The role of retromer complex in the unconventional trafficking of CFTR

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The role of retromer complex in the unconventional trafficking of CFTR

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The Master's Thesis
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

Ye Jin Kim

December 2022

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December 2022

ACKNOWLEDGEMENTS

졸업이라는 단어가 낯설고 실감이 나지 않지만, 학위 과정 동안 도움 주시고 기다려 주신 많은 분들께 정말 감사드립니다.
먼저 실험실 생활의 기회를 주시고 실험적으로도, 인성적으로도 성장할 수 있도록 여러 방면에서 많은 가르침을 주시고, 부족했던 저의 모습에도 믿고 기다려 주신 이민구 교수님 감사드립니다.

그리고 제가 졸업하기까지 실질적으로 많은 도움을 주시고 활발하게 토의하며 여러가지 문제에 있어서 항상 해결사가 되어 주셨던 노신혜 선생님께도 정말 많이 감사드립니다.

실험실에 적응할 수 있도록 도와 주신 김연정 선생님 엄소원 선생님, 항상 활발하게 소통하시는 송민석 선생님, 언제나 많이 도와주려고 하셨던 심주리 선생님, 편하게 대해 주셨던 유정우 선생님, 실험실 적응에 도와주셨던 김민재 선생님, 실험실을 위해 힘써 주시는 신선미 선생님, 도움이 필요할 때 흔쾌히 도와 주신 신동훈 선생님, 이영채 선생님, 저의 활기를 찾아 주셨던 은성호 선생님 감사합니다.
실험실 적응부터 시작하여 너무 많은 도움을 주시고 좋은 친구가 되어 주신 서수경 선생님께도 감사드립니다.

마지막으로 제가 어떤 길을 택해도 지지해주시고 응원해주신 부모님, 항상 제 편이 되어줬던 언니 에게도 감사함을 전합니다.

앞으로 실험실에서 배웠던 것들을 잊지 않고 잘 새기며 더욱 성장할 수 있도록 노력하겠습니다.

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ABSTRACT

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The most common disease-causing mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene is ΔF508-CFTR. Due to its folding defect, ΔF508-CFTR is rapidly decomposed by endoplasmic reticulum-associated degradation (ERAD) and is unable to reach the plasma membrane. Although our group has reported that ΔF508-CFTR can restore its function and reach the plasma membrane via the unconventional pathway under the ER stress or ER-to-Golgi blockade condition, the precise mechanism of this unconventional protein secretion (UPS) pathway remains to be further elucidated. In this study, we found that the retromer complex and sorting nexin (SNX) 27 play a crucial role in the unconventional trafficking of CFTR. The gene silencing results reveled that SNX27 and the vacuolar protein sorting subcomplex (VPS) 26-29-35, components of the retromer complex, are involved in the UPS of CFTR. We also found that SNX27 and CFTR physically interact and this interaction is augmented when UPS is activated. These results suggest that retromer components and SNX27 are required for the UPS of CFTR, which is mediated by the physical interaction between SNX27 and CFTR.

Key words : CFTR, unconventional protein secretion (UPS), retromer, SNX27

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I. INTRODUCTION

In general, proteins formed in the rough endoplasmic reticulum (ER) are destined to either be a part of the membrane via the ER-to-Golgi pathway or be secreted from the cell membrane out of the cell.[1] Proteins undergo ER quality control (ERQC) to determine if they have a folding defect; those with a folding defect are decomposed by ER-associated protein degradation (ERAD) and are unable to reach the Golgi apparatus.[2] Folding defects in proteins, such as cystic fibrosis transmembrane conductance regulator (CFTR), may lead to disorders.[3]

CFTR is a cAMP-activated anion channel responsible for chloride and bicarbonate transport at the apical membrane of epithelial cells.[4] ΔF508-CFTR, which has a deletion of the 508th phenylalanine, is the most common disease-causing mutation in the CFTR gene.[3] ΔF508-CFTR is degraded by ERAD and unable to reach the plasma membrane.[2] However, despite having a folding defect, ΔF508-CFTR can reach the plasma membrane under ER stress via unconventional protein secretion (UPS). Moreover, CFTR can function to some extent when it reaches the cell membrane via UPS. CFTR undergoes core glycosylation (band B) under ER stress, whereas CFTR undergoes complex glycosylation (band C) through conventional trafficking.[5]

ER stress can be induced by the overexpression of a dominant-negative mutant form of ADP-ribosylation factor 1 (ARF1), ARF1-Q71L.[6] ARF1 recruits the COP1 coat protein from the Golgi apparatus to the ER.[7] However, ARF1-Q71L induces the blockade of retrograde transport.[8] The proteins are accumulated in the Golgi apparatus and ER-Golgi intermediate compartment (ERGIC) by the overexpression of ARF1-Q71L, followed by ER-to-Golgi blockade.[8] The UPS mechanism of CFTR involves type 4 UPS bypassing the Golgi apparatus.[9] The UPS of CFTR is related to specific autophagy and an endosomal sorting complex required for transport (ESCRT).[10]

The retromer complex is a crucial protein coat of the endosomal protein sorting and trafficking machinery.[11] The retromer complex is assembled on endosomes and consists of the vacuolar protein sorting subcomplex 26-29-35 (VPS 26-29-35).[12] Moreover, the retromer complex is known to interact strongly with some sorting nexins (SNXs) found in mammals.[11] As SNXs have a Phox homology (PX) domain in common, they associate with the membrane through the PX domain or interact with other proteins using the specific motif of each SNX.[13] The retromer complex forms tubular vesicles that return the cargo proteins to the trans-Golgi network (TGN) or the plasma membrane.[14,15]

In this study, we assessed the role of the retromer complex and SNX27 in the UPS of CFTR. We found that SNX27 interacts with CFTR through its PDZ domain. This novel finding will help understand the precise unconventional trafficking process of CFTR and contribute to the treatment of diseases associated with protein secretion.

II. MATERIALS AND METHODS

1. Cell culture, plasmids, siRNAs, and transfection

HeLa and HEK293 cells were maintained in Dulbecco's modified Eagle medium (Gibco, #11995-065, Carlsbad, CA, USA) containing 10% fetal bovine serum (Gibco, #26140-079, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Gibco, #15140-122, Carlsbad, CA, USA). The cells were grown in a 5% CO2 incubator at 37℃.

pCMV-CFTR and HA-CFTR have been described previously.[5, 22] The coding region of ARF1-Q71L-HA was synthesized and inserted into pcDNA3. pCMV6-VPS26-GFP and pCMV-SNX27-GFP were commercially purchased (Origene #NM_004896, #NM_030918, Rockville, MD, USA), and the coding regions of VPS26 and SNX27 were ligated into the pCMV6-Entry vector by using BamH1 and Xho1 restriction enzyme sites. Domain-specific SNX27 constructs tagged with His_6 were generated by inserting the PCR fragments into the bacterial expression vector pET-28a(+) by using Nco1 and Xho1 restriction enzyme sites. The coding region of CFTR-Ct-30 amino acids was ligated into the pGEX4T1 vector.

Scrambled siRNA was purchased from Bioneer (Daejeon, Korea), and siRNAs targeting each gene were purchased from Dharmacon (ON-TARGETplus human SMARTpool siRNAs; VPS26 gene ID: 9559, SNX27 gene ID: 81609, VPS29 gene ID: 51699, VPS35 gene ID: 55737, SNX17 gene ID: 9784; Lafayette, CO, USA).

Plasmids were transfected into HeLa and HEK293 cells by using Lipofectamine LTX Reagent (Invitrogen, #15338-100, Carlsbad, CA, USA), and siRNAs were transfected into HEK293 cells by using RNAiMAX transfection reagent (Invitrogen, #13778-150, Carlsbad, CA, USA), according to the manufacturer's protocol.

2. Chemicals and antibodies

Dynasore (Sigma Aldrich, #D7695) and Ponceau S solution (Sigma Aldrich, #P7170) were purchased commercially.

Anti-CFTR M3A7 (Millipore, #05-583, Billerica, MA, USA), anti-CFTR ACL006 (Alomone Labs, #ACL006, Jerusalem, Israel), anti-aldolase A (Santa Cruz, #sc-390733, Dallas, TX, USA), anti-HA (Cell Signaling Technology, #2367, Danvers, MA, USA), anti-DYK (Cell Signaling Technology, #2368), anti-Myc (Cell Signaling Technology, #2276), anti-SNX27 (Abcam, #ab77799), anti-VPS26 (Abcam, #ab23892), anti-VPS29 (Abcam, #ab236796), and anti-VPS35 (Abcam, #ab10099) were purchased commercially.

3. RNA extraction and quantitative polymerase chain reaction (qPCR)

RNA was extracted from the cells using an RNA extraction kit (Bioneer, #K-3140, Daejeon, Korea), according to the manufacturer's protocol. cDNA synthesis from the extracted RNA was performed using cDNA EcoDry Premix (Takara, #639549, San Jose, CA, USA), according to the manufacturer's protocol.

The primer sequences used for qPCR were as follows: GAPDH, forward primer 5′- GTC TCC TCT GAC TTC AAC AGC G-3′, reverse primer 5′- ACC ACC CTG TTG CTG TAG CCA A -3′; SNX27, forward primer 5′- ATG GTG TGT CCG ACG TAG AGC T -3′, reverse primer 5′- TGT CCA TGC CAA CCT TTG CTG C -3′; VPS26, forward primer 5′- GAG GCT AGA ACA CCA AGG AAT TAG -3′, reverse primer 5′- CTG CTC TGA GTC AGT TCT CCA G -3′; VPS29, forward primer 5′- CTC AAG ACT CTG GCT GGT GAT G -3′, reverse primer 5′- CTG TCC AAC AGT CAC AAC TTT CTG -3′; VPS35, forward primer 5′- TGC TGA TGA GCA GAG CCT TGT G -3′, reverse primer 5′- CAG TGT GAA GCG AAT CCG CTG A -3′; and SNX17, forward primer 5′- GCT GCA AAG CTG GAT CTT CCA G -3′, reverse primer 5′- GGT GAC AGA CAC ATA AGG CAG C -3′.

4. Surface biotinylation and immunoblotting

For the biotinylation assay, HEK293 cells grown in six-well plate were washed twice with cold phosphate-buffered saline (PBS). The transmembrane proteins of the cells were biotinylated with Sulfo-NHS-SS-Biotin (Thermo Pierce, #21331, Waltham, MA, USA) in cold PBS (0.3 mg/ml biotin in PBS) for 30 min on ice in the dark. Following this, the cells were incubated with quenching buffer containing 1% BSA in cold PBS for 10 min in the dark and washed thrice with cold PBS. The cells were harvested with lysis buffer containing 150 mM NaCl, 20 mM Tris (pH 7.4), 1 mM EDTA, 1% (v:v) NP40, 0.5% (v:v) sodium deoxycholate, and a protease inhibitor (Roche, #04693159001). The harvested cells were homogenized with a sonicator for 20 s and centrifuged at 13,200 rpm for 20 min at 4°C. The supernatant of the lysate was collected, and 300 µg of the lysate was incubated at 4°C overnight with 300 µl of 10% streptavidin agarose resin (Thermo Pierce, #20349). The biotinylated protein-bound resin was centrifuged and washed four times with lysis buffer. Biotinylated proteins were eluted with 2x sodium dodecyl sulfate (SDS) sample buffer containing dithiothreitol (DTT, 0.02 g/ml) at 38°C for 40 min and then separated by SDS-polyacrylamide gel electrophoresis (PAGE). The separated proteins were transferred to a nitrocellulose membrane, and the membrane was blotted with the appropriate primary antibodies and HRP-conjugated secondary antibodies in 5% skim milk.

5. Immunoprecipitation assay

HEK293 cells were transfected for 24 h and, washed thrice with cold PBS. The cells were harvested with lysis buffer containing 150 mM NaCl, 20 mM Tris (pH 7.4), 1 mM EDTA, 1% (v:v) NP40, and a protease inhibitor (Roche, #04693159001); homogenized with a sonicator for 20 s and centrifuged at 13,200 rpm for 20 min at 4°C. The supernatant of the lysate was collected, and 400 µg of the lysate was incubated at 4°C overnight with 300 µl of 5% Ez view red anti-HA affinity gel (Millipore, #E6779, Billerica, MA, USA). The protein-bound resin was centrifuged,

washed four times with lysis buffer, and eluted with 2x SDS sample buffer containing DTT (0.02g/ml) at 38°C for 40 min. Following this, it was separated by SDS-PAGE and immunoblotted as described previously.

6. Pull-down assay

The recombinant proteins were produced in the BL-21(DE3) *Escherichia coli* strain. The recombinant fusion proteins were induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 30°C for 6 h. The proteins were then purified using Glutathione Sepharose™ beads (GE Healthcare, #17-0756) or Nickel-Nitrilotriacetic acid agarose (Ni-NTA agarose) beads (QIAGEN, #30210), according to the manufacturer's protocol. The elution buffer of His_6-fusion proteins was replaced with a dialysis buffer containing 50 mM Tris (pH 7.4), 150mM NaCl, and 0.05% (v:v) NP40. Following this, 10μg of the eluted His_6-fusion proteins were incubated with Glutathione Sepharose™ bead-bound GST-fusion proteins overnight at 4°C. The bead-bound proteins were then washed four times with dialysis buffer, and eluted in 2x SDS sample buffer and immunoblotted, as described previously.

7. Immunofluorescence assay

HeLa cells were incubated on an 18-mm coverslip and transfected for 24 h. The cells were fixed with 4% paraformaldehyde for 7 min and permeabilized with 0.2% Triton X-100 in PBS for 5 min. Following this, the cells were washed thrice with PBS and incubated with blocking buffer containing 1% BSA and 5% horse serum for 1 h at room temperature. The cells were incubated with the appropriate primary antibodies for 1 h at room temperature and washed thrice with PBS. Then, the cells were stained with secondary antibodies conjugated with a fluorophore for 30 min at room temperature and washed thrice with PBS. The sample was mounted on a slide glass with mounting medium (Agilent Dako, #S3025, Santa Clara, CA, USA), and the images were captured using a confocal microscope (LSM 980, Carl Zeiss, Berlin, Germany) with a 63× 1.4 numerical aperture oil objective lens.

8. Statistical analysis

The degree of colocalization between CFTR and SNX27 was quantified with Manders' colocalization coefficients (MCC)[35] using ZEN 2012 software (black edition; Carl Zeiss, Berlin, Germany).

The results of multiple experiments are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using a two-tailed Student's t-test or one-way analysis of variance followed by Tukey's multiple comparison test as appropriate, using GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

III. RESULTS

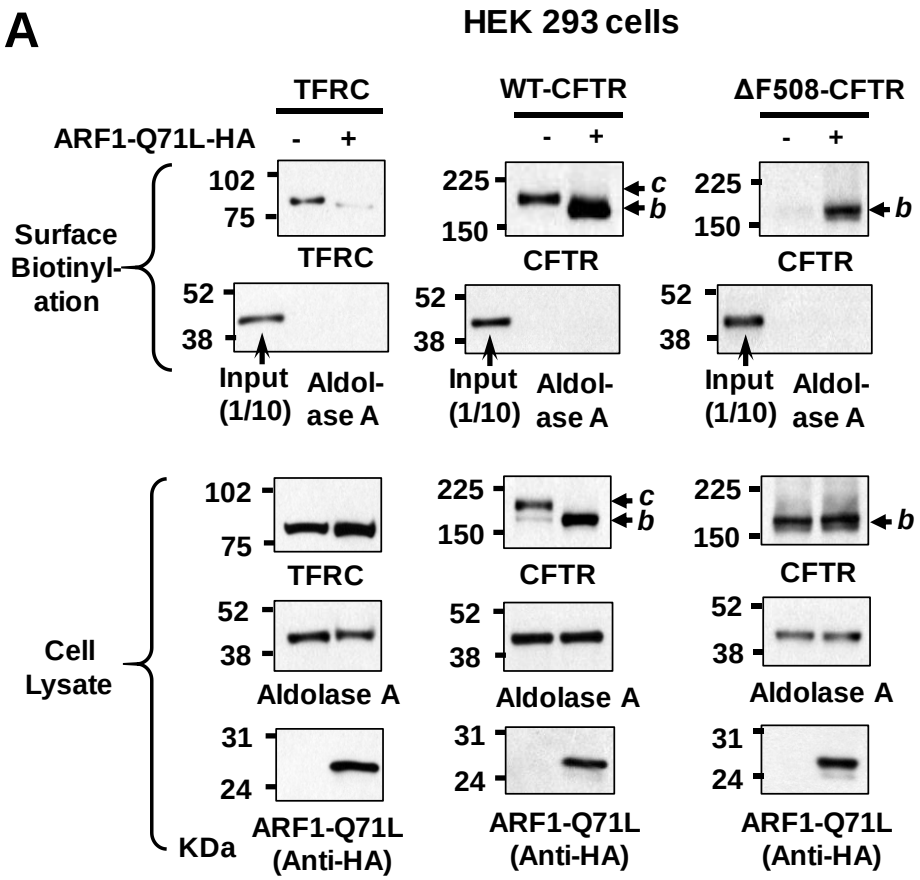
1. The retromer complex and SNX27 are involved in the UPS, but not in the conventional protein secretion, of CFTR.

We initially conducted a control experiment for this study. The dominant-negative ARF1 mutant ARF1-Q71L is capable of inducing ER stress by ER-to-Golgi blockade.[5] Unconventional trafficking of ΔF508-CFTR was induced by the overexpression of ARF1-Q71L. In the control experiment, ARF1-Q71L induced the cell surface expression of core-glycosylated ΔF508-CFTR and core-glycosylated WT CFTR but abolished the cell surface expression of transferrin receptor, a non-UPS cargo protein (Figure 1A, B).

Next, we assessed the effects of retromer complex components on the UPS of CFTR. The silencing of VPS26, VPS29, VPS35, and SNX27 genes inhibited the unconventional trafficking of ΔF508-CFTR caused by the overexpression of ARF1-Q71L (Figure 2A-H). The silencing of each retromer component was validated by real time qPCR-based mRNA quantification (Figure 2I).

To assess the effects of other SNXs on the UPS of CFTR, we performed a surface biotinylation assay with silencing of the SNX17 gene, which has a similar construct to the SNX27 gene but without the PDZ domain. ARF1-Q71L-induced cell surface expression of ΔF508-CFTR was unaffected by the knockdown of SNX17 (Figure 3A, B). qPCR-based mRNA quantification confirmed the knockdown of SNX17 (Figure 3C).

However, as the silencing of genes involved in endosomal protein recycling may affect endocytosis, the surface biotinylation assay was performed by treatment with dynasore, which blocks endocytosis,[16] in order to assess the data without considering the impact of endocytosis. We found that the amount of ΔF508-CFTR on the cell membranes reduced similarly to that without dynasore treatment under the retromer complex and SNX27 knockdown condition (Figure 4A, B). SNX17 did not affect the surface expression of ΔF508-CFTR when endocytosis was blocked (Figure 4C, D). This result confirmed that VPS26-29-35 and SNX27, but not SNX17, participate in the UPS of ΔF508-CFTR.



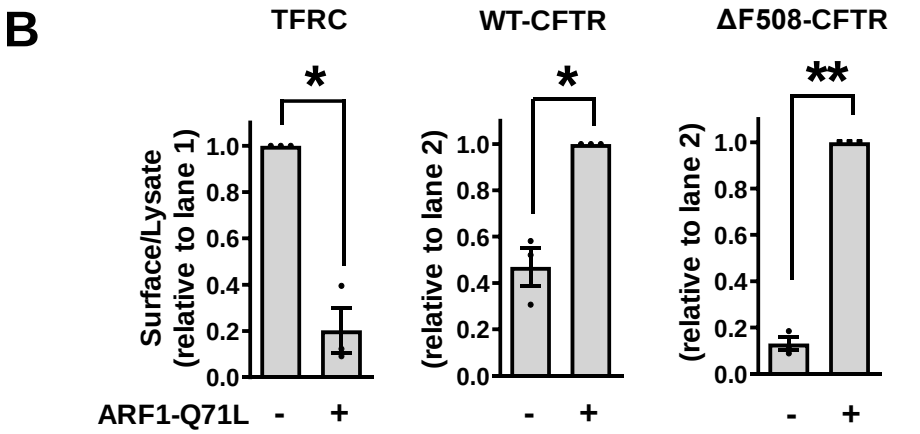
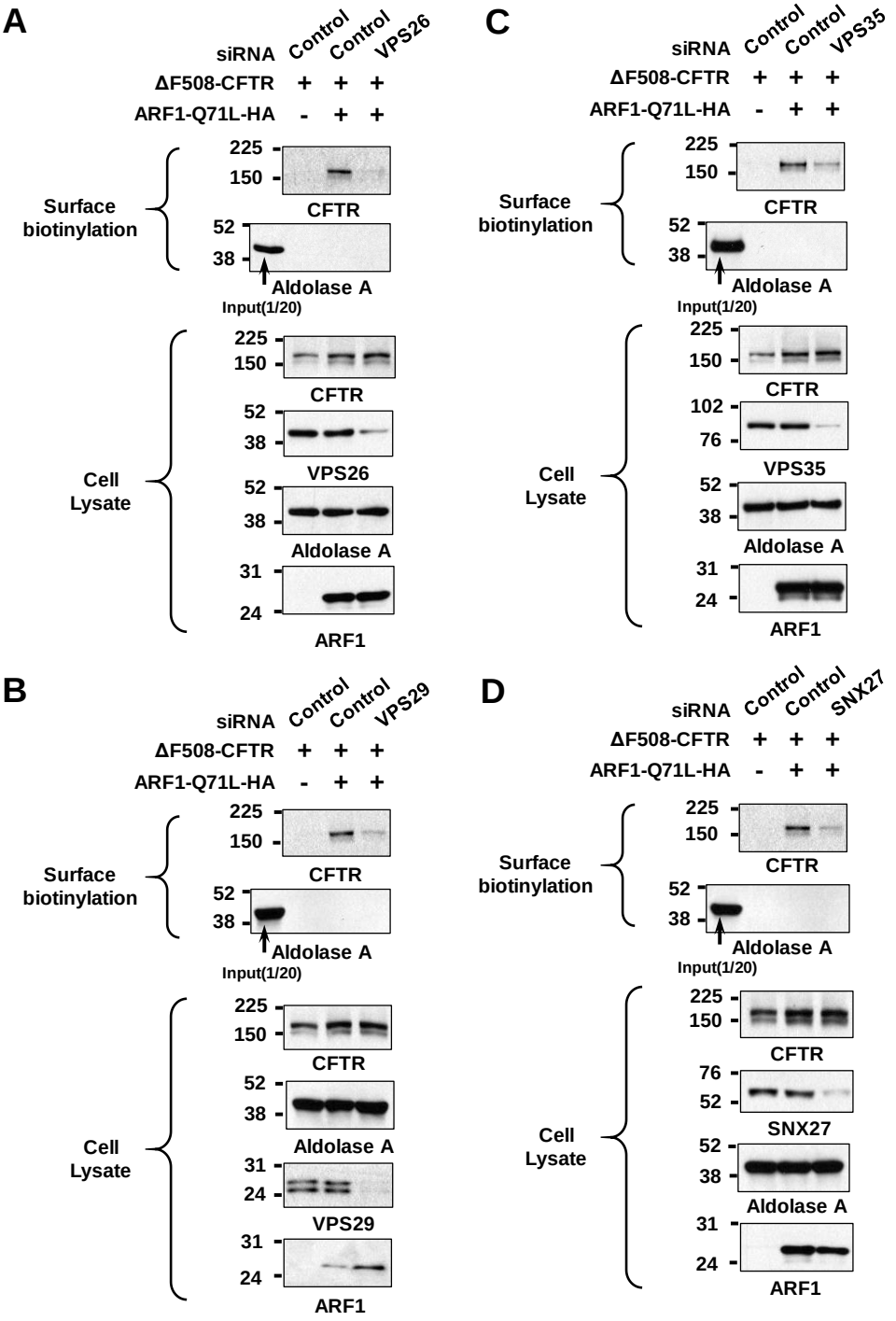


Figure 1. CFTR, but not the transferrin receptor (TFRC), undergoes UPS under the ER stress condition. (A and B) A biotinylation assay was performed to assess the effects of ER-to-Golgi blockade on the secretion of the transmembrane proteins CFTR and TFRC. HEK293 cells were transfected with plasmids encoding WT-CFTR, ΔF508-CFTR, and TFRC (24 h). In some cells, ARF1-Q71L was transfected together to induce ER-to-Golgi blockade. The results shown in A are representative of surface biotinylation assays, and the data of multiple experiments are summarized in B (n = 3). Bar graph data are presented as the mean ± SEM. **P* < 0.05, ***P* < 0.01. Data were analyzed using a two-tailed Student's *t*-test.



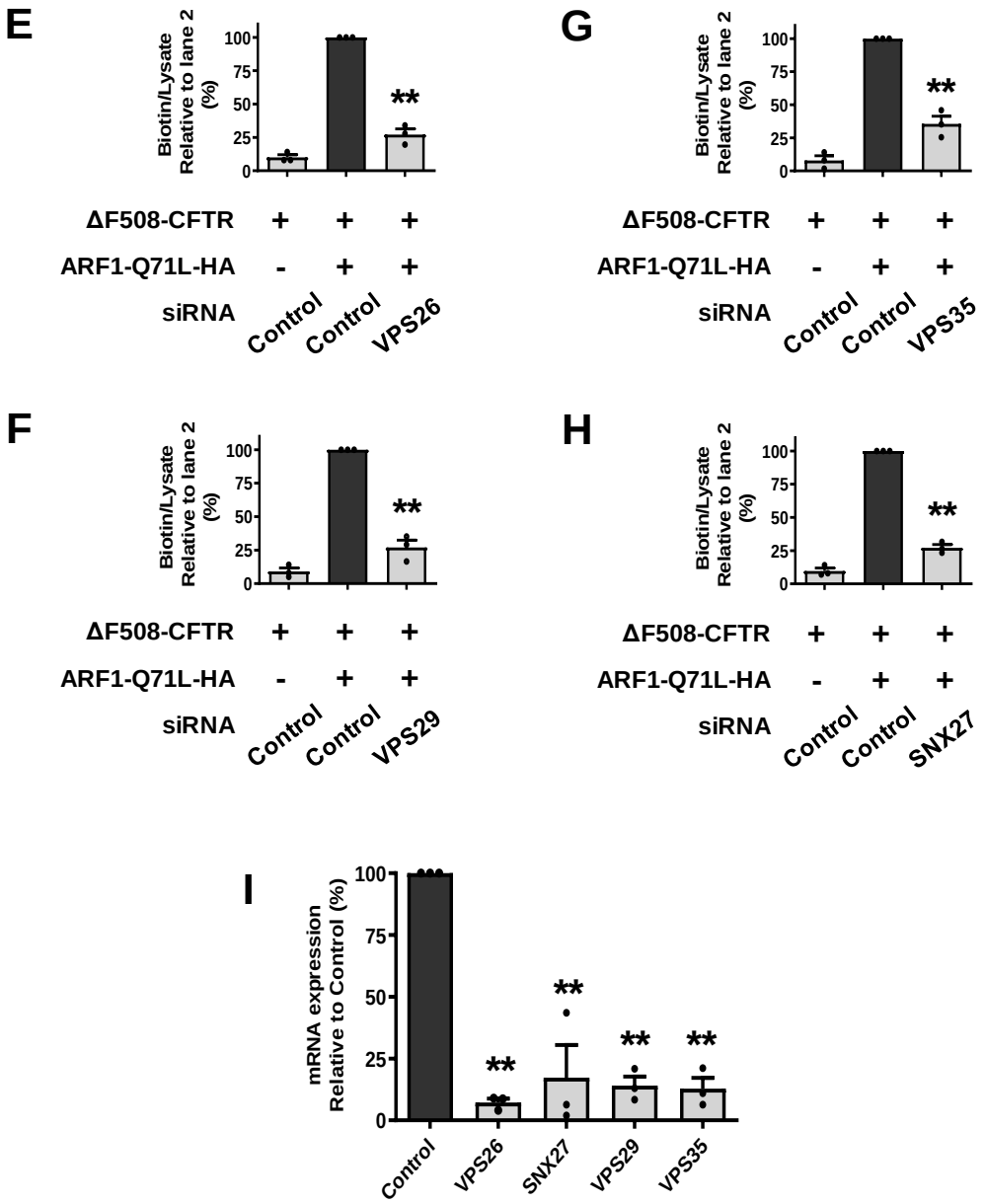


Figure 2. The retromer complex participates in the unconventional trafficking of ΔF508-CFTR. (A-H) Effects of retromer complex silencing on the UPS of ΔF508-CFTR. HEK293 cells were transfected with control siRNA or retromer complex-specific siRNA (50 nM each, 48 h). After 24 h, the cells were transfected with plasmids encoding ΔF508-CFTR. In some cells, ARF1-Q71L was cotransfected to induce ER stress (24 h). Following this, the cell surface biotinylation assay was performed. Representative blots are shown in A-D. The data of multiple experiments are summarized in E-H (n = 3). **(I)** Efficacy of siRNAs knockdown. HEK293 cells were transfected with control or siRNAs against each retromer complex component gene (50 nM each, 48 h). mRNA samples were extracted, and a quantitative polymerase chain reaction assay was performed. Bar graph data are presented as the mean ± SEM. $^{**}P < 0.01$. Data were analyzed using one-way analysis of variance, followed by Tukey's multiple comparison test.

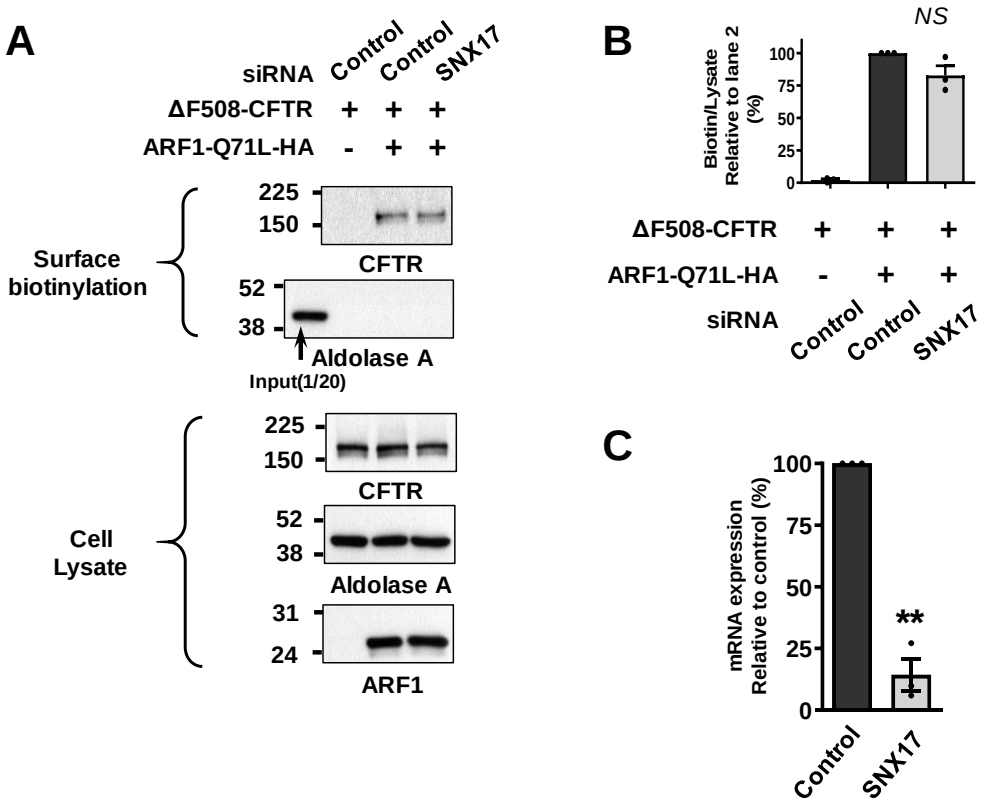


Figure 3. The UPS of CFTR is not inhibited by SNX17 gene silencing. (A and B) Effects of SNX17 gene silencing on the unconventional trafficking of ΔF508-CFTR. The cell surface biotinylation assay was performed using HEK293 cells transfected with control siRNA or SNX17 siRNA (50 nM, 48 h) together with plasmids encoding ΔF508-CFTR (24 h). In some cells, ARF1-Q71L was coexpressed to induce ER stress. Representative blots are presented in A. The data of multiple experiments are summarized in B (n = 3). **(C)** Efficacy of SNX17 siRNA knockdown. Bar graph data are presented as the mean ± SEM. ***P* < 0.01. Data were analyzed using one-way analysis of variance, followed by Tukey's multiple comparison test (B) or a two-tailed Student's *t*-test (C).

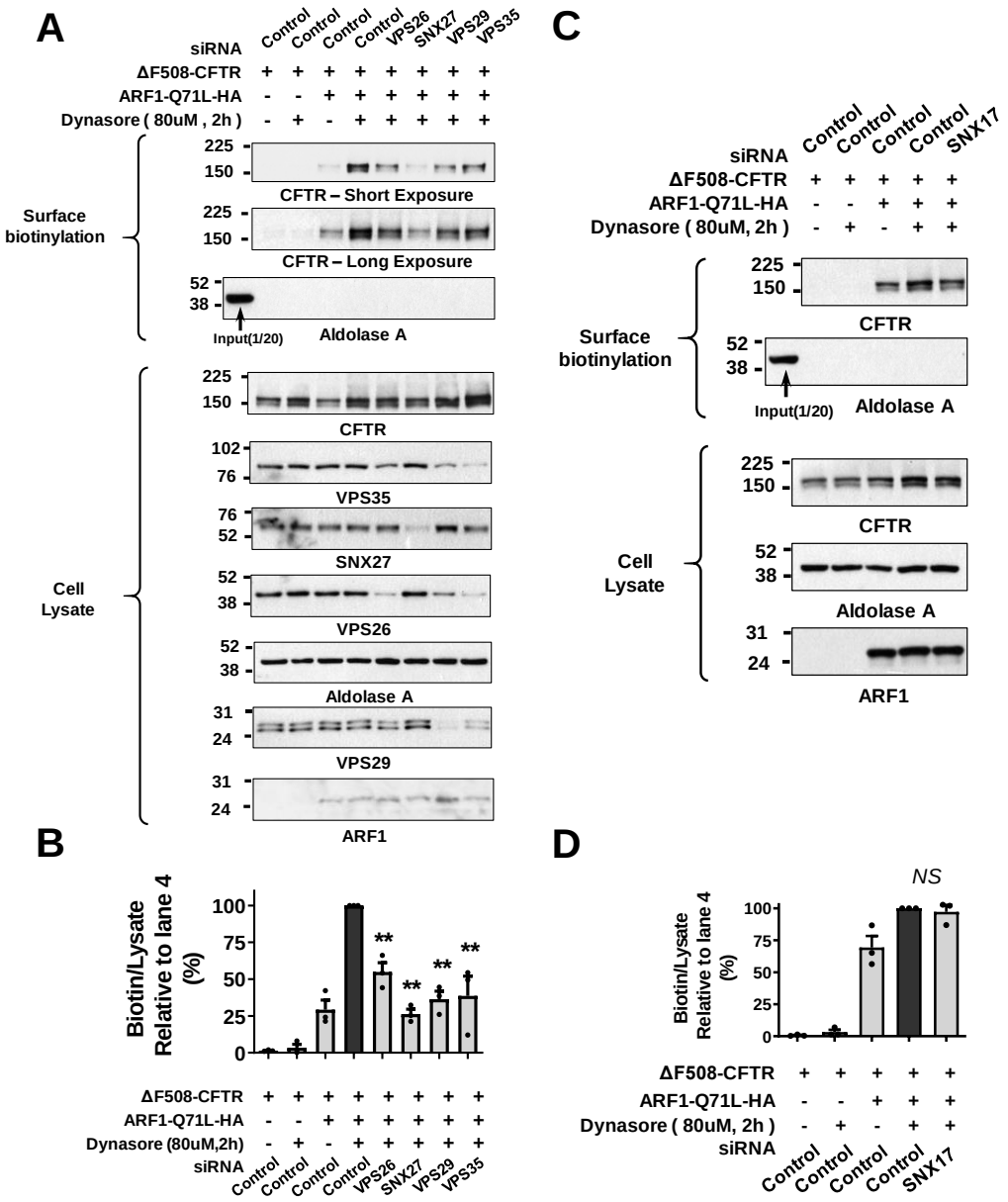
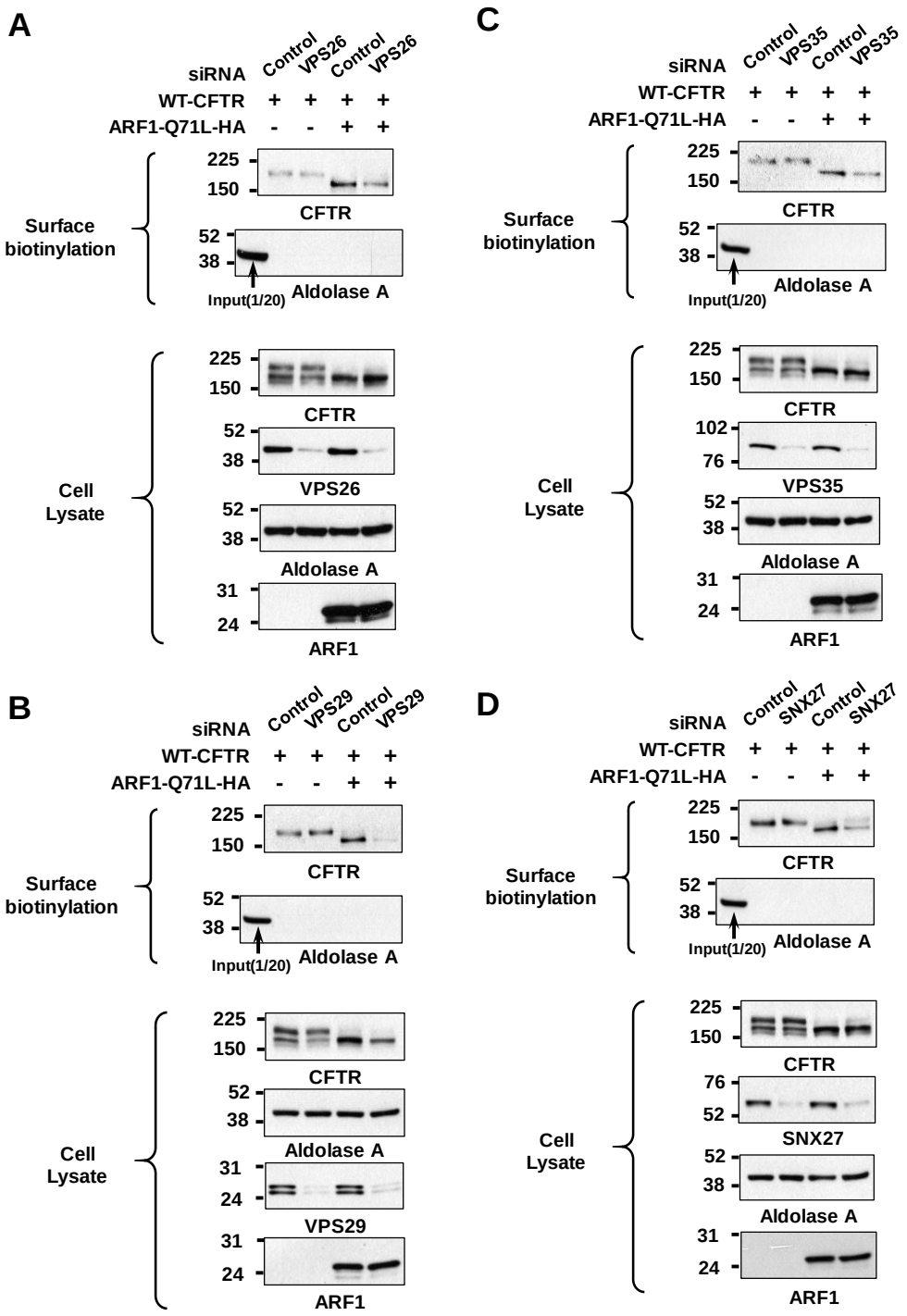


Figure 4. Retromer and SNX27, but not SNX17, are involved in the UPS of ΔF508-CFTR when endocytosis is blocked. (A-D) A biotinylation assay was performed to assess the effects of retromer complex component gene and SNX silencing on the UPS of ΔF508-CFTR when endocytosis was blocked. HEK293 cells were transfected with control siRNA or target siRNAs (50 nM each, 48 h) together with plasmids encoding ΔF508-CFTR (24 h). ARF1-Q71L was cotransfected in some cells to induce ER stress, and the cells were treated with dynasore (80 μM, 2 h) to block endocytosis. Representative blots are presented in A and C. The data of multiple experiments are summarized in B and D (n = 3). Bar graph data are presented as the mean ± SEM. $^{**}P < 0.01$. Data were analyzed using one-way analysis of variance, followed by Tukey's multiple comparison test.

Next, a surface biotinylation assay was performed to assess the effects of retromer complex and SNX27 gene silencing on the conventional protein secretion of CFTR. Interestingly, retromer complex and SNX27 gene silencing did not affect the conventional secretion of the complex glycosylated form (band C) of WT-CFTR (Figure 5A-H). To determine if the same results would be obtained if endocytosis was blocked, surface biotinylation was performed with dynasore treatment. When the cells were treated with dynasore, the surface expression of WT-CFTR was not affected by the silencing of the retromer complex gene (Figure 6A, B).

Knockdown of retromer component genes and SNX27 abolished the surface expression of the core glycosylated form (band B) of WT-CFTR under the ARF1-induced ER-to-Golgi blockade condition. These results indicate that the retromer complex is crucial for the UPS, but not the conventional secretion, of CFTR.



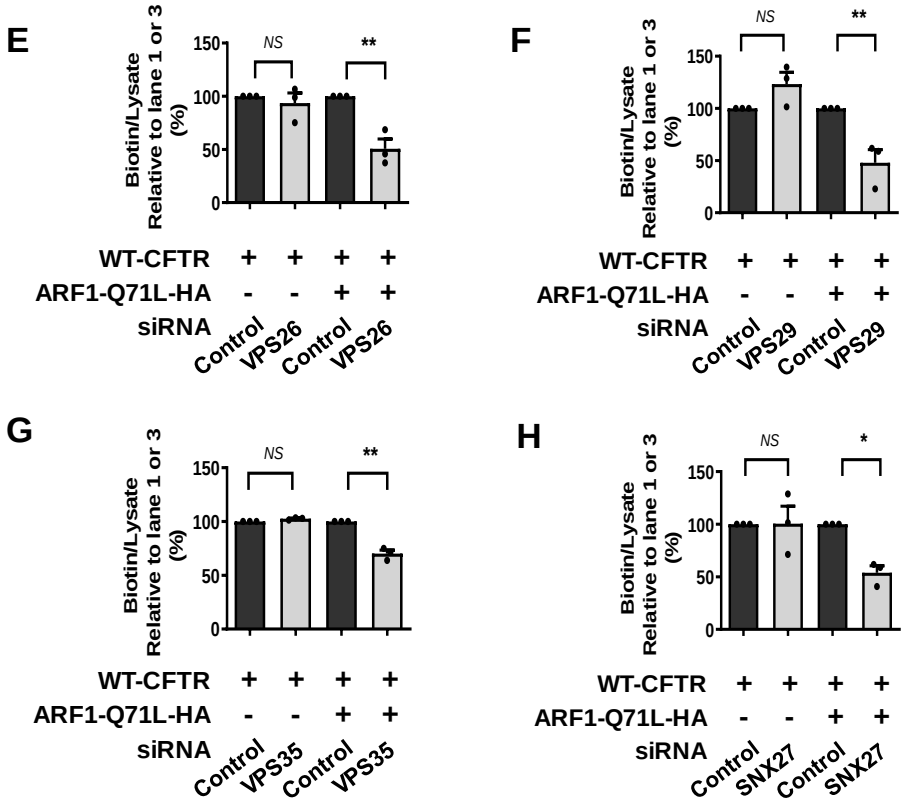


Figure 5. Knockdown of retromer components and SNX27 does not affect the conventional secretion of CFTR. (A-H) Effects of retromer complex component and SNX27 gene silencing on the conventional trafficking of WT-CFTR. HEK293 cells were transfected with control siRNA or target siRNAs (50 nM each, 48 h) together with plasmids encoding WT-CFTR (24 h). In some cells, ARF1-Q71L was cotransfected for ER-to-Golgi blockade. Representative blots are presented in A-D. The data of multiple experiments are summarized in E-H (n = 3). Bar graph data are presented as the mean ± SEM. Data were analyzed using one-way analysis of variance, followed by Tukey's multiple comparison test.

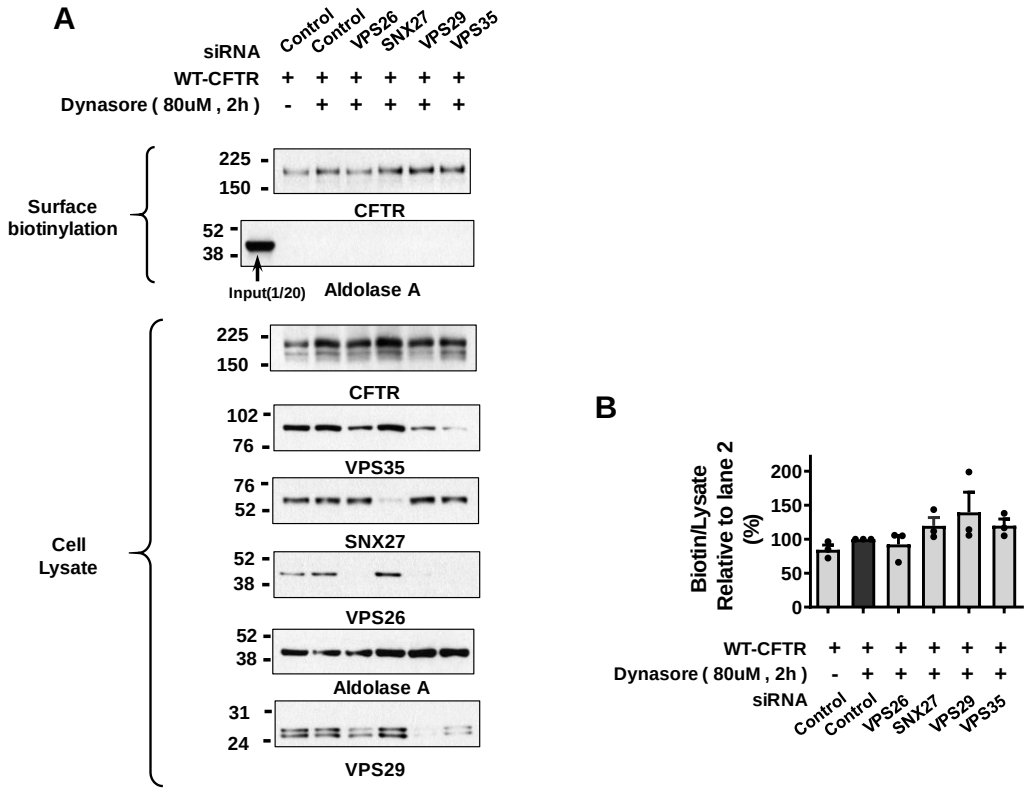


Figure 6. WT-CFTR is unaffected by the knockdown of retromer complex component genes under the endocytosis blockade condition. (A and B) Effects of retromer complex component gene silencing on the conventional trafficking of WT-CFTR during endocytosis blockade. HEK293 cells were transfected with control siRNA or target siRNAs (50 nM each, 48 h) together with plasmids encoding WT-CFTR (24 h). Some cells were treated with dynasore to block endocytosis (80 μM, 2 h). Representative blots are presented in A. The data of multiple experiments are summarized in B (n=3). Bar graph data are presented as the mean ± SEM. Data were analyzed using one-way analysis of variance, followed by Tukey's multiple comparison test.

2. SNX27 and CFTR physically interact via the PDZ domain, and the interaction is augmented under the ER-to-Golgi blockade condition

Immunoprecipitation was performed to confirm the interaction between CFTR and SNX27 and assess the mechanism by which the retromer complex affects the unconventional trafficking of CFTR. In the case of ΔF508-CFTR, the interactions with SNX27 significantly increased when ER stress was induced by ARF1-Q71L (Figure 7A, B). As both CFTR and SNX27 have a PDZ domain,[5,13] we hypothesized that they would interact with each other via the PDZ domain.

To determine whether this interaction involves the PDZ domain of SNX27 and CFTR, we performed immunoprecipitation between SNX27 and ΔC-term-CFTR, in which the C-terminal portion with the PDZ domain was removed. We found that CFTR, in which the C-terminal portion was not removed, interacted with SNX27. ARF1-Q71L augmented the interaction between SNX27 and CFTR. On the other hand, ΔC-term-CFTR and SNX27 hardly interacted, regardless of ER stress (Figure 7C, D).

Next, a pull-down assay was performed to confirm whether the aforementioned interaction occurs through the PDZ domain. Figure 8A and B illustrate the structures of CFTR and SNX27 and the constructs generated for the protein interaction studies. Pull-down assays between a His-tagged fusion recombinant protein expressing each domain of SNX27 and GST-fusion recombinant protein expressing 30 amino acids of the C-terminus of CFTR were performed. As expected, the PDZ domain and full-length constructs of SNX27 strongly interacted with CFTR-Ct-30. The structure-deleted PDZ domain of SNX27 abolished the interaction with CFTR-Ct-30 (Figure 8C).

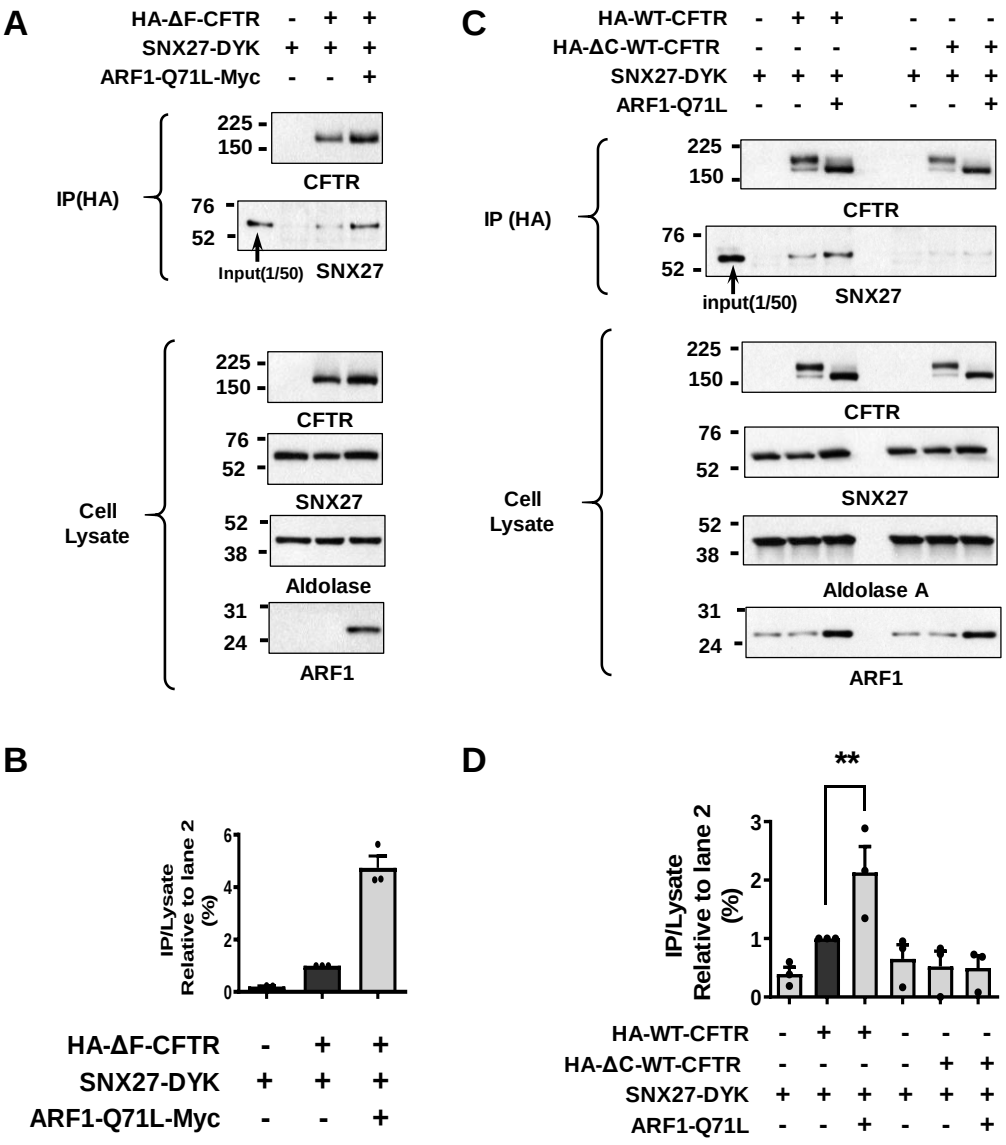
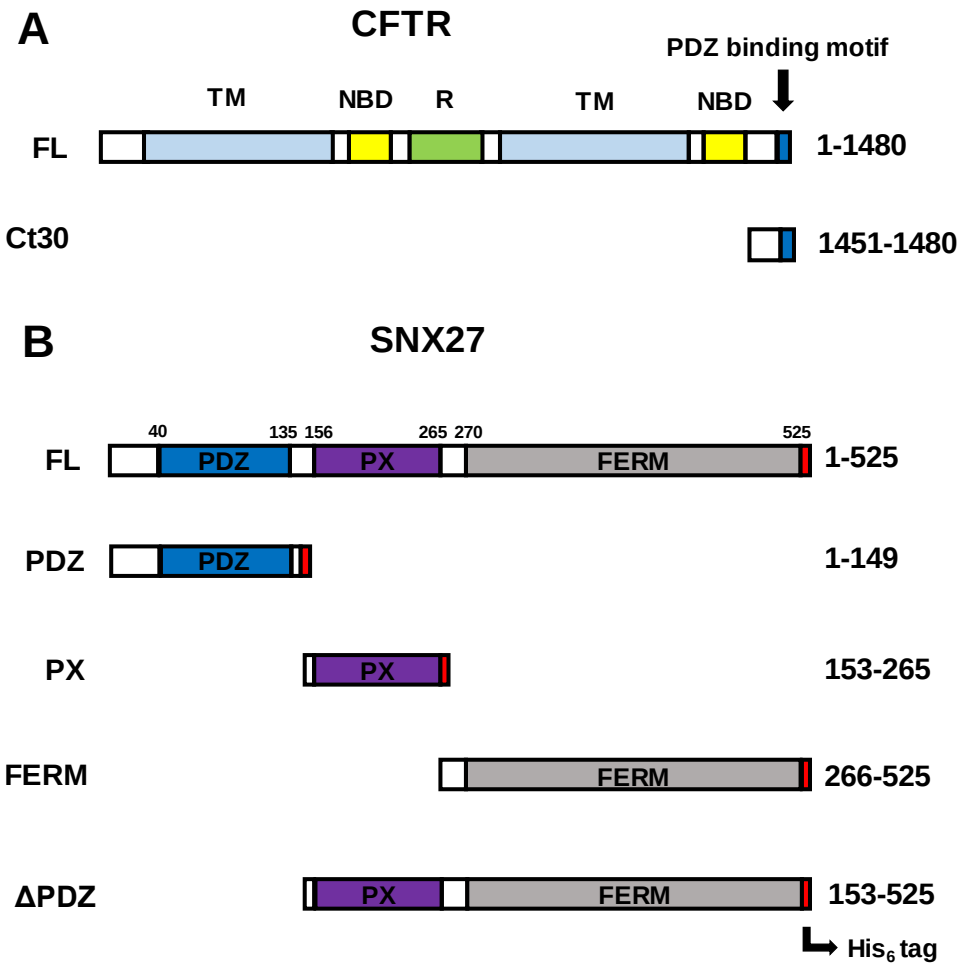


Figure 7. CFTR and SNX27 physically interact, and the interaction is augmented when CFTR UPS is activated. (A-D) An immunoprecipitation assay of SNX27 and CFTR was performed using anti-DYK antibodies. HEK293 cells were transfected with plasmids encoding DYK-tagged SNX27 (SNX27-DYK) alone or with HA-CFTR (24 h). ARF1-Q71L was coexpressed in some cells to induce UPS. The results presented in A and C are representative of the immunoprecipitation assay. The data of multiple experiments are summarized in B and D (n = 3). Bar graph data are presented as the mean ± SEM. $^{**}P < 0.01$. Data were analyzed using one-way analysis of variance, followed by Tukey's multiple comparison test.



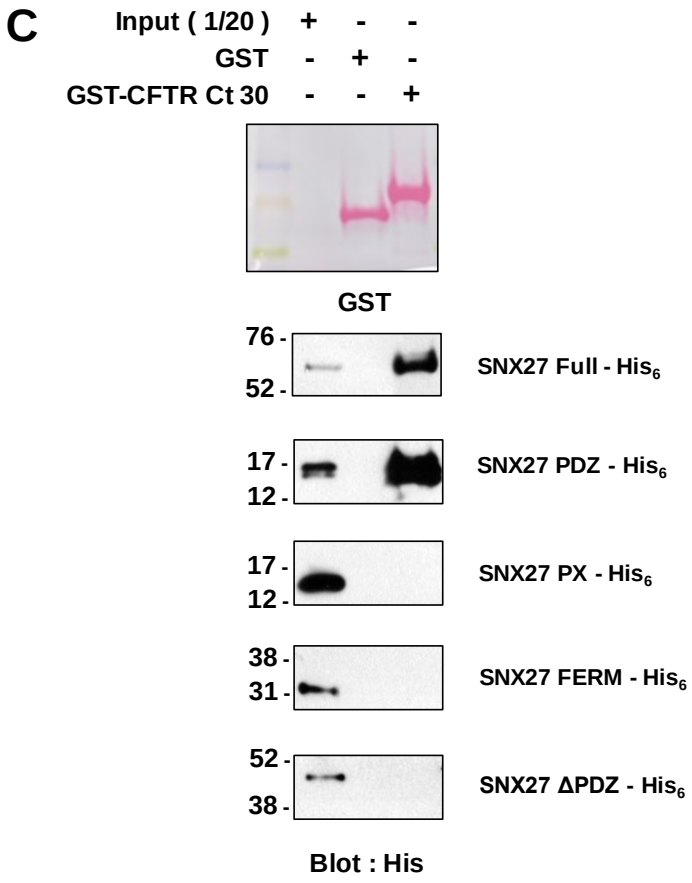


Figure 8. CFTR and SNX27 directly interact via the PDZ domain. (A and B) Schematic diagram of CFTR and SNX27 constructs used in pull-down assays. FL, full-length; TM, transmembrane domain; NBD, nucleotide-binding domain; R, regulatory domain; PDZ, PSD-95/discs large/ZO-1; PX, Phox homology; FERM, Four-point-one, ezrin, radixin, moesin. **(C)** Pull-down assay was performed with the His_6-fused domain of SNX27 proteins and GST-fused CFTR C-terminal 30 amino acids. The input GST-fused proteins were visualized by Ponceau S staining, and each domain of SNX27 was immunoblotted with anti-His antibodies.

An immunofluorescence assay was performed to assess the localization of SNX27 and ΔF508-CFTR in the cells. The colocalization of ΔF508-CFTR and SNX27 increased when ARF1-Q71L-induced unconventional trafficking was activated. In control cells, SNX27 fraction of ≈30% colocalized with ΔF508-CFTR. Under the ER stress condition, >80% of SNX27 colocalized with ΔF508-CFTR (Figure 9A, B). Significantly increased colocalization was noted between SNX27 and ΔF508-CFTR, supporting the idea that the retromer complex participates in the UPS of ΔF508-CFTR.

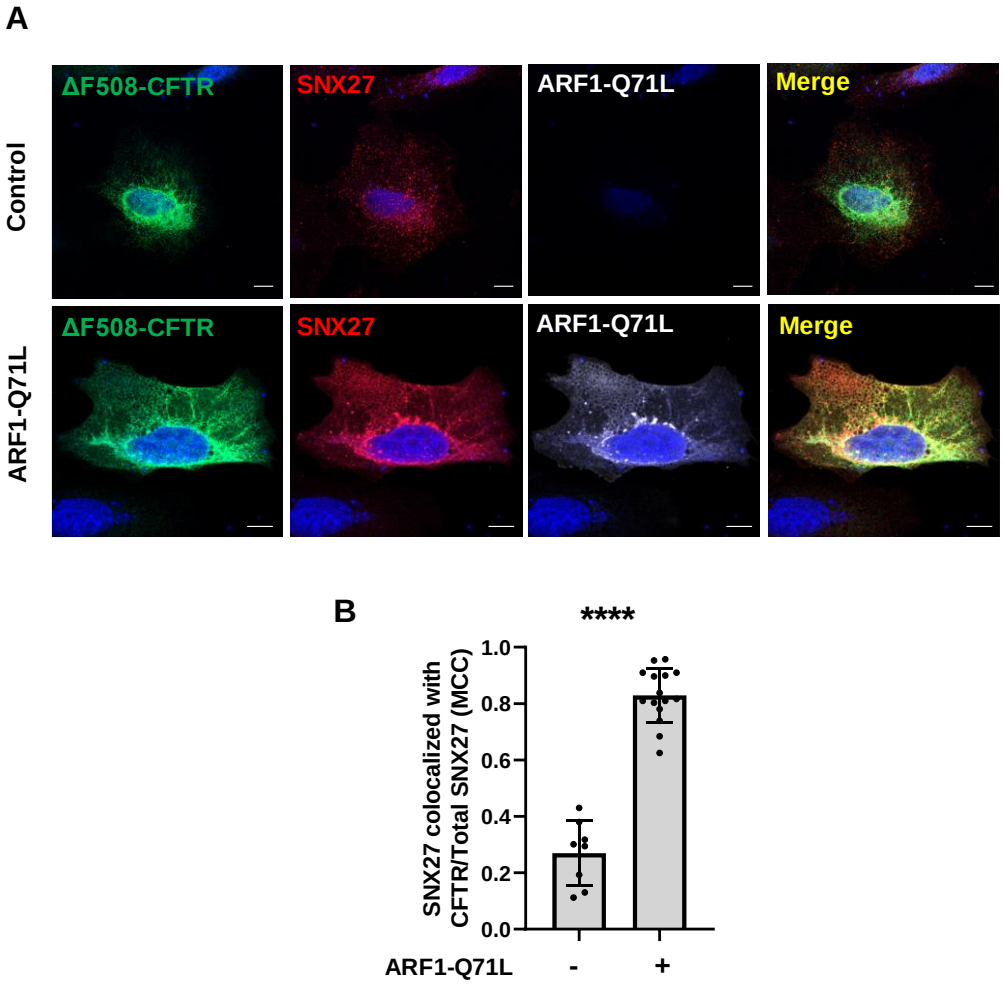


Figure 9. The colocalization of SNX27 and CFTR is augmented when CFTR UPS is activated. (A and B) Immunofluorescence analysis of SNX27 and ΔF508-CFTR in cells**.** HeLa cells were transfected with plasmids encoding ΔF508-CFTR, and some cells were coexpressed with ARF1-Q71L. ΔF508-CFTR was stained with anti-CFTR (green, Alexa Fluor 488) antibodies and endogenous SNX27 with anti-SNX27 (red, Alexa Fluor 568) antibodies. Representative images are presented in A. The colocalization data of ΔF508-CFTR and SNX27 using Manders' colocalization coefficient (MCC) are summarized in B (n > 7). Scale bar: 10 μm. Bar graph data are shown as the mean ± SEM. Data were analyzed using two-tailed Student's *t*-test.

IV. DISCUSSION

Most secretory proteins travel via a well-documented conventional secretory pathway from the ER to the Golgi apparatus. A recent study revealed that many secretory proteins are involved in several alternative processes, collectively known as UPS pathways. UPS facilitates the secretion of proteins from the ER to the cell membrane via a Golgi-independent pathway.[9] The UPS pathways can be classified into four types according to the distinct features of the cargo protein.[9] Of these, the UPS of CFTR belongs to type IV UPS and is related to Golgi reassembly and stacking protein (GRASP), autophagy, and ESCRT.[5,10] Moreover, the activation of the kinase domain of inositol-requiring enzyme 1α (IRE1α) has been reported to be involved.[17] However, the mechanism underlying the UPS pathway of CFTR is not well understood. For instance, the process of directing each protein to a specific destination and the types of vesicles involved remain unclear. In this study, we verified that the retromer complex components and SNX27 play a significant role in the UPS of CFTR. In particular, SNX27 directly interacts with CFTR via the PDZ domain in this process.

The retrograde transport of cargo proteins between the endosome and TGN or recycling to the plasma membrane is mediated by a highly conserved complex, known as the retromer complex.[14,15] However, the results of the surface biotinylation assay performed by silencing of the retromer complex components genes demonstrated that the retromer complex is involved in CFTR trafficking under the UPS conditions induced by ARF1-Q71L (Figure 2). The same result was noted even when endocytosis was blocked by dynasore treatment (Figure 4). Interestingly, the retromer complex was not involved in the conventional trafficking of CFTR (Figure 5). The retromer complex is involved in the sorting of CIMPR, sortilin, SORL1, wntless, and other physiologically important membrane proteins.[18] These results suggest that the retromer complex is involved in the sorting of CFTR. Moreover, tubules are formed because of the oligomerization of SNX dimer.[18] SNX27 is involved in

the trafficking of membrane proteins, such as the glutamine transporter ASCT2 and AMPA receptor.[19,20]

In our study, the SNX27 gene was the most relevant gene involved in the UPS of CFTR. Immunoprecipitation results revealed that the interaction between SNX27 and CFTR is stronger than usual under ER stress and that ΔC-term-CFTR and SNX27 do not interact, regardless of ER stress (Figure 7). There are various types of SNX, and each has different domains. Generally, SNX have a PX domain. SNX27 characteristically has a PDZ domain and a FERM domain.[13] SNX17 has a FERM domain but no PDZ domain,[21] it does not affect the UPS of ΔF508-CFTR (Figure 3, 4). Thus the PDZ domain interactions identified here are important in CFTR and SNX27. As expected, these data confirm that CFTR directly interacts with the PDZ domain (Figure 8). Although VPS26 does not interact directly with CFTR, CFTR appears to interact with VPS26 via the SNX27 PDZ domain.

The unconventional trafficking of CFTR occurs via a GRASP-dependent pathway, and the PDZ domain of CFTR interacts with the PDZ domain of GRASP55.[5] A recent study revealed that GRASP55 interacts with transmembrane emp24 domain-containing protein 3 (TMED3) and that the interaction increases under ER stress.[22] TMED3 plays a critical role in the ER stress-associated UPS of ΔF508-CFTR.[22,23] However, the interaction between GRASP55 and SNX27 or TMED3 and SNX27 during UPS ramains unknown. Moreover, SNX27 has been shown to interact with GTPases, such as kRAS, through its FERM domain.[24] Hence, we can expect the formation of a complex between ARF1 and SNX27. The interaction between ARF1 and SNX27 remains unknown. Therefore, further research is warranted to assess these interactions in the future.

In the immunofluorescence assay, we assessed the colocalization of CFTR and SNX27. The colocalization of ΔF508-CFTR and SNX27 increased under the ARF1-Q71L-induced ER stress condition (Figure 9). Interestingly, the localization of SNX27 and ARF1-Q71L also overlappeds considerably. SNX27 is originally assembled in the endosomes.[13] However, further investigation is warranted to assess the location in the cell where SNX27 interacts with CFTR, the route taken by CFTR to reach the membrane through UPS, and

the rescue of ΔF508-CFTR trafficking with SNX27.

Various cargo proteins, including CFTR, are involved in UPS.[9] For example, pendrin functions as a cargo protein associated with IRE1α kinase activation, similar to CFTR.[17] The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein also has an unconventional trafficking pathway.[22] The MTSC motif of the SARS-CoV-2 spike protein interacts with the PDZ domain of SNX27. The mutant form of the spike protein that has lost the ability to bind to SNX27 reduces the surface expression of the spike protein.[25,26] SNX27 also interacts with angiotensin-coverting enzyme 2 (ACE2) and is related to the entry of SARS-CoV-2.[27] Based on the results of these studies, it is important to thoroughly assess whether other UPS cargoes or viruses are affected by retromer complexes.

The recycling endosome is also involved in the pathway that transports cargo proteins to the plasma membrane.[28] Ras-associated binding protein 11 (Rab11) mediates the slow endocytic recycling process when recycling endosomes recycle cargo proteins to the plasma membrane from early endosomes through vesicle trafficking.[29] Previous studies have reported that numerous Rab proteins are involved in the autophagy pathway and the UPS of other cargo proteins, in addition to the UPS of CFTR.[10,30,31] It is well known that Rab11a participates in the cellular trafficking of CFTR.[32,33] Rab11b also participates in the conventional trafficking of CFTR.[34] It would be interesting to study the interaction between Rab11 and the trafficking of CFTR.

We have been conducting research to assess the exact mechanism of UPS. Based on the finding, we were able to elucidate the role of the retromer complex and prove that CFTR and SNX27 interact directly. It is worthwhile to further assess whether the other UPS cargo proteins mentioned above are also related to retromers. Combined results from these studies may helpful in assess which route UPS cargo proteins use to reach the membrane. This understanding will further promote the search for new methods to treat various diseases and may serve as a springboard for other studies on the mechanism of UPS.

V. CONCLUSION

The precise mechanism of unconventional protein secretion (UPS) pathway are not well delineated. In this study, we assessed the role of the retromer complex and SNX27 in the UPS of CFTR. In conclusion, the present findings revealed that

1. The retromer complex and SNX27 play a significant role in the UPS, but not in the conventional protein secretion, of CFTR.
2. SNX27 and CFTR interact with each other via their PDZ domains and the interaction is augmented when CFTR UPS is activated.
3. The colocalization of ΔF508-CFTR and SNX27 increased under the ARF1-Q71L-induced ER stress condition.

These findings will help elucidate the mechanism underlying the unconventional trafficking of CFTR and will be helpful in the treatment of various diseases associated with protein secretion.

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ABSTRACT (IN KOREAN)

CFTR의 비전형적 수송에서 retromer 복합체의 역할

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김 예 진

단백질이 합성되어 세포의 세포막이나 각 기관으로 이동하는 경우, 보통 소포체와 골지체를 통해 이동하게 된다. 이 때 단백질들은 단백질 접힘에 문제가 있는지 확인하기 위해 ERQC 과정을 거치게 된다. 단백질 접힘에 이상이 있는 경우에는 ERAD에 의해 분해되고 골지체를 통과할 수 없게 된다. 단백질 접힘에 이상이 있는 단백으로 인해 질병이 발생할 수 있는데 대표적인 예시로는 CFTR이 있다. CFTR은 고리형 아데노신 일인산으로 인해 활성화되어 상피세포에서 염화이온과 탄산수소염을 운반하는 단백질이다. 질병을 일으키는 가장 흔한 CFTR 유전자의 돌연변이는 508번째 페닐알라닌이 제거된 CFTR이다. 평소에 이 돌연변이는 단백질 접힘에 이상이 생겨서 ERAD에 의해 분해되어 세포막으로 도달할 수 없게 된다. 그럼에도 불구하고 소포체에 스트레스가 주어지는 상황이나 소포체에서 골지체로 가는 경로가 막힌 상황에서 GRASP 의존 비전형적 세포막 수송을 통해 이 돌연변이 CFTR이 세포막으로 도달할 수 있으며 일정 수준 이상의 기능을 회복할 수 있음을 발견하였다. 하지만 아직 이 비전형적 세포막

수송의 정확한 메커니즘은 밝혀져 있지 않다. 해당 연구는 retromer 복합체와 Sorting nexin 27이 CFTR의 비전형적 세포막 수송에 중요한 역할을 한다는 것을 확인하였다. Retromer 복합체를 구성하는 VPS26·29·35와 SNX27 유전자의 발현을 억제하였을 때 CFTR의 비전형적 세포막 수송이 현저히 감소하는 것을 확인할 수 있었다. 특히 CFTR의 세포막 수송 과정에서 SNX27과 CFTR은 각각이 가지고 있는 PDZ 도메인을 통해 서로 물리적으로 결합한다는 것 또한 확인할 수 있었고 이 결합은 비전형적 세포막 수송이 활성화되었을 때 더 강해진다는 것을 확인할 수 있었다. 이 결과를 통해 SNX27과 CFTR의 결합을 통해 매개되는 CFTR의 비전형적 세포막 수송에 retromer 복합체의 구성요소들이 중요하다는 것을 알 수 있었다.

핵심되는 말 : 비전형적 단백수송, 낭포성 섬유증, Retromer, SNX27

PUBLICATION LIST

1. Park H*, Seo SK*, Sim JR*, Hwang SJ*, **Kim YJ***, Shin DH, et al. TMED3 Complex Mediates ER Stress-Associated Secretion of CFTR, Pendrin, and SARS-CoV-2 Spike. Adv Sci (Weinh) 2022:e2105320.(* equal contribution)