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Pro^{178} and Pro^{183} of Selenoprotein S Are Essential Residues for Interaction with p97(VCP) during Endoplasmic Reticulum-associated Degradation*

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Background: SelS is a binding partner of p97(VCP) for endoplasmic reticulum-associated degradation (ERAD).
Results: The proline mutants at SelS positions at 178 or 183 did not interact with p97(VCP).
Conclusion: Both Pro^{178} and Pro^{183} of SelS are essential for SelS function during ERAD.
Significance: The essential SelS amino acid residues for binding to p97(VCP) are identified.

During endoplasmic reticulum (ER)-associated degradation, p97(VCP) is recruited to the ER membrane through interactions with transmembrane proteins, such as selenoprotein S (SelS), selenoprotein K (SelK), hrd1, and gp78. SelS has a single-spanning transmembrane domain and protects cells from ER stress-induced apoptosis through interaction with p97(VCP). The cytosolic tail of SelS consists of a coiled-coil domain, a putative VCP-interacting motif (VIM), and an unpronounced glycine- and proline-rich secondary structure. To understand the regulatory mechanism of SelS during ER stress, we investigated the interaction of the protein with p97(VCP) using mouse neuroblastoma cells and human embryonic kidney 293 cells. The SelS expression level increased when ER stress was induced. In addition, the effect of ER stress was enhanced, and recruitment of p97(VCP) to the ER membrane was inhibited in SelS knockdown cells. The effect of SelS knockdown was rescued by ectopic expression of SelS U188C. p97(VCP) interacted with SelS U188C and was recruited to the ER membrane. The expression of SelS[ΔVIM], which is a VIM deletion mutant of SelS, also showed both a recovery effect and an interaction with p97(VCP) in cells. However, mutants in which the proline residue positions 178 or 183 of SelS were changed to alanine or were deleted did not interact with p97(VCP). The proline mutants did not rescue ER stress in SelS knockdown cells. These results suggest that both Pro^{178} and Pro^{183} of SelS play important roles in the translocation of p97(VCP) to the ER membrane and protect cells from ER stress.

The endoplasmic reticulum (ER)[2] is a eukaryotic cell organelle that has various functions, including protein synthesis and the quality control of proteins. Newly synthesized proteins undergo folding and post-translational modifications in the ER (1). Multiple proteins and enzymes, such as chaperones, oxidoreductases, and protein modification enzymes, are involved in the proper folding of proteins. However, some proteins may not reach their native folded state. An elimination process for these proteins, called ER-associated degradation (ERAD), is an important mechanism for protein quality control (2, 3). This mechanism enables the ER to distinguish properly and improperly folded proteins. In addition, ERAD retains the improperly folded proteins in the ER and leads them to degradation by a ubiquitin-proteasome pathway. The ERAD process includes transporting misfolded proteins from the ER membrane to the cytosol by either retrotranslocation or dislocation (2, 4). This transport is followed by polyubiquination of these proteins. The polyubiquitin chain provides a binding site for the p97(VCP) complex so that the protein can be removed from the membrane. The ubiquitinated proteins are recognized and eventually degraded by proteasomes (3). Therefore, ERAD is essential for restoring ER stress by degrading misfolded proteins. Abnormal ERAD is involved in the pathogenesis of diseases related to ER stress-induced apoptosis, such as neurodegenerative diseases and diabetes (5, 6).

SelS, which is also known as VIMP (VCP-interacting membrane protein), is a selenoprotein that contains a single selenocysteine (Sec, U) at position 188 as a C-terminal penultimate residue (7–9). SelS is localized to the ER membrane and is an essential component of the ERAD complex, together with SelK, p97(VCP), Derlin-1 or -2, and E3 ligases (10). SelS has a short N-terminal ER-luminal tail and a longer cytosolic tail from amino acids 49–189. The transmembrane (TM) domain is predicted in the region of positions 26–48 (7, 9). A coiled-coil domain is predicted in the cytosolic tail of SelS and may be involved in dimerization of SelS or in binding to other proteins (11, 12). A VIM has been predicted (region of positions 78–88) in the coiled-coil domain, (9, 13) (see Fig. 3*A*). A pronounced secondary structure does not exist in the C-terminal end, which is downstream of the coiled-coil domain. This unstructured

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[2] The abbreviations used are: ER, endoplasmic reticulum; SelS, selenoprotein S; SelK, selenoprotein K; ERAD, ER-associated degradation; Sec, selenocysteine; Tm, tunicamycin; Tg, Thapsigargin; VIM, VCP-interacting motif; TM, transmembrane; MTT, 3-[4,5–2-yl]-2,5-diphenyl tetrazolium bromide.

region is rich in glycine and proline (12) and may be required for the interaction with p97(VCP) (9). SelS binds to the cytosolic p97(VCP) and Derlin-1 or -2. The Derlins contain a four-transmembrane domain, which interacts with the SelS transmembrane domain (11, 14). SelS does not interact with E3 ubiquitin ligase, which has been identified as a p97(VCP)-binding partner (15, 16).

Similar to SelS, SelK is a selenoprotein located in the ER membrane (7, 17). SelK contains a single Sec at position 92 as a C-terminal penultimate residue, but it has no VIM and coiled-coil domain (13). Recent studies suggest that SelK is an ER stress-regulated protein and plays an important role in resisting ER stress-mediated apoptosis (18). SelK is also involved in the ERAD complex and interacts with p97(VCP) and Derlins (10, 13).

p97(VCP) has two ATP-binding domains, flexible N-terminal domains responsible for co-factors and substrate binding, and a short C-terminal domain (19). p97(VCP) has a homohexameric double-ring structure. This structure is involved in multiple cellular functions, including ERAD, mitochondrial-associated degradation, and DNA repair (20–22). p97(VCP) interacts with a number of binding partners and co-factors, and this binding plays important roles in its activity and different subcellular locations (22). When p97(VCP) is located in the ER membrane by its binding partners, it has an essential function for maintenance of ER homeostasis and regulation of ER stress through the ERAD (20–23).

During ERAD, p97(VCP) plays a key role transporting misfolded proteins from the ER to the cytosol (11, 24). This mechanism represents a determinant for misfolded protein degradation, such as CD3δ. CD3δ is ubiquitinated and degraded by proteasomes (25, 26).

SelS plays an important role restoring ER stress (12). We now report that SelS has critical functions in ERAD, such as degradation of CD3δ, through interaction with p97(VCP). However, the SelS-dependent protective mechanisms against ER stress-induced apoptosis remain unclear. To understand the regulatory function of SelS in ERAD, we identified the effect of the protein on ER stress and the essential residues of SelS for binding to p97(VCP) in this study.

EXPERIMENTAL PROCEDURES

Cell Culture and ER Stress Induction—Mouse neuroblastoma (N2a) cells and human embryonic kidney 293 (HEK293) cells were cultured in DMEM (Invitrogen) that was supplemented 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, and HEK293 cells were seeded at a density of 3×10^5 in 60-mm dishes. At 24 h after seeding, the cells were treated with 1 μg/ml tunicamycin (Tm) and 100 nM thapsigargin (Tg) or DMSO (Sigma-Aldrich) (18, 27) for various times.

RNA Interference—The siSelSs used in this study were designed by Invitrogen. The three sequences for mouse were as follows: siSelS 1, 5′-GGA AGA UCU AAA UGC CCA AGU UGA A-3′; siSelS 2, 5′-AGC CUU UGC GAG GAG GUG GUU AUA A-3′; and siSelS 3, 5′-CAU GCA AGA AGG CAG AAG UUA CAA A-3′. The sip97(VCP)s were designed by Shanghai GenePharma Co., Ltd. The three sequences for mouse were as follows: sip97(VCP) 1, 5′-GAG CUG AUU UGA CAG AAA UTT AUU UCU GUC AAA UCA GCU CTT-3′; sip97(VCP) 2: 5′-GAG CAC AUG UGA UAG UUA UTT AUA ACU AUC ACA UGU GCU CTT-3′; and sip97(VCP) 3: 5′-CCU GAA AUC AUG AGC AAA UTT AUU UGC UCA UGA UUU CAG GTT-3′. A stealth negative control siRNA was also obtained from Invitrogen.

Construction of SelS and SelK Mutants—Using human SelS cDNA, the PCR was performed in a Mini Cycler for Sec to Cys mutation. The primers used are shown in Table 1. The PCR products were cloned into the BamHI and XhoI sites of the pcDNA 3.1 His C vector. The mutant was designated SelS U188C. This mutant was then used as a template DNA for the following SelS mutants. The primers that were designed for SelS[1–122], SelS[1–144], SelS[1–167], SelS[1–176], SelS[1–187], and SelS[ΔTM] are shown in Table 1. The PCR products were cloned into the BamHI and XhoI sites of the pcDNA 3.1 His C vector. All primers used for constructing SelS[ΔVIM], SelS[Δ177–179], SelS[Δ180–185], SelS[Δ178P], SelS[Δ183P], SelS[Δ184S], SelS[Δ185S], SelS[P178A], SelS[P183A], and SelS[P178, 183A] are shown in Table 1. The human SelK (HA-TEV-SelK) was kindly provided as a gift by Dr. Vadim N. Gladyshev (Harvard Medical School) (10). Using HA-TEV-SelK, PCR was performed in a Mini Cycler for the Sec to Cys mutation. The primers used are shown in Table 2. The PCR products were cloned into the BamHI and XhoI sites of the pcDNA 3.1 His C vector. The mutant was designated SelK U92C. This mutant was then used as a template DNA for the following SelK mutants. All primers designed for mtSelK1[P66,67,70,71A] and mtSelK2[P83,85,86,87A] are shown in Table 2. All SelS and SelK mutants were constructed by sequential site-directed mutagenesis reactions using QuikChange site-directed mutagenesis according to the manufacturer's instructions (Stratagene). All SelS mutants were confirmed by sequencing (Macrogen, Seoul, Korea).

CD3δ Expression Plasmid—The pYR-CD3δ-FLAG was a kind gift from Dr. J. B. Yoon (Yonsei University, Seoul, Korea). The pYR-CD3δ-FLAG contained a tetracycline-regulated promoter (25, 28). N2a cells were co-transfected with pYR-CD3δ-FLAG and the pTet-off (Clontech), the plasmids encoding tetracycline-controlled transactivator, to express CD3δ-FLAG. Doxycycline, the tetracycline-controlled transactivator inhibiter, and MG132 were purchased from Sigma.

Transfections—For transfection into N2a and HEK293 cells, the cells were seeded at a densities of 1×10^6 and 3×10^5 cells in 60-mm dishes, respectively. At 12 h after seeding, the cells were transfected with siRNA or plasmids using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Stealth negative control siRNAs (Invitrogen) were used as controls (29, 30).

Isolation of RNA and RT-PCR Analysis—Total RNA was extracted from N2a cells with TRIzol (Invitrogen), and 1 μg of RNA was subjected to RT-PCR using oligo(dT) primer and SuperScript® III reverse transcriptase (Invitrogen). RT-PCR analysis of the resulting cDNA preparation was performed using a PCR Premix, Sapphire (Super Bio Co., Seoul, Korea) (30) and the following primers (forward and reverse, respectively): SelS, 5′-GAG GCT TTA GCA GCT GCT CG-3′, 5′-GTC AGA GGG TTA TTA CCA CCT CC-3′; XBP-1,

TABLE 1

Oligonucleotide sequences for SelS mutants used in this study

F indicates forward, and R indicates reverse. GenBank™ accession number NM_018445.4.

SelS mutants	Primer (5′ →3′)
Cloning for SelS U188C	
SelS U188C-F	GGCGGATCCATGGAACGCCAAGAGGAGTC
SelS U188C-R	GCGCTCGAGTTAGCCGCATCCGCCAGATG
Cloning for mutants of SelS	
SelS [1–122]-F	GGCGGATCCATGGAACGCCAAGAGGAGTC
SelS [1–122]-R	CGCTCGAGTTACATGCTGTCCCACATTTCAATC
SelS [1–144]-F	GGCGGATCCATGGAACGCCAAGAGGAGTC
SelS [1–144]-R	CGCTCGAGTTAGGAAGGCCCAGGACTGTCTTCC
SelS [1–167]-F	GGCGGATCCATGGAACGCCAAGAGGAGTC
SelS [1–167]-R	CGCTCGAGTTAAGACAACGGGTTATAACCTCCTCCC
SelS [1–176]-F	GGCGGATCCATGGAACGCCAAGAGGAGTC
SelS [1–176]-R	GCGCTCGAGTTACCAGGAGCAAGCTCCGCC
SelS [1–185]-F	GGCGGATCCATGGAACGCCAAGAGGAGTC
SelS [1–185]-R	GCGCTCGAGTTAAGATGACGGGCCTCTGCG
SelS [1–187]-F	GGCGGATCCATGGAACGCCAAGAGGAGTC
SelS [1–187]-R	GCGCTCGAGTTATCCGCCAGATGACGGGCC
SelS [ΔTM]-F	GGCGGATCCTCCGCCCGGCTAAGAGCCTT
SelS [ΔTM]-R	GCG TCGAGTTAGCCGCATCCGCCAGATG
Deletion or point mutants of SelS	
SelS [ΔVIM]-F	GATGTTGTTGTTAAAATGCAAGAAGAACTAAATGC
SelS [ΔVIM]-R	GCATTTAGTTCTTCTTGGTATTTAACAACAACATC
SelS [Δ177–179]-F	GGAGCTTGCTCCTGGCGCAGAGGCCCGTCATCTGG
SelS [Δ177–179]-R	CCAGATGACGGGCCTCTGCGCCAGGAGCAAGCTCC
SelS [Δ180–182]-F	GCTCCTGGAGACCTGGACCGTCATCTGGCGGATGC
SelS [Δ180–182]-R	GCATCCGCCAGATGACGGTCCAGGTCTCCAGGAGC
SelS [Δ183–185]-F	CCTGGACGCAGAGGCGGCGGATGCGGCTAACTCG
SelS [Δ183–185]-R	CGAGTTAGCCGCATCCGCCGCCTCTGCGTCCAGG
SelS [Δ178P]-F	GGAGCTTGCTCCTGGAGAGGACGCAGAGGCCCG
SelS [Δ178P]-R	CGGGCCTCTGCGTCCTCTCCAGGAGCAAGCTC
SelS [Δ183P]-F	CCTGGACGCAGAGGCTCATCTGGCGGATGC
SelS [Δ183P]-R	GCATCCGCCAGATGAGCCTCTGCGTCCAGG
SelS [Δ184S]-F	GGACGCAGAGGCCCGTCTGGCGGATGCGGC
SelS [Δ184S]-R	GCCGCATCCGCCAGACGGGCCTCTGCGTCC
SelS [Δ185S]-F	CGCAGAGGCCCGTCAGGCGGATGCGGCTAACTCG
SelS [Δ185S]-R	CGAGTTAGCCGCATCCGCCTGACGGGCCTCTGCG
SelS [P178A]-F	GGAGCTTGCTCCTGGAGAGCTGGACGCAGAGGC
SelS [P178A]-R	GCCTCTGCGTCCAGCTCTCCAGGAGCAAGCTCC
SelS [P183A]-F	GGACGCAGAGGCGCGTCATCTGGCGGATGC
SelS [P183A]-R	GCATCCGCCAGATGACGCGCCTCTGCGTC
SelS [P178,183A]-F	GCTCCTGGAGAGCTGGACGCAGAGGCGCGTCATCTGGCGG
SelS [P178,183A]-R	CCGCCAGATGACGCGCCTCTGCGTCCAGCTCTCCAGGAGC

TABLE 2

Oligonucleotide sequences for the SelK mutants used in this study

F indicates forward, and R indicates reverse. GenBank™ accession number BC013162.

SelK mutants	Primer (5′ → 3′)
Cloning for SelK U92C	
SelK U92C-F	GCGGATCCATGGTTTACATCTCGAACGG
SelK U92C-R	GCCTCGAGTTACCTTCCGCATCCACCAGCC
Point mutants of SelK	
mtSelK 1-F	GATGGAAGAGGGGCAGCAGGAAACGCTGCCCGAAGAATGGG
mtSelK 1-R	CCCATTCTTCGGGCAGCGTTTCCTGCTGCCCCTCTTCCATC
mtSelK 2-F	CATCTGCGTGGCGCTAGTGCCGCTGCAATGGCTGGTGG
mtSelK 2-R	CCACCAGCCATTGCAGCGGCACTAGCGCCACGCAGATG

5′-GAA CCA GGA GTT AAG AAC ACG-3′, 5′-AGG CAA CAG TGT CAG AGT CC-3′; and GAPDH, 5′-CTG CAC CAC CAA CTG CTT AGC-3′, 5′-CTT CAC CAC CTT CTT GAT GTC-3′. The PCR products were loaded on a 2% agarose gel and were visualized by ethidium bromide staining.

Subcellular Fractionation—The cells were lysed using a ProteoJET membrane protein extraction kit (31). The cells were harvested with cold PBS, washed with cell wash solution, scraped with cell permeabilization buffer that contained protease inhibitors, and incubated at 4 °C for 10 min. Permeabilized cells were centrifuged at 16,000 × *g* for 15 min at 4 °C. The supernatants were collected to isolate the cytosolic fraction. The membrane protein extraction buffer was added to the pellet, which was then incubated on ice for 30 min and centrifuged at 16,000 × g for 15 min at 4 °C. After another centrifugation, supernatants were collected to isolate the membrane fraction. These cytosolic and membrane fractions were used for immunoblotting.

Antibodies and Immunoblot Analysis—The cells were then lysed as described in Ref. 32. The protein concentrations in the whole cell lysates and subcellular fractions were determined using Bradford reagent (Sigma-Aldrich). These lysates were separated on 8–12% SDS-PAGE, and the separated proteins were then transferred to a PVDF membrane and probed with specific antibodies. Antibodies were obtained from the following sources: anti-His and anti-HA antibodies were

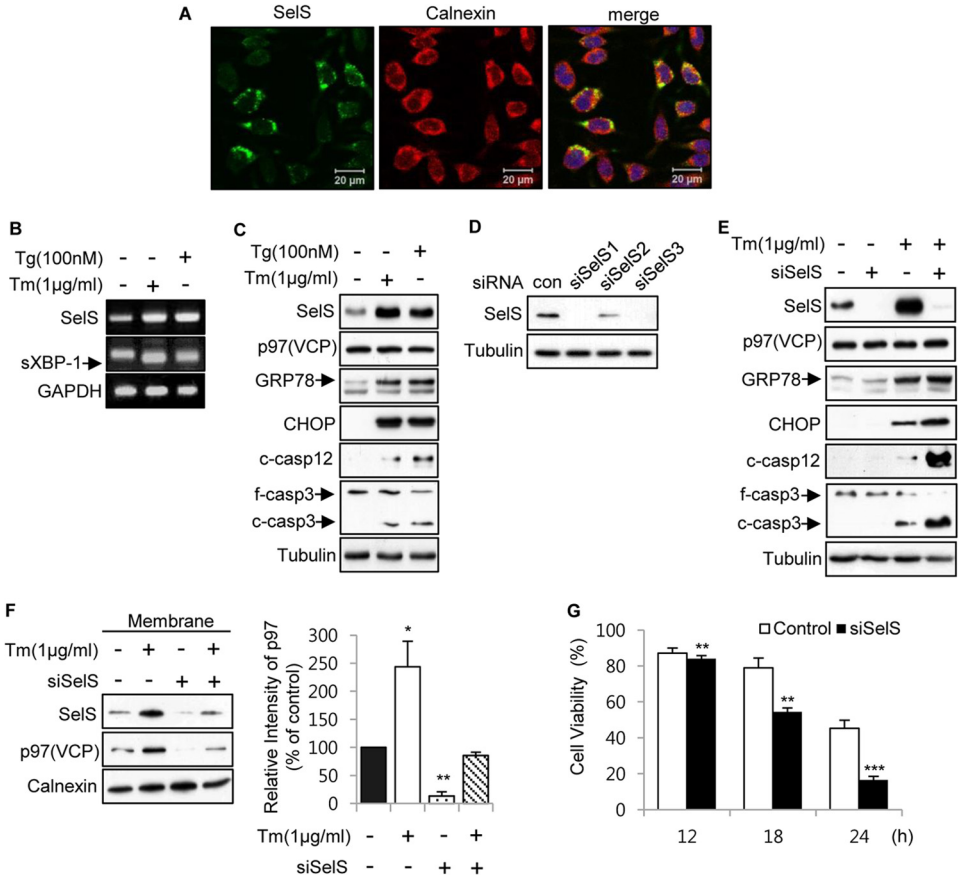

FIGURE 1. **SelS suppression enhances ER stress-induced apoptosis and inhibits translocation of p97(VCP).** *A*, confocal images from N2a cells were immunostained for SelS (*green*) and calnexin (*red*), which are endoplasmic reticulum markers. *B*, total RNAs isolated from N2a cells cultured in treatment with 1 μg/ml Tm or 100 nM Tg for 24 h was subjected to RT-PCR analysis for SelS, splicing XBP-1 (*sXBP-1*), and GAPDH mRNA. *C*, N2a cells in *B* were assessed by Western blotting. Whole cell lysates were detected by specific antibodies. *D*, N2a cells were transiently transfected with control siRNA (*con*) or siSelSs. Cells were harvested 48 h after transfection with three independent siSelSs. SelS expression was subsequently analyzed by Western blotting. Tubulin served as a loading control. *E*, N2a cells were transiently transfected with siSelS1 (*siSelS*) or control siRNA. These cells were treated with 1 μg/ml Tm for 24 h. N2a cells were assessed by Western blotting. Whole cell lysates were detected by specific antibodies. *F*, membrane fraction was prepared from N2a cells were transiently transfected with siSelS or control siRNA. These cells were assessed by Western blotting (*left panel*). p97(VCP) expression is represented as a percentage of the control in the membrane fraction (*right panel*). The graph indicates the results from three independent experiments (**, $p < 0.005$; *, $p < 0.05$). The *error bars* represent S.D., and the *p* values represent comparisons with the control. *G*, N2a cells were transfected with siSelS or control siRNA. These cells were treated with Tm (1 μg/ml) in a time-dependent manner. Cell viabilities were investigated using the MTT assay. Cell viability is expressed as the percentage relative to that of untreated cells. The graph indicates the results from six independent experiments (***, $p < 0.0001$; **, $p < 0.005$). The *error bars* in the graph represent S.D., and the *p* values represent comparisons of siSelS with control siRNA (*Control*).

obtained from ABM; anti-FLAG antibody was obtained from Sigma; anti-grp78, anti-Ub antibody, anti-α-tubulin, and anti-CHOP antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-caspase3 antibody was obtained from Cell Signaling Technology (Danvers, MA); anti-caspase12 antibody was obtained from Abcam (Cambridge, MA); anti-p97(VCP) antibody was obtained from Abnova; and anti-calnexin antibody was obtained from BD Biosciences (San Diego, CA).

We prepared rabbit polyclonal antibodies against SelS. To prepare these antibodies, two antigenic peptides were prepared (Peptron, Daejeon, Korea), and then a rabbit was injected with these peptides. The amino acid sequences of these peptides were: ^{128}KSYKGNAKKPQEEDSPG^{142} and ^{174}SWRPGRRGPSSGG^{187}.

Immunoprecipitation—Immunoprecipitation was performed as described previously with a slight modification (31). The proteins were precleared with protein G-agarose for 1 h at 4 °C, which was followed by incubation with 0.5 μg of His antibody overnight at 4 °C. Immune complexes were further incubated with protein G-agarose for 2 h at 4 °C and then washed with lysis buffer (150 mM EDTA, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 0.3% Nonidet P-40, with 50 mM Tris, pH 7.4, and 1 mM DTT) three times. For immunoblotting, proteins were boiled with SDS-PAGE sample buffer for 5 min. The samples were loaded onto SDS-PAGE gels, transferred to a PVDF membrane, and incubated with primary antibody at 4 °C overnight. 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 siSelS or plasmids. Then the cells were treated with 1 μg/ml Tm (Sigma-Aldrich) for 6 h after transfection. The medium was replaced with a medium containing 5 mg/ml of MTT at the indicated time points, and the cells were further incubated for 2 h at 37 °C. After incubation, DMSO was then added 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—Mutant SelS-transfected HEK293 cells 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-His antibody and anti-p97(VCP) antibody in 0.1% BSA overnight at 4 °C and washed three times with PBS, respectively, which was followed by incubation with a secondary rabbit FITC antibody and mouse Alexa Fluor 546 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 using mounting solution. Immunofluorescence was examined using a fluorescence microscope (Zeiss LSM 700 META).

Data Analysis and Statistics—All of the results are represented in this study as the means and standard deviations of the control value. Statistical comparisons from at least three independent experiments were determined using Student's *t* tests. *p* values < 0.05 were considered significant.

RESULTS

Recruitment of p97(VCP) to the ER membrane Is Regulated by SelS expression—SelS functions in cell survival by regulating ER stress (33). ER stress has been suggested to be involved in neurodegenerative disorders, such as Alzheimer disease and Parkinson disease (34). N2a cells have been useful for elucidating several neurodegenerative disorders (35). Therefore, we used these cells to investigate the function of SelS in ER stress. SelS is an ER membrane protein and a member of the ERAD complex (11). We also confirmed that SelS was localized in the ER membrane of N2a cells (Fig. 1*A*). In addition, we found that the mRNA levels of SelS and sXBP-1 (splicing XBP-1) increased in N2a cells when treated with 1 μg/ml Tm or 100 nM Tg for 24 h (Fig. 1*B*). We also found that the expression levels of SelS, glucose-regulated protein 78 (GRP78, an ER chaperone), C/EBP-homologous protein (CHOP, a pro-apoptosis protein), cleaved caspase12 (c-casp12, an ER stress-dependent pro-apoptosis protein), and full-length and cleaved caspase3 (f- and c-casp3, respectively, critical executioners of apoptosis) increased in N2a cells when treated with 1 μg/ml Tm or 100 nM Tg for 24 h. It has been previously reported that the expression of such proteins increases (27, 33, 36, 37) (Fig. 1*C*). In this experiment, SelS knockdown cells were prepared by introducing three independent siRNAs against SelS into cells. SelS expression was efficiently reduced by siSelS1 and siSelS3 in N2a cells for 72 h (Fig. 1*D*). SelS knockdown cells were more sensitive to ER stress compared with that of control cells (Fig. 1*E*). However, the p97(VCP) expression level was not changed by SelS knockdown (Fig. 1, *C* and *E*). Nevertheless, the amount of p97(VCP) in the membrane fraction increased with SelS during the ER stress condition, as shown in Fig. 1*F*. Localization of p97(VCP) in the membrane did not increase in SelS knockdown cells (Fig. 1*F*); therefore, the cytosolic p97(VCP) level was higher in SelS knockdown cells than that in control cells under ER stress conditions (data not shown). SelS-suppressed cells increased CHOP, c-casp12, and c-casp3 expression under the ER stress conditions, as shown in Fig. 1*E*. Furthermore, SelS knockdown cells were more sensitive to the ER stress compared with that of control cells (Fig. 1*G*).

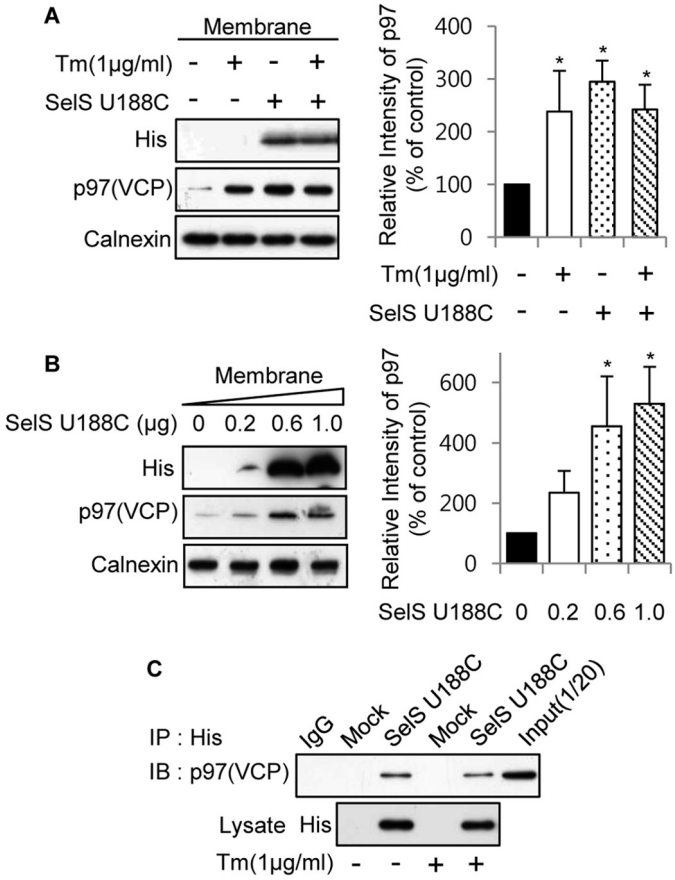

FIGURE 2. **Translocation of p97(VCP) requires interaction with SelS.** *A*, in the membrane fraction, N2a cells were transfected with the pcDNA3.1 control vector or with an expression vector containing His-tagged SelS U188C (1.0 μg). These cells were treated with or without Tm for 24 h. Lysates were detected by specific antibodies (*left panel*). p97(VCP) expression is represented as a percentage of the control in the membrane fraction (*right panel*). The graph indicates the results from three independent experiments (**, $p <$ 0.005; *, $p < 0.05$). The *error bars* represent S.D., and the *p* value represents a comparison with the control. *B*, in the membrane fraction, an increasing amount (0, 0.2, 0.6, and 1.0 μg) of His-tagged SelS U188C plasmid was transfected into untreated N2a cells. These cells were assessed by Western blotting (*left panel*). p97(VCP) expression is represented as a percentage of the control in the membrane fraction (*right panel*). The graph indicates the results from three independent experiments (**, $p < 0.005$; *, $p < 0.05$). The *error bars* represent S.D., and the *p* value represents a comparison with the control. *C*, N2a cells were transfected with His-tagged SelS U188C or with empty vector for the control (*Mock*). Cells were treated with or without Tm for 24 h. Lysates and immunoprecipitation samples (anti-His antibody) were analyzed by Western blotting with the indicated antibodies. *IP*, immunoprecipitation; *IB*, immunoblot.

Additionally, translocation of p97(VCP) to the membrane is important for protecting cells from ER stress (20–23). SelS plays a role in ERAD by its association with Derlins and by its ability to interact with p97(VCP) (11). In this study, we confirmed that SelS U188C was also able to recruit p97(VCP). Sec was replaced with Cys in the SelS U188C mutant. The expression of recombinant wild-type SelS was not easy to control because the codon for Sec, UGA, was often recognized as a stop codon (38). Cys^{188} in SelS U188C formed a disulfide bond with Cys^{174}, which is consistent with the selenosulfide bond in wild-type SelS (9, 39). We confirmed that the physiological effect of

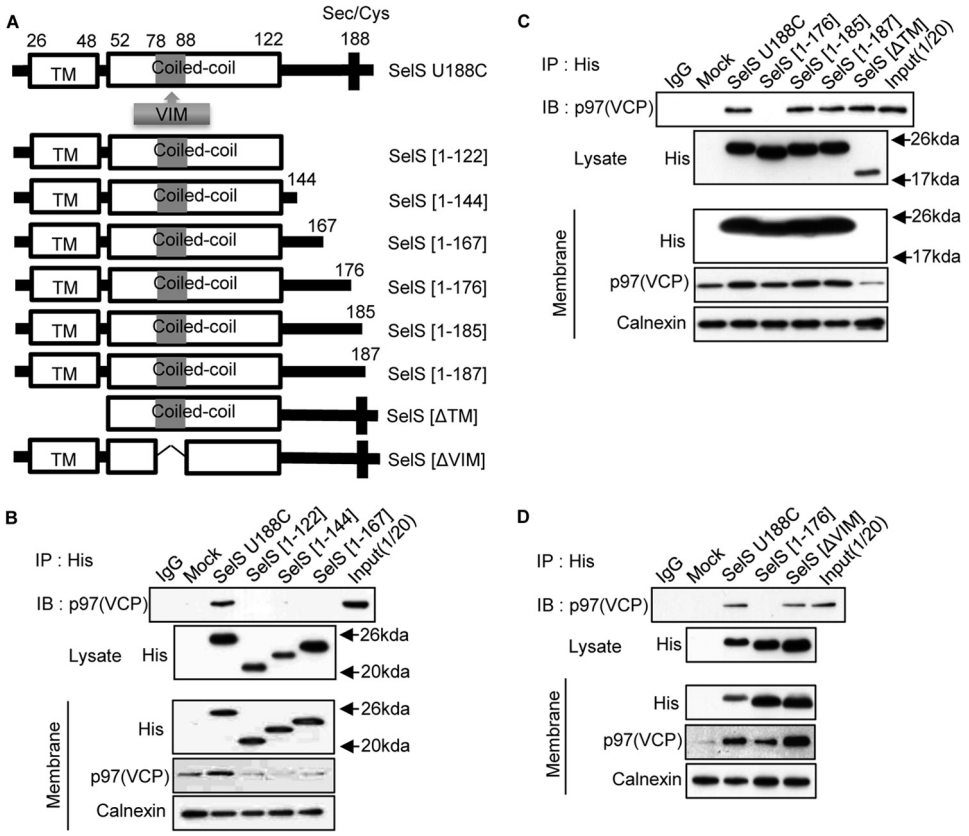

FIGURE 3. **Interaction between SelS mutants and p97(VCP).** *A*, schematic representation of the domain organization of SelS. The following elements of the domain structure are marked: TM region, coiled-coil domain (coiled-coil), and the tagged pcDNA 3.1 vector (His). The locations of Sec or Cys residues are indicated as *vertical bars*. *B–D*, N2a cells were transfected with His-tagged SelS U188C, which indicated mutants of SelS or the empty vector for the control (*Mock*). Lysates and immunoprecipitation samples (anti-His antibody) were analyzed by Western blotting with the indicated antibodies (*upper panels*). Translocation of p97(VCP) was compared with the membrane fraction and with transfection of SelS U188C or with the indicated SelS mutants (*lower panels*). *IP*, immunoprecipitation; *IB*, immunoblot.

SelS U188C was consistent with endogenous SelS (see Fig. 7). Therefore, we used SelS U188C in this study and found that translocation of p97(VCP) to the membrane was enhanced in N2a cells that overexpressed SelS U188C in both resting and ER stress conditions (Fig. 2*A*). Moreover, translocation of p97(VCP) was enhanced by the increased expression level of SelS U188C in the cells, regardless of ER stress (Fig. 2*B*). The interaction of SelS U188C with p97(VCP) was not dependent on ER stress (Fig. 2*C*). These results indicate that suppressing SelS enhances ER stress-induced apoptosis and inhibits translocation of p97(VCP) to the membrane. In addition, translocation of p97(VCP) to the membrane required the expression of SelS U188C, regardless of ER stress.

Both Pro^{178} and Pro^{183} Residues of SelS Are Essential for Interacting with p97(VCP)—As described above, we found that translocation of p97(VCP) to the ER membrane was regulated by an interaction with SelS. However, the regulatory mechanism for this interaction remains unknown. We next aimed to identify the binding site of SelS to p97(VCP). Human SelS has a VIM, a coiled-coil domain interface, and an unstructured C-terminal region with an internal selenosulfide bond (10, 13). To find the binding region or SelS residue to p97(VCP), we constructed various plasmids that contained His-tagged SelS mutants that were defective in VIM or TM or were downstream of the coiled-coil domain (Fig. 3*A*). The deletion mutants were designated SelS[1–122], SelS[1–144], SelS[1–167], SelS[1–176], SelS[1–185], SelS[1–187], SelS[ΔTM], and SelS[ΔVIM]. To test whether the mutants interacted with p97(VCP), N2a cells were transfected with His-tagged SelS U188C and the deletion mutants. The interaction was then determined by immunoprecipitation of the cell lysates with anti-His antibody. The presence of p97(VCP) was determined by Western blot analysis using anti-p97(VCP) antibody. The membrane fraction was also prepared using a membrane protein extraction kit (31) and analyzed by immunoblotting using anti-p97(VCP) antibody. SelS[1–122], SelS[1–144], and SelS[1–167] did not interact with p97(VCP) and could not recruit p97(VCP) to the membrane (Fig. 3*B*). This result indicates that the binding site for p97(VCP) might be downstream of amino acid 168 on the C-terminal end. SelS[1–185], SelS[1–187], and SelS[ΔVIM] interacted with p97(VCP) and recruited p97(VCP) to the membrane. In the case of SelS[ΔTM], the mutant was able to interact with p97(VCP), although the complex was localized only in the cytosol because of deletion of the transmembrane domain. However, SelS[1–176] did not interact with p97(VCP), and p97(VCP) could not be recruited to the membrane (Fig. 3*C*). Unexpectedly, deletion of VIM did not affect the binding activity of SelS. Thus, SelS[ΔVIM] maintained the function of p97(VCP) binding (Fig. 3*D*). These results suggest that the region between amino acids 177 and 185 is important for reg-

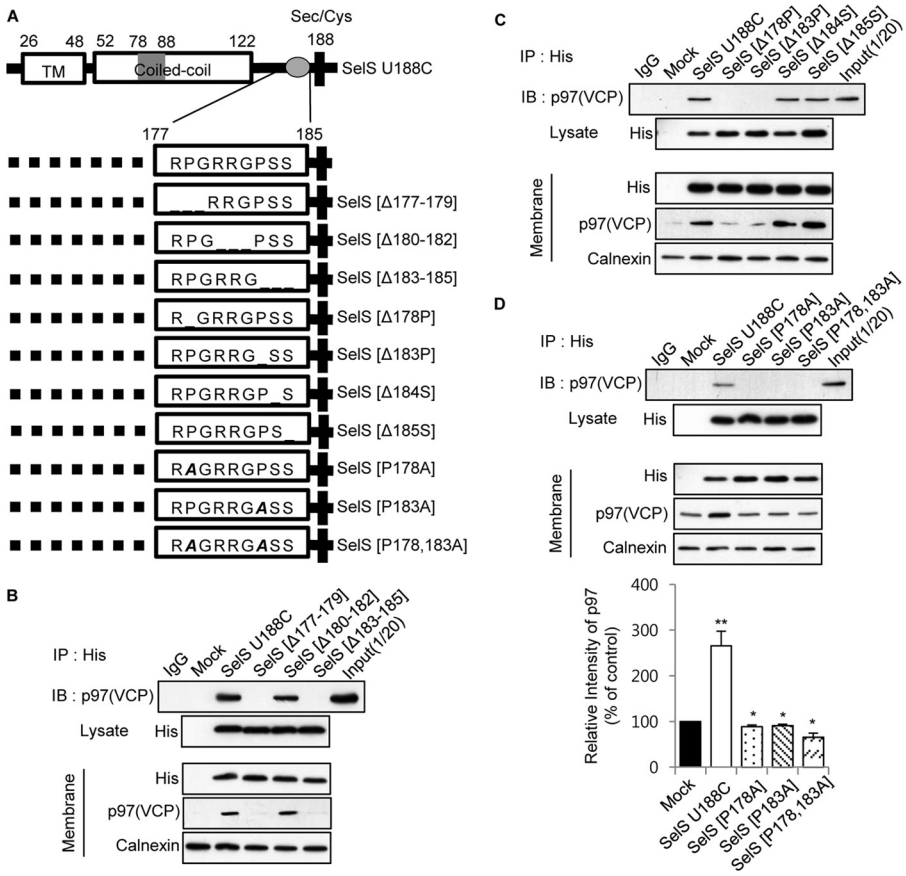

FIGURE 4. **Both Pro^{178} and Pro^{183} of SelS are essential for p97(VCP) binding.** *A*, schematic representation of the domain organization of SelS. The following elements of the domain structure are marked as in Fig. 3. The *lower panel* displays the mutant form of SelS, the constructs that code 11 sites of the cytosolic tail region (178–185). *B* and *C*, N2a cells were transfected with His-tagged SelS U188C, which indicates the SelS mutants or the empty vector for the control (*Mock*). Lysates and immunoprecipitation samples (anti-His antibody) were analyzed by Western blotting with the indicated antibodies (*upper panels*). Translocation of p97(VCP) was compared by the membrane fraction with transfection of SelS U188C or with the indicted SelS mutants (*lower panels*). *D*, N2a cells were transfected with His-tagged SelS U188C, which indicates the SelS mutants or the empty vector for the control (*Mock*). Lysates and immunoprecipitation samples (anti-His antibody) were analyzed by Western blotting (*top panel*). Translocation of p97(VCP) was compared by the membrane fraction with the transfection of SelS U188C or with the indicted SelS mutants (*middle panel*). p97(VCP) expression is represented as a percentage of the control in the membrane fraction (*bottom panel*). The graph indicates the results from three independent experiments (**, $p < 0.005$; *, $p < 0.05$). The *error bars* represent S.D., and the *p* values represent comparisons with the control. *IP*, immunoprecipitation; *IB*, immunoblot.

ulating the translocation of p97(VCP) to the membrane. Next, we further constructed deletion or point mutants in this region to find specific interaction sites for translocation of p97(VCP), as shown in Fig. 4*A*. The sequence between amino acids 177 and 185 of human SelS is RPGRRGPSS. Among the deletion mutants in this sequence, only SelS[Δ180–182] had the ability to interact with p97(VCP) (Fig. 4*B*). Additionally, we observed that the proline residues at Pro^{178} and Pro^{183} were both essential for binding to p97(VCP). Thus, when either of the residues was deleted or changed to alanine, the mutant lost the ability to bind to p97(VCP) and to recruit the protein to the membrane (Fig. 4, *C* and *D*). SelS is distributed throughout the ER membrane, whereas p97(VCP) is primarily in the cytosol (11). To confirm the interaction between SelS mutants and p97(VCP), HEK293 cells were transfected with plasmids encoding SelS U188C, SelS[ΔVIM], or SelS[P178,183A], and localization of the proteins was observed (Fig. 5). All SelS mutants formed aggregates around the nucleus, which were reminiscent of the ER structure. The endogenous p97(VCP) of cells expressing SelS U188C or SelS[ΔVIM] co-localized with SelS mutants in dotted structures (Fig. 5, *A* and *B*). However, the endogenous

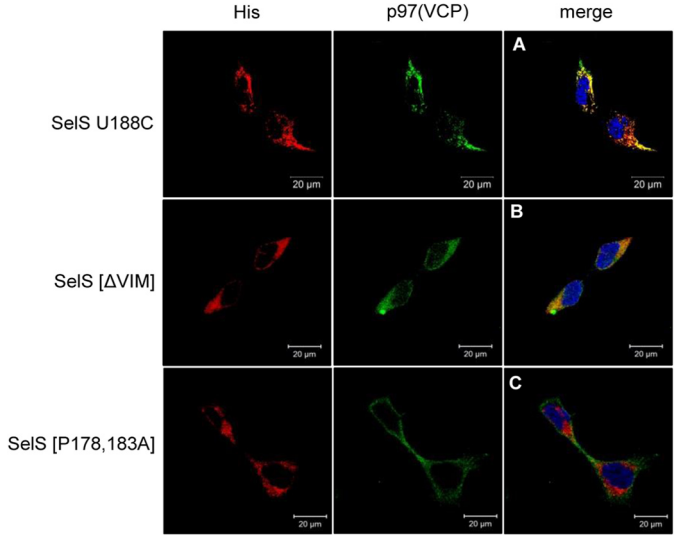

FIGURE 5. **Co-localization of endogenous p97(VCP) and SelS mutants in HEK293 cells.** HEK293 cells were transfected with His-tagged SelS U188C or with the indicated SelS mutants. Cells were fixed and stained for His (*red*), p97(VCP) (*green*), and DAPI (*blue*). Images of the samples were obtained using a confocal microscope. *Panels A, B,* and *C* are explained under "Results."

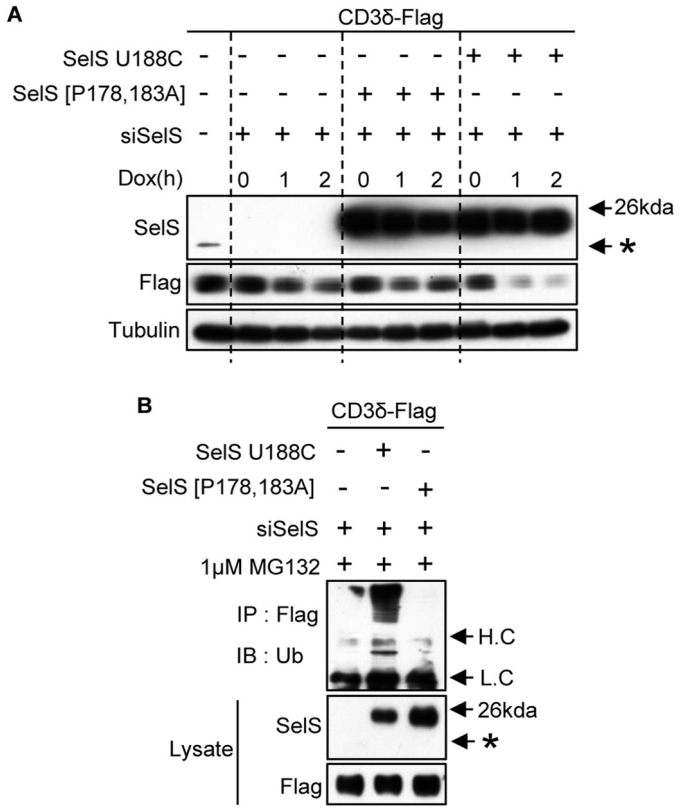

FIGURE 6. **Both Pro^{178} and Pro^{183} of SelS are important for ERAD substrate degradation.** *A*, N2a cells were co-transfected with siSelS and His-tagged SelS U188C or SelS[P178,183A] for 16 h and then co-transfected with CD3δ-FLAG and pTet-off. At 40 h after transfection, the cells were treated with 50 μg/ml doxycycline (*Dox*) for the indicated times. Whole cell lysates were detected by specific antibodies. *B*, N2a cells were co-transfected with siSelS and SelS U188C or SelS[P178,183A] for 16 h and then co-transfected with CD3δ-FLAG 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. * indicates endogenous SelS. *IP*, immunoprecipitation; *IB*, immunoblot.

cytosolic p97(VCP) did not co-localize with SelS[P178,183A] (Fig. 5*C*). These results suggest that both Pro^{178} and Pro^{183} in the cytosolic tail of SelS play key roles in interacting with p97(VCP) and in translocating p97(VCP) to the ER membrane.

SelS Has a Function in ERAD by Interacting with p97(VCP) on the ER Membrane—The p97(VCP) complex plays an important role in ERAD. When degraded misfolded proteins (ERAD substrates, such as CD3δ) are translocated through the ER membrane, the p97(VCP) complex hands them to the proteosome (21, 24, 40). SelS interacts with p97(VCP), and this interaction recruits p97(VCP) to the ER membrane (15, 16). Therefore, we examined the function of SelS in the ERAD pathway. Using the CD3δ-FLAG Tet-off system (25), we observed degradation of CD3δ in N2a cells. These cells were co-transfected with siSelS and SelS U188C/SelS[P178,183A] or siSelS alone. After a 24-h co-transfection, we blocked synthesis of CD3δ by treating the cells with 50 μg/ml doxycycline for the indicated times. Fig. 6 shows that CD3δ was slightly degraded or not in siSelS alone cells and siSelS + SelS[P178,183A] cells. However, degradation of CD3δ increased significantly in siSelS + SelS U188C co-transfected cells (Fig. 6*A*). Moreover, we found that ubiquitination of CD3δ only occurred in siSelS + SelS U188C co-transfected cells (Fig. 6*B*). These data suggest that the interaction between SelS and p97(VCP) is essential for ERAD function and that both Pro^{178} and Pro^{183} residues of SelS play a key role in ERAD through interaction with p97(VCP).

Silencing of p97(VCP) increases ER stress (23), and we found the function of p97(VCP) in ER stress and ER stress-mediated apoptosis (Fig. 7). In this experiment, p97(VCP) knockdown cells were prepared by introducing three independent siRNAs against p97(VCP) into cells. p97(VCP) expression was efficiently reduced by sip97(VCP)1 and sip97(VCP)2 in N2a cells after 72 h (Fig. 7*A*). Although endogenous SelS or SelS mutants (SelS U188C or SelS[P178,183A]) increased by treatment with Tm (1 μg/ml) or by transfection with His-tagged SelS mutants, ER stress-mediated apoptosis remained high in p97(VCP) knockdown cells (Fig. 7*B*). For these experiments, we knocked down SelS in N2a cells and then ectopically expressed the His-tagged human SelS mutants, followed by treatment with Tm (1 μg/ml). CHOP, c-casp12, and c-casp3 were expressed by Tm treatment, and its expression increased following SelS knockdown. These increases in pro-apoptosis molecule expression were restored by SelS U188C (Fig. 7*C*). However, CHOP, c-casp12, and c-casp3 expression were not repressed in cells that expressed proline mutants (Fig. 7*C*), SelS[P178A], SelS[P183A], or SelS P178,183A], which did not interact with p97 (VCP) (Fig. 4*D*). CHOP, c-casp12, and c-casp3 expression was restored in cells that expressed SelS[ΔVIM], which interacted with p97(VCP) (Fig. 3*D*). These results indicate that SelS mutants that were capable of interacting with p97(VCP) repressed pro-apoptosis molecule expression. However, CHOP, c-casp12, and c-casp3 expression did not decrease in the cells that expressed SelS[ΔTM] (Fig. 7*C*) because the transmembrane domain was deleted in this mutant, and the complex was localized only in the cytosol. CHOP, c-casp12, and c-casp3 expression of cells that expressed SelS U188C or SelS[ΔVIM] was similar to that of control cells. The viability of cells expressing SelS U188C or SelS[ΔVIM] was also the same as that of control cells. Overexpression of proline mutants, SelS[P178A], SelS[P183A], and SelS[P178,183A], could not rescue the SelS silencing effects (Fig. 7*D*).

Proline Residues of SelK Are Also Essential for Interacting with p97(VCP)—SelK has been reported as another ER selenoprotein that interacts with ERAD components, including p97(VCP) (10). Although SelK does not have a predicted VIM or a coiled-coil domain (13), there is a similarity in domain organization between SelS and SelK. In particular, the cytosolic tails of both selenoproteins have a region termed the glycine-rich region that has an undefined secondary structure and is rich in glycine, proline, and positively charged amino acids (10). We found that Pro^{178} and Pro^{183} of SelS, on the glycine-rich region, were essential residues for interacting with p97(VCP) (Fig. 4). To determine whether proline residues in the cytosolic tail were also important for SelK binding to p97(VCP), we constructed three plasmids that contained His-tagged SelK mutants that were changed from Sec to Cys (SelK U92C) or from Pro to Ala, in the cytosolic tail of SelK (mtSelK1, Pro to Ala at residues 66, 67, 70, and 71; mtSelK2, Pro to Ala at residues 83, 85, 86, and 87) (Fig. 8*A*). SelK U92C interacted with p97(VCP), whereas the interaction of the mutants mtSelK1 and mtSelK2 was very weak or not

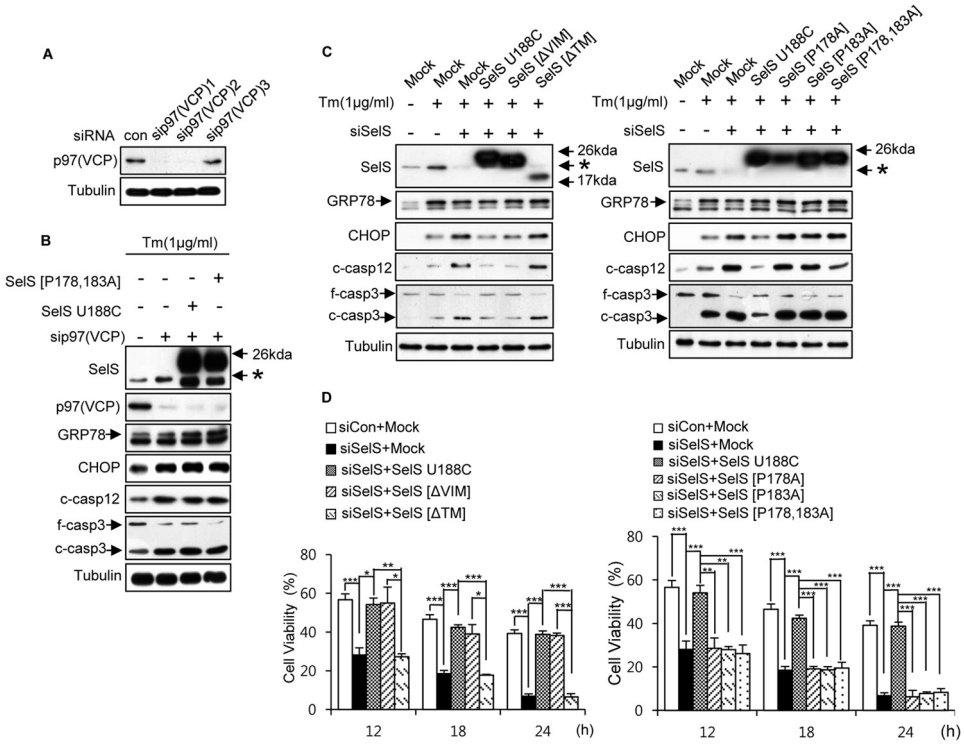

FIGURE 7. **SelS proline mutants cannot recover the effect of SelS knockdown on ER stress-induced cell death and apoptosis.** *A*, N2a cells were transiently transfected with control siRNA (*con*) or sip97(VCP)s. Cells were harvested 48 h after transfection with three independent sip97(VCP)s. p97(VCP) expression was subsequently analyzed by Western blotting. Tubulin served as a loading control. *B*, N2a cells were co-transfected with sip97(VCP) and His-tagged SelS U188C/SelS[P178,183A] or with sip97(VCP) alone. These cells were treated with Tm for 24 h. * indicates endogenous SelS. *C*, N2a cells were co-transfected with siSelS and His-tagged SelS U188C/indicated mutants of SelS or siSelS alone. These cells were treated with Tm for 24 h. *B* and *C*, cell lysates isolated from these cells were analyzed by Western blotting with the indicated antibodies. * indicates endogenous SelS. *D*, N2a cells were co-transfected with siSelS and the indicated SelS or siSelS mutants alone. These cells were treated with Tm in a time-dependent manner. Cell viability was determined by the MTT assay. Cell viability is expressed as the percentage relative to that of untreated cells. The graph indicates the results from six independent experiments (***, $p < 0.0001$; **, $p < 0.005$; *, $p < 0.05$). The *error bars* represent S.D., and the *p* values represent comparisons with each control group.

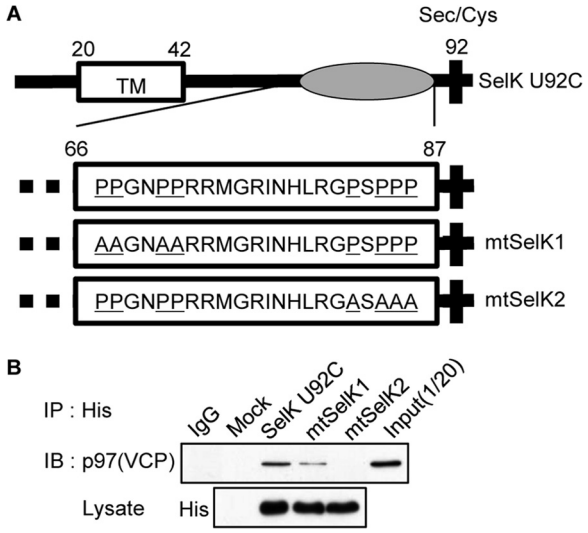

FIGURE 8. **SelK proline residues are essential for the interaction with p97(VCP).** *A*, schematic representation of the domain organization of SelK. The following elements of the domain structure are marked: TM region and the tagged pcDNA 3.1 vector (*His*). Locations for Sec or Cys residues are shown in *bold*. The *lower panel* displays the SelK mutant form, the constructs that code 22 sites of the cytosolic tail region (66–87). *B*, N2a cells were transfected with His-tagged SelK U92C, proline mutant SelK (mtSelK1 or mtSelK2), or the empty vector for the control (*Mock*). Lysates and immunoprecipitation samples (anti-His antibody) were analyzed by Western blotting with the indicated antibodies. *IP*, immunoprecipitation; *IB*, immunoblot.

detected (Fig. 8*B*). These results suggest that proline residues of SelK are also important to interact with p97(VCP).

DISCUSSION

We demonstrated that both proline residues 178 and 183 of SelS are essential for its function in ERAD via p97(VCP) binding. A consensus sequence, RX_5AAX_2R (where *X* indicates any residue), has been defined as the core of a p97(VCP)-binding motif called VIM (41–43), and human SelS has a predictable VIM between residues 78 and 88 (9). However, the VIM-deleted SelS mutant (SelS[ΔVIM]) was unexpectedly able to interact with p97(VCP) and to recruit the protein to the ER membrane (Figs. 3*D* and 5). We found that Pro^{178} and Pro^{183} of SelS were essential residues for interacting with p97(VCP) (Fig. 4, *C* and *D*). According to the predicted structure of the cytosolic region, SelS had an intrinsically disordered region with a reductase function, and a selenosulfide bond was expected between Cys^{174} and Sec^{188} in the intrinsically disordered region (9). Proline residues have an important role in the structural property of this region (44–46). Therefore, mutations of either Pro^{178} or Pro^{183} might cause a structural alteration, which might include a selenosulfide bond in the region, such that SelS loses p97 (VCP) binding activity.

SelK is another selenoprotein that contains a Sec at the penultimate position in the cytosolic domain (7, 12, 47) and is involved in various cellular events, such as protection against

oxidative stress (48, 49), calcium regulation, and the ERAD pathway (10, 50). SelK is an ER protein, and a single-pass transmembrane domain was predicted in this molecule (13). Similar to SelS, SelK is regulated by ER stress and interacts with p97(VCP) (10). However, there is no sequence similarity between SelK and SelS (10). For example, VIM and cysteine residues are not observed in SelK. Nevertheless, both proteins have a G-rich domain, which is rich in glycine and proline in the cytosolic tail (10, 13). Together with our findings that both prolines at positions 178 and 183 are crucial for the interaction between SelS and p97(VCP) (Fig. 4), the prolines at the cytosolic tail of SelK are also essential for the interaction of SelK with p97(VCP) (Fig. 8). The G-rich region of both proteins may be primarily involved in recruiting p97(VCP) to the ER membrane. Further studies are required to understand whether p97(VCP) binds proline directly or through the proline-mediated structure of the cytosolic tail.

Various adaptor proteins interact with p97(VCP). These p97(VCP) complexes have different functions in cellular pathways (51, 52). p97(VCP) was critical during the ER stress response in N2a cells, particularly in the ERAD, Thus, the silencing effect of p97(VCP) significantly increased ER stress-mediated apoptosis in N2a cells (Fig. 7*B*). The co-factors of p97(VCP), Ufd1, and Npl4 are essential for p97(VCP)-mediated ubiquitin-dependent processes (53). Thus, in the first step, p97(VCP) was translocated to the ER membrane through the interaction with increased SelS (Figs. 1*F*, 2, and 5). Second, p97(VCP) extracts the misfolded protein from the ER to the cytosol (12). Finally, Fas-associated factor 1 (FAF1) selectively binds to the N-terminal domain of p97(VCP), and this interaction regulates the recruitment of polyubiquitinated misfolded proteins (25). These ERAD steps are required to translocate p97(VCP), and these steps play a key role restoring ER stress (Figs. 6 and 7) (20–23).

When misfolded proteins are translocated through the ER membrane to the cytosol, the FAF1-p97(VCP)-Npl4-Ufd1 complex delivers ubiquitinated misfolded proteins to proteasomes (21, 24, 40). The interaction between p97(VCP) and FAF1 is regulated by the S3/S4 loop structure of FAF1 (25). Pro^{620} in the loop structure plays a key role in this interaction. Moreover, it is known that p97(VCP) adaptor proteins, such as p47, have the S3/S4 loop. In addition, Pro^{344} in the loop is an essential residue for p97(VCP) binding (54). These prolines are important for p97(VCP) binding proteins because the N-terminal domain of p97(VCP) requires an S3/S4 loop structure for binding. The precise structure of the cytosolic tail in SelS and the function of Cys^{174}–Sec^{188} selenosulfide-bond in SelS remain unclear. Therefore, further investigation is required.

It has been demonstrated that the accumulation of misfolded proteins in the ER induces apoptosis via ER stress (55). ERAD is the last chance to recover from ER stress through degradation of misfolded proteins. Additionally, translocation of p97(VCP) to the ER membrane is an essential step for ERAD (12, 40). We report here that Pro^{178} and Pro^{183} of SelS are essential residues that recruit p97(VCP) to the ER membrane.

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