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Selenoprotein S-dependent Selenoprotein K Binding to p97(VCP) Protein Is Essential for Endoplasmic Reticulum-associated Degradation*

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Cytosolic valosin-containing protein (p97(VCP)) is translocated to the ER membrane by binding to selenoprotein S (SelS), which is an ER membrane protein, during endoplasmic reticulum-associated degradation (ERAD). Selenoprotein K (SelK) is another known p97(VCP)-binding selenoprotein, and the expression of both SelS and SelK is increased under ER stress. To understand the regulatory mechanisms of SelS, SelK, and p97(VCP) during ERAD, the interaction of the selenoproteins with p97(VCP) was investigated using N2a cells and HEK293 cells. Both SelS and SelK co-precipitated with p97(VCP). However, the association between SelS and SelK did not occur in the absence of p97(VCP). SelS had the ability to recruit p97(VCP) to the ER membrane but SelK did not. The interaction between SelK and p97(VCP) did not occur in SelS knockdown cells, whereas SelS interacted with p97(VCP) in the presence or absence of SelK. These results suggest that p97(VCP) is first translocated to the ER membrane via its interaction with SelS, and then SelK associates with the complex on the ER membrane. Therefore, the interaction between SelK and p97(VCP) is SelS-dependent, and the resulting ERAD complex (SelS-p97(VCP)-SelK) plays an important role in ERAD and ER stress.

The endoplasmic reticulum (ER)[3] is a cellular organelle whose functions include the synthesis and quality control of proteins. Newly synthesized proteins undergo folding and post-translational modifications in the ER (1). Chaperones and protein modification enzymes are involved in proper protein folding before the proteins are delivered to their functional destinations. However, some proteins may not reach their native folded state. The removal of these proteins from the ER, a process known as ER-associated degradation (ERAD), is essential for maintaining the homeostasis of ER function and protein quality control (2, 3). The ERAD system, as a quality control mechanism for proteins, eliminates misfolded proteins and prevents their accumulation in the ER. The ERAD process involves transporting misfolded proteins from the ER membrane to the cytosol either by retrotranslocation or dislocation (2, 4). This transport is followed by the polyubiquitination of these proteins. The polyubiquitin chain forms a binding site for the p97(VCP) complex so that the protein can be removed from the membrane. The ubiquitinated proteins are then degraded by proteasomes (3). Therefore, ERAD can directly regulate physiological processes that occur in the ER, and, furthermore, ERAD is essential for relieving ER stress by degrading misfolded proteins. Abnormal ERAD is involved in the pathogenesis of diseases that are related to ER stress-mediated apoptosis, such as neurodegenerative diseases, cardiac hypertrophy, and diabetes (5–7).

p97(VCP) is a highly abundant protein with many roles in diverse biological processes that include ERAD, mitochondrial-associated degradation, and DNA repair (8–10). p97(VCP) has two ATP-binding domains, flexible N-terminal domains that are responsible for co-factor and substrate binding, and a short C-terminal domain. p97(VCP) has a homohexameric double ring structure (11). The interaction of p97(VCP) with its binding partners and co-factors plays a key role in its activity and subcellular location (10). When p97(VCP) is relocalized to the ER membrane via its interaction with its binding partners, such as SelS, gp78, and Hrd1 (7, 12–14), it has an essential function in the maintenance of ER homeostasis and the regulation of ER stress through ERAD (15).

It has recently been demonstrated that, during ERAD, p97(VCP) forms a complex with derlins, SelS, and E3 liagases that plays an important role in the extraction of misfolded proteins from the ER to the cytosol (12, 16, 17). The association of p97(VCP) with derlins depends on the SelS. However, the association of Hrd1 with derlins occurs independently of SelS (16, 17). gp78 and SelS recruit p97(VCP) to the ER membrane, which mediates the ERAD machinery. Both gp78 and SelS have the VCP-interacting motif (VIM) (12, 13). gp78 needs the VIM

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[3] The abbreviations used are: ER, endoplasmic reticulum; SelS, selenoprotein S; SelK, selenoprotein K; p97(VCP), valosin-containing protein; ERAD, endoplasmic reticulum-associated degradation; Sec, selenocysteine; Tm, tunicamycin; CHX, cycloheximide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

for interaction with p97(VCP) (13), whereas SelS does not require the VIM to recruit p97(VCP) to the ER membrane (12). During ERAD, recruitment of p97(VCP) to the ER membrane is important for ERAD substrate degradation. This mechanism determines the fate of misfolded proteins such as CD3δ, whose misfolded form is ubiquitinated and degraded by proteasomes (18, 19). CD3δ has been known as a substrate for Hrd1- and gp78-mediated ERAD (14, 20). We also found that CD3δ was the substrate for SelS-mediated ERAD (12).

SelS, which is also known as VCP-interacting membrane protein, is a selenoprotein that contains a single selenocysteine (Sec, U) at position 188, which is the penultimate C-terminal residue (21–23). SelS is an important component of the ERAD complex and is localized to the ER membrane together with SelK (24). Similarly to SelS, SelK is a selenoprotein that contains a single Sec at position 92 as the penultimate C-terminal residue, but it has no VIM or coiled-coil domain (Fig. 3, *A* and *B*) (25). Both SelS and SelK are involved in the ERAD complex and associate with p97(VCP) and derlins (12, 24, 25). It is well known that SelS functions in cell survival by regulating ER stress (17, 26). Recent studies have suggested that SelK is also an ER stress-regulated protein and plays an important role in resisting ER stress-mediated apoptosis (27).

SelS, SelK, and p97(VCP) are important components of the ERAD complex. Both the 178th and 183rd proline residues of SelS and SelK proline residues at the 83rd, 85th, 86th, and 87th positions are essential for the interaction of these proteins with p97(VCP) (12). However, the way in which these proteins interact during ERAD remains unclear. In this study, the interrelationship of SelS, SelK, and p97(VCP) in ERAD was examined. The results show that both SelS and SelK are required for ERAD when present in a complex with p97(VCP) and that the binding of SelK to p97(VCP) is SelS-dependent.

Experimental Procedures

Cell Culture and ER Stress Induction—Mouse neuroblastoma (N2a) cells and HEK293 cells were cultured in DMEM (Invitrogen) with 10% fetal bovine serum (Invitrogen) at 37 °C in 5% CO_2. To induce ER stress, N2a cells were seeded at a density of 1×10^6 cells, and HEK293 cells were seeded at a density of 3×10^5 cells in 60-mm dishes. 24 h after seeding, the cells were treated with 1 μg/ml tunicamycin (Tm) and 100 nM thapsigargin or dimethyl sulfoxide (Sigma-Aldrich) (27, 28) for 24 h.

RNA Interference—The siSelS used in this study was designed by Invitrogen. The sequence for mouse was as follows: siSelS, 5′-GGA AGA UCU AAA UGC CCA AGU UGA A-3′. sip97(VCP) was designed by Shanghai GenePharma Co., Ltd. The sequence for mouse was as follows: sip97(VCP), 5′-GAG CUG AUU UGA CAG AAA UTT AUU UCU GUC AAA UCA GCU CTT-3′ (12). The siSelKs were designed by Shanghai GenePharma Co., Ltd. The sequences for human and mouse were as follows: siSelK (human), 5′-GCA GAA GGA AAC CAU CAA G-3′ (3′ UTR); siSelK (mouse), 5′-GAA GAG GCT ACG GGA GCT CC-3′. A stealth negative control siRNA was also obtained from Invitrogen.

Construction of SelS and SelK Mutants—The human SelS plasmids His-SelS (Sec^{188} changed to Cys) and His-mtSelS (Pro^{178} and Pro^{183} changed to Ala) were constructed as described previously (Fig. 3*B*) (12). The human SelK (HA-TEV-SelK) construct was a gift from Dr. Vadim N. Gladyshev (Harvard Medical School) (24). This plasmid was named HA-SelK WT. Point mutations in HA-SelK WT at Pro^{83}, Pro^{85}, Pro^{86}, and Pro^{87} to Ala (HA-mtSelK) were generated by site-directed mutagenesis reactions using QuikChange site-directed mutagenesis according to the instructions of the manufacturer (Fig. 3*A*) (Stratagene). The pEGFP-C2 vector was used as a template to change HA-TEV-tagged SelK to GFP-tagged SelK. The primers for GFP-SelK were as follows: GFP-SelK forward, 5′-GA GAA TTC ATG GTG AGC AAG GGC GAG GAG C-3′; GFP-SelK reverse, 5′-CAT TCT AGA CTT GTA CAG CTC GTC CAT GCC-3′. The PCR products were cloned into the EcoRI and XbaI sites of the HA-SelK and HA-mtSelK constructs. These plasmids were designated GFP-SelK WT and GFP-mtSelK (Fig. 7*A*). All SelS and SelK mutants were confirmed by sequencing (Macrogen, Seoul, Korea).

CD3δ Expression Plasmid—The pYR-CD3δ-FLAG construct was a gift from Dr. J. B. Yoon (Yonsei University, Seoul, Korea). The pYR-CD3δ-FLAG construct contains a tetracycline-regulated promoter (18, 29). The pYR-CD3δ-FLAG and pTet-off (Clontech) plasmids, which encodes a tetracycline-controlled transactivator, were co-transfected into N2a cells to express CD3δ-FLAG. Doxycycline, a tetracycline analog that inhibits the transactivator, and MG132 were purchased from Sigma.

Cycloheximide Chase Assay—To determine the degree of CD3δ-FLAG degradation, cycloheximide (CHX) chase analysis was performed according to the method described by Ballar *et al.* (13), with a slight modification. CHX was purchased from Sigma.

Transfections—For transfections, 1×10^6 N2a cells or 3×10^5 HEK293 cells were seeded in 60-mm dishes. 12 h after seeding, these cells were transfected with siRNAs or plasmids using Lipofectamine 2000 transfection reagent (Invitrogen) according to the instructions of the manufacturer. Stealth negative control siRNAs (Invitrogen) were used as controls (30–32).

Subcellular Fractionation—The cells were lysed using a ProteoJET membrane protein extraction kit (33). The membrane fractionation was performed as described previously (12, 33), and the intensity was determined densitometrically using ImageJ software.

Construction and Purification of the GST Fusion p97(VCP) Protein—Full-length human p97(VCP) was cloned into pET-28a, which was a gift from Dr. E. E. Kim (Korea Institute of Science and Technology) (34). This plasmid was used as a template for a GST fusion p97(VCP). The primers that were designed for GST-p97 were as follows: GST-p97 forward, 5′-GC GGA TCC ATG GCT TCT GGA GCC GAT TC-3′; GST-p97 reverse, 5′-CG GTC GAC TTA GCC ATA CAG GTC ATC ATC-3′. The PCR product was cloned into the BamHI and SalI sites of a pGEX-4T-3 vector. This plasmid was designated GST-p97 (Fig. 4*A*). GST-p97 was confirmed by sequencing (Macrogen, Seoul, Korea). The GST and GST-p97 proteins were expressed in *Escherichia coli* with 1 mM isopropyl 1-thio-β-D-galactopyranoside induction for 6 h at 18 °C. The protein was lysed by sonication. The lysis buffer contained 50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.5% Nonidet P-40, 4 μg/ml aprotinin, 4 μg/ml leupeptin, and 1 mM PMSF. The prepared cell

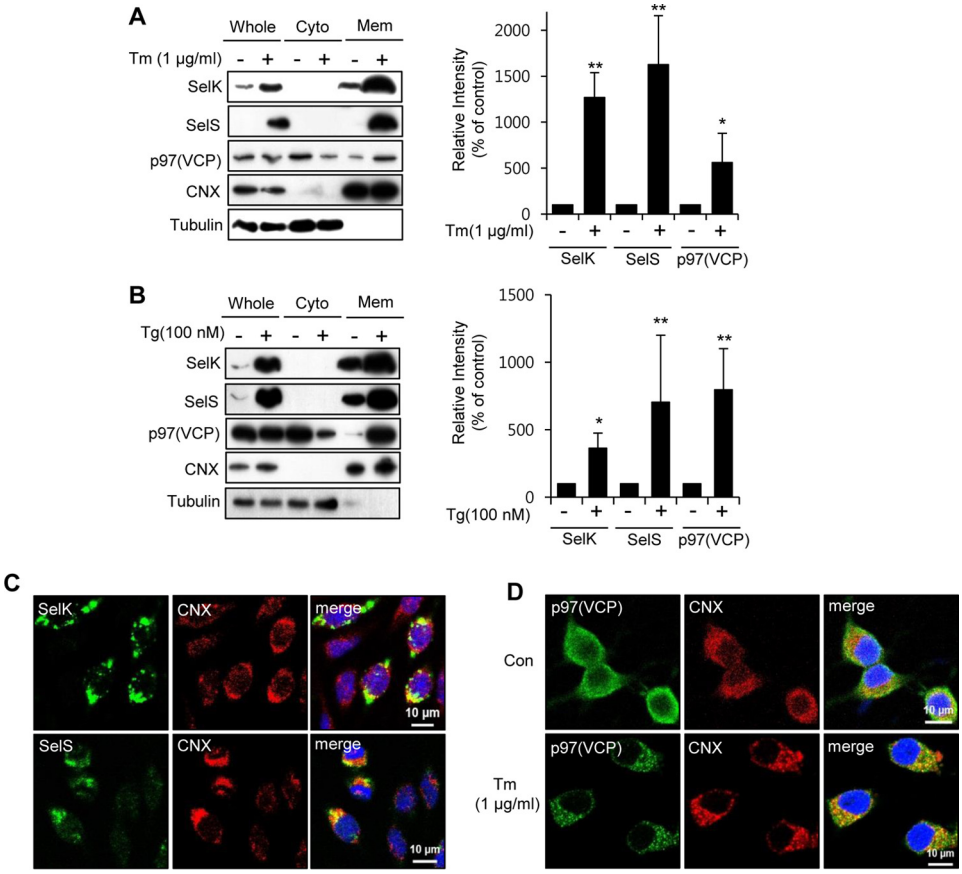

FIGURE 1. **Localization of SelS and SelK and the translocation of p97(VCP).** *A* and *B*, N2a cells were treated with 1 μg/ml Tm (*A*) or 100 nM thapsigargin (*Tg*, *B*) for 24 h and subjected to total cell extracts (*whole*)/cytosol (*Cyto*)/membrane (*Mem*) fraction analysis. These fractions were assessed by Western blotting for SelK, SelS, p97(VCP), and Calnexin (*CNX*), which is an endoplasmic reticulum marker (*left panel*). The expression of SelK, SelS, and p97(VCP) is represented as a percentage of the control for each membrane fraction (*right panel*). The *graphs* indicate results from four independent experiments (**, $p < 0.005$; *, $p < 0.05$). *Error bars* represent mean ± S.D., and the *p* values represent comparisons with the control. *C*, confocal images from N2a cells that were immunostained for SelS or SelK (*green*) and Calnexin (*red*). These cells were treated with 1 μg/ml Tm for 24 h. *D*, confocal images from N2a cells that were treated with 1 μg/ml Tm or dimethyl sulfoxide (*Con*) and immunostained for p97(VCP) (*green*) and Calnexin (*red*).

lysates were incubated with glutathione beads (Invitrogen) for 2 h at 4 °C. The GST beads were washed with wash buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.05% SDS, and 0.5% Nonidet P-40 and then eluted with elution buffer containing 50 mM Tris-HCl (pH 8.0), 20 mM KCl, 1 mM DTT, and 20 mM glutathione for 10 min at 37 °C.

GST Pulldown Assay—N2a cells were transfected with His-SelSs or HA-SelKs in 60-mm dishes. The cells were lysed with lysis buffer (150 mM EDTA, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 0.3% Nonidet P-40 with Tris-HCl (pH 7.4) and 1 mM DDT). After the purification of GST and GST-p97 proteins as described above, the purified GST proteins were preincubated with N2a cell lysates and rotated for 16 h at 4 °C. Glutathione beads were added to the mixtures and rotated for an additional 30 min at room temperature. The beads were washed and then eluted. The eluted products were visualized using Coomassie Blue staining or Western blotting.

Antibodies and Immunoblot Analysis—The cells were then lysed as described in Ref. 35. The immunoblot analysis was performed as described previously (12). Antibodies were obtained from the following sources. The anti-His and anti-HA antibodies were obtained from ABM. The anti-FLAG and anti-SelK antibodies were obtained from Sigma. The anti-caspase3 antibody was obtained from Cell Signaling Technology (Danvers, MA). The anti-caspase12 antibody was obtained from Abnova. The anti-GRP78, anti-Ub, anti-α-tubulin, anti-GFP, anti-GST, and anti-C/EBP-homologous protein (CHOP) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-calnexin antibody was obtained from BD Biosciences. The anti-SelS antibody was used as described previously (12).

Immunoprecipitation—For immunoprecipitation, lysates were mixed with antibodies for 16 h at 4 °C. Immune complexes were incubated with protein G-agarose for 2 h at 4 °C, washed with lysis buffer three times, and boiled with SDS sample buffer for 5 min (12, 31). The samples were loaded onto SDS-PAGE gels, transferred to a PVDF membrane, and incubated with primary antibody for 16 h at 4 °C. After further incubation with an HRP-conjugated secondary antibody for 1 h at room temperature, immunoreactive bands were visualized using a West Pico enhanced ECL detection kit (Pierce).

MTT Assay—For the MTT assay, N2a cells were seeded at 3×10^5 cells/well in 12-well plates. Separate plates of cells were transfected with siRNAs or plasmids. The cells were then treated with 1 μg/ml Tm for 6 h after transfection. The medium was replaced with medium containing 5 mg/ml of MTT at the indicated time points, and the cells were incubated further for 2 h at 37 °C. After the incubation, dimethyl sulfoxide was added

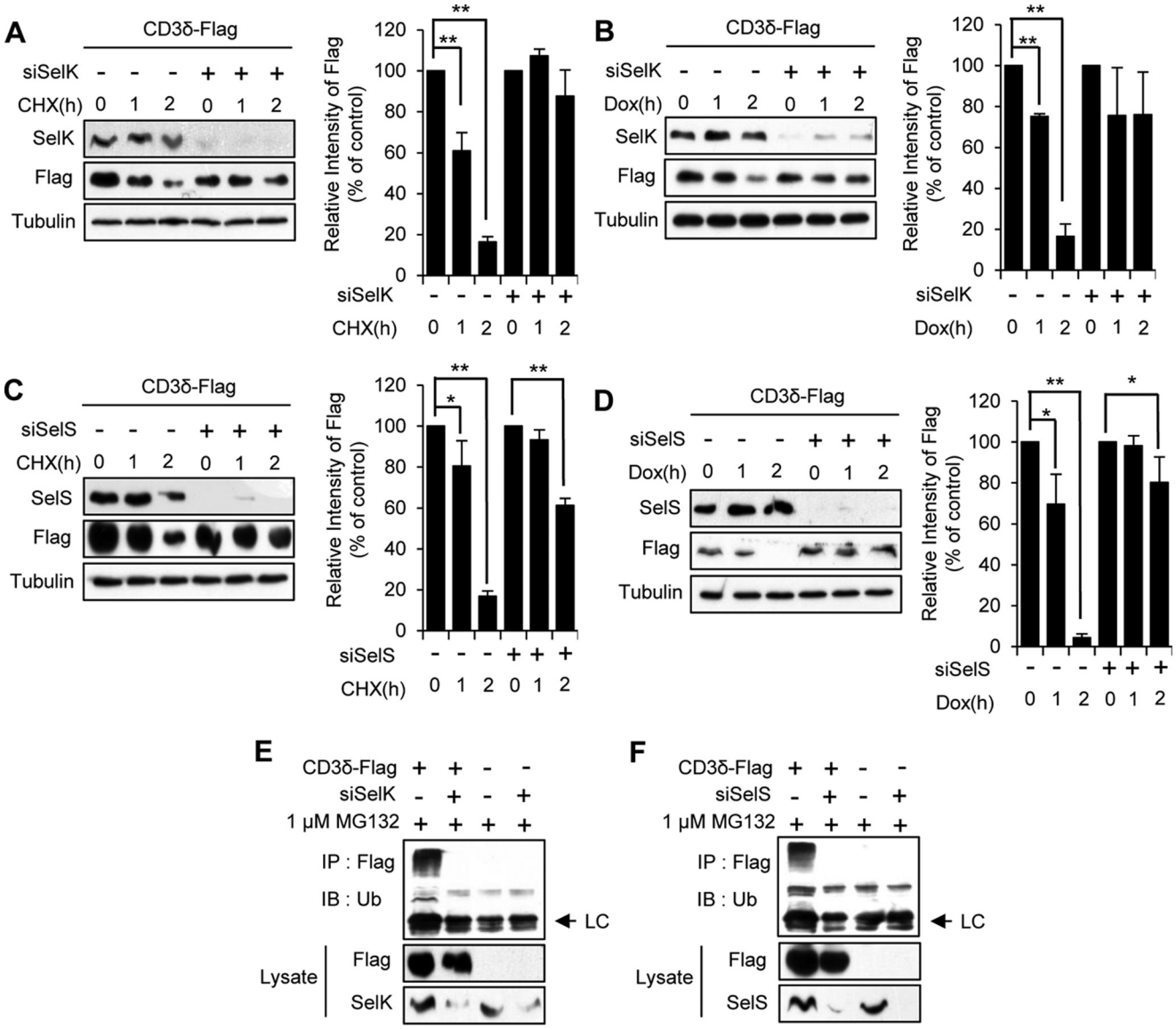

FIGURE 2. **Both SelS and SelK are important for ERAD substrate degradation.** *A–D*, N2a cells were transfected with siSelK (mouse) or control siRNA (*A* and *B*) or siSelS or control siRNA (*C* and *D*) for 16 h and then co-transfected with CD3δ-FLAG and pTet-off. 24 h after co-transfection, the cells were treated with 10 μg/ml CHX (*A* and *C*) or 50 μg/ml doxycycline (*Dox*, *B* and *D*) for the indicated times. *A–D*, *left panels*, whole cell lysates were analyzed by Western blotting with the indicated antibodies. *Right panels*, the expression of CD3δ-FLAG is represented as a percentage of the control for CHX- or doxycycline-treated cells. The *graphs* indicate the results from three independent experiments (**, $p < 0.005$; *, $p < 0.05$). *Error bars* represent mean ± S.D., and the *p* values represent comparisons with the control. *E* and *F*, N2a cells were transfected with siSelK (mouse) or control siRNA (*E*) or siSelS or control siRNA (*F*) for 16 h and then co-transfected with CD3δ-FLAG and pTet-off or control vector and pTet-off. These cells were harvested after a 12-h incubation with 1 μM MG132. Lysates and immunoprecipitation samples (anti-FLAG antibody) were analyzed by Western blotting with the indicated antibodies. *LC*, light chain; *IP*, immunoprecipitation; *IB*, immunoblot; *Ub*, ubiquitin.

to dissolve the insoluble product into a colored solution. The absorbance of the solution at 570 nm was measured using an automated microplate reader.

Confocal Microscopy—Cells that were treated with siRNAs or transfected with plasmids were washed with PBS, fixed with 4% formaldehyde for 10 min at room temperature, permeabilized with 0.1% Triton X-100 for 5 min, and incubated with 2% BSA for 1 h to block nonspecific staining. Cells were then immunostained with anti-HA, anti-His, anti-calnexin, anti-SelS, anti-p97(VCP), and anti-SelK antibodies in 0.1% BSA for 16 h at 4 °C and washed three times with PBS. The wash was followed by incubation with a secondary rabbit Alexa Fluor 488 antibody and a mouse Alexa Fluor 568 antibody (Invitrogen) for 1 h at room temperature. To visualize nuclei, the cells were stained with DAPI for 5 min. Finally, the cells were mounted onto slides and visualized using a fluorescence microscope (Zeiss LSM 700 META).

Data Analysis and Statistics—All data are presented as the mean ± S.D. of the control value. Statistical comparisons from at least three independent experiments were determined using Student's *t* test. $p < 0.05$ was considered significant.

Results

ER Stress Enhances the Expression of SelS and SelK and the Translocation of p97(VCP) to the ER Membrane—SelS and SelK are involved in cell survival during ER stress as participants in

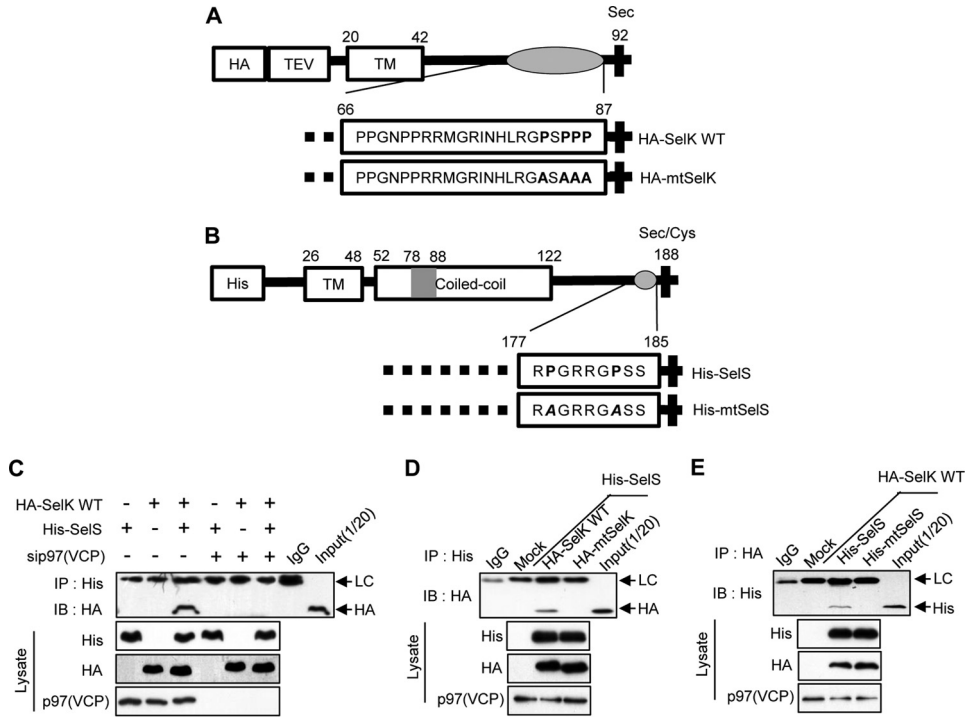

FIGURE 3. **SelK interacts with SelS through p97(VCP).** *A*, schematic of the domain organization of SelK. The following elements of the domain structure are marked: the transmembrane domain (*TM*) region and the tagged pCI-Toxo-SECIS vector (*HA-TEV*). The location of the Sec residue is indicated as a *vertical bar*. The *bottom* displays the SelK mutant form, the construct that encodes 22 residues of the cytosolic tail region (66–87). *B*, schematic of the domain organization of SelS. The following elements of the domain structure are marked: the TM region, the coiled-coil domain, the 78th to 88th residues (VIM), and the tagged pcDNA 3.1 vector (*His*). The locations of Sec and Cys residues are indicated as *vertical bars*. The *bottom* displays the mutant form of SelS, the construct that encodes 11 residues of the cytosolic tail region (178–185). *C*, N2a cells were transfected with His-SelS, HA-SelK WT, or both His-SelS and HA-SelK WT for 16 h and then transfected with sip97(VCP) or control siRNA. Lysates and immunoprecipitation (*IP*) samples (anti-His antibody) were analyzed by Western blotting with the indicated antibodies. *IB*, immunoblot; *LC*, light chain. *D*, N2a cells were transfected with His-SelS or an empty vector as the control (*Mock*) for 16 h and then transfected with HA-SelK. Lysates and immunoprecipitation samples (anti-His antibody) were analyzed by Western blotting with the indicated antibodies. *E*, N2a cells were transfected with HA-SelK WT or an empty vector as the control for 16 h and then transfected with His-SelSs. Lysates and immunoprecipitation samples (anti-HA antibody) were analyzed by Western blotting with the indicated antibodies.

ERAD with p97(VCP) (12, 24, 26). The location of p97(VCP) is important for its subcellular function (10), and the protein is translocalized to the ER membrane via its interaction with SelS to participate in ERAD during ER stress (9, 12). It has been reported that the expression of SelS and SelK is increased in various cell types that have been treated with various ER stress inducers, such as Tm and thapsigargin (24, 27, 36). In this study, increased expression levels were observed for both selenoproteins in N2a cells that had been treated with 1 μg/ml Tm or 100 nM thapsigargin for 24 h, and the translocation of p97(VCP) to the ER membrane was increased (Fig. 1, *A* and *B*). Therefore, the cytosolic p97(VCP) level of control cells was higher than that of cells under ER stress conditions. However, the amount of p97(VCP) was not changed in whole cell lysates of both cells. The localization of the selenoproteins and p97(VCP) was confirmed by an immunofluorescence assay. Endogenous SelS and SelK were found around the nucleus in a pattern that resembled an ER structure (Fig. 1*C*). Cytosolic p97(VCP) was translocalized to the ER membrane upon treatment with 1 μg/ml Tm (Fig. 1*D*).

Both SelK and SelS Participate in ERAD Substrate Degradation—In the degradation pathway for misfolded proteins, ERAD substrates, such as CD3δ, are translocated from the ER to the cytosol by the SelS-p97(VCP) complex, which delivers the substrates to the proteasome (18, 29, 37, 38). SelK is another known component of the complex. In this study, to understand the interrelationship between SelK and SelS in the ERAD pathway, the degradation of CD3δ in SelS or SelK knockdown N2a cells was examined using a CHX chase assay and the CD3δ-FLAG Tet-off system (Fig. 2). After 24 h of co-transfection with CD3δ-FLAG and siSelS or siSelK (mouse), the global translation was blocked by treatment with 10 μg/ml CHX (Fig. 2, *A* and *C*), or the transcription of CD3δ-FLAG was blocked by treating the cells with 50 μg/ml doxycycline (Fig. 2, *B* and *D*) for the indicated times, and the degree of CD3δ degradation was determined. Similar results of CD3δ degradation were obtained from both the CHX chase assay and the experiment using the Tet-off system. A previous study has reported that CD3δ was not degraded in SelS knockdown cells. In this study, not only was SelS required for protein degradation but SelK was as well. Compared with control cells, the degradation of CD3δ was decreased significantly decreased in both SelK and SelS knockdown cells (Fig. 2, *A*–*D*). Additionally, CD3δ was not ubiquitinated in the absence of SelS or SelK (Fig. 2, *E* and *F*). These results suggest that both SelS and SelK are required for protein degradation through ERAD.

p97(VCP) Is Required for the Association of SelK with SelS—Recent studies have suggested that SelS and SelK interacted with p97(VCP) (24, 25, 39). A previous study has reported that both Pro^{178} and Pro^{183} in the cytosolic tail of SelS and proline residues at the 83rd, 85th, 86th, and 87th positions in the C-terminal region of SelK were essential for the interaction of these

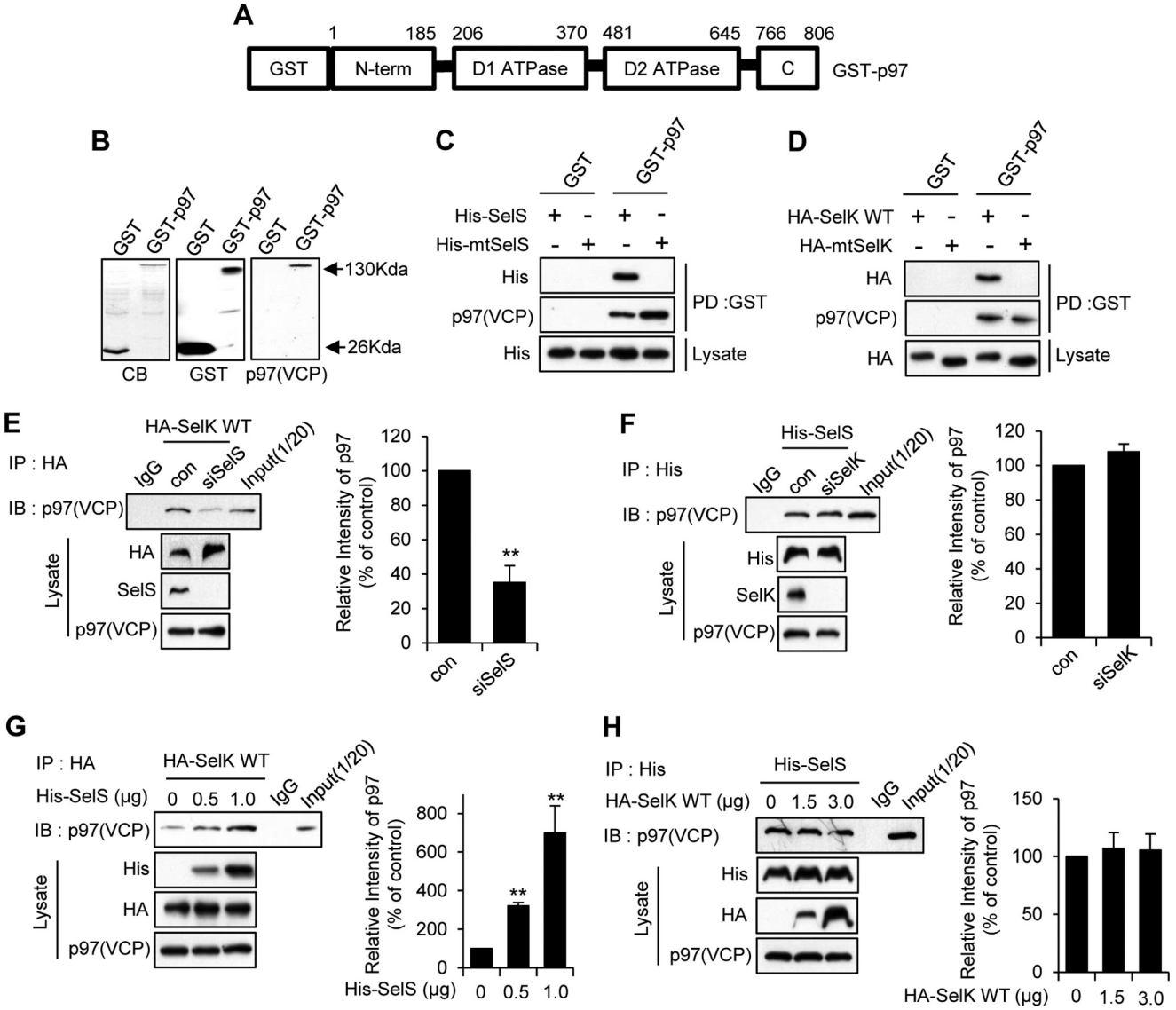

FIGURE 4. **A direct interaction between SelK and p97(VCP) depends on SelS.** *A*, schematic of the domain organization of p97(VCP). The following elements of the domain structure are marked: the N-terminal region (*N-term*), ATPase domain D1 (*D1 ATPase*), ATPase domain D2 (*D2 ATPase*), C-terminal region (*C*), and the tagged pGEX-4T-3 vector (*GST*). *B*, GST and GST-p97 were purified and visualized by Coomassie Blue staining (*CB*) and Western blotting (anti-GST, anti-p97(VCP) antibodies). *C*, purified proteins were incubated with N2a cell lysates in which His-SelS was overexpressed. *PD*, pulldown. *D*, purified proteins were incubated with N2a cells lysates in which HA-SelK was overexpressed. Each mixture and lysate was analyzed by Western blotting with the indicated antibodies. *E*, N2a cells were transfected with HA-SelK WT for 16 h and then transfected with siSelS or control siRNA. *IP*, immunoprecipitation; *IB*, immunoblot; *con*, control. *F*, N2a cells were transfected with His-SelS for 16 h and then transfected with siSelK (mouse) or control siRNA. *G*, increasing amounts (0, 0.5, and 1.0 μg) of His-SelS were transfected into HA-SelK WT-transfected N2a cells. *H*, increasing amounts (0, 1.5, and 3.0 μg) of HA-SelK WT were transfected into His-SelS-transfected N2a cells. *E–H*, lysates and immunoprecipitation samples (anti-HA and anti-His antibodies) were analyzed by Western blotting with the indicated antibodies (*left panels*). The levels of p97(VCP) are represented as a percentage of the control for each the immunoprecipitation samples (*right panels*). The *graphs* indicate results from three independent experiments (**, $p < 0.005$; *, $p < 0.05$). *Error bars* represent mean ± S.D., and the *p* values represent comparisons with the control.

proteins with p97(VCP) (12). To understand the inter-regulatory mechanism among these three proteins (SelS, SelK, and p97(VCP)) in ERAD, their interactions were examined using wild-type or mutant forms of the selenoproteins. The plasmids used in this experiment are described in Fig. 3, *A* and *B* (HA-TEV-tagged SelK (*HA-SelK WT*) is wild-type SelK containing Sec^{92}; HA-TEV-tagged SelK mutant (*HA-mtSelK*) is a SelK that is unable to interact with p97(VCP); His-tagged SelS (*His-SelS*) is SelS; and His-tagged SelS mutant (*His-mtSelS*) is a SelS that is unable to interact with p97(VCP)). A previous study has found that the ability of p97(VCP) to interact with a His-SelS in which Sec^{188} was replaced by Cys^{188} and the physiological effect thereof was the same as that of endogenous SelS containing Sec (12). It has been reported that the Cys^{188} in His-SelS forms a disulfide bond with Cys^{174}, which is consistent with the selenosulfide bond that is formed by Sec^{188} in wild-type SelS (23, 40). When His-SelS and HA-SelK WT were co-transfected into N2a cells, the interaction between His-SelS with HA-SelK WT was observed. However, the interaction did not occur in the absence of p97(VCP) (Fig. 3*C*). The interaction was determined by the immunoprecipitation of cell lysates with an anti-His antibody, and the presence of HA-SelK WT was determined by Western

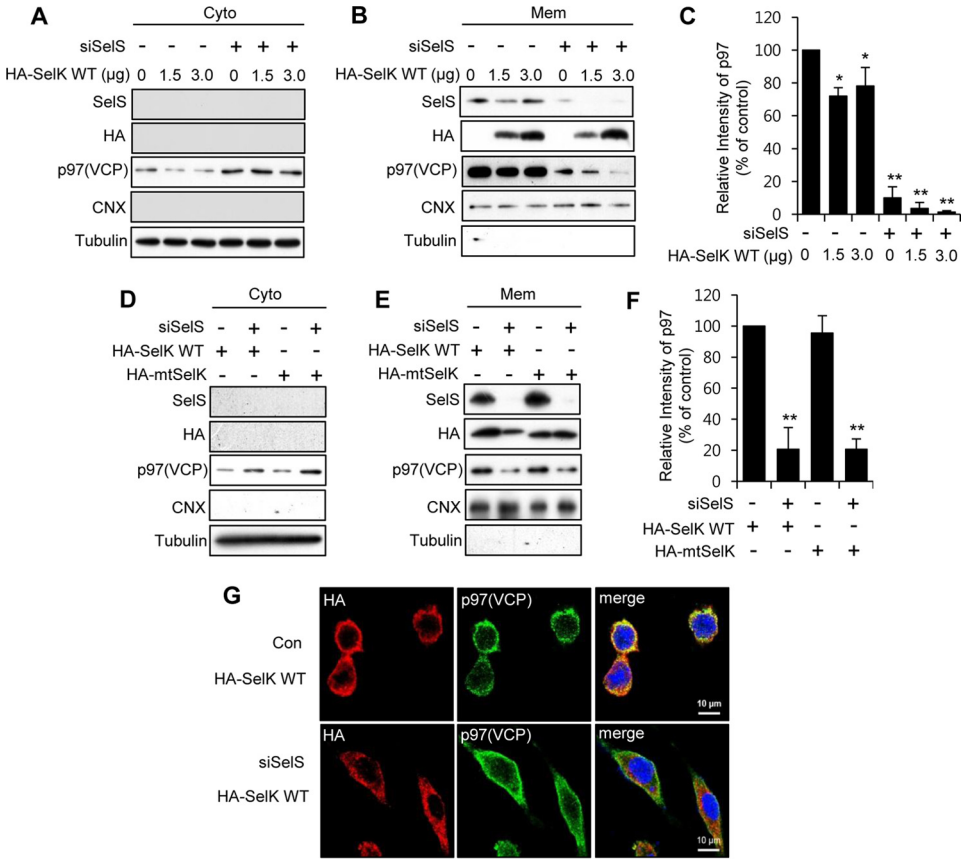

FIGURE 5. **The translocation of p97(VCP) is regulated by SelS.** *A* and *B*, increasing amounts (0, 1.5, and 3.0 μg) of HA-SelK WT were transfected into N2a cells, which were then transfected with siSelS or control siRNA. Samples from (*A*) the cytosolic fraction (*Cyto*) and (*B*) the membrane fraction (*Mem*) were assessed by Western blotting. N2a cells were then co-transfected with siSelS or control siRNA and HA-SelK WT or HA-mtSelK. *CNX*, Calnexin. *D* and *E*, samples from (*D*) the cytosolic fraction and (*E*) the membrane fraction were detected by the indicated antibodies. *C* and *F*, p97(VCP) expression is represented as a percentage of the control in the membrane fraction. The *graph* indicates results from three independent experiments (**, $p < 0.005$; *, $p < 0.05$). *Error bars* represent mean ± S.D., and the *p* values represent comparisons with the control. *G*, N2a cells were co-transfected with siSelS or control siRNA (*Con*) and HA-SelK WT. Cells were fixed and stained for HA (*red*), p97(VCP) (*green*), and DAPI (*blue*). Images of the samples were obtained using a confocal microscope.

blot analysis using an anti-HA antibody. His-SelS did not interact with HA-mtSelK, which lacked the ability to interact with p97(VCP) (Fig. 3*D*). Additionally, HA-SelK WT interacted with His-SelS but not with His-mtSelS, which is unable to interact with p97(VCP) (Fig. 3*E*). These results indicate that the interaction of SelS with SelK occurred via the binding of each protein to p97(VCP).

The Interaction between p97(VCP) and SelK Is Regulated by SelS—To determine whether p97(VCP) interacted with SelS and SelK directly, a protein-protein interaction assay was performed with purified GST or a GST-tagged p97(VCP) (GST-p97) fusion protein that was expressed in *E. coli* (Fig. 4, *A* and *B*). After preincubating the purified protein with N2a cell lysates containing overexpressed His-SelS, His-mtSelS, HA-SelK WT, or HA-mtSelK, each mixture was incubated with glutathione beads, followed by an elution with reduced glutathione. The ability of the proteins to interact was then determined by Western blot analysis (Fig. 4, *C* and *D*). As shown in Fig. 4, *C* and *D*, interactions between His-SelS or HA-SelK WT with GST-p97 were observed. However, neither His-mtSelS nor HA-mtSelK interacted with GST-p97 *in vitro*. These observations suggest that both SelS and SelK interacted directly with p97(VCP) and that the proline residues were essential for the interaction. However, the interaction between SelK and p97(VCP) was decreased by 65% in SelS knockdown cells (Fig. 4*E*), whereas the interaction of p97(VCP) with His-SelS was not affected by the absence of SelK (Fig. 4*F*). Moreover, the interaction of p97(VCP) with HA-SelK WT was enhanced by increasing the expression level of His-SelS (Fig. 4*G*). However, the interaction between p97(VCP) and His-SelS was not affected by increasing the expression level of HA-SelK WT (Fig. 4*H*). These results indicate that the interaction of SelS with p97(VCP) is SelK-independent, whereas the interaction between SelK and p97(VCP) is SelS-dependent. Therefore, p97(VCP) binding to SelS is followed by the interaction of SelK with the SelS-p97(VCP) complex.

The Translocation of p97(VCP) to the ER Membrane Is Regulated by SelS, Not SelK—The degradation of ERAD substrates requires p97(VCP), whose translocation to the ER membrane from the cytosol is essential for the delivery of ERAD substrates to the proteasome (10). Additionally, both Pro^{178} and Pro^{183} on SelS are essential for the translocation of p97(VCP) (12). As shown in Fig. 2, not only was the SelS-p97(VCP) complex required for the degradation of CD3δ, an ERAD substrate, but so was SelK. To understand whether SelK is also involved in recruiting p97(VCP) to the ER membrane, we examined the translocation of p97(VCP) in N2a cells by immunoblotting with the membrane fraction and by immunofluorescence assay.

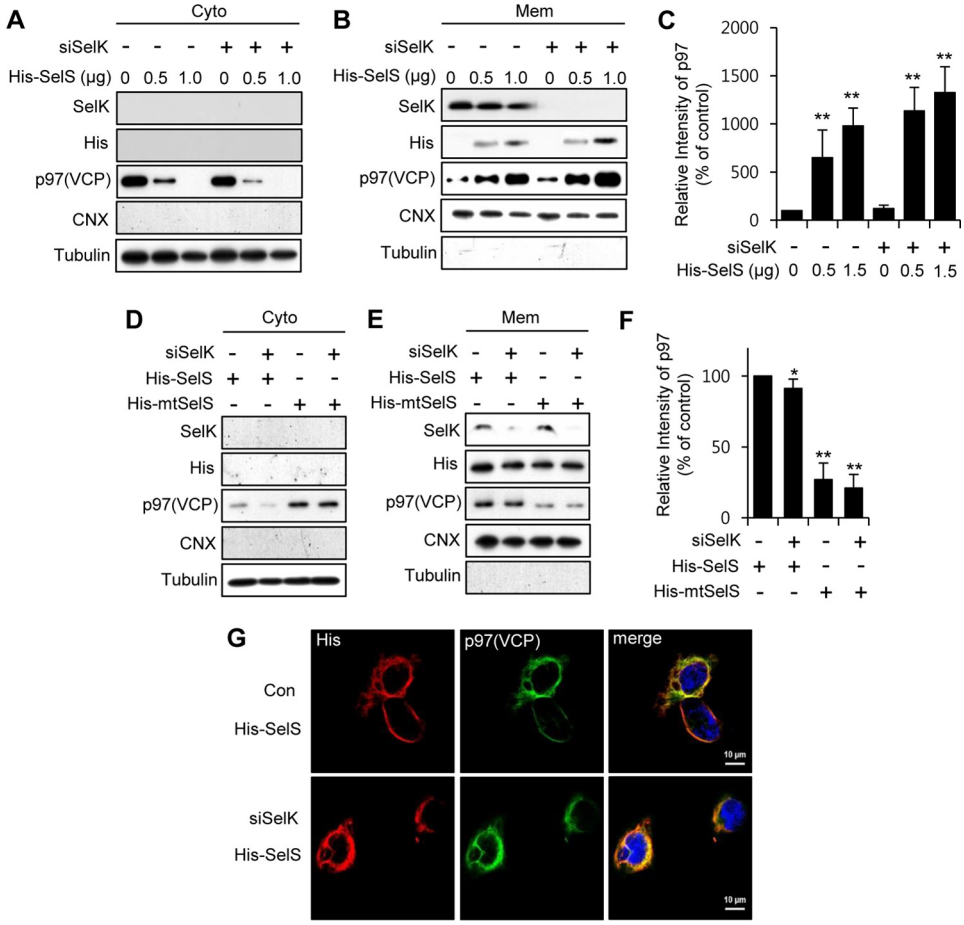

FIGURE 6. **SelK is not involved in the translocation of p97(VCP).** *A* and *B*, increasing amounts (0, 0.5, 1.0 μg) of His-SelS were transfected into HEK293 cells, which were then transfected with siSelK (human) or control siRNA. Samples from (*A*) the cytosolic fraction (*Cyto*) and (*B*) the membrane fraction (*Mem*) were detected by Western blotting. HEK293 cells were co-transfected with siSelK (human) or control siRNA and His-SelS or His-mtSelS. *CNX*, Calnexin. *D* and *E*, samples from (*D*) the cytosolic fraction and (*E*) the membrane fraction were detected by the indicated antibodies. *C* and *F*, p97(VCP) expression is represented as a percentage of the control in the membrane fraction. The *graphs* indicate results from three independent experiments (**, $p < 0.005$; *, $p < 0.05$). *Error bars* represent mean ± S.D., and the *p* values represent comparisons with the control. *G*, HEK293 cells were co-transfected with siSelK (human) or control siRNA (*Con*) and His-SeS. Cells were fixed and stained for His (*red*), p97(VCP) (*green*), and DAPI (*blue*). Images of the samples were obtained using a confocal microscope.

When SelS was knocked down, the translocation of cytosolic p97(VCP) to the ER membrane was decreased regardless of SelK concentration (Fig. 5). The expression level of p97(VCP) in SelS knockdown cells was higher in the cytosol but lower in the membrane fraction than in control cells and was not affected by HA-SelK expression (Fig. 5, *A–C*). The translocation of p97(VCP) to the membrane was regulated by SelS and unaffected by either HA-SelK or HA-mtSelK (Fig. 5, *D–F*). The effects of SelS or SelK on the translocation of p97(VCP) to the ER membrane were confirmed by an immunofluorescence assay using N2a cells that were co-transfected with HA-SelK WT and control siRNA or siSelS (Fig. 5*G*). HA-SelK WT was observed around the nucleus in a pattern that resembled an ER structure. p97(VCP) co-localized with HA-SelK in cells that expressed control siRNA (Fig. 5*G*, *top panels*) but not in cells that expressed siSelS (Fig. 5*G*, *bottom panels*). The amount of cytosolic p97(VCP) was decreased under these conditions, whereas the protein level increased with increasing SelS expression regardless of SelK expression (Fig. 6, *A–C*). The translocation of p97(VCP) did not occur when cells were transfected with His-mtSelS, which was not able to interact with p97(VCP) (Fig. 6, *D–F*). Therefore, the translocation of p97(VCP) to the membrane was regulated by SelS, and it was enhanced by increasing the expression level of SelS. SelK was not involved in this process. A previous study has shown that the overexpression of His-SelS altered ER morphology, resulting in a dotted structure, and that His-SelS co-localized with p97(VCP) (12). Furthermore, regardless of whether His-SelS was transfected with siSelK (human) into HEK293 control cells, p97(VCP) always co-localized with His-SelS around the nucleus (Fig. 6*G*).

Both SelK and SelS Are Essential for p97(VCP)-dependent ERAD—As described above, although SelK was not involved in the translocation of p97(VCP) to the ER membrane, SelK interacted with the SelS-p97(VCP) complex and was required for its ERAD activity (Figs. 2 and 4–6). Degradation and ubiquitination of CD3δ in SelS knockdown cell occurred only by expression of His-SelS (12). To understand the function of SelK, recombinant plasmids were constructed that contained GFP-tagged SelK (GFP-SelK WT) or GFP-tagged mtSelK (GFP-mtSelK) (Fig. 7*A*), and the ERAD response was determined using SelK knockdown N2a cells. mtSelK is a mutant that does not interact with p97(VCP). After the co-transfection of CD3δ-FLAG with GFP-SelK WT or GFP-mtSelK into endogenous

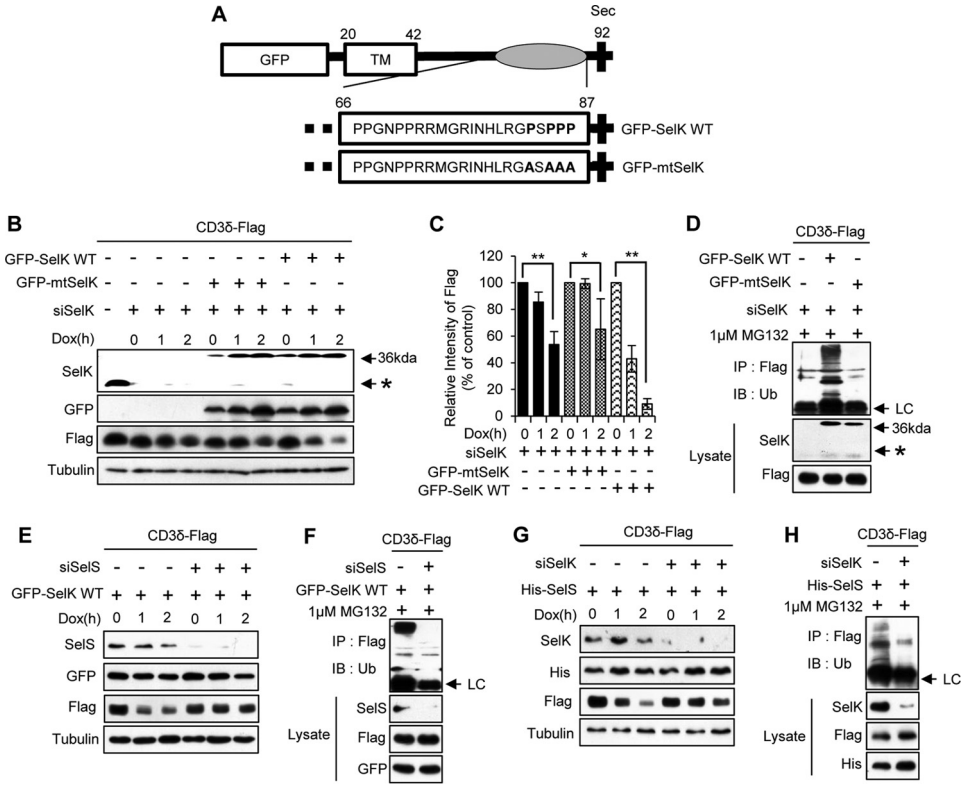

FIGURE 7. **Both SelK and SelS are essential for a p97(VCP)-dependent ERAD system.** *A*, schematic of the domain organization of GFP-tagged SelK. The various elements of the domain structure are marked as in Fig. 3*A*. The HA-TEV tag on pCI-Toxo-SECIS-SelK was replaced with GFP. *B* and *D*, N2a cells were co-transfected with siSelK (mouse) and GFP-SelK WT or GFP-mtSelK for 16 h. *Dox*, doxycycline; *IP*, immunoprecipitation; *IB*, immunoblot; *Ub*, ubiquitin; *LC*, light chain. *E* and *F*, N2a cells were co-transfected with siSelS or control siRNA and GFP-SelK WT for 16 h. *G* and *H*, N2a cells were co-transfected with siSelK (mouse) or control siRNA and His-SelS for 16 h. *B–H*, all cells were co-transfected with CD3δ-FLAG and pTet-off to determine the expression of CD3δ-FLAG. *B*, *E*, and *G*, 40 h after the first co-transfection, cells were treated with 50 μg/ml doxycycline for the indicated times. *C*, Quantification of CD3δ-FLAG level from the experiment shown in *B*. The *graph* indicates results from three independent experiments (**, $p < 0.005$; *, $p < 0.05$). *Error bars* represent mean ± S.D., and the *p* values represent comparisons with the control. *D*, *F*, and *H*, cells were harvested after 12 h of incubation with 1 μM MG132. Lysates and immunoprecipitation samples (anti-FLAG antibody) were analyzed by Western blotting with the indicated antibodies. The *asterisks* indicate endogenous SelK.

SelK-deficient N2a cells, the cells were treated with 50 μg/ml doxycycline for the indicated times. As shown in Fig. 7, *B* and C, some CD3δ was degraded in cells that were transfected with siSelK (mouse) or siSelK (mouse) + GFP-mtSelK. However, a significant amount of CD3δ was degraded in cells that were co-transfected with siSelK (mouse) and GFP-SelK WT. Moreover, the ubiquitination of CD3δ occurred only in cells that were co-transfected with siSelK (mouse) and GFP-SelK WT (Fig. 7*D*). These data show that the interaction of SelK with p97(VCP) is necessary for the degradation of ERAD substrates. However, ERAD activity was not observed in the absence of SelS (Fig. 7, *E* and *F*). CD3δ was not degraded or ubiquitinated even though GFP-SelK WT was overexpressed in SelS knockdown cells. One possible explanation is that SelK was not able to recruit p97(VCP) to the ER membrane (Figs. 5 and 6). CD3δ was also not degraded or ubiquitinated in the absence of SelK even though SelS was overexpressed (Fig. 7, *G* and *H*). These results suggest that both SelS and SelK are essential for ERAD, although only SelS recruits p97(VCP) to the ER membrane. Therefore, p97(VCP) is translocated to the ER membrane via its interaction with SelS, and the interaction of SelK with the complex follows. The SelS-p97(VCP)-SelK complex as a whole is necessary for ERAD.

Both SelS and SelK Respond to ER Stress by Forming the ERAD Complex—As described above, the translocation of p97(VCP) to the ER membrane and the formation of the SelS-p97(VCP)-SelK complex are essential for ERAD (Figs. 3, 4, and 7). The accumulation of misfolded proteins in the ER induces apoptosis via ER stress (41). Therefore, the effects of SelK and SelS on ER stress and ER stress-mediated apoptosis were determined. CHOP, c-casp12, and c-casp3 were activated by Tm treatment, and their expression was increased by SelK knockdown. The increased expression of pro-apoptotic factors was reversed by the presence of GFP-SelK WT, whereas it was not reversed in cells that were transfected with GFP-mtSelK (Fig. 8*A*). The repressive effect of GFP-SelK WT on the expression of pro-apoptotic molecules was not observed in SelS knockdown cells. Although the expression levels of GFP-SelK WT or GFP-mtSelK were increased, high levels of ER stress-mediated apoptotic factors remained in SelS knockdown cells (Fig. 8*B*). Moreover, these factors were still highly expressed even when SelK knockdown N2a cells were transfected with His-SelS or His-mtSelS (Fig. 8*C*). When both SelS and SelK were knocked down, the increase in CHOP, c-casp12, and c-casp3 expression was only reversed in the cells that were co-transfected with His-SelS and GFP-SelK WT (Fig. 8, *D* and *E*). Neither His-SelS

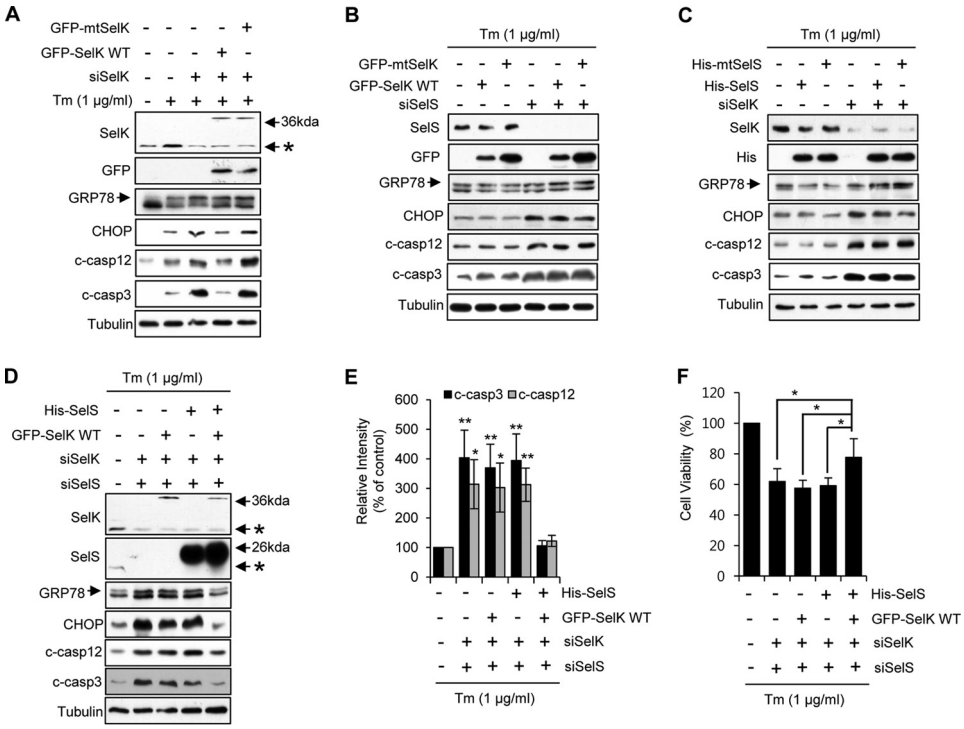

FIGURE 8. **Physiological effects of SelK and SelS on ER stress.** *A*, N2a cells were co-transfected with siSelK (mouse) and GFP-tagged SelK WT/mtSelK or with siSelK (mouse) alone. These cells were treated with Tm for 24 h. *B*, N2a cells were co-transfected with siSelS or control siRNA and GFP-SelK WT or GFP-mtSelK. *C*, N2a cells were co-transfected with siSelK (mouse) or control siRNA and His-SelS or His-mtSelS. *B* and *C*, cells were treated with Tm for 24 h. *A–C*, cell lysates that were isolated from these cultured cells were analyzed by Western blotting with the indicated antibodies. The *asterisks* indicate endogenous SelK. *D*, N2a cells were co-transfected with siSelS and siSelK (mouse) for 16 h and then transfected with GFP-SelK WT alone, His-SelS alone, or GFP-SelK WT and His-SelS together. These cells were treated with Tm for 24 h. Cell lysates were analyzed by Western blotting with the indicated antibodies. The *asterisks* indicate endogenous SelK or SelS. *E*, Quantification of protein levels from experiment shown in *D*. The *graph* shows results from three independent experiments (**, $p < 0.005$; *, $p < 0.05$). *Error bars* represent mean ± S.D., and the *p* values represent comparisons with the control group. *F*, the same culture conditions were used here as in *D*. Cell viability was determined by MTT assay. Cell viability is expressed as a percentage relative to that of non-transfected cells. The *graph* shows results from six independent experiments (**, $p < 0.005$; *, $p < 0.05$). *Error bars* represent mean ± S.D., and the *p* values represent comparisons with the control group. CHOP is the abbreviation for C/EBP-homologous protein.

nor GFP-SelK WT alone was able to reduce the expression of pro-apoptotic factors in the dual knockdown cells. The viability of cells expressing both His-SelS and GFP-SelK WT was also similar to that of control cells. The overexpression of His-SelS alone or GFP-SelK WT alone could not rescue the effects of the absence of both SelS and SelK (Fig. 8*F*). These results indicate that both SelS and SelK are essential for regulating ER stress in ERAD in the form of the SelS-p97(VCP)-SelK complex.

Discussion

Although SelS and SelK have no significant similarity to each other in their amino acid sequences, they could be assigned to a single protein family on the basis of their topology, including a single transmembrane domain and a glycine-rich region that contains an unusually high amount of glycine and proline. Moreover, homologs of both SelS and SelK contain Sec in the second or third position from the C-terminal end. The function of their Sec residues still remains unclear. Both SelS and SelK are localized to the ER membrane and belong to a group of type III transmembrane proteins that contain a single transmembrane domain and a C-terminal end that faces the cytosol. The C-terminal portions of the proteins have glycine-rich regions and are highly unstructured (12, 24). The proline residues in the glycine-rich regions of SelS and SelK have been reported previously to be essential for the binding of each protein to p97(VCP) (12). This interaction is required for ER stress regulation and for the degradation of ERAD substrates such as CD3δ (Figs. 2, 7, and 8).

Although both SelS and SelK have recently been implicated in p97(VCP)-dependent ERAD (12, 24, 42), the relationship between the ERAD component proteins SelS, SelK, and p97(VCP) is unclear. A recent report has shown that the translocation of p97(VCP) to the ER membrane was enhanced in cells that overexpressed SelS regardless of ER stress status (12). It is well known that SelS plays important roles in the translocation of p97(VCP) to the ER membrane and in the regulation of ERAD functions. However, the function of SelK during ERAD remains unclear. According to recent studies, SelS and SelK have been shown to associate with each other through their transmembrane domains, although the association might also involve additional proteins (24, 43). In this study, p97(VCP) was required for the interaction of SelS with SelK (Fig. 3). Consistent with the roles of SelS and SelK in ERAD (Fig. 2), the expression of both proteins was up-regulated by ER stress (Fig. 1).

Various co-factors interact with p97(VCP), and these p97(VCP) complexes function in many different cellular processes (16, 44, 45). Among these cellular functions, it is known that p97(VCP) is essential to the ER stress response, particularly

in ERAD. The knockdown of p97(VCP) significantly increases ER stress-mediated apoptosis in N2a cells (12). In this study, the relationship between p97(VCP), SelS, and SelK in ERAD was observed under ER stress. The initial translocation of p97(VCP) to the ER membrane is necessary for ERAD, and this activity could possibly be due to the interaction of the complex with SelS, which was highly expressed during ER stress (Figs. 1, 4, and 5).

The translocated p97(VCP) then interacted with SelK, whose expression levels were also increased by ER stress, and this formed the SelS-p97(VCP)-SelK complex (Figs. 1 and 3–5). It is also known that misfolded proteins are extracted by p97(VCP) from the ER to the cytosol, where they are ubiquitinated by E3 ligases such as Hrd1 and gp78 (13, 14, 17). Finally, Fas-associated factor 1 (FAF1) binds to the N terminus of p97(VCP), and this interaction recruits the polyubiquitinated misfolded protein (18). The ubiquitinated protein is eventually degraded by the proteasome (46). The SelS-p97(VCP)-SelK complex was required for ERAD activation, resulting in the restoration of cells after ER stress (Figs. 7 and 8).

In this study, we found that both the translocation of p97(VCP) to the ER membrane and the interaction between p97(VCP) and SelK were regulated by SelS (Figs. 3–7). However, the translocation was not completely diminished by SelS knockdown (Fig. 5*B*). It might be due to other binding factors for p97(VCP). It has been suggested that gp78 may also be a player in the recruitment of p97(VCP) to the ER membrane (13).

SelS has been suggested to be involved in neurodegenerative disorders such as Alzheimer disease and Parkinson disease by regulating ER stress (47). Recently, it has been reported that SelS was involved not only in recruiting p97(VCP) to the ER membrane but also in regulating ER shape (48). SelS also regulates the early phase of adipogenesis through dexamethasone-induced SelS degradation (49). Furthermore, SelS is known to be associated with the maintenance and intracellular membrane-based transport of multiprotein complexes (39). SelK has been reported to regulate Ca^{2+} flux during immune responses and to protect cells from ER stress-mediated apoptosis (27, 50). However, the way in which SelS and SelK interact and the subsequent effect on the ER stress response is not well understood. This study found that SelS was required for the interaction between SelK and p97(VCP), resulting in the formation of the SelS-p97(VCP)-SelK complex. This interaction was a prerequisite for the degradation of ERAD substrates and the restoration of cells after ER stress.

Author Contributions—I. Y. K. conceived and coordinated the study and wrote the paper. J. H. L. designed, performed, and analyzed the experiments. K. J. P., J. K. J., Y. H. J., K. Y. K., J. H. K., and S. R. L. provided technical assistance and contributed to the preparation of mutants and antibodies.

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