise) in 96-well flat-bottom plates and incubated at 37°C, 5% CO₂ for 30 min. Cells were pulsed with 1 µCi of [3H]tdT (Amersham/GE healthcare) overnight, and radioactive thymidine incorporation was subsequently measured on a beta scintillation counter 1450 MicroBetaTM (Wallac, Turku, Finland).

Western blotting and antibodies. Typically 5µg and/or 2.5µg of cell lysates in Tris-saline pH 7.5, 1% Triton X-100 containing protease inhibitor mixture were separated by SDS-PAGE. Proteins from the gel were transferred to nylon membrane (Immobilon P Millipore). Membranes were incubated overnight at 4°C, with: mouse anti-SOD2 antibody (Abcam), rabbit anti-actin antibody (Sigma), anti- Lamp-1 (Iowa hybridoma bank), and 1:5000 dilution of secondary antibodies HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Immunoreactive bands were visualized using enhanced chemiluminescence Western Lightning™ (PerkinElmer). In some cases membranes were stripped using Restore™ western blot stripping buffer (Pierce) and incubated at room temperature for 120 min, followed by incubation with anti-actin antibody as a loading control and appropriate secondary HRP-conjugated antibody.

Real time PCR. Total RNA was isolated using TRIzol followed by RNAse clean-up and treated with DNAseI. Total RNA (5 µg) was reverse transcribed using the Superscript II RT kit and random hexamers as primers (Invitrogen). All PCR reactions were done in triplicates using ABI Prism 7700 Sequence Detector (Applied Biosystems). SOD2 and 18SrRNA were amplified using TaqMan Universal PCR master mix (Applied Biosystems), and the average threshold cycles (Ct) of the triplicates were used to compare the relative abundance of the mRNA. Ct of 18SrRNA was used to normalize all samples. Primers and TaqMan probe for SOD2 were designed using Primer Express Software as following: forward: 5’-ctgtctataacaggccacct-3’, reverse: 5’-ggtctcagctcaactc-3’, TaqMan probe 5’-FAM-aacaagcgtcatc-MGB-3’. Primers and probe for 18SrRNA were previously reported [15]. TaqMan probe for SOD2 was designed to span the junction between 2 exons in order to avoid amplification of genomic DNA. This was not possible for 18SrRNA which has no introns. The DNA contamination in these samples was excluded by amplifying control samples treated identically with the exception of the reverse transcriptase step.

Cycloheximide chase experiment. 5x10^6 primary mouse fibroblasts, or mouse fibroblast cell lines were incubated in complete RPMI 1640 media.
with cycloheximide (0.2mg/ml). Cell lysates were processed for immunoblotting with mouse anti-SOD2 antibody at 0, 1, 6, 24hrs. Equivalent amounts of cell lysates were analyzed at each time point by Western blot.

**Superoxide dismutase activity assay.** 2x10⁷ GILT-/- and WT mouse fibroblast cells were homogenized in 1ml of cold buffer (20mM HEPES, 1mM EGTA, 210mM mannitol, and 70mM sucrose), pH 7.2, using dounce homogenizer. Protein concentration of the cytoplasmic lysate was measured using BCA™ protein assay kit (Pierce). SOD activity assay was performed as suggested by the supplier (Cayman Chemical). This protocol is based on tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. When measuring SOD2 activity, in order to separate cytosolic and mitochondrial SOD activity 3mM potassium cyanide was added to some sample wells. When measuring SOD1 activity fractions containing SOD1 (cytosolic) were separated from fractions containing SOD2 (mitochondrial) by centrifugation at 10,000xg as recommended by manufacturer’s protocol. Absorbance was read at 450 nm using a plate reader SpectraMax 190 (Molecular Devices) and data were analyzed using SofMaxPro software (Molecular Devices).

**ROS detection.** Changes in intracellular ROS concentration were assessed with a method adapted from Bass et al. [16]. GILT-/- and WT mouse fibroblast cell lines were starved overnight in phenol red-free medium with 1% FBS. Fresh medium and 10µg/ml of dihydroethidium (DHE) (Molecular Probes) was added and incubated for 5 minutes at room temperature (preliminary kinetics experiment suggested 5 minutes to be the optimal incubation time). Cells were washed again in PBS, resuspended in cold PBS/0.5%BSA buffer and immediately analyzed by flow cytometry.

**RESULTS**

GILT-deficient mouse fibroblasts show increased proliferation. GILT-/- T cells display stronger proliferative responses upon stimulation with anti-CD3 [11]. Hence, we examined whether GILT might affect cellular proliferation in general. To address this question, we tested the proliferation capabilities of fibroblast cell lines (Fig.1a) and primary fibroblasts (Fig.1b) isolated from GILT-/- and GILT WT mice. Because high density of fibroblast may induce contact inhibition of growth [17] we titrated the numbers of fibroblasts used in our proliferation assay. Our data indicate that irrespective of the concentration of cells (1x10⁵, 1x10⁶, 1x10⁷) in 96-well plates incubated overnight, proliferation of GILT-/- and GILT WT fibroblasts was significantly different. GILT-/- fibroblasts persistently showed at least two fold increased proliferation. Both GILT-/- and GILT WT fibroblasts were generated by transfection with Large T antigen of SV40 and the increased proliferation in GILT-/- could be a consequence of random insertion of the gene coding for SV40 large T antigen, an oncogene known to deregulate cell cycle and cause cellular proliferation. To exclude such a possibility we tested primary mouse fibroblasts from GILT-/- and GILT WT mouse spleens (Fig1b). GILT-/- primary fibroblasts also showed increased proliferation when compared to GILT WT primary fibroblasts. The identical findings were observed if fibroblasts were treated with EGF that is known to induce cellular proliferation (Fig1c). Thus, GILT-/- mouse fibroblast (MFs) cell lines display increased levels of both basal and EGF-induced proliferation. This effect could be ascribed directly to the presence or absence of GILT, since transfection of GILT-/- fibroblast cell line with either hGILT (not shown) or mGILT reverses the phenotype (Fig. 1d). GILT-/- fibroblasts transfected with GILT have decreased proliferation levels similar to WT fibroblasts.

**SOD 2 is down regulated in GILT-/- mouse fibroblasts.** Most of the GILT is sequestered in endosomal/lysosomal compartment, therefore its effect on fibroblast proliferation is likely to be exerted by affecting the stability and/or activity of other endosomal/lysosomal proteins. To this end, we first analyzed contents of Lamp-1/cathepsin D+ Percoll fractions in GILT-/- and GILT WT fibroblast cell lines by 2D gel electrophoresis followed by qualitative and quantitative assessment of protein spots. Of the several protein spots that were differentially displayed in two samples the most intriguing was identification of mitochondrial SOD2 (gi:17390379). SOD2 was almost absent from heavy fractions of GILT-/- mouse fibroblasts in
comparison to heavy fractions of GILT WT mouse fibroblasts (data not shown). The finding of SOD2 in these fractions is not surprising since endosomal/lysosomal and mitochondrial fractions isolated using Percoll gradient have overlapping density. However, different levels of SOD2 in GILT WT and GILT-/- cell lines were unexpected given their distinct subcellular localization. In order to exclude the possibility of altered intracellular localization of SOD2 or different density of GILT-/- and GILT WT lysosomes, SOD2 protein levels were determined in whole cell lysates using semi-quantitative Western blot. As shown in Fig. 2a SOD2 expression was significantly lower in both GILT-/- fibroblast cell line and GILT-/- primary mouse fibroblasts. The lack of GILT in fibroblasts does not correlate with alteration of the expression of other mitochondrial protein such as Hsp60 (data not shown).

To test whether the activity of SOD2 is decreased in GILT-/- cells, mouse fibroblasts were tested by an in vitro SOD assay based on tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The lysates of cells contain both cytosolic SOD1 and mitochondrial SOD2, thus in order to distinguish between these two enzymes, we added potassium cyanide that inhibits the activity of SOD1 but not of SOD2. Our data clearly show that GILT-/- mouse fibroblast cell line (Fig. 2b) as well as primary mouse fibroblasts (Fig. 2c) has decreased activity of SOD2. Although the role of SOD2 appears to be intrinsic for cellular survival, cytoplasmic copper/zink SOD (SOD1) is far more abundant in the cell accounting for 90% of the total SOD activity [18], [19]. Thus, we tested the protein expression of SOD1 in GILT-/- and GILT WT fibroblast cell line and primary fibroblasts (Fig. 2d). While SOD2 levels were significantly decreased in GILT-/- fibroblast cell line and primary fibroblasts, SOD1 levels remain similar in GILT-/- and GILT WT cells. Furthermore the activity of SOD1 in GILT-/- fibroblast cell line and primary fibroblasts also remains similar (Supplementary Fig.1). Thus, SOD2 levels and activity are specifically affected by GILT.

Reconstitution of GILT recovers SOD2 activity. Difference in SOD2 protein expression and activity in GILT-/- and GILT WT cells could be a consequence of GILT presence or a random event. To distinguish between these possibilities, we assessed SOD2 activity in GILT-/- fibroblasts reconstituted with mouse GILT. As shown in Fig. 3 SOD2 activity of transfectants is increased to the level of WT fibroblasts. These data suggest that the presence of GILT correlates with the decreased expression and activity of mitochondrial enzyme SOD2.

SOD2 protein stability is decreased in GILT-/- mouse fibroblasts. Our data indicate that lack of GILT correlates with lower activity and the amount of SOD2 in the fibroblasts. Decreased expression of SOD2 in GILT-/- fibroblasts may be due to down-regulation of Sod2 gene expression and/or due to instability of SOD2 protein in the absence of GILT. Thus, we first measured relative levels of SOD2 mRNA in GILT-/- and GILT WT mouse fibroblast cell line, by quantitative PCR. As shown in Fig. 4a mRNA SOD2 levels were only moderately decreased in GILT-/- mouse fibroblasts when compared with GILT WT cells.

However, this very moderate decrease of SOD2 at the level of gene expression does not preclude the possibility that SOD2 protein is less stable in GILT-/- fibroblasts. In order to test the stability of SOD2 protein in GILT-/- and WT mouse fibroblasts, we treated both GILT-/- and GILT WT fibroblasts with the protein synthesis inhibitor-cyclohexamide. Cell aliquots were collected at different time points up to 48h of treatment with cycloheximide. Protein concentration of each sample was determined and equal amount of protein was loaded per well. Data shown in Fig. 4b indicate that SOD2 stability is reduced in GILT-/- mouse fibroblast cell line as well as in GILT-/- primary mouse fibroblasts (Fig.4c), although the amounts of SOD2 in GILT-/- cell line and primary cells differ. The observed difference is likely to originate from different lengths that cells were kept under cell culture conditions. It is well known that cell culture and hyperoxic environment impose oxidative stress [20]. Cell line has been kept in tissue culture much longer than primary fibroblasts and therefore has been exposed to additional extracellular oxidative stress that may have destabilized SOD2 further than in primary fibroblasts. Our data suggest a possible dual regulation of GILT on SOD2 expression: at the level of gene expression and also through its effect on the stability of SOD2 protein.
GILT-deficient mouse fibroblast cell line has increased superoxide anion levels. As the stability and function of an important antioxidant SOD2 is decreased in GILT-/- mouse fibroblasts, it is possible that the levels of endogenous ROS particularly superoxide anion are increased. It has been shown that moderate increase of endogenous ROS can trigger signaling that induces cell proliferation [21], [22], [23]. Therefore, we tested levels of superoxide anion and ROS in GILT-/- and GILT WT fibroblast cell lines. We used dihydroethidium (DHE) (Fig.5) and 2',7'dichlorodihydrofluorescein diacetate (DCF-DA) (data not shown), oxidation-sensitive dyes, to detect intracellular ROS. DHE is recognized and widely used as a probe relatively specific for O$_2^\cdot$ and shows little oxidation by H$_2$O$_2$, ONOO', or HOCl, while DCF-DA is less specific and is used to assay cellular peroxides, superoxide anions. DCF-DA reacts even faster with various cellular radicals (RO$_2^\cdot$, RO, NO$_2^\cdot$, CO$_3^\cdot$, OH And ONOO') thus it is not very specific probe and easily oxidizes [24],[20]. Fibroblasts were incubated with DHE in the RPMI for 5 minutes and immediately analyzed by flow cytometry. Our data indicate that GILT-/- mouse fibroblasts have higher levels of intracellular O$_2^\cdot$ (Fig. 5a). However, transfection with either hSOD2 (Fig.5b) or mGILT (Fig.5c) decreases levels of O$_2^\cdot$ to the level seen in GILT WT fibroblasts, suggesting that this is the component of the mechanism of increased cellular proliferation.

Restoring SOD2 levels in GILT-/- fibroblasts restores proliferation. We have shown that GILT affects fibroblast proliferation and the levels of SOD2 expression. The key question is whether these two phenotypes are related. If SOD2 expression is the mechanism of GILT-regulated fibroblast proliferation, then reconstitution of SOD2 levels in GILT-/- cells (Fig.6a) should reverse proliferation to GILT WT levels. Indeed, the proliferation of GILT-/- fibroblasts, reconstituted with human SOD2 (two independently made transfectants show the same phenotype) was markedly lower than in GILT-/- and similar to GILT WT fibroblasts. In addition, our data indicate that SOD2 activity is reconstituted in transfectant cells (Fig.6b). Thus, regulation of SOD2 expression and activity appears to be the major mechanism of GILT-induced inhibition of fibroblast proliferation.

**DISCUSSION**

In this study we demonstrate that GILT-deficient mouse fibroblasts have increased levels of proliferation. Surprisingly, our data indicate that GILT deficiency affects the stability and activity of a major antioxidant mitochondrial enzyme SOD2. GILT-/- fibroblasts have 2-4 fold lower SOD2 activity due to decreased half-life of SOD2. Partial loss of SOD2 activity leads to the rise in superoxide anion as measured by DHE dye and possibly other ROS produced by mitochondrial oxidative phosphorylation. Reconstitution of GILT-/- fibroblasts with GILT reverses the SOD2 activity and the proliferation to the levels closely resembling that of GILT WT fibroblasts. These data suggest that the presence of GILT is necessary to maintain the levels of mitochondrial enzyme SOD2 and cellular proliferation. Transfection of human SOD2 into GILT-/- fibroblasts restored proliferation to WT levels suggesting that SOD2 is a key step linking GILT and its effects on cellular proliferation.

SOD2 is an antioxidant enzyme responsible for the dismutation of the superoxide radical into hydrogen peroxide [20], [25]. Although superoxide radicals have short life and also spontaneously dismutate into hydrogen peroxide, deletion of Sod2 gene in mice is perinatal lethal [26], [27], severely reduces life span in Drosophila [28], and mice genetically modified to lack SOD2 in specific tissues show various pathologies [29], [30], [31]. In addition to SOD2 located in mitochondria, there are two additional forms of SOD in cells of aerobic organisms, namely SOD1 and SOD3. SOD1 is primarily cytosolic (although a fraction is also found in the intermembrane space of mitochondria) [32], and SOD3 is extracellular enzyme. SOD1 constitutes up to 90% of total activity in most cells and tissues and is considered a principal scavenger in the cell. Inspite of that, genetic inactivation of SOD1 results in relatively mild phenotype [33]. Therefore, we tested the levels and the activity of SOD1 in our model system. The levels of SOD1 were unaffected by the absence of GILT. This finding is in agreement with previous findings that cytosolic SOD1 cannot compensate for the loss of mitochondrial SOD2 and vice versa [34], [35]. Thus, in spite of additional systems for removal of ROS (SOD1, catalase,
glutathione peroxidase) [36], SOD2 plays a distinct and crucial role in removal of ROS generated by respiratory chain in mitochondria. Therefore, SOD2 is an important regulator of generation of endogenous ROS generation [34].

Several studies indicated that the balance between ROS production and antioxidant defenses is a factor influencing cell growth and differentiation [28]. ROS were shown to be involved in processes of cell growth and proliferation as well as apoptosis in various cell types [37], [38]. Since superoxide anion is the major target for SOD2 action we used fairly specific fluorescent probe dihydroethidium (DHE) to compare the overall levels of O$_2^-$ in GILT-/-- vs. GILT WT fibroblasts. Our data indicate that O$_2^-$ levels are increased in GILT-/-- cells. However, one has to be aware of caveats of this approach. DHE is a non-fluorescent cell permeant that undergoes oxidation to a fluorescent product 2-hydroxyethidium, that intercalates into nuclear DNA, and shows strong fluorescence upon interaction with O$_2^-$ [55]. It shows little oxidation by H$_2$O$_2$, ONOO- or HOCl. DHE can spontaneously oxidize or be oxidized by singlet O$_2$ and if cytochrome c is released into cytosol by mitochondria [39], it can also oxidize DHE [24], [20]. Although we think the O$_2^-$ is most likely source of DHE fluorescence in our model system, we cannot exclude other, above-mentioned factors. More detailed study is underway to dissect further details of moderately increased oxidative stress in GILT-/-- fibroblasts. SOD2 activity is directly linked to the degree of cell differentiation and inversely related to proliferation in several different systems [40], [41], [42], [43]. Most types of tumor cell lines have reduced levels of SOD2 in comparison to their normal cell counterparts. Several studies have shown that tumorigenicity of various tumor cell lines is decreased post transfection with SOD2 cDNA [44], [45], [46]. We hypothesize that in our system, moderately increased endogenous ROS in GILT-/-- fibroblasts due to lower SOD2 activity stimulate signaling pathways involved in regulation of cellular proliferation.

The precise mechanism of GILT-SOD2 interaction is currently under study. While GILT resides in the endosomal compartment, SOD2 is a resident protein of mitochondriae. A remote possibility exists that these two proteins may interact directly either during their transport through endoplasmic reticulum and Golgi or perhaps transiently co-localize in yet another vesicular compartment. Intracellular immunofluorescence confocal microscopy (Supplementary Fig.2) showed that the majority of GILT and SOD2 reside in their respective compartments (lysosomes and mitochondria). However, the resolution of this method does not completely exclude the possibility that small fractions of these molecules co-localize.

The alternative possibility is that GILT and SOD2 interact indirectly. GILT may limit the source of cysteine necessary for glutathione synthesis and that may cause an overall redox balance alteration that may affect the activity of SOD2. Currently we do not know the exact mechanism of regulation of SOD2 expression by GILT. However, it is possible that increased amounts of O$_2^-$ act as scavengers for nitric oxide (NO) and turns it into a shorter-lived and less reactive peroxynitrite (ONOO$^-$) NO has been indicated to stimulate SOD2 expression and activity [47], [48] via Ras/Erk1/2 pathway [49]. Lower availability of NO may contribute to decreased SOD2 expression. Either alternatively or perhaps in addition, through interaction of as yet unidentified signaling pathway, or through the altered overall redox status of the cell, GILT may affect the activity of transcription factors such as nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) that are involved in expression of mitochondrial proteins. Binding of NRF-2 to DNA is inhibited by ROS due to oxidation of essential thiol groups, thus redox changes within the cell may affect the supply of SOD2 [50]. NRF transcription factors are not the sole potential regulators of SOD2 expression, increased oxidative stress has been implicated in cellular signaling and activation of NFkB, AP-1, AP-2 [51], [52], [53], [54]. All these transcription factors have been found to be redox sensitive and are involved in SOD2 transcription/expression. Therefore GILT might at least in part affect the expression of SOD2 through this mechanism because we have seen small decrease of SOD2 mRNA. In addition, GILT may affect expression of other proteins that influence the stability of SOD2 protein.

The overall picture is that at the steady state in normal fibroblasts both GILT and SOD2 are expressed. The role of SOD2 is to scavenge superoxide anions (generated in respiratory chain
reaction within mitochondriae) and dismutate them into peroxide that will eventually be turned into water through the catalase or peroxidase action. In GILT-deficient cells less SOD2 protein is expressed and its activity is decreased as well. As a result increased levels of ROS (superoxide anions and hydrogen peroxide) exist in these cells. Consequently certain signaling pathways may have activated GILT-deficient cells in the GILT WT cells.

Our data reveal an unexpected correlation between lysosomal thiol reductase and mitochondrial reactive oxygen species scavenger. Both GILT and SOD2 are involved in regulation of cellular proliferation of fibroblasts. Overall, the importance of this study lies in the fact that GILT-/- cells can be used as a model to study novel intracellular communication pathways between lysosomal and mitochondrial compartments. In addition, our results support the role of reactive oxygen species not solely as factors with negative consequence for cell survival but also as mediators of cellular signaling. In many diseases redox processes gone “awry” are cause for further damage of cellular structures and tissues. Learning how to control and modify these events may lead to prevention or invention of drugs that control negative consequences of our aerobic existence.

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**FOOTNOTES**

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Abbreviations used in this paper: GILT, Gamma IFN-inducible lysosomal thiol reductase; WT, wild type; KO, knockout; SOD2, superoxide dismutase 2; ROS, reactive oxygen species, EGF, epidermal growth factor, FBS-fetal bovine serum, MFs-mouse fibroblast cell line, PMFs- primary mouse fibroblast
FIGURE LEGENDS

FIGURE 1. GILT-/- mouse fibroblasts have increased proliferation. a. GILT WT and GILT-/- Mouse Ear Fibroblasts (MFs) were incubated at different concentrations (10^3, 10^4, 10^5), in 96-well plate in serum-free RPMI medium with 1µCi/well of [3H]dT overnight. Cells were harvested and [3H]dT incorporation was subsequently measured on a beta scintillation counter. Proliferation intensity is presented as counts per minute (CPM). *p<0.05 probability associated with a Student’s paired t test. One representative experiment of three is shown. Results are stated as a mean+/− s.e.m. b. Primary fibroblasts isolated from the spleens of GILT WT and GILT-/- mice were grown for 10 days in RPMI/10%FBS. 24h before proliferation assay they were placed in serum-free RPMI medium. Following day [3HdT] was added as in (a) GILT-/- primary fibroblasts show approximately one third higher proliferation when compared to GILT WT fibroblasts. c. GILT WT and GILT-/- MFs +/- 100ng/ml of mouse rEGF were handled the same way as in a. GILT-/- MFs retain higher proliferative ability either in presence or absence rEGF. d. GILT-/- MFs transfected with empty vector pCDNA3.1 retain higher proliferation when compared with GILT WT MFs transfected with the same vector. When GILT is reconstituted into GILT-/- MFs proliferation is decreased to the levels of GILT WT MFs. This is a representative experiment with one of three different transfectants.

FIGURE 2. SOD2, but not SOD1 protein expression and activity is decreased in GILT-/- MFs and primary fibroblasts. a. Protein concentrations of cell lysates in TS/1%Triton-x with protease inhibitor cocktail were determined by BCA protein assay and either 5 or 2.5µg/well were loaded onto 12% SDS-PAGE gels. Proteins were transferred onto nylon membrane and SOD2 was detected by anti-SOD2 antibody by chemiluminescent method. Following the detection of SOD2, the membranes were stripped and re-incubated with anti-actin antibody as a loading control. b. and c. MFs and PMFs respectively, were lysed and equal amount of total proteins were assayed for SOD2 activity as described in Experimental Procedures. d. Cells were prepared the same way as in a. except that samples were run on 15% SDS-PAGE gel and SOD1 was detected by anti-SOD1 antibody by chemiluminescent method.

FIGURE 3. SOD2 activity is reconstituted in GILT-/- MFs transfected with mouse GILT. GILT-/- MFs were transfected by Lipofectamine method with pCDNA3.1Zeo.mGILT construct. GILT expressing clones were selected in selective medium containing Zeocin. SOD2 activity was tested in untransfected GILT-/-, GILT WT and GILT-/- MFs transfected with mGILT as described in Experimental Procedures. GILT-/- MFs transfected with vector only have low SOD2 activity as GILT-/- MFs (data not shown).

FIGURE 4. SOD2 mRNA is less abundant and protein stability is decreased in GILT-/- MFs. a. SOD2 gene expression is approximately 35% decreased in GILT-/- MFs SOD2 when compared to GILT WT MFs. Real time/Quantitative PCR reaction was performed using SybrGreen PCR kit (Applied Biosystems). This is representative one of three experiments. b. MFs lysates were processed for immunoblotting with mouse anti-SOD2 antibody after 0, 1, 6, and 24h incubation of cells with 200 µg/ml of cycloheximide. Membranes were stripped and incubated with anti-actin antibody as a loading control. Film was scanned by phosphomager and bands quantified. c. PMF lysates were treated the same way as MFs in (b).

FIGURE 5. Reconstitution of mGILT and hSOD2 in GILT-deficient mouse fibroblasts decreases endogenous ROS. Cells were starved overnight in phenol red-free medium with 1%FBS. 5µg/ml of HE was added to cells and incubated for 5 min. Cells were washed again in PBS, resuspended in PBS/0.5%BSA buffer and immediately analyzed by flow cytometry. Y-axis-cell count, X-axis FL-2 channel. GILT WT MFs incubated with DHE-grey-filled dotted line, GILT-/- fibroblasts incubated with DHE-thick black line, Transfectants GILT-/-/tfmGILT (b) and GILT-/-/tfhSOD2 (c) –thin dotted line.
FIGURE 6. Proliferation is down modulated in GILT-/- MFs reconstituted with hSOD2. GILT-/- MFs were reconstituted with human SOD2 gene in pCDNA3.1 vector. GILT WT and GILT-/- and GILT-/- hSOD2 transfecant were incubated overnight with 1µCi of [3H]dT and [3H]dT incorporation was subsequently measured on a beta scintillation counter. Proliferation intensity is presented as counts per minute (CPM). b. Equal amount of total proteins of lysates from GILT-/- MFs reconstituted with hSOD2 were assayed for SOD2 activity as described in Experimental Procedures.
Figure 1.
a. WT KO WT KO WT KO WT KO

5µg 2.5µg

Loaded total protein/lane

SOD2 (23-25kD)

Actin (42kD)

b. WT SOD2 KO SOD2

*S<0.0001

MFs

PMFs

c. WT SOD2 KO SOD2

*p<0.05

MFs

PMFs

d. SOD1 (19kD)

Actin (42kD)
Figure 3.
Figure 4.
Figure 5.
Figure 6.

a.

b.
An unexpected functional link between lysosomal thiol reductase and mitochondrial manganese superoxide dismutase
Branka Bogunovic, Milica Stojakovic, Leonard Chen and Maja Maric

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