Antivirus activity, but not thioreductase activity, is conserved in interferon-gamma-inducible GILT protein in arthropod

Mai Izumida[a], Hideki Hayashi[b], Chris Smith[a,c,d], Fumito Ishibashi[e], Koushirou Suga[e,f], Yoshinao Kubo[a,g]*,

[a]Department of Clinical Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan

[b]Medical University Research Administrator, Nagasaki University School of Medicine, Nagasaki, Japan

[c]School of Tropical Medicine and Global Health, Nagasaki University, Japan

[d]Department of Clinical Research, London School of Hygiene and Tropical Medicine, United Kingdom

[e]Graduate School of Fisheries and Environmental Sciences, Nagasaki University, Nagasaki, Japan

[f]Organization for Marine Science and Technology, Nagasaki University, Nagasaki, Japan

[g]Program for Nurturing Global Leaders in Tropical Medicine and Emerging Communicable Diseases, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

*Corresponding author. Department of Clinical Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852-8523, Japan.

E-mail address: yoshinao@nagasaki-u.ac.jp (Y. Kubo).

Highlights

- Thiolreductase active motif of mammalian GILT protein is CXXC, but the GILT homolog of black tiger shrimp has CXXS instead of CXXC.
- Black tiger shrimp GILT does not have thiolreductase activity.
- Black tiger shrimp GILT significantly inhibits amphotropic murine leukemia virus infection through complex formation with the viral envelope protein.

ABSTRACT

We have previously reported that gamma-interferon inducible lysosomal thiolreductase (GILT) functions as a host defense factor against retroviruses by digesting disulfide bonds on viral envelope proteins. GILT is widely conserved even in plants and fungi as well as animals. The thiolreductase active site of mammalian GILT is composed of a CXXC amino acid motif, whereas the C-terminal cysteine residue is changed to serine in arthropods including shrimps, crabs, and flies. GILT from *Penaeus monodon* (PmGILT) also has the CXXS motif instead of the CXXC active site. We demonstrate here that a human GILT mutant (GILT C75S) with the CXXS motif and PmGILT significantly inhibits amphotropic murine leukemia virus vector infection in human cells without alterning its expression level and lysosomal localization, showing that the C-terminal cysteine residue of the active site is not required for the antiviral activity. We have reported that human GILT suppresses HIV-1 particle production by digestion of disulfide bonds on CD63. However, GILT C75S mutant and PmGILT did not digest CD63 disulfide bonds, and had no effect on HIV-1 virion production, suggesting that they do not have thiolreductase activity. Taken together, this study found that antiviral activity, but not thiolreductase activity, is conserved in arthropod GILT proteins. This finding provides a new insight that the original function of GILT might be inhibition of viral infection.

Keywords: GILT, antiviral activity, thiolreductase activity

1. Introduction

Redox regulation is involved in many biological events including virus infection. There are many lines of evidence showing that inhibitors of thioredoxin (TRX) and protein disulfide isomerase (PDI) attenuate human immunodeficiency virus type 1 (HIV-1) infection and these enzymes catalyze disulfide bond digestion of HIV-1 envelope protein (Env), showing that TRX and PDI are involved in infection by HIV-1 (Lundberg et al., 2019; Reiser et al., 2012 and 2016; Stantchev et al., 2012; Bi et al., 2011; Khan et al., 2011; Papandreou et al., 2010; Bilington et al., 2007; Ou et al., 2006; Barbouche et al., 2003 and 2005; Markovic et al., 2004; Gallina et al., 2002; Fenouillet et al., 2001; Ryser et al., 1994). It has been reported that changes in the extracellular redox environment by a membrane-impermeable sulfhydryl blocker or specific antibody inhibitors of TRX alter cellular localization of CD4 and strongly inhibit HIV-1 infection (Moolla et al., 2016; Matthias et al., 2002). TRX reduces disulfide bonds on a CD4 recombinant protein, and HIV gp120 protein cannot bind to oxidized CD4 recombinant protein (Cerutti et al., 2014). Glutaredoxin-1 also catalyzes the reduction of HIV-1 gp120 and CD4 disulfides and its inhibition by an inhibitor or antibody suppresses HIV-1 infection (Auwerx et al., 2009). A disulfide bond in retroviral envelope glycoprotein is digested after its interaction with the cell surface receptor (Smith et al., 2007). Furthermore, disulfide bonds or PDI are involved in other viral infections including hepatitis B virus (Perez-Vargas et al., 2021), SARS-CoV-2 (Mancek-Keber et al., 2021), and astrovirus (Aguilar-Hernandez et al., 2020). These results indicate that the redox condition of cellular infection receptors and viral envelope proteins are key regulators of various virus infections.

We have reported that human γ-interferon (γ-IFN)-inducible lysosomal thiolreductase (GILT) functions as a host antiviral factor (Kubo et al., 2016). GILT was initially identified as a

cellular factor that contributes to the generation of peptides presented onto major histocompatibility complexes (MHCs) by reducing disulfide bonds of antigen proteins (Ewanchuk et al., 2018; Hastings, 2013; Hastings et al., 2006; West et al., 2013). GILT reduces disulfide bonds of antigen proteins to enhance their degradation by endosome proteases including cathepsins. In GILT-deficient mice, the helper T-cell response against mouse herpes virus is not induced (Maric et al., 2001) and thus, GILT plays an important role in the initiation of adaptive immunity.

Lower animals, plants, and fungi contain GILT homologs (Fig. 1), which do not have adaptive immunity, suggesting that these GILT homologs have other unknown function(s) (Kongton et al., 2014; Kongton et al., 2011). We have found that human GILT directly restricts HIV-1, vesicular stomatitis virus, and murine leukemia virus infections as one of γ-IFN-induced innate immune factors by digesting disulfide bonds on viral envelope glycoproteins (Kubo et al., 2016). Consistently, it has been reported that human GILT restricts severe acute respiratory syndrome-coronavirus, Ebola virus, and Lassa virus infections (Chen et al., 2019). GILT silencing abrogates antivirus activity of γ-IFN in certain human cell lines, and mouse embryonic fibroblasts (MEFs) from GILT-deficient mice are more susceptible to infection by murine leukemia virus (MLV) (Kubo et al., 2016) or dengue virus (Teramoto et al., 2013) than MEFs from wild type mice. Porcine GILT inhibits replication of porcine reproduction and respiratory syndrome virus (Guo et al., 2018). It has been also reported that GILT homologs function as a host defense factor against many infectious agents in lower animals as follows: Mosquito GILT homolog reportedly inhibits *Plasmodium* sporozoite transmission (Schleicher et al., 2018). Bacterial growth is elevated in GILT homolog-deficient fruit flies versus wild type flies (Kongton et al., 2014). Shrimp GILT homolog restricts white spot syndrome virus infection (Thipwong et al., 2019). Planarian GILT homolog is

required for gram-negative bacterial clearance (Gao et al., 2021). Therefore, it is thought that lower animal GILT homologs function as host defense factors against infectious agents like mammalian GILT, and hosts subsequently evolved to utilize GILT to generate peptides presented onto MHCs.

Like other thiolreductases including TRX and PDI, mammalian GILT has a CXXC motif in the thiolreductase active site (Arunachalam et al., 2000: Phan et al., 2000). GILT mutants containing amino acid substitutions at the conserved cysteine residues do not have thiolreductase activity (Arunachalam et al., 2000: Phan et al., 2000). However, GILT proteins isolated from arthropods including flies, shrimps, and crabs have a CXXS motif instead of CXXC (Fig. 1A) (Kongton et al.,2014; Kongton et al., 2011; Huang et al., 2015). In addition, the C-terminal cysteine residue of the thiolreductase active motif is changed to other amino acids in planarian (Gao et al., 2021) and fungi (Gao et al., 2011). It has been reported that a human GILT mutant containing CXXS motif does not have thiolreductase activity (Schleicher et al., 2018; Arunachalam et al., 2000). These reports prompted us to herein address the hypothesis that arthropod GILT containing a CXXS motif has antiviral activity but not thiolreductase activity, which might suggest that GILT originally functions as a host defense factor against infectious agents without thiolreductase activity. To assess the hypothesis, we analyzed antiviral activity of the black tiger shrimp GILT and a human GILT mutant that contains an amino acid substitution of the C-terminal cysteine residue in the active site CXXC to serine.

2 Results

2.1. The thiolreductase active motif is not conserved

Examination of the amino acid sequences of GILT proteins from vertebrate species shows that CXXC motif of the thiolreductase active site is conserved, whereas the C-terminal cysteine residue of the GILT thiolreductase active motif from shrimps, crabs, insects, planarian, and fungi are changed to serine, threonine, or alanine (Fig. 1). This result suggests that the latter GILT proteins might not have thiolreductase activity.

2.2. *Penaeus monodon GILT has antiviral activity*

To assess whether the CXXS motif-containing GILT protein inhibits retrovirus infection, the GILT protein-coding sequence of black tiger shrimp (*Penaeus monodon*) (PmGILT) was artificially synthesized, because it has been reported that PmGILT has antiviral activity (Thipwong et al., 2019). The complete amino acid sequence is annotated in supplementary Fig. 1. We also constructed a human GILT mutant having an amino acid substitution of the cysteine residue at the position 75 to serine (GILT C75S) (Kubo et al., 2016). A human GILT DSC mutant containing amino acid substitutions of cysteine residues at the positions 72 and 75 was also constructed. The GILT C75S and DSC mutants do not reportedly have thiolreductase activity (Arunachalam et al., 2000; Phan et al., 2000). To detect the GILT proteins, we generated plasmids encoding C-terminally FLAG-tagged human GILT wild type (Wt), DCS, C75S, and PmGILT. When HeLa cells were transduced by an MLV vector expressing GILT Wt to generate stably expressing cells, mature GILT protein (20 kDa) was detected at a much lower level than in transiently transfected 293T cells by western blotting using the antibody recognizing the mature GILT protein (Fig. 2A).

Because high proportion of precursor GILT is cleaved, the level of precursor GILT was too low to detect in the transfected 293T cells. This result suggests that GILT Wt has cytotoxity or inhibits cell growth as reported (Barjaktarevic et al., 2006). Thus, HeLa cells were transiently transfected by pcDNA3.1, GILT Wt, DCS, C75S, or PmGILT expression plasmid, and then inoculated with an amphotropic MLV vector encoding LacZ marker gene 24 h after the transfection. Transduction titers were estimated by X-Gal staining of the inoculated cells. GILT C75S and PmGILT significantly reduced transduction titers as GILT Wt in a dose-dependent manner (Fig. 2B). Transfection of GILT DCS mutant expression plasmid at 300 ng attenuated the infection, but the inhibitory effect was much lower than those of GILT Wt, C75S, and PmGILT. When cells were transfected with the expression plasmid at 300 ng, the amounts of these GILT proteins were similar (Fig. 2C). The higher amount of expression plasmid induced the higher level of GILT protein (Fig. 2D). These results indicate that GILT C75S and PmGILT inhibit amphotropic MLV infection as efficiently as the Wt GILT, but not DCS mutant.

The molecular sizes of the precursor, signal peptide-digested, and mature human GILT proteins are predicted to be 30, 25, and 20 kDa, respectively (supplementary Fig. 2A). A 28 kDa band was detected in mock transfected-HeLa cells, showing that it is a nonspecific band. When HeLa cells were transfected with the human GILT Wt expression plasmid, intensity of the 28 kDa band was significantly elevated (Figs. 2C and D). Because the GILT protein was C-terminally tagged with FLAG and the C-terminal prodomain is cleaved during its maturation process, and because the GILT protein is glycosylated, the 28 kDa band represented signal peptide-cleaved GILT protein, and mature 20 kDa protein could not be detected by anti-FLAG antibody.

The molecular sizes of precursor, signal peptide-cleaved PmGILT, and C-terminal prodomain-cleaved mature proteins are expected to be 24, 22, and 15 kDa, respectively (supplementgary Fig. 2B). When HeLa cells were transfected with the PmGILT expression plasmid, proteins at 24 and 22 kDa were detected (Fig. 2C). If the PmGILT C-terminal domain is digested like human GILT, then mature protein cannot be detected. Since the arginine residue at the C-terminal prodomain cleavage site in human GILT is substituted by threonine in PmGILT (supplementary Fig. 1), the C-terminal domain of PmGILT protein may not be digested. To assess this hypothesis, an expression plasmid of human GILT mutant containing an amino acid substitution of the arginine residue by threonine (K232T) was constructed. HeLa cells were transfected with the GILT Wt or K232T expression plasmids, and cell lysates prepared from the transfected cells were analyzed by western blotting using anti-GILT antibody. Matute GILT protein was detected in GILT Wt- and K232T-transfected cells, suggesting that the substitution of the arginine residue by threonine does not affect the cleavage (supplementary Fig. 2C).

2.3. PmGILT and GILT C75S do not inhibit HIV-1 virion production

We have reported that GILT decreases HIV-1 Gag protein level through reduction of CD63 disulfide bonds (Kubo et al., 2016). To assess whether PmGILT and GILT C75S decrease Gag protein level, COS7 cells were transfected with VSV-G-pseudotyped HIV-1 vector construction plasmids together with pcDNA3.1, GILT Wt, DCS, C75S, or PmGILT expression plasmid, and culture supernatants from the transfected cells were inoculated into TE671 cells to estimate transduction titers. As we reported (Kubo et al., 2016), GILT Wt attenuated transduction titers, but not GILT DSC, C75S, and PmGILT (Fig. 3A). Then, cell lysates prepared from the

transfected cells were analyzed by western blotting using anti-HIV-1 p24 antibody. Amount of HIV-1 Gag protein in the GILT Wt-transfected cells was much lower than that in cells transfected with pcDNA3.1, GILT DSC, C75S, or PmGILT (Fig. 3B), showing that GILT DSC, C75S, and shrimp GILT do not decrease HIV-1 Gag level, unlike GILT Wt.

We have previously shown that GILT decreases HIV-1 Gag level through digestion of disulfide bonds within CD63 (Kubo et al., 2016), which is involved in HIV-1 virion formation (Chen et al., 2008; Fu et al., 2015; Li et al., 2014). To assess whether PmGILT digests CD63 disulfide bonds, COS7 cells that stably express C-terminally HA-tagged CD63 (CD63-HA) (Kubo et al., 2016) were transfected with pcDNA3.1, GILT Wt, DSC, C75S, or PmGILT. The transfected cells were treated with biotin-maleimide (2 mM) to biotinylated free cysteine residues of cell surface proteins, and cell lysates were prepared from the treated cells. Biotinylated proteins were precipitated using avidin-agarose, and analyzed by western blotting using HA antibody. CD63-HA protein was detected in the precipitates prepared from GILT Wt-transfected cells, but not from cells transfected with GILT DSC, C75S, and PmGILT (Fig. 3C). This result indicates that PmGILT does not digest CD63 disulfide bonds as well as the GILT DCS and C75S mutants, suggesting that PmGILT does not have thiolreductase activity, as reported (Schleicher et al., 2018; Arunachalam et al., 2000).

To examine the effect of PmGILT on HIV-1 replication, CD4-expressing HeLa cells were transfected with pcDNA3.1, GILT Wt, C75S, or PmGILT expression plasmid, and the NL4-3 strain of HIV-1 was inoculated into these transfected cells 2 days after the transfection. The amounts of p24 protein in culture supernatants of the inoculated cells were measured by ELISA. GILT C75S

and PmGILT reduced p24 levels, but less efficiently than GILT Wt (Fig. 3D). Taken together, these results show that PmGILT inhibits HIV-1 replication, but does not have thiolreductase activity.

Human GILT protein is localized to endosomes or lysosomes and can approach to CD63 that is an endosome/lysosome marker. It is possible that PmGILT is not localized to endosomes or lysosomes and the disulfide bonds on CD63 are not digested in human cells. To assess the possibility, cellular localization of PmGILT was analyzed. HeLa cells were transfected with expression plasmids of C-terminally DsRed-tagged PmGILT (PmGILT-DsRed) and GFP-tagged CD63 (Kubo et al., 2016). PmGILT-DsRed was co-localized with CD63-GFP (Fig. 4), showing that PmGILT protein is localized to endosomes or lysosomes and co-localized with CD63-GFP in human HeLa cells like hGILT (Kubo et al., 2016).

2.4. Complex formation of GILT C75S and PmGILT with substrate MLV Env protein

Despite lacking thiolreductase activity, PmGILT and GILT C75S were able to inhibit amphotropic MLV vector infection. How do they suppress viral infection? In the cascade of disulfide bond digestion, GILT Wt firstly binds to a substrate protein via disulfide bond, and then the disulfide bond between GILT and substrate proteins is digested (Maric et al., 2001). It has been reported that the GILT C75S mutant binds to a substrate protein via a disulfide bond, but cannot proceed to the next step. Thus, complex of a substrate protein with GILT C75S is formed. To examine whether GILT C75S and PmGILT bind to amphotropic MLV Env protein, HeLa cells were transfected with the pcDNA3.1, GILT Wt, C75S, or PmGILT expression plasmid, and then incubated with amphotropic MLV vector at 37 °C for 5 h. Cell lysates were prepared from the incubated cells. GILT proteins were precipitated using anti-FLAG antibody, and precipitates were

analyzed by western blotting using anti-MLV SU antibody. MLV SU protein was detected in the precipitate prepared from GILT C75S- (Fig. 5 left panel) and PmGILT- (Fig. 5 right panel) expressing cells, but not from GILT Wt-expressing cells. This result indicates that GILT C75S and PmGILT form complexes with MLV SU protein.

2.5. PmGILT inhibits baculovirus infection in arthropod cells

Because baculovirus can infect shrimp cells (Anoop et al., 2021), impact of PmGILT on baculovirus vector infection in arthropod Sf9 cells. Sf9 cells were transfected with an expression plasmid of a replication-competent baculovirus vector encoding the LacZ gene together with empty or PmGILT expression plasmid. The LacZ activities were measured in the culture supernatant of the transfected cells 3 days after the transfection. The LacZ activities in PmGILT-transfected cells were lower than those in the empty plasmid-transfected cells (Fig. 6 left panel). Protein bands with expected molecular sizes were detected in the transfected cells by western blotting using anti-FLAG antibody (Fig. 6 right panel). These results show that PmGILT inhibits baculovirus replication in Sf9 cells.

3. Discussion

This study found that PmGILT inhibits amphotropic MLV Env protein-mediated infection. PmGILT has the CXXS motif instead of the CXXC thiolreductase active site, and the protein did not digest disulfide bonds on CD63. It has been already reported that fly GILT with a CXXS motif does not have thiolreductase activity (Schleicher et al., 2018). The C-terminal cysteine residue in the active motif is required for thiolreductase activity (Arunachalam et al., 2000; Phan et

al., 2000). GILT proteins from shrimps, crabs, insects, and planarian function as host defense factor against infectious agents, but should not have thiolreductase activity. Thus, thiolreductase activity of GILT is not required for its function as a host defense factor. As GILT DCS mutant did not have antiviral activity, the N-terminal cysteine residue of the thiolreductase active site is required for the antiviral activity.

The thiolreductase activity of GILT is required for the inhibition of virion production, but not for the inhibition of Env-mediated entry. GILT C75S mutant and PmGILT attenuated amphotropic MLV vector infection like hGILT Wt, showing that thiolreductase activity is not necessary for the inhibition of viral entry into host cells. However, GILT C75S did not digest CD63 disulfide bonds and did not inhibit HIV-1 virion production. GILT C75S and PmGILT have less efficient antiviral activity against HIV-1 replication than GILT Wt. These results indicate that the thiolreductase activity of GILT is required for the inhibition of HIV-1 virion production.

PmGILT inhibits baculovirus replication in arthropod Sf9 cells. Baculovirus can infect shrimps (Anoop et al., 2021) and encodes sulfhydryl oxidase P33. The deletion of P33 impairs baculovirus replication (Wu and Passarelli, 2010). *Per os* infectivity factors (PIFs) are components of the viral envelope complex. Recently it has been reported that PIF-5 protein is oxidized by viral sulfhydryl oxidase P33 to form disulfide bonds and all of cysteine-to-serine mutants of PIF-5 lost oral infectivity without affecting viral production and morphogenesis, showing that disulfide bonds on PIF-5 is required for oral infection (Zhang et al., 2020). These results indicate that formation of disulfide bonds is required for baculovirus replication. PmGILT may inhibit baculovirus vector infection by disruption of the disulfide bond formation.

PmGILT inhibits viral infection through binding to viral Env protein. It has been reported that GILT C75S mutant binds to its substrate protein via a disulfide bond (Maric et al., 2001). Since a complex of GILT C75S or PmGILT with MLV surface (SU) proteins was detected, it is thought that GILT C75S and PmGILT inhibit MLV Env-mediated infection by complex formation with MLV Env protein.

Inconsistent with our result, Chen et al. have reported that GILT C75S mutant does not inhibit infection by SARS-CoV, Ebola virus, and influenza A virus (Chen et al., 2019). However, in their study, the mutant restricts Lassa virus infection. GILT expression significantly inhibited amphotropic MLV infection in COS7, HeLa, and TE671 cells, but moderately in 293T cells (Kubo et al., 2016). Thus, GILT antiviral activity may depend on cell lines and on virus types used.

It has been shown that mammalian GILT is involved in the initiation of adaptive immunity (Hastings et al., 2006). GILT is required for the generation of peptides presented on MHC by digestion of disulfide bonds of antigen proteins. Therefore, the thiolreductase activity of mammalian GILT is absolutely needed for this function. However, lower animals have GILT homologs but do not adaptive immunity. Their expression is elevated by lipopolysaccharide or bacterial infection in lower animals (Cui et al., 2011 and 2012; Kongton et al., 2011 and 2014; Huang et al., 2015; Ren et al., 2015; Cao et al., 2018; Fu et al., 2019; Pang et al., 2019), suggesting that GILT homologs of lower animals have another unknown function in innate immunity. Furthermore, the thiolreductase active CXXC motif of mammalian GILT is not conserved in arthropod GILT homologs. These results prompted us to speculate that thiolreductase is not required for the unknown function of GILT. Indeed, mosquite GILT with the CXXS sequence does not have thiolreductase activity (Schleicher et al., 2018). In contrast, GILT C75S and PmGILT

inhibited viral infection in human and arthropod cells, respectively. Many other arthropod GILT homologs have CXXS sequence like PmGILT. These results show that the function of arthropod GILT homologs is antiviral activity, but not thiolreductase.

As GILT with thiolreductase activity has higher antiviral activity, the CXXC motif rather than CXXS is conserved in mammals. In higher animals that have adaptive immunity, GILT is utilized to generate peptides presented on MHC, in addition to direct inhibition of viral infection. Taken together, GILT is a key factor involved in both innate and adaptive immunities and bridges between these immune activities.

4. Materials and methods

4.1. Cells

African green monkey COS7, human 293T, human HeLa, and human TE671 cells are routinely maintained in our laboratory. They were cultured in Dulbecco's modified Eagle's medium (Wako) with 8% fetal bovine serum (FBS) and 1% penicillin-streptomycin. COS7 cells stably expressing C-terminally HA-tagged CD63 were constructed in our previous study (Kubo et al., 2016). Arthropod Sf9 cells were purchased from TaKaRa Bio, and culture in BacPAK Grace's basic medium (Clontech) with 10% FBS.

4.2. Plasmids

The MLV Gag-Pol expression plasmid was purchased from TaKaRa. An expression plasmid of the MLV vector genome encoding the LacZ marker gene was kindly provided by Dr. D. Baltimore (Mann et al., 1983). The amphotropic MLV Env protein expression plasmid was

constructed in our laboratory (Kubo et al., 2007). The HIV-1 Gag-Pol-Tat-Rev expression plasmid was kindly provided by Dr. D. Trono (Naldini et al., 1996). The LacZ-encoding HIV-1 vector genome expression plasmid was obtained from Dr. L. Chang through the AIDS Research and Reference Reagent Program, NIAID, NIH, USA (Chang et al., 1999). The human GILT expression plasmid was constructed from GILT cDNA prepared from HeLa cells by RT-PCR (Kubo et al., 2016). The expression plasmids of GILT DCS and C75S mutants were constructed by PCR-mediated mutagenesis (TaKaRa). Nucleotide sequences of the mutants were confirmed using the BigDye terminator (Applied Biosystems). The PmGILT coding sequence was artificially synthesized (Eurofins Genomics), and subcloned into pTargeT mammalian expression plasmid (Promega). The HIV-1 NL4-3 infectious DNA was kindly obtained from Dr. A. Adachi (Adachi et al., 1987). Expression plasmids of C-terminally DsRed-tagged PmGILT and C-terminally GFP-tagged CD63 were constructed in our laboratory (Kubo et al., 2016). To construct the expression plasmid of C-terminally FLAG-tagged PmGILT in Sf9 cells, its coding sequence was subcloned to pIX-3 (Clontech).

4.3. GILT amino acid sequences of many species

Amino acid sequences were obtained from GenBank for GILT from human (AF097362) (Arunachalam et al., 2000), golden snub-nosed monkey (XM_010386631), cow (NM_001101251) (Zimin et al., 2009), horse (XM_023625483), sheep (HM017967) (Ai et al., 2011), pig (EF644197) (Dan et al., 2008), mouse (AF309649), rat (BC099774), bat (JX853968), chicken (JX843734) (Yang et al., 2013), Chinese soft-shelled turtle (NM_001317044) (Fu et al., 2019), African clawed frog (JN202725) (Cui et al., 2011), silver carp (KU935714) (Cao et al., 2018), golden pompano

(MF929063) (Zhu et al., 2018), largemouth bass (KR270996) (Yang et al., 2015), goldfish (HQ610621) (Li et al., 2015), Atlantic salmon (NM_001314702) (Leong et al., 2010), zebrafish (BC083267) (Cui et al., 2012), Chinese cuttlefish (MH513610) (Pang et al., 2019), Pacific white shrimp (HQ317497) (Qin et al., 2018), black tiger shrimp (EU837195) (Kongton et al., 2011), planarian (MH602290) (Gao et al., 2021), water strider (LT986713), fruit fly (NM_142030) (Sim et al., 2015), melon fly (GBXI01010654) (Sim et al., 2015), yellow sugarcane aphid (GGMS01003461), Asian longhorned beetle (GALX01001173), mud crab (KJ728650) (Huang et al., 2015), sea cucumber (KM201329) (Ren et al., 2015), thale cress (NM_117365) (Meyer et al., 1999), and three species of fungi (XM_007814565, XM_035462522, and XM_022536862) (Gao et al., 2011; Moore et al., 2016).

4.4. Amphotropic MLV vector

To construct a replication-defective amphotropic MLV vector, 293T cells were transfected with the MLV Gag-Pol (1 μg), amphotropic Env protein (1 μg), and LacZ-encoding MLV vector genome (1 μg) expression plasmids using the Fugene transfection reagent (5 μL) (Promega) in a 6-cm dish. Culture media of the transfected cells were changed to fresh media 24 h after the transfection to remove the transfected DNA and transfection reagent. Culture supernatants of the transfected cells were inoculated to transfected HeLa cells in the presence of polybrene (4 μg/mL) (Sigma-Aldrich). Target HeLa cells were transfected with indicated expression plasmid. The total amount of transfected expression plasmid was adjusted to 300 ng with pcDNA3.1. The transfected HeLa cells were inoculated with the amphotropic MLV vector 24 h after the transfection.

The inoculated cells were stained with X-Gal (Wako), and numbers of blue cells in 8 randomly selected microscopic fields were counted and the total numbers of blue cells were compared.

Target HeLa cells were transfected with pcDNA3.1, GILT Wt, C75S, DCS, or PmGILT expression plasmid using the transfection reagent (3 μL) in a 3-cm culture dish. Total amounts (300 ng) of transfected plasmids were adjusted with pcDNA3.1. The transfected cells were washed with media 3 times to remove the transfected DNA and transfection reagent 24 h after the transfection, and inoculated with culture supernatants of the amphotropic MLV vector-producing cells.

4.5. HIV-1 vector

To construct amphotropic MLV-pseudotyped HIV-1 vector, COS7 cells were transfected with HIV-1 Gag-Pol-Tat-Rev (1 μg), LacZ-encoding HIV-1 vector genome (1 μg), and amphotropic Env (1 μg) expression plasmids together with pcDNA3.1, GILT Wt, C75S, DSC, or PmGILT expression plasmid (1 μg) using the Fugene transfection reagent (5 μL) in a 6-cm dish. Culture media of the transfected cells were changed to fresh media to remove the transfected DNA and transfection reagent 24 h after the transfection, and the transfected cells were cultured for an additional 24 h. Culture supernatants of the transfected cells were inoculated to 293T or TE671 cells in the presence of polybrene (4 μg/mL). The inoculated cells were stained with X-Gal, and the numbers of blue cells in 8 randomly selected microscopic fields were counted, and the total numbers of blue cells were compared.

4.6. Western blotting

Protein samples were separated by SDS-polyacrylamide gel electrophoresis (BioRad), and then transferred onto a PVDF membrane (Millipore). The membrane was treated with 10% skimmed milk in PBS, and then with appropriate antibody, followed with HRP-conjugated secondary antibody. The antibody-bound proteins on the membrane were visualized using the ECL reagent (BioRad). Mouse anti-FLAG, mouse anti-HA (Convance), mouse anti-actin (Santa Cruz Biotechnology), goat anti-GILT (Santa Cruz Biotechnology), and goat anti-MLV SU (ViroMed Biosafety Laboratories) antibodies were used in this study as the first antibodies. HRP-conjugated anti-mouse IgG antibody and protein G (BioRad) were used as the secondary antibody. No protein bands were detected with western blotting using anti-HIV-1 p24 or anti-HA antibody (supplementary Fig. 1).

4.7. Cellular localization of PmGILT

HeLa cells were transfected with the expression plasmids of C-terminally DsRed-tagged PmGILT (PmGILT-DsRed) and C-terminally GFP-tagged CD63 (CD63-GFP). The transfected cells were permeabilized with methanol, and then were treated with DAPI. The cells were observed under a confocal fluorescence microscopy (KEYENCE).

4.8. HIV-1 replication

Human 293T cells were transfected with HIV-1 NL4-3 infectious DNA (3 μg) using the FuGene transfection reagent (5 μL) in a 6-cm culture dish. Culture media were changed with fresh media 24 h after the transfection, and the transfected cells were cultured for additional 24 h. Culture

supernatants (100 μL) of the transfected cells were inoculated to transfected HeLa cells expressing CD4. Amounts of HIV-1 p24 in the culture media were measured by ELISA (ZeptoMetrix).

4.9. Immunoprecipitation

HeLa cells were transfected with the amphotropic MLV Env protein expression plasmid (1.5 μg) together with human GILT Wt or PmGILT expression plasmid (1.5 μg) in a 6-cm dish. Culture media of the transfected cells were changed to culture supernatants of amphotropic MLV vector-producing cells in the presence of polybrene, and the inoculated cells were incubated at 37°C for 5 h. Cell lysates were prepared from the inoculated cells using the immunoprecipitation solution (Sigma-Aldrich). The cell lysates were incubated with mouse anti-FLAG antibody and anti-mouse IgG-agarose beads (Sigma-Aldrich) at 4°C for 24 h. The agarose beads were washed with the immunoprecipitation solution 5 times, and proteins bound to the beads were eluted with SDS-containing sample buffer. The elution was analyzed by western blotting.

4.10. Analysis of CD63 disulfide bonds

COS7 cells stably expressing C-terminally HA-tagged CD63 were transfected with pcDNA3.1, GILT Wt, C75S, DCS, or PmGILT expression plasmid (3 μg) using the Fugene transfection reagent (5 μL) in a 6-cm dish. The cells were treated with biotin-maleimide (2 mM) (Sigma-Aldrich) for 1 h at 37°C to biotinylate free cysteine residues of proteins, and cell lysates were prepared from the treated cells. Biotinylated proteins were precipitated using avidin-agarose (Thermo Scientific), and the precipitates were analyzed by western blotting.

4.11. Baculovirus vector

Sf9 cells were transfected with pBacPAK9 that express LacZ gene-encoding baculovirus vector (Clontech) together with empty or PmGILT expression plasmid using the Bacfectin transfection reagent (Clontech). To express PmGILT protein in Sf9 cells, pIEx-3 expression plasmid was used in this study. The FLAG-tagged PmGILT sequence was subcloned into pIEx-3 plasmid. pIEx-3 itself was used as a negative control (empty). LacZ activities of the culture media were measured 3 days after the transfection by the highly sensitive β-galactosidase assay kit (Stratagene).

4.12. Statistics

Differences between two groups of data were determined using Student's *t*-test. Statistical significance was set at $p < 0.05$ for all tests.

Declarations of interest

None

Acknowledgments

We thank Dr. D. Trono for the HIV-1 Gag-Pol-Tat-Rev expression plasmid, Dr. D. Baltimore for the LacZ-encoding MLV vector genome expression plasmid, Dr. L. Chang for the LacZ-encoding HIV-1 vector genome expression plasmid, and Dr. A. Adachi for the HIV-1 NL4-3 plasmid. We also thank Dr. K. Ariyoshi for valuable discussion and Ms. S. Koyama for assistance.

Funding

This study is supported by a grant-in-aid from the Japan Society for the Promotion of Science (18K16773 and 19K07593) and by the Asahi Kasei Medical Co., LTD.

References

Adachi, A., Koenig, S., Gendelman, H.E., Daugherty, D., Gattoni-Celli, S., Fauci, A.S., Martin, M.A., 1987. Productive, persistent infection of human colorectal cell lines with human immunodeficiency virus. J. Virol. 61, 209-213.

Aguilar-Hernandez, N., Meyer, L., Lopez, S., DuBois, R.M., Arias, C.F., 2020. Protein disulfide isomerase A4 is involved in genome uncoating during human astrovirus cell entry. Viruses 13, 53.

Ai, H.X., Zhang, Z.Z., Shen, Y.F., Zhang, J.X., Zhou, X.M., Min, C., Zhu, S.Y., Zhang, S.Q., 2011. Molecular structure, phylogenetic analysis, tissue distribution, and function characterization of interferon-gamma-inducible lysosomal thiol reductase (GILT) gene in sheep. Vet. Immunol. Immunopathol. 140, 329-334.

Anoop, B.S., Puthumana, J., Sukumaran, V., Vazhappilly, C.G., Kombiyil, S., Philip, R., Singh, I.S.B., 2021. A novel approach of transducing recombinant baculovirus into primary lymphoid cells of Penaeus Monodon for developing continuous cell line. Mar. Biotechnol. 23, 517-528.

Arunachalam, B., Phan, U.T., Geuze, H.J., Cresswell, P., 2000. Enzymatic reduction of disulfide bonds in lysosomes: characterization of a gamma-interferon-inducible lysosomal thiol reductase (GILT). Proc. Natl. Acad. Sci. USA 97, 745-750.

Auwerx, J., Isacsson, O., Soderlund, J., Balzarini, J., Johansson, M., Lundberg, M., 2009. Human glutaredoxin-1 catalyzes the reduction of HIV-1 gp120 and CD4 disulfides and its inhibition reduces HIV-1 replication. Int. J. Biochem. Cell Biol. 41, 1269-1275.

Barbouche, R., Lortat-Jacob, H., Jones, I.M., Fenouillet, E., 2005. Glycosaminoglycans and protein difulfide isomerase-mediated reduction of HIV Env. Mol. Pharmacol. 67, 1111-1118.

Barbouche, R., Miquells, R., Jones, I.M., Fenouillet, E., 2003. Protein-disulfide isomerase-mediated reduction of two disulfide bonds of HIV envelope glycoprotein 120 occurs post-CXCR4 binding and is required for fusion. J. Biol. Chem. 278, 3131-3136.

Barjaktarevic, I., Rahman, A., Radoja, S., Bogunovic, B., Vollmer, A., Vukmanovic, S., Maric, M., 2006. Inhibitory role of IFN-gamma-inducible lysosomal thiol reductase in T cell activation. J. Immunol. 177, 4369-4375.

Bi, S., Hong, P.W., Lee, B., Baum, L.G., 2011. Galectin-9 binding to cell surface protein disulfide isomerase regulates the redox environment to enhance T-cell migration and HIV entry. Proc. Natl. Acad. Sci. USA 108, 10650-10655.

Billington, J., Hicking, T.P., Munro, G.H., Halai, C., Chung, R., Dodson, G.G., Daniels, R.S., 2007. Stability of a receptor-binding active human immunodeficiency virus type 1 recombinant gp140 trimer conferred by intermonomer disulfide bonding of the V3 loop: differential effects of protein disulfide isomerase on CD4 and coreceptor binding. J. Virol. 81, 4604-4614.

Cao, F., Wu, H., Lv, T., Yang, Y., Li, Y., Liu, S., Hu, L., Xu, X., Ma, L., Zhang, X., Li, J., Bi, X., Gu, W., Zhang, S., 2018. Molecular and biological characterization of gamma-interferon-inducible lysosomal thiol reductase in silver carp (*Hypophthalmichthys molitrix*). Fish Shellfish Immunol. 79, 73-78.

Cerutti, N., Killick, M., Jugnarain, V., Papathanasopoulos, M., Capovilla, A., 2014. Disulfide reduction in CD4 domain 1 or 2 is essential for interaction with HIV glycoprotein 120

(gp120), which impairs thioredoxin-derived CD4 dimerization. J. Biol. Chem. 289, 10455-10465.

Chang, L.J., Urlacher, V., Iwakuma, T., Cui, Y., Zucali, J., 1999. Efficacy and safety analysis of a recombinant human immunodeficiency virus type 1 derived vector system. Gene Ther. 6, 715-728.

Chen, D., Hou, Z., Jiang, D., Zheng, M., Li, G., Zhang, Y., Li, R., Lin, H., Chang, J., Zeng, H., Guo, J., Zhao, X., 2019. GILT restricts the cellular entry mediated by the envelope glycoproteins of SARS-CoV, Ebola virus and Lassa fever virus. Emerg. Microbes Infect. 8, 1511-1523.

Chen, H., Dziuba, N., Friedrich, B., von Linderm, J., Murray, J.L., Rojo, D.R., Hodge, T.W., O'Brien, W.A., Ferguson, M.R., 2008. A critical role for CD63 in HIV replication and infection of macrophages and cell lines. Virology 379, 191-196.

Cui, X.W., Ji, C.B., Cao, X.G., Fu, Z.Y., Zhang, S.Q., Guo, X.R., 2012. Molecular and biological characterization of interferon-gamma-inducible-lysosomal thiol reductase gene in zebrafish (*Danio rerio*). Fish Shellfish Immunol. 33, 1133-1138.

Cui, X.W., Xiao, W., Ke, Z., Liu, X., Xu, X.Z., Zhang, S.Q., 2011. Cloning and expression analysis of interferon-gamma-inducible-lysosomal thiol reductase gene in South African clawed frog (*Xenopus laevis*). Int. Immunoparmacol. 11, 2091-2097.

Dan, W.B., Wang, S.L., Liang, J.Q., Zhang, S.Q., 2008. Molecular cloning and expression analysis of porcine gamma-interferon-inducible lysosomal thiol reductase (GILT). Vet. Immunol. Immunopathol. 126, 163-167.

Ewanchuk, B.W., Yates, R.M., 2018. The phagosome and redox control of antigen processing. Free Radic. Biol. Med. 125, 53-61.

Fenouillet, E., Barbouche, R., Courageot, J., Miguelis, R., 2001. The catalytic activity of protein disulfide isomerase is involved in human immunodeficiency virus envelope-mediated membrane fusion after CD4 cell binding. J. Infect. Dis. 183, 744-752.

Fu, E., Xie, Y., Mu, D., Liu, W., Jin, F., Bai, X., 2015. Tetraspanin CD63 is a regulator of HIV-1 replication. Int. J. Clin. Exp. Pathol. 8, 1184-1198.

Fu, J., Chen, S., Zhao, X., Luo, Z., Zou, P., Liu, Y., 2019. Identification and characterization of the interferon-gamma-inducible lysosomal thiol reductase gene in Chinese soft-shelled turtle, *Pelodiscus sinensis*. Dev. Comp. Immunol. 90, 55-59.

Gallina, A., Hanley, T.M., Mandel, R., Trahey, M., Broder, C.C., Viglianti, G.A., Ryser, H.J., 2002. Inhibitors of protein-disulfide isomerase prevent cleavage of disulfide bonds in receptor-bound glycoprotein 120 and prevent HIV-1 entry. J. Biol. Chem. 277, 505790-50588.

Gao, L., Li, A., Lv, Y., Huang, M., Liu, X., Deng, H., Liu, D., Zhao, B., Liu, B., Pang, Q., 2021. Planarian gamma-interferon-inducible lysosomal thiol reductase (GILT) is required for gram-negative bacterial clearance. Dev. Comp. Immunol. 116, 103914.

Gao, Q., Jin, K., Ying, S.H., Zhang, Y., Xiao, G., Shang, Y., Duan, Z., Hu, X., Xie, X.Q. et al., 2011. Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. PLoS Genet. 7, e1001264.

Guo, J., Zhou, M., Liu, X., Pan, Y., Yang, R., Zhao, Z., Sun, B., 2018. Porcine IFI30 inhibits PRRSV proliferation and host cell apoptosis in vitro. Gene 649, 93-98.

Hastings, K.T., 2013. GILT: Shaping the MHC class II-restricted peptidome and CD4(+) T cell-mediated immunity. Front. Immunol. 4, 429.

Hastings, K.T., Lackman, R.L., Cresswell, P., 2006. Functional requirements for the lysosomal thiol reductase GILT in MHC class II-restricted antigen processing. J. Immunol. 177, 8569-8577.

Hoskins, R.A., Carlson, J.W., Wan, K.H., Park, S., Mendez, I., Galle, S.E. et al., 2015. The release 6 reference sequence of the *Drosophila melanogaster* genome. Genome Res. 25, 445-458.

Huang, W.S., Duan, L.P., Huang, B., Zhou, L.H., Liang, Y., Tu, C.L., Zhang, F.F., Nie, P., Wang, T., 2015. Identification of three IFN-gamma inducible lysosomal thiol reductase (GILT)-like genes in mud crab *Scylla paramamosain* with distinct gene organizations and patterns of expression. Gene 570, 78-88.

Khan, M.M., Simizu, S., Lai, N.S., Kawatani, M., Shimizu, T., Osada, H., 2011. Discovery of a small molecule PDI inhibitor that inhibits reduction of HIV-1 envelope glycoprotein gp120. ACS Chem. Biol. 6, 245-251.

Kongton, K., McCall, K., Phongdara, A., 2014. Identification of gamma-interferon-inducible lysosomal thiol reductase (GILT) homologues in the fruit fly *Drosophila melanogaster*. Dev. Comp. Immunol. 44, 389-396.

Kongton, K., Phongdara, A., Tonganunt-Srithaworn, M., Wanna, W., 2011. Molecular cloning and expression analysis of the interferon-γ-inducible lysosomal thiol reductase gene from the shrimp *Penaeus monodon*. Mol. Biol. Rep. 38, 3463-3470.

Kubo, Y., Izumida, M., Yashima, Y., Yoshii-Kamiyama, H., Tanaka, Y., Yasui, K., Hayashi, H., Matsuyama, T., 2016. Gamma-interferon-inducible, lysosome/endosome-localized thiolreductase, GILT, has anti-retrovirl activity and its expression is counteracted by HIV-1. Oncotarget 7, 1255-71273.

Kubo, Y., Tominaga, C., Yoshii, H., Kamiyama, H., Mitani, C., Amanuma, H., Yamamoto, N., 2007. Characterization of R peptide of murine leukemia virus envelope glycoproteins in syncytium formation and entry. Arch. Virol. 152, 2169-2182.

Leong, J.S., Jantzen, S.G., von Schalburg, K.R., Cooper, G.A., Messmer, A.M., Liao, N.Y., Munro, S., Moore, R., Holt, R.A., Jones, S.J., Davidson, W.S., Koop, B.F., 2010. *Salmon salar* and *Esox lucius* full-length cDNA sequences reveal changes in evolutionary pressures on a post-tetraploidization genome. BMC Genomics 11, 279.

Li, F., Li, J., Wang, Z.G., Liu, H.Z., Zhao, Y.L., Zhang, J.X., Zhang, S.Q., Liu, J.P., 2015. Identification of interferon-gamma-inducible-lysosomal thiol reductase (GILT) gene in goldfish (*Carassius auratus*) and its immune response to LPS challenge. Fish Shellfish Immunol. 42, 465-472.

Li, G., Endsley, M.A., Somasunderam, A., Gbota, S.L., Mbaka, M.I., Murray, J.L., Ferguson, M.R., 2014. The dual role of tetraspanin CD63 in HIV-1 replication. Virol. J. 8, 23.

Lundberg, M., Mattsson, A., Reiser, K., Holmgren, A., Curbo, S., 2019. Inhibition of the thioredoxin system by PX-12 (1-methylpropyl 2-imidazolyl disulfide) impedes HIV-1 infection in TZM-bl cells. Sci. Rep. 9, 5656.

Mancek-Keber, M., Hafner-Bratkovic, I., Lainscek, D., Bencina, M., Govednik, T., Orehek, S., Plaper, T., Jazbec, V., Bergant, V., Grass, V., Pichlmair, A., Jerala, R., 2021. Disruption of disulfides within RBD of SARS-CoV-2 spike protein prevents fusion and represents a target for viral entry inhibition by registered drugs. FASEB J. 35, e21651.

Mann, R., Mulligan, R.C., Baltimore, D., 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell 33, 153-159.

Maric, M., Arunachalam, B., Phan, U.T., Dong, C., Garrett, W.S., Cannon, K.S., Alfonso, C., Karlsson, L., Flavell, R.A., Cresswell, P., 2001. Defective antigen processing in GILT-free mice. Science 294, 1361-1365.

Markovic, I., Stantchev, T.S., Fields, K.H., Tiffany, L.J., Tomic, M., Weiss, C.D., Broder, C.C., Strebel, K., Clouse, K.A., 2004. Thiol/disulfide exchange is a prerequisite for CXCR4-tropic HIV-1 envelope-mediated T-cell fusion during viral entry. Blood 103, 1586-1594.

Matthias, L.J., Yam, P.T., Jiang, X.M., Vandegraaff, N., Li, P., Poumbourios, P., Donoghue, N., Hogg, P.J., 2002. Disulfide exchange in domain 2 of CD4 is required for entry of HIV-1. Nature Immunol. 3, 727-732.

Mayer, K., Schuller, C., Wambutt, R., Murphy, G., Volckaert, G., Pohl, T., Dusterhoft, A. et al., 1999. Sequence and analysis of chromosome 4 of the plant *Arabidopsis thaliana*. Nature 402, 769-777.

Moolla, N., Killick, M., Papathanasopoulos, M., Capovilla, A., Thioredoxin (Trx1) regulates CD4 membrane domain localization and is required for efficient CD4-dependent HIV-1 entry. Biochim. Biophys. Acta 1860, 1854-1863.

Moore, G.G., Mack, B.M., Beltz, S.B., Gilbert, M.K., 2016. Draft genome sequence of an aflatoxigenic *Aspergillus* species, *A. bombycis*. Genome Biol. Evol. 8, 3297-3300.

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F.H., Verma, I.M., Trono, D., 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272, 263-267.

Ou, W., Silver, J., 2006. Role of protein disulfide isomerase and other thiol-reactive proteins in HIV-1 envelope protein-mediated fusion. Virology 350, 406-417.

Pang, Z., Zhang, Y., Liu, L., 2019. Identification and functional characterization of interferon-gamma-inducible lysosomal thiol reductase (GILT) gene in common Chinese cuttlefish *Sepiella japonica*. Fish Shellfish Immunol. 86, 627-634.

Papandreou, M.J., Barbouche, R., Guleu, R., Rivera, S., Fantini, J., Khrestchatisky, M., Jones, I.M., Fenouillet, E., 2010. Mapping of domains on HIV envelope protein mediating association with calnexin and protein-disulfide isomerase. J. Biol. Chem. 285, 13788-13796.

Perez-Vargas, J., Teppa, E., Amirache, F., Boson, B., Pereira de Oliveira, R., Combet, C., Bockmann, A., Fusil, F., Freitas, N., Carbone, A., Cosset, F.L., 2021. A fusion peptide in preS1 and the human protein disulfide isomerase ERp57 are involved in hepatitis B virus membrane fusion process. Elife 10, e64507.

Phan, U.T., Arunachalam, B., Cresswell, P., 2000. Gamma-interferon-inducible lysosomal thiol reductase (GILT). Maturation, activity, and mechanism of action. J. Biol. Chem. 275, 25907-25914.

Qin, Z., Babu, V.S., Wan, Q., Zhou, M., Liang, R., Muhammad, A., Zhao, L., Li, J., Lan, J., Lin, L., 2018. Transcriptome analysis of Pacific white shrimp (*Litopenaeus vannamei*) challenged by Vibrio parahaemolyticus reveals unique immune-related genes. Fish Shellfish Immunol. 77, 164-174.

Reiser, K., Francois, K.O., Schols, D., Bergman, T., Jornvall, H., Balzarini, J., Kalsson, A., Lundberg, M., 2012. Thioredoxin-1 and protein disulfide isomerase catalyze the reduction of similar disulfides in HIV gp120. Int. J. Biochem. Cell Biol. 44, 556-562.

Reiser, K., Mathys, L., Curbo, S., Pannecouque, C., Noppen, S., Liekens, S., Engman, L., Lundberg, M., Balzarini, J., Karlsson, A., 2016. The cellular thioredoxin-1/thioredoxin reductase-1

driven oxidoreduction represents a chemotherapeutic target for HIV-1 entry inhibition. PLoS One 11, e0147773.

Ren, C., Chen, T., Jiang, X., Luo, X., Wang, Y., Hu, C., 2015. The first echinoderm gamma-interferon-inducible lysosomal thiol reductase (GILT) identified from sea cucumber (*Stichopus monotuberculatus*). Fish Shellfish Immunol. 42, 41-49.

Ryser, H.J., Levy, E.M., Mandel, R., DiSciullo, G.J., 1994. Inhibition of human immunodeficiency virus infection by agents that interfere with thiol-disulfide interchange upon virus-receptor interaction. Proc. Natl. Acad. Sci. USA 91, 4559-4563.

Schleicher, T.R., Yang, J., Freudzon, M., Rembisz, A., Vraft, S., Hamilton, M., Graham, M., Mlambo, G., Tripathi, A.K., Li, Y., Cresswell, P., Sinnis, P., Dimopoulos, G., Fikrig, E., 2018. A mosquito salivary gland protein partially inhibits *Plasmodium* sporozoite traversal and transmission. Nature Comm. 9, 2908.

Sim, S.B., Calla, B., Hall, B., DeRego, T., Geib, S.M., 2015. Reconstructing a comprehensive transcriptome assembly of a white-pupal translocated strain of the pest fruit fly *Bactrocera cucurbitae*. Gigascience 4, 14.

Smith, J.G., Cunningham, J.M., 2007. Receptor-induced thiolate couples Env activation to retrovirus fusion and infection. PLoS Pathog. 3, e198.

Stantchev, T.S., Paciga, M., Lankford, C.R., Schwartzkopff, F., Broder, C.C., Clouse, K.A., 2012. Cell-type specific requirements for thiol/disulfide exchange during HIV-1 entry and infection. Retrovirology 9, 97.

Teramoto, T., Chiang, H.S., Takhampunya, R., Manzano, M., Padmanabhan, R., Maric, M., 2013. Gamma interferon-inducible lysosomal thioreductase (GILT) ablation renders mouse fibroblasts sensitive to dengue virus replication. Virology 441, 146-151.

Thipwong, J., Saelim, H., Panral, T., Phongdara, A., 2019. *Penaeus monodon* GILT enzyme restricts WSSV infectivity by reducing disulfide bonds in WSSV proteins. Dis. Aquat. Organ. 135, 59-70.

West, L.C., Grotzke, J.E., Cresswell, P., 2013. MHC class II-restricted presentation of the major house dust mite allergen Der p 1 is GILT-dependent: implications for allergic asthma. PLoS One 8, e51343.

Wu, W., Passarelli, A.L., 2010. Autographa California multiple nucleopolyhedrovirus Ac92 (ORF92, P33) is required for budded virus production and multiply enveloped occlusion-derived virus formation. J. Virol. 84, 12351-12361.

Yang, L., Cao, X., Ji, X., Liu, H., Wu, H., Gu, W., Zhang, S., 2013. Molecular structure, tissue distribution and functional characterization of interferon-gamma-inducible lysosomal thiol reductase (GILT) gene in chicken (*Gallus gallus*). Vet. Immunol. Immunopathol. 153, 140-145.

Yang, Q., Zhang, J., Hu, L., Lu, J., Sang, M., Zhang, S., 2015. Molecular structure and functional characterization of the gamma-interferon-inducible lysosomal thiol reductase (GILT) gene in largemouth bass (*Microptenus salmoides*). Fish Shellfish Immunol. 47, 689-696.

Zhang, H., Kuang, W., Chen, C., Shang, Y., Ma, X., Deng, F., Wang, H., Wang, M., Hu, Z., 2020. Per os infectivity factor 5 identified as a substrate of P33 in the baculoviral disulfide bond formation pathway. J. Virol. 94, e00615-20.

Zhu, K., Yu, W., Guo, H., Zhang, N., Guo, L., Liu, B., Jiang, S., Zhang, D., 2018. Genomic structure, expression pattern and polymorphisms of GILT in golden pompano *Trachinotus ovatus*. Gene 665, 18-25.

Zimin, A.V., Delcher, A.L., Florea, L., Kelley, D.R., Schatz, M.C., Puiu, D., Hanrahan, F., Pertea, G., Van Tassell, C.P., Sonstegard, T.S., Marcais, G., Roberts, M., Subramanian, P., Yorke, J.A., Salzberg, S.L., 2009. A whole-genome assembly of the domestic cow, *Bos taurus*. Genome Biol. 10, R42.

Figure legends

Fig. 1 Thiolreductase active motifs of GILT from select species. Blue letters indicate conserved cysteine residues. Red letters show amino acid residues different from conserved cysteine residues.

Fig. 2 Amino acid sequences of human and black tiger shrimp GILT protein. (A) Arrows indicate cleavage sites of signal peptide, N-terminal prodomain, and C-terminal prodomain. Letters shaded in grey indicate identical amino acid residues. Conserved cysteine residues are indicated by dark shading. Dots indicate deletions. Boxed amino acid motifs indicate N-glycosylation sites. (B) Precursor and processed polypeptides of human and black tiger shrimp GILT proteins are indicated.

Fig. 3 PmGILT inhibits amphotropic MLV vector infection. (A) Cell lysates were prepared from empty (control) or GILT-encoding retrovirus vector-transduced HeLa cells. Cell lysates were also prepared from 293T cells transiently transfected with pcDNA3.1 (control) or GILT Wt expression plasmid. The cell lysates were analyzed by western blotting using anti-GILT antibody. (B) Amphotropic MLV vector was inoculated to HeLa cells transiently transfected by indicated expression plasmids. Total amount of transfected DNA was adjusted with pcDNA3.1. Blue cell numbers in pcDNA3.1-transfected cells were always set to 1, and relative values to blue cell numbers in pcDNA3.1 alone-transfected cells are indicated. Error bars indicate standard deviations, and asterisks show statistically significant differences compared to pcDNA3.1-transfected cells (n=3). (C and D) HeLa cells were transfected with indicated expression plasmid, and cell lysates prepared from the transfected cells were analyzed by western blotting using anti-FLAG antibody.

Fig. 4 PmGILT is localized to endosomes or lysosomes. HeLa cells were transfected with the expression plasmids of C-terminally DsRed-tagged PmGILT (PmGILT-DsRed) and C-terminally GFP-tagged CD63 (CD63-GFP), an endosome/lysosome marker. The transfected cells with treated with DAPI, and observed under a confocal fluorescence microscope.

Fig. 5 PmGILT does not have thiolreductase activity. (A) COS7 cells were transfected with the amphotropic MLV-pseudotyped HIV-1 vector construction plasmids together with indicated expression plasmid. Culture supernatants of the transfected cells were inoculated to TE671 cells. Blue cell numbers in cells inoculated with culture supernatant from the pcDNA3.1-transfected cells were always set to 1, and relative values to the blue cell numbers are indicated. Error bars indicate standard deviations, and asterisks show statistically significant differences compared to pcDNA3.1-transfected cells (n=3). (B) Cell lysates prepared from the transfected COS7 cells were analyzed by western blotting using anti-HIV-1 p24, anti-FLAG, or anti-actin antibody. (C) COS7 cells expressing C-terminally HA-tagged CD63 were transfected with indicated expression plasmid. Biotinylated proteins (upper panel) and cell lysates (lower panel) were analyzed by western blotting using anti-HA, anti-FLAG, or anti-actin antibody. (D) CD4-expressing HeLa cells were transfection with the indicated expression plasmids, and inoculated with the replication-competent NL4-3 HIV-1. Amounts of HIV-1 p24 protein in culture supernatants were measured by ELISA. Error bars indicate standard deviations (n=3).

Fig. 6 PmGILT forms a complex with MLV Env protein. HeLa cells were transfected with the indicated expression plasmids, and incubated with amphotropic MLV vector at 37°C for 5 h.

FLAG-tagged GILT protein was precipitated by anti-FLAG antibody. The precipitates (upper panel) and cell lysates (lower panel) were analyzed by western blotting using anti-FLAG, and MLV gp70, or anti-actin antibody.

Fig. 7 Mechanism of GILT antiviral activity. Firstly, the conserved N-terminal cysteine residue of the thiolreductase active motif of mammalian and shrimp GILTs binds to a cysteine residue of a substrate protein. This complex formation is sufficient for the infection inhibition. Finally, disulfide bonds of a substrate protein are digested by mammalian GILT, but not by shrimp GILT. This disulfide bond digestion induces inhibition of virion production and generation of peptides presented onto MHC.