Subcellular localization of ERGIC-53 under endoplasmic reticulum stress condition

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Newly synthesized glycoproteins destined for secretion are transported from the endoplasmic reticulum (ER), through the Golgi and toward the cell surface. In this secretion pathway, several intracellular ER- or Golgi-resident transmembrane proteins serve as cargo receptors. ER–Golgi intermediate compartment (ERGIC)-53, VIP36 and VIPL, which have an L-type lectin domain within the luminal portion, participate in the vectorial transport of glycoproteins via sugar–protein interactions. To understand the nature of these receptors, monoclonal antibodies were generated against human ERGIC-53, VIP36 and VIPL using 293T cells expressing these receptors on cell surfaces. These cells were used to immunize rats and for screening antibody-producing clones. Flow cytometric analysis and immunoprecipitation studies showed that the obtained monoclonal antibodies bound specifically to the corresponding cargo receptors. Immunostaining of HeLa cells using the monoclonal antibodies showed that the localization of ERGIC-53 changed from relatively broad distribution in both the ER and the Golgi under normal conditions to a compact distribution in the Golgi under ER stress conditions. This redistribution was also observed by the overexpression of ERGIC-53 and abrogated by co-expression with VIPL but not VIP36. Real-time polymerase chain reaction revealed that ERGIC-53 along with several chaperone proteins was up-regulated after tunicamycin treatment; however, the expression of VIPL was unchanged. Furthermore, ERGIC-53 co-precipitated with VIPL but not VIP36, indicating that ERGIC-53 may interact with VIPL in the ER, which may regulate the localization of ERGIC-53 inside cells. Taken together, these observations provide new insights into the regulation of these cargo receptors and the quality control of glycoproteins within cells.

Keywords: cargo receptor / ERGIC-53 / ER stress / glycoprotein quality control / VIPL

Introduction

In many cases, glycosylation directly affects the properties of proteins and cells, sometimes with important biological consequences. The biological functions of glycosylation are mostly mediated outside the cell (Ashwell and Morell 1974; Crocker and Varki 2001; Ley 2003). In contrast, oligosaccharides, especially N-glycans, function as tags for the quality control of glycoproteins within cells. Recently, the biological mechanisms regulating folding, transport and endoplasmic reticulum (ER)-associated degradation of glycoproteins have become better understood (Helenius and Aebi 2004; Moremen and Molinari 2006; Ruddock and Molinari 2006; Yamamoto 2009). The extension of protein folding, or the onset of disposal, is regulated by several ER- and Golgi-resident sugar-binding proteins (called lectins), which recognize specific N-glycan structures attached to proteins. We recently determined the sugar-binding specificity of the intracellular cargo receptors, ER–Golgi intermediate compartment (ERGIC)-53, VIP36 and VIPL, using a flow cytometry-based method incorporating soluble lectin tetramers (Kawasaki et al. 2007, 2008; Yamaguchi et al. 2007; Yamamoto and Kawasaki 2010) and frontal affinity chromatography (Kamiya et al. 2005, 2008). We found that progressive trimming of terminal sugar residues is required to recruit cargo receptors. However, the intracellular localization of these receptors is necessary if we are to understand their biological roles, particularly with respect to the transport of cargo proteins. The cargo receptors, ERGIC-53, VIP36 and VIPL, are homologous at the amino acid level; however, monoclonal antibodies that distinguish between these cargo receptors have not yet been reported. VIP36 localization to either the pre-Golgi secretory pathway or the post-Golgi pathway was shown using endogenously expressed Myc-tagged or FLAG-tagged receptors and anti-tag antibodies (Fiedler et al. 1994; Fullekrug et al. 1999), and a recent report identified VIP36 mainly on the cell surface (Shirakabe et al. 2011). ERGIC-53, which was first identified as a marker of the ERGIC, is distributed between the ER and the Golgi (Vollenweider et al. 1998; Appenzeller et al. 1999), whereas VIPL localized predominantly in the ER and may be a non-cycling ER-resident protein (Nuber, Mitrovic, et al. 2003); however, there may be the possibility that exogenously expressed tagged protein does not co-localized with endogenous...
one or that overexpression causes altered intracellular localization, since the intracellular localization of proteins is regulated by cytoplasmically associated coat proteins. Furthermore, up- or down-regulation accompanied by cellular activation (or other cell responses) cannot be monitored using exogenously expressed tagged proteins, since regulated transcription depends on both cis-regulatory elements on the gene and trans-elements such as miRNAs. Therefore, to rule out these artifacts and to identify the endogenous receptors involved in the transport of glycoproteins within the cells, it is important to track endogenous ERGIC-53, VIP36 and VIPL, using specific monoclonal antibodies that recognize these proteins in their native conformation.

Here, we describe an easy and simple method of generating monoclonal antibodies against the intracellular cargo receptors, ERGIC-53, VIP36 and VIPL, using a cell surface display technique (Lemaire et al. 2011). Using these monoclonal antibodies, we elucidated the intracellular distribution of endogenous ERGIC-53 changed under conditions of ER stress induced by tunicamycin or dithiothreitol (DTT). The same distribution of ERGIC-53 to the Golgi was also caused by overexpression of ERGIC-53, which was abrogated by co-expression with VIP36, but not by co-expression with VIP36. Furthermore, ERGIC-53 co-precipitated with VIP36 but not VIP36, indicating that ERGIC-53 can interact with VIP36 in the ER, which may regulate the localization of ERGIC-53 inside cells. These observations provide new insights into the regulation of these cargo receptors during the quality control of glycoproteins within cells, particularly under conditions of ER stress.

Results
Production of monoclonal antibodies against ERGIC-53, VIP36 and VIPL
The cDNAs coding the luminal portion of human ERGIC-53, VIP36 and VIPL were cloned into pFLAG-CMV-CD8TM to express the proteins on mammalian cell surfaces as FLAG-tagged CDrp-fusion proteins. To confirm the expression of the FLAG-tagged proteins on cell surfaces, 293T cells transfected with each plasmid were stained with an anti-FLAG antibody and analyzed by flow cytometry. More than 97% of the transfected cells displayed FLAG-tagged ERGIC-53, VIP36 or VIPL on the cell surface (Figure 1). Next, we immunized a rat with a mixture of transfected cells expressing ERGIC-53, VIP36 and VIPL and used harvested spleen cells to produce monoclonal antibodies against all three proteins. Screening of the culture supernatant from each cloned hybridoma was performed by staining each of the FLAG-tagged ERGIC-53, VIP36 and VIPL-expressing cell lines with each of the supernatants and analyzing them by flow cytometry. Of the 384 cloned cells screened, several clones were identified as producing antibodies specific for FLAG-tagged ERGIC-53, VIP36 or VIPL. Three monoclonal antibodies, named ER1, ER2 and ER3, bound to FLAG-tagged ERGIC-53-expressing 293T cells, but not to cells expressing FLAG-tagged VIP36 or VIPL. The VIP1 monoclonal antibody was specific for FLAG-tagged VIP36-expressing cells, and two monoclonal antibodies, VL1 and VL2, were specific for VIPL-expressing cells (Figure 2A). To further confirm the specificity of the antibodies, ER1, VIP1 and VL1, we performed immunoprecipitation of ERGIC-53, VIP36 and VIPL from cell lysates expressing myc-tagged ERGIC-53, VIP36 or VIPL, respectively. Though VIP36 dimmer was partially detected, myc-tagged ERGIC-53, VIP36 and VIPL were precipitated with ER1, VIP1 and VL1, respectively (Figure 2B). Monoclonal antibodies, ER1, ER2, ER3, VIP1, VL1 and VL2 were purified from the hybridoma culture supernatants and all were identified as rat IgG2a with κ light chains (data not shown).

Distinct intracellular distribution of ERGIC-53 after tunicamycin or DTT treatment
Labeled ER1, VIP1 and VL1 monoclonal antibodies were used to study the intracellular localization and distribution of endogenous ERGIC-53, VIP36 and VIPL in HeLa cells by immunocytochemistry. HeLa cells were fixed with paraformaldehyde, permeabilized with TX-100 and then stained with the anti-ERGIC-53, anti-VIP36 and anti-VIPL monoclonal antibodies. The microscopic observation of endogenous VIPL showed that the protein localized in the ER and co-localized with the ER marker protein, calnexin (CNX; data not shown), which is consistent with a previous report showing that HA-tagged VIPL localized to the ER (Nufer, Mitrovic, et al. 2003). Endogenous ERGIC-53 (Figure 3, control) and VIP36 (data not shown) were distributed around the ER and the Golgi, and partially co-localized with both the ER marker protein, CNX and the Golgi marker protein, Golgi 58K. These data are also in good agreement with previous studies (Vollenweider et al. 1998; Fülek et al. 1999; Shirakabe et al. 2011). Next, we observed the distribution of ERGIC-53 in HeLa cells after the induction of ER stress. Tunicamycin causes ER stress by causing the accumulation of non-glycosylated proteins in the ER lumen (Morris et al. 1997). After treatment with 5 µg/mL of tunicamycin for 24 h, the ERGIC-53, which distributed in both the ER and the Golgi under normal conditions, became mainly localized to the Golgi apparatus with a morphological change to form a compact shape [Figure 3, tunicamycin (TM)]. The same distribution of ERGIC-53 was observed when the cell was treated with 2 mM DTT for 24 h, which also causes ER stress to the cell (Oliveira et al. 2009; Figure 3, DTT). Overexpression of ERGIC-53 also caused a similar distribution of the protein to the Golgi within the cell, whereas the localization of ERGIC-53 to the ER was enhanced by co-expression with VIPL (Figure 4A) but not by co-expression with VIP36 (Figure 4B), which was also reported by Nufer, Mitrovic, et al. (2003). These results suggest that the distribution of ERGIC-53 is strictly regulated under ER stress conditions and VIPL may be involved in regulating the localization of ERGIC-53 inside the cells.

Expression of ERGIC-53 and VIP36, but not VIPL, is up-regulated by tunicamycin
An immunofluorescence study revealed a curious distribution of ERGIC-53 induced by tunicamycin treatment. Under ER stress conditions, several chaperones and intracellular lectins, which are associated with ER-associated protein degradation
ERAD), are up-regulated via the unfolded protein response in the ER (Schroder and Kaufman 2005). To determine whether ERGIC-53, VIP36 and VIPL were similarly up-regulated in HeLa cells under the same conditions, the tunicamycin-mediated induction of the mRNAs encoding these proteins was examined by real-time polymerase chain reaction (PCR). Immunoglobulin-binding protein (a major protein of the Hsp70 family), CNX and calreticulin, which are both calcium-dependent lectins, were up-regulated more than 10-fold after 24 h of tunicamycin treatment (Figure 5), which is in agreement with previous reports (Zhang and Kaufman 2004; Hebert and Molinari 2007; Coe et al. 2008). ER degradation enhancing α-mannosidase-like protein 1 and the ubiquitin ligase subunit FBG3, which were both involved in ERAD, are also induced ~4-fold 14 h after tunicamycin treatment (Hosokawa et al. 2001; Ilyin et al. 2002). VIP36, ERGIC-53 and MCFD2 (which is associated with ERGIC-53) mRNAs were also increased 2–6-fold after 14 h and by >6-fold after 42 h (Figure 5). In contrast, VIPL mRNA levels did not change, even after 24 h and increased <2-fold after 42 h (Figure 5). The same results were obtained when HEK293 cells and human testicular carcinoma (DU145) cells

Fig. 1. Expression of intracellular cargo receptors on the cell surface of 293T cells. (A) Topology of the cargo receptor–CD8α fusion protein. The luminal part of the cargo receptor is fused with the membrane proximal, transmembrane and intracellular domains of CD8α. (B) 293T cells expressing each FLAG-tagged cargo receptor–CD8α fusion protein were stained with or without (none) an anti-FLAG monoclonal antibody (anti-FLAG Ab) or an isotype-matched control monoclonal antibody (isotype Ab), followed by PE-labeled goat anti-mouse IgG F(ab′)2.
Fig. 2. Specificity of the generated monoclonal antibodies. (A) 293T cells expressing cargo receptor-CD8α fusion proteins were stained with each monoclonal antibody (ER1, ER2, ER3, VL1, VL2 and VIP1) followed by staining with PE-labeled anti-mouse IgG. Lines indicate staining with a secondary antibody alone. Filled histograms show staining with the indicated monoclonal antibodies. (B) Cell lysates from 293T cells expressing myc-tagged cargo receptors and immunoprecipitates of the cell lysates with a VIP1, VL1 or ER1 monoclonal antibody were electrophoresed under reduced conditions, blotted onto a membrane and stained with an anti-myc antibody. The white arrow head indicates myc-tagged ERGIC-53, and the black arrowhead indicates myc-tagged VIPL and VIP36.
were treated with tunicamycin under the same conditions (data not shown). This suggests that different expression levels of ERGIC-53 and VIPL (i.e. a different ratio of ERGIC-53 to VIPL) could be induced during ER stress, which may be a reason for the redistribution of ERGIC-53 to the Golgi.

**ERGIC-53 co-precipitated with VIPL but not VIP36**

The relationship of VIPL with the distribution of ERGIC-53 made us speculate that ERGIC-53 may interact with VIPL, either directly or indirectly, in the ER, which then regulates the localization of ERGIC-53 inside the cells. Next, we performed immunoprecipitation experiments using cell lysates expressing myc-tagged ERGIC-53 and/or HA-tagged VIPL and anti-myc and anti-HA antibodies. When the lysate of cells expressing myc-ERGIC-53 plus HA-VIPL was precipitated with an anti-HA antibody, myc-ERGIC-53 was co-precipitated (Figure 6A, lane 12). However, the signal corresponding to myc-ERGIC-53 was detected neither from the cells expressing HA-VIPL only (Figure 6A, lane 10) nor from the cells expressing myc-ERGIC-53 only (Figure 6A, lane 11), though HA-VIPL was actually precipitated with an anti-HA antibody (Figure 6B, lane 10). Similarly, when lysate...
Fig. 4. Intracellular distribution of overexpressed ERGIC-53 in HeLa cells with or without co-expression with VIPL or VIP36. After HeLa cells were transfected with plasmid(s) for 24 h, cells were fixed and co-stained with an anti-CNX antibody and an anti-ERGIC-53 antibody ER1 (ERGIC-53) for the visualization of the distribution of ERGIC-53 in the ER or co-stained with an anti-Golgi 58K protein antibody (Golgi 58K) and an anti-ERGIC-53 antibody ER1 (ERGIC-53) for the visualization of the distribution of ERGIC-53 in the Golgi. The nucleus was stained with DAPI and a merged image is shown in the right panel. White scale bars: 10 µm. (A) HeLa cells were transfected with pRcCMV1-ERGIC-53 (upper two rows, ERGIC-53) or co-transfected with both pRcCMV1-ERGIC-53 and pRcCMV1-VIPL (lower two rows, ERGIC-53 and VIPL). (B) HeLa cells were transfected with pRcCMV1-ERGIC-53 (upper two rows, ERGIC-53) or co-transfected with both pRcCMV1-ERGIC-53 and pRcCMV1-VIP36 (lower two rows, ERGIC-53 and VIP36).
of cells expressing myc-ERGIC-53 plus HA-VIPL was precipitated with an anti-myc antibody, HA-VIPL was co-precipitated (Figure 6B, lane 8). However, the signal corresponding to HA-VIPL was not detected from the cells expressing myc-ERGIC-53 only (Figure 6B, lane 7) and from the cells expressing HA-VIPL only (Figure 6B, lane 6), even though myc-ERGIC-53 was equally precipitated with an anti-myc antibody (Figure 6A, lane 7). This indicates that the precipitation of myc-ERGIC-53 depends on the presence of HA-VIPL and vice versa. The same data were obtained when we precipitated endogenous VIP36 from cell lysates expressing myc-ERGIC-53 using an anti-VIPL antibody, VL1 (Figure 2B, lane 12). These data together indicated that ERGIC-53 interacts with VIPL, either directly or indirectly.

To further confirm the specificity of the interaction between ERGIC-53 and VIPL, we analyzed the interaction between ERGIC-53 and VIP36 by co-precipitation from myc-ERGIC-53 and FLAG-tagged VIP36 (FLAG-VIP36)-expressing 293T cells. We did not observe any co-precipitation with myc-ERGIC-53 and FLAG-VIP36 (Figure 6C and D). These data suggested that ERGIC-53 specifically interacted with VIPL but not VIP36.

To address the physiological relevance of ERGIC-53/VIPL association, we investigated the interaction of ERGIC-53 with VIPL or VIP36 by the surface plasmon resonance (SPR) method using their luminal portions. However, no significant binding of ERGIC-53 to either VIPL or VIP36 was observed (Figure 7). This finding may provide two possible explanations. First, the interaction between ERGIC-53 and VIPL may be mediated by the transmembrane or cytoplasmic domains of these cargo receptors. Second, ERGIC-53 may interact with VIPL indirectly via another molecule.

**Discussion**

In the present study, we successfully generated monoclonal antibodies specific for ERGIC-53, VIP36 and VIPL, which are cargo receptors distributed on the membranes of the ER and the Golgi. For this purpose, we expressed the ER- and the Golgi-resident receptors on the cell surface of 293T cells and these cells were used for both immunization and screening of monoclonal antibodies. As shown in the present study, this procedure has many advantages when preparing monoclonal antibodies without the need for the purification of the immunized protein. All of the established monoclonal antibodies (ER1, ER2, ER3, VIP1, VL1 and VL2) could be used for immunoprecipitation and immunostaining. Western blotting and immunostaining with each of the antibodies were further performed to test the reactivity of each monoclonal antibody with FLAG-tagged proteins transferred on polyvinylidene fluoride (PVDF) membrane. Although FLAG-tagged proteins were clearly stained by an anti-FLAG antibody, not all monoclonal antibodies reacted with denatured FLAG-tagged ERGIC-53, VIP36 or VIPL; even when the antibody concentration was increased to 50 µg/mL (data not shown), indicating these antibodies reacted with target proteins with native conformation. Thus, this method appears to generate monoclonal antibodies that recognize proteins in the native conformation.

In this study, we identified the intracellular localization of endogenous cargo receptors using established monoclonal antibodies. VIP36 and VIPL have the same cytoplasmic ER exit motif (KRFY) on their C termini, but VIP36 has an additional arginine at the fifth position from the C terminus, creating a diarginine ER localization motif (RKR). As a result, VIPL is a resident of the ER (Neve et al. 2003; Nufer, Mitrovic, et al. 2003), unlike ERGIC-53 and VIP36, which cycle in the secretory pathway. In the case of ERGIC-53, the overexpression of ERGIC-53 in the cell interfered with the intracellular distribution of the protein itself (Figure 4). This appeared to occur physiologically when ER stress was triggered in the cell. When cells were treated with tunicamycin or DTT, ERGIC-53, which distributed in both the ER and the
Golgi under normal conditions, became mainly localized to the Golgi apparatus (Figure 3). Further, we also demonstrated that co-expression with VIPL caused increased localization of ERGIC-53 to the ER (Figure 4A). Real-time reverse transcriptase–PCR indicated that the levels of ERGIC-53 mRNA increased greatly upon ER stress, whereas VIPL mRNA levels did not change, even after 24 h (Figure 5). These observations made us speculate that ERGIC-53 may interact with VIPL in the ER and such interaction causes distinct intracellular distribution of ERGIC-53 under the ER stress condition. Interestingly, orthologs for human ERGIC-53 and VIPL are broadly distributed in many eukaryotes from human to fission yeast, whereas VIP36 is restricted to higher organisms (Ilyin et al. 2002), which suggested that ERGIC-53 and VIPL might be functionally associated each other. Based on this hypothesis, we immunoprecipitated myc-tagged ERGIC-53 and HA-tagged VIPL from cell lysates using anti-myc and anti-HA antibodies. As shown in Figure 6A and B, myc-tagged ERGIC-53 co-precipitated with VIPL and vise versa. This supports the hypothesis that ERGIC-53 interacts with VIPL, either directly or indirectly. To further confirm the interaction between ERGIC-53 and VIPL, we also investigated the interaction of ERGIC-53 with VIPL by the SPR method using their soluble forms. However, no significant binding of ERGIC-53 to VIPL was observed (Figure 7). The result indicates that the interaction between ERGIC-53 and VIPL is not mediated via the luminal portions or that ERGIC-53 interacts with VIPL indirectly via another molecule. One of the latter possibilities is that glycoproteins containing both M8B and M9 high-mannose-type glycans mediate the interaction between ERGIC-53 and VIPL, since ERGIC-53 and VIPL bind strongly to M8B and M9 glycans, respectively (Yamaguchi et al. 2007; Kawasaki et al. 2008). However, ERGIC-53 could interact with VIPL but not VIP36, which also binds to high-mannose-type glycans, indicating that the interaction between ERGIC-53 and VIPL may be mediated

Fig. 6. ERGIC-53 co-precipitated with VIPL, but not with VIP36. Cell lysates expressing myc-tagged ERGIC-53 and/or HA-tagged VIPL were immunoprecipitated with an anti-myc antibody (lanes 5–8) or an anti-HA antibody (lanes 9–12) and the precipitates were stained with (A) an anti-myc antibody or (B) an anti-HA antibody, respectively. Expression of myc-ERGIC-53 and/or HA-VIPL in 293T cells were confirmed by western blotting of the cell lysates using anti-myc and anti-HA antibodies, respectively (lanes 1–4). The black arrow heads in (A) and (B) indicate myc-tagged ERGIC-53 and HA-tagged VIPL, respectively. Cell lysates expressing myc-tagged ERGIC-53 and/or FLAG-tagged VIP36 were immunoprecipitated with an anti-myc antibody (lanes 5–8) or an anti-FLAG antibody (lanes 9–12) and the precipitates were stained with (C) an anti-myc antibody or (D) an anti-FLAG antibody, respectively. Expression of myc-ERGIC-53 and/or FLAG-VIP36 in 293T cells were confirmed by western blotting of the cell lysates using anti-myc and anti-FLAG antibodies, respectively (lanes 1–4). The black arrow heads in (C) and (D) indicate myc-tagged ERGIC-53 and FLAG-tagged VIP36, respectively.
via transmembrane or cytoplasmic domains. Anyway, it is necessary to clarify the underlying mechanisms involved.

The main role of ERGIC-53 is to transport correctly folded glycoproteins from the ER, through the ERGIC, to the Golgi (Moussalli et al. 1999; Nufer, Kappeler, et al. 2003). This process depends on N-glycans attached on proteins, since ERGIC-53 preferentially binds to M8B high-mannose-type oligosaccharides (Kawasaki et al. 2008). However, in the ER stress condition, such as tunicamycin or DTT treatment, misfolded or unfolded proteins may be also transported to the Golgi because of a bystander effect. Thus, the Golgi-localization of ERGIC-53 under the ER stress condition might help to prevent misfolded or unfolded proteins in the ER from being transported to the Golgi. Further extensive studies are necessary to clarify the underlying mechanisms involved and to shed further light on the biological role of these cargo receptors.

Materials and methods

Animals

Lewis rats were purchased from Japan SLC, Inc. (Shizuoka, Japan) and kept in our own animal facility under specific pathogen-free conditions. Animal experiments were conducted in accordance with a comprehensive, high-quality animal care program, which approved by the Animal Experiment Committee of the Graduate School of Frontier Sciences of the University of Tokyo guided by the Bioscience Committee of the University of Tokyo.

Cells

HeLa, 293T and DU145 cells were obtained from the Cell Resource Center for Biochemical Research (Tohoku University, Miyagi, Japan) and maintained in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA), 100 µg/mL penicillin, 100 U/mL streptomycin, 2 mM glutamine, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 50 mM 2-mercaptoethanol under 5% CO2 at 37°C. PAI cells (kindly provided by Dr Junken Aoki. Tohoku University, Sendai, Japan) were maintained in the RPMI-1640 medium supplemented as Dulbecco’s modified Eagle’s medium.

Construction of plasmids encoding ERGIC-53-, VIP36- and VIPL-CD8α fusion proteins and their expression in 293T cells

A DNA fragment consisting of the membrane proximal (14 amino acids), transmembrane and intracellular regions of mouse CD8α (CD8TM) was generated by PCR amplification using the plasmid pBlueScript II SK(+) (Stratagene, La Jolla, CA) containing the coding sequence of mouse CD8α as a template and primers, 5′-TTGCGGCCGCTCAGTGAAGGGAGCCCGGAT-3′ and 5′-TGTCAGTACACAAATTCTCTGAAG-3′. The PCR products were cloned into pRcCMV1 (Invitrogen), pFLAG-CMV-3 (Sigma-Aldrich) and pMyc-CMV (Kawasaki et al. 2008) digested with NotI and XbaI. The cloned plasmid, pRc-CMV-CD8TM, allows the expression of proteins on cell surfaces as a CD8α-fusion protein. In the same manner, pFLAG-CMV-CD8TM and pMyc-CMV-CD8TM allow the expression of proteins on the cell surface as N-terminal FLAG- or Myc-tagged CD8α-fusion proteins. The cDNAs encoding the luminal parts of the type-I transmembrane proteins, ERGIC-53, VIP36 and VIPL were generated.
by PCR using plasmid pBlueScript II SK (+)-ERGIC-53, -VIP36 and -VIPL, respectively, and the primers listed below:

FLAG-ERGIC-53-forward:
5′-ACGCGGCCGCAAGCAAGCTTGGG-3′

ERGIC-53-forward:
5′-CAGCGGCCGCAAGCAAGCTTGGG-3′

ERGIC-53-reverse:
5′-GAGCGGCCGCAAGCAAGCTTGGG-3′

FLAG-VIP36-forward:
5′-TAAATGTTGTTTTATGTTGTTT-3′

VIP36-forward:
5′-GAAAGCTTATGCGGAGAAGGT-3′

VIP36-reverse:
5′-AGCGGCGCCCGCCCGCAGCGGCT-3′

FLAG-VIP36-forward:
5′-ACGCGGCCGCAAGCAAGCTTGGG-3′

VIPL-forward: 5′-TAAATGTTGTTTTATGTTGTTT-3′

VIPL-reverse: 5′-ACGCGGCCGCAAGCAAGCTTGGG-3′

Flow cytometry
Flow cytometry was performed to confirm the expression of FLAG-tagged CD8α fusion proteins on the surface of 293T cells. For staining with an anti-FLAG antibody (Sigma-Aldrich), 2 × 10^5 cells were incubated with 10 μg/mL of a primary antibody for 30 min on ice, then the cells were washed twice with Hank’s balanced salt solution (Nissui Pharmaceuticals, Tokyo, Japan) containing 0.1% BSA (Wako, Osaka, Japan) and 0.1% sodium azide (Wako) [fluorescence-activated cell sorting (FACS) buffer]. Washed cells were stained with 10 μg/mL of fluorescein isothiocyanate-labeled goat anti-mouse IgG F(ab′)2 (Beckman Coulter, Fullerton, CA) or R-phycocerythrin (PE)-labeled goat anti-mouse IgG F(ab′)2 (Beckman Coulter), for 30 min on ice. The cells were washed twice with FACS buffer and suspended in FACS buffer containing 1 μg/mL of propidium iodide. The fluorescence intensity of stained cells was measured using a FACS Calibur flow cytometer and the data were analyzed using CellQuest software (BD Biosystems, San Jose, CA).

Generation of hybridomas and screening
Before immunization, 293T cells (5 × 10^7) expressing FLAG-tagged ERGIC-53-, VIP36- or VIPL-CD8α on the cell surface were emulsified with complete Freund’s adjuvant (Difco Laboratories, Detroit, MI; 38:62, v/v; total volume = 1.3 mL). Two female Lewis rats were immunized in the footpad with the emulsified immunogen mixture (100 μL/footpad). For the booster immunizations, the same amount of immunogen mixture emulsified with Freund’s incomplete adjuvant (Difco Laboratories) was injected three times at 10-day intervals. Animals were sacrificed 3 days after the last injection and common iliac lymph node cells were collected and fused at a 2:1 ratio with the non-Ig producing mouse myeloma cell line PAI using polyethylene glycol 1500 (50% w/v in 75 mM HEPES, pH 7.2; Roche, Mannheim, Germany). After fusion, hybridomas were selected in the hypoxanthine–aminopterin–thymidine medium containing 50–100 U/mL mouse IL-6 (culture supernatant from mouse IL-6-transfected X63 cells, a generous gift from Dr H. Karasuyama, Tokyo Medical and Dental University, Tokyo, Japan). For the first screening, hybridoma culture supernatants were assayed for binding to 293T cells co-transfected with pFLAG-CMV-CD8TM-ERGIC-53, -VIP36 and -VIPL by flow cytometry. For the second screening, culture supernatants were assayed for binding to 293T cells expressing N-terminal FLAG-tagged ERGIC-53-, VIP36- or VIPL-CD8α. For the final screening, 293T cells expressing ERGIC-53-, VIP36- or VIPL-CD8α were used.

Purification of monoclonal antibodies
The isotype of the antibodies was determined using a rat MonoAb ID/SP kit (Zymed Laboratories, San Francisco, CA). All antibodies generated in this study were rat IgG2a. The culture supernatants from hybridoma producing monoclonal antibodies, ER1, ER2, ER3, VIP1, VL1 and VL2, were concentrated with ammonium sulfate, dialyzed against 10 mM sodium phosphate, pH 7.4, containing 137 mM NaCl and 2.68 mM KCl (PBS(−)) and purified by affinity chromatography using Hi-Trap Protein G HP (GE Healthcare, Buckinghamshire, UK). Approximately 20–30 mg of a purified antibody was purified from 600 mL of the hybridoma culture supernatant.

Immunoprecipitation
Twenty-four hours after transfection of pMyc-CMV-ERGIC-53, -VIP36 or -VIPL with or without pH-A-CMV-VIP36 or pFLAG-CMV-VIP36, 293T cells (2.0 × 10^6 cells) were washed with PBS(−) and suspended in lysis buffer [50 mM Tris–HCl, pH 8.0, containing 150 mM NaCl, 0.5% Triton X-100, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mg/mL leupeptin] at 2.0 × 10^6 cells/mL. After 1 h incubation on ice, the lysate was cleared by centrifugation at 12,000 × g for 20 min. The supernatant was then mixed with protein G-Sepharose beads (GE Healthcare) to which antibodies had been bound and incubated with rotation at 4°C for 18 h. The beads were washed three times with wash buffer (50 mM Tris–HCl, pH 7.5, containing 150 mM NaCl, 0.1% Triton X-100, 1 mM PMSF and 5 mM iodoacetamide). In the case of co-immunoprecipitation experiment, anti-HA antibody HA-7 (Sigma-Aldrich), anti-FLAG antibody (Sigma-Aldrich) and anti-Myc antibody which was purified from the culture supernatant of hybridoma clone 9E10 purchased from the American type culture collection (Manassas, VA) were used.

Polyacrylamide gel electrophoresis and western blotting
Precipitated proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis according to the method of Laemmli (1970) and transferred to PVDF
membrane (Millipore, Bedford, MA) at 15 V for 45 min using semi-dry blotting system (Bio-Rad Laboratories, Hercules, CA). The membrane was incubated with 20 mM Tris–HCl, pH 7.5, containing 150 mM NaCl, 0.005% Tween-20 [tris-buffered saline (TBS)-T] and 3% BSA for 1 h and then incubated with biotinylated anti-Myc, anti-FLAG or an anti-HA antibody for 1 h at 25°C followed by washing three times for each 15 min with TBS-T. Next, the membrane was incubated with horseradish peroxidase-conjugated streptavidin (SA) for 1 h at 25°C, followed by washing three times for each 15 min with TBS-T. Finally, the membrane was processed using enhanced chemiluminescence (ECL; GE Healthcare) and exposed to ECL films (GE Healthcare).

**Immunocytochemistry**

HeLa cells were cultured in 8-well culture slides (BD Biosciences, San Jose, CA) coated with 20 µg/mL of fibronectin and then treated with 5 µg/mL of tunicamycin in 0.05% dimethyl sulfoxide (DMSO) for 24 h. As a control, DMSO was added at a final concentration of 0.05% to the cells instead of tunicamycin for the indicated times. The cells were also treated with 2 mM DTT for 24 h. After treatment, the cells were fixed with 4% paraformaldehyde in PBS for 15 min, washed three times with PBS and permeabilized with 0.1% Triton X-100/PBS for 60 min. After blocking with 5% goat serum (Sigma-Aldrich) for 60 min, the cells were incubated with 5 µg/mL of an anti-CNX antibody (Abcam, Cambridge, UK) for the staining of the ER and 5 µg/mL of an anti-Golgi 58K protein antibody (Sigma-Aldrich) for the staining of the Golgi apparatus, respectively, and followed by incubation with 5 µg/mL of an Alexa Fluor 488-labeled antimouse antibody. After that, cells were stained with 8 µg/mL of a biotin-labeled anti-ERGIC-53 antibody (ER1) followed by incubation with 5 µg/mL of Alexa Fluor 568-labeled SA. Finally, the nucleus were stained with 4’,6-diamidino-2-phenylindole (DAPI). The coverslips were mounted in Immersion oil (Leica Microsystems, Wetzlar, Germany) and the cells were observed under a confocal laser scanning microscope LSM510 (Carl Zeiss, Gottingen, Germany) with LSM image browser software (Carl Zeiss).

**Quantitative real-time PCR**

HeLa cells were treated with 5 µg/mL of tunicamycin for 14, 24 and 48 h and then harvested with PBS/EDTA and washed twice with PBS. Poly-A⁺ RNA was then extracted using a µMACS mRNA isolation kit (Miltenyi Biotec, Germany). Each cDNA was generated using a SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer’s instructions using 0.2 mg of poly-A⁺ RNA. Quantitative real-time PCR was performed in a Smart cycler real-time PCR system (Takara Bio, Kyoto, Japan) using SYBR premix Ex Taq (Takara Bio). The standard curve for each gene was constructed using serial dilutions of control cDNA. The amplification conditions were: 35 cycles of denaturation at 95°C for 15 s followed by annealing at 63°C for 15 s and extension at 72°C for 30 s. The relative amounts of RNA for the target gene transcripts were normalized against an endogenous gene, β-actin. Primer details are listed in Table I. The P-values were calculated using a paired Student’s t-test and statistical significance was determined when a P-value was <0.05.

**SPR analysis of the interaction of ERGIC-53 with VIPL or VIP36**

SPR experiments were performed with the Biacore 3000 system (Biacore AB, Uppsala, Sweden). Biotinylated recombinant soluble VIPL (Yamaguchi et al. 2007) or VIP36 (Kawasaki et al. 2007) were immobilized to SA-coupled sensor chip SA (Biacore) until the resonance units reached approximately saturation. The binding of soluble ERGIC-53 (Kawasaki et al. 2008) to VIPL or VIP36 was measured in 10 mM 2-(morpholino)ethanesulfonic acid (MES)–NaOH, pH 7.0, containing 150 mM NaCl, 0.005% Tween-20 and 1.0 mM CaCl₂ at 25°C with a flow rate of 20 µL/min. ERGIC-53 at 0.1, 0.25 and 0.5 mg/mL was applied to the sensor chip. The regeneration of the chip surface was carried out with 10 mM MES–NaOH, pH 7.0, containing 150 mM NaCl, 0.005% Tween-20 and 10 mM EDTA.

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**Table I. Primers used for quantitative reverse transcriptase–PCR**

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<th>Gene</th>
<th>Forward primer</th>
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Conflict of interest

None declared.

Abbreviations

CNX, calnexin; DAPI, 4’,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERGIC, ER and Golgi intermediate compartment; FACS, fluorescence-activated cell sorting; GAPDH, glyceraldehyde phosphate dehydrogenase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(morpholino)ethanesulfonic acid; PCR, polymerase chain reaction; PE, P-phycoerythrin; PMSF, phenylmethylsulfonyl fluoride; PVDP, polyvinylidene fluoride; SA, streptavidin; SPR, surface plasmon resonance; TBS, tris-buffered saline; TM, tunicamycin.

References


