VPS29–VPS35 intermediate of retromer is stable and may be involved in the retromer complex assembly process

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Abstract
Retromer is a complex of proteins that functions in the endosome-to-Golgi retrieval cargo transport pathway. VPS35 works as the central subunit of retromer to recognize the cargos and binds with VPS29 and VPS26 via distinct domains. We show that deficiency of VPS35 or VPS29 accompanies degradation of other subunits, whereas VPS26 deficiency had no effect on VPS29 and VPS35 levels. Although VPS35 forms VPS26–VPS35 and VPS29–VPS35 sub-complexes with similar efficiency in vitro, VPS26–VPS35 was more easily degradable by the ubiquitin–proteasome-system than VPS29–VPS35. These results indicate that VPS29 and VPS35 form a biologically stable sub-complex in vivo.

1. Introduction
Retromer is an evolutionarily conserved protein complex that functions in the retrieval transport of cargo proteins from the endosome to trans-Golgi network (TGN) [1,2]. Initially, subunits of the retromer complex were identified as components of the sorting machinery required for the endosome-to-Golgi retrieval of the carboxypeptidase Y receptor VPS10 in Saccharomyces cerevisiae [3]. The components of retromer are highly conserved from yeast to mammals and consist of cargo-recognition sub-complexes containing VPS35, VPS29, VPS26, and sorting nexin (SNX) dimers of SNX1 and SNX2 and possibly SNX5 and SNX6 [2,4,5]. These two sub-complexes assemble into a multimeric membrane coat in endosomes. VPS35, the largest subunit of retromer, acts as the center of the cargo-recognition complex to independently interact with VPS26 and VPS29 in retromer, and interact with cargos, i.e., cation-independent mannose 6-phosphate receptor (CI-M6PR), Wntless, and DMT1 [2,6,7]. VPS35 binds with VPS26 via N-terminal PRLYL motif, and with VPS29 via C-terminal region, and can form sub-complexes with either of them in vitro [11–13]. CI-M6PR, which functions as a
lyosomal enzyme receptor, is one of the representative cargos of retromer [2]. Upon dissociation of CI-M6PR from its ligands in endosomes, CI-M6PR is transported back to TGN via the retromer complex through interaction between the C-terminal cytoplasmic domain of CI-M6PR and the VPS35 subunit [14]. In addition to retromer components and cargos, VPS35 interacts with the GTP-bound form of Rab7 via N-terminal conserved region of VPS35, and the retromer is consequently recruited to the endosome via this interaction [15].

Recently, a number of reports revealed that a point mutation in the VPS35 gene (D620N) causes late-onset, autosomal dominant Parkinson's disease [16–18]. The mutant form of VPS35 causes defects in cathepsin D trafficking [19] and alters association with the actin-nucleating Wiskott-Aldrich syndrome and SCAR homolog (WASH) complex [20,21]. These molecular-based studies suggest that VPS35 D620N induces a loss-of-function pathogenesis.

We performed knockdown of retromer components using siRNA to elucidate the differences of retromer component deficiency. We found that in the absence of VPS29 expression, the VPS26–VPS35 sub-complex is very fragile and easily ubiquitylated and degraded by proteasomes. This finding indicates that VPS29–VPS35 dimer formation may be the initial step of retromer assembly in vivo.

2. Materials and methods

2.1. Plasmid DNA construction

Human VPS35, VPS35 D620N, VPS29, VPS26A, and mCherry were cloned by PCR using VPS35 expression plasmids, cDNA of HeLa cell or pmCherry vector as templates. VPS35 wild-type or mutants, and mCherry were ligated into pcDNA3.1-Hyg (+) vector (Life Technologies, Carlsbad, CA, USA) to construct N-terminal mCherry-tagged VPS35 expression plasmids. VPS29 or VPS26A were cloned into p3xFLAG-CMV-7.1 or p3xFLAG-CMV-14 vectors (Sigma–Aldrich, St. Louis, MO, USA) for N-terminal FLAG-tagged or C-terminal FLAG-tagged fusion protein expression, respectively.

2.2. Cell culture and transfection

HeLa cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 100 units/mL penicillin/streptomycin in a humidified 37 °C incubator supplemented with 5% CO2/95% O2. Plasmid DNAs were transfected into cells using Lipofectamine 2000 Reagent (Life Technologies) according to the manufacturer's instructions. For RNA interference experiments, ON-TARGETplus human siRNA oligos (Thermo Scientific Dharmacon, Waltham, MA, USA; GE Dharmacon, Lafayette, CO, USA) for VPS35 (L-010894-00-0005), VPS29 (L-009764-01-0005), VPS26A (L-013195-00-0005), VPS26B (L-015152-00-0005), SNX1 (L-017518-00-0005), SNX2 (L-017520-00-0005), and scrambled siRNA (D-001810-10-20) were transfected into cells using Lipofectamine RNAiMAX Reagent (Life Technologies) according to the manufacturer's instructions.

2.3. Antibodies and reagents

Anti-VPS35 (NB100-1397, Novus Biologicals, Littleton, CO, USA) antibodies were obtained from Novus Biologicals. Anti-VPS29 (ab98892), anti-VPS26 (ab181352), and anti-cation independent Mannose 6 Phosphatase Receptor antibodies (ab2733, ab124767) were from Abcam (Cambridge, UK). Anti-SNX1 (611482), anti-SNX2 (611308), and anti-EEA1 antibodies (610457) were from BD Transduction Laboratories (San Jose, CA, USA). The anti-TGN46 antibody (T7576) was from Sigma–Aldrich. The anti-EEA1 antibody (2411), and anti-LC3B antibody (3868) were from Cell Signaling Technology (Boston, MA, USA). The anti-Cathepsin D antibody (sc6486) was from Santa Cruz Biotechnology (Dallas, Texas, USA). The anti-Actin antibody (MAB1501R) was from Millipore (Billerica, MA, USA). Anti-RFP (M208-3) and anti-Multi Ubiquitin antibodies (D058-3) were obtained from Medical & Biological Laboratories Co. Ltd. (Nagoya, Japan). Alexa Fluor 488-conjugated, Alexa Fluor 594-conjugated, and horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. Peptstatin A (4397-v), E-64-d (4321-v), and MG-132 (aldehyde, 3175-v) were purchased from Peptide Institute, Inc. (Osaka, Japan).

2.4. Western blotting

Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris–HCl pH 8.0, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany, 11873580001) and the 14,000xg supernatants were used as cell lysates. Cell lysates and immunoprecipitants were resolved by SDS–PAGE and transferred onto PVDF membranes. The blots were incubated with primary antibodies followed by HRP-conjugated secondary antibodies. For detection, ECL prime Detection Kit (GE Healthcare, Little Chalfont, UK, RP2232) or SuperSignal West Dura Extended Substrate (Thermo Scientific, Rockford, IL, USA, 34075) were used as chemiluminescent substrates and images were analyzed using LAS4000 mini (GE Healthcare).

2.5. Quantitative real-time PCR analysis

mRNA was isolated using TRIzol reagent (Invitrogen, 15596026). cDNA was prepared using the ReverTra Ace qPCR RT Master Mix (TOYOBO Co. Ltd., Osaka, Japan, FSQ-201) according to the manufacturer's protocol. Quantitative real-time PCR was performed using the 7500 Fast Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA) and the Fast SYBR Green Master Mix (Life Technologies, 4385612) according to the manufacturer's instructions.

2.6. Immunoprecipitation

For siRNA-treated cells, cells expressed mCherry-VPS35, mCherry-VPS35 D620N, N-terminal or C-terminal FLAG-tagged VPS29 or N-terminal or C-terminal FLAG-tagged VPS26A at 24 h after siRNA transfection. Forty-eight hours after plasmid transfection, cells were lysed in NP-40 lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA and 1% NP-40) containing protease and phosphatase inhibitor cocktail (Roche Diagnostics, 11873580001; 04906837001) for 10 min on ice. The 14,000xg supernatants were incubated with anti-RFP mAb magnetic beads (MBL, M165-9) or the anti-FLAG antibody followed by Dynabeads protein G-Sepharose (Life Technologies, 2411), and the anti-FLAG antibody followed by Dynabeads protein G-Sepharose (Life Technologies, 2411).

2.7. Immunofluorescence staining

Cells grown on glass-chamber slides were transfected with siRNAs. Forty-eight hours after transfection, cells were fixed with 4% paraformaldehyde. To detect intracellular localization of VPS35, CI-M6PR, EEA1, and TGN46, cells were incubated with primary antibodies followed by Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies. Fluorescence staining was visualized using an Olympus FX1000 confocal microscope. For the quantification of colocalization of CI-M6PR with EEA1 or TGN46, the colocalization area of CI-M6PR with EEA1 or TGN46...
were quantified using a set of single confocal images by image calculator of imageJ software. Percentages of colocalization were determined by calculation of the colocalization area over total CI-M6PR area.

2.8. Electron microscopy

Cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h, and post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h. Fixed cells were dehydrated and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed using a Hitachi HT7700 transmission electron microscope.

2.9. Statistics

All data are expressed as means ± S.D. Differences between groups were examined for statistical significance using Student t test. A P value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Knockdown of VPS35 impairs not only retrograde transport but also anterograde transport of CI-M6PR

To assess the function of the endosome-to-Golgi retrograde transport, we knocked down VPS35 using a specific siRNA oligo for human VPS35 in HeLa cells (Fig. 1). To optimize the experimental condition, cells were harvested at 12 h, 24 h, 48 h, and 72 h after siRNA transfection (Fig. 1a). Expression levels of VPS35 were dramatically decreased and reached their lowest level 48 h after transfection and maintained this level until 72 h after transfection. Therefore, we decided to use knockdown in cells 48–72 h after transfection.

In control cells, CI-M6PR localized to both endosomes and trans-Golgi at perinuclear regions (with EEA1: 11.6% and with TGN46: 15.5%, Fig. 1b–d). In VPS35 knockdown cells, CI-M6PR was significantly enriched at trans-Golgi, while its localization to endosomes was not altered (colocalization with TGN46: 21.4% and with EEA1: 14.3%). The distribution of CI-M6PR indicates that VPS35 knockdown induces a defect in the shuttling of CI-M6PR resulting in an accumulation in the trans-Golgi. However, we expected CI-M6PR accumulation at endosome rather than trans-Golgi by VPS35 knockdown, because of retromer functions retrograde transport from endosome to trans-Golgi. It has been previously reported that knockdown of VPS35 induces the mis-sorting of DMT1, a cargo of retromer to lysosome [6] and the accumulation of Cathepsin D precursor form, a ligand of CI-M6PR [19,22]. Cathepsin D is synthesized as an inactive precursor and becomes a mature active form in the lysosome. Thus, VPS35 knockdown influences not only the retrograde vesicular transport pathway, but also anterograde transport pathway. In VPS35 knockdown cells, we also observed an accumulation of the precursor form of Cathepsin D, while the levels of the mature form were comparable (Fig. 1a). These results indicate that only the transport of de novo synthesized Cathepsin D was influenced by VPS35 knockdown in our study. On the other hand, Cathepsin D existing at endosomes might be probably transported to lysosomes and became mature-form. CI-M6PR existing at endosome might also be transported to lysosome and degraded, and apparently showed the accumulation in trans-Golgi. Electron microscopy analysis revealed that abnormal endosome-lysosome-like vesicles accumulated in
the perinuclear region of VPS35 knockdown cells (Fig. 1e). These phenotypes indicate that VPS35 knockdown induced the defect of intracellular trafficking of CI-M6PR.

### 3.2. Knockdown of VPS35 and VPS29, but not of VPS26, reduces the levels of its binding partners

Interestingly, we found that the levels of VPS26A were significantly reduced and the levels of VPS29 were slightly reduced by VPS35 knockdown (Fig. 2a). However, levels of SNX1 and SNX2, further components of the retromer, were not affected by VPS35 knockdown. VPS35 acts as a central subunit of the VPS26–VPS29–VPS35 hetero-trimeric complex. It is possible that without formation of the trimeric complex with VPS35, VPS26 and VPS29 subunits are intrinsically fragile proteins. We examined the effects of VPS26A and VPS29 knockdown using specific siRNA on the levels of retromer components. The levels of VPS26A and VPS29 were significantly reduced in VPS29 knockdown cells. However, VPS26A knockdown had almost no effects on VPS29 and VPS35 levels. In addition, knockdown of SNX1 and SNX2, the other retromer complex components, also had no effects on VPS26A, VPS29, and VPS35 levels. Because SNXs are recruited to the endosome independently of the VPS26–VPS29–VPS35 complex [23], SNXs may not have an effect on the stability of cargo-recognition complex components.

There are two paralogs of VPS26 that exist in mammalian cells, VPS26A and VPS26B [24]. VPS26B is found in the cytoplasm with low levels at the plasma membrane, while VPS26A is predominantly associated with endosomal membranes in Hela cells [24]. The difference in phenotypes observed between knockdown of VPS26A and of the other two subunits may be caused by the existence of another VPS26 paralog, VPS26B. We therefore tested knockdown of VPS26B using a specific oligo (Fig. 2b and c). Similar to VPS26A knockdown, VPS26B knockdown did not influence the levels of VPS29 and VPS35. In addition, the levels of VPS29 and VPS35 were not significantly reduced in VPS26A and VPS26B double-knockdown cells. These results indicate that expression levels of VPS29 and VPS35 influence the levels of the other subunit of cargo-recognition complex, but VPS26 does not.

To clarify which protein degradation system participates in the degradation of retromer components in knockdown cells, we examined the effect of proteasome and lysosome inhibitors in each knockdown condition. The degradation of VPS35 in VPS29 knockdown cells, and VPS26A in VPS29 or VPS35 knockdown cells were inhibited by MG–132, a proteasome inhibitor, but not inhibited by pepstatin A plus E64d, a lysosome inhibitor cocktail (Fig. 2d) [25]. LC-3-II, an autophagosomal marker protein, was accumulated by both pepstatin A plus E64d treatment and MG–132 treatment. MG–132 inhibits not only proteasome but also inhibit certain lysosomal hydrolases such as cathepsins [25]. However, neither of these inhibitors affected the levels of VPS29 in VPS26 or VPS35 knockdown cells. It is possible that another protein degradation system is involved in the degradation of VPS29 in VPS26 or VPS35 knockdown conditions. These results indicate that VPS26 and VPS35 can easily be degraded by the proteasome in VPS29 knockdown cells.

### 3.3. VPS29 and VPS35 stably form an intermediate sub-complex of the retromer

Using isothermal titration calorimetry, Norwood and her colleagues reported that in vitro interactions of VPS35 with VPS29 and VPS26 are independent of each other [26]. However, it is possible that VPS26–VPS35 and VPS29–VPS35 are biologically different in stability, even if the efficiency of the sub-complex formation is comparable. To confirm whether the difference of effects by VPS29 or VPS35, and VPS26 knockdown on levels of binding partners are caused by the stability of sub-complexes, we performed immunoprecipitation experiments. mCherry-VPS35–, FLAG-VPS29–, and FLAG–VPS26A-expressing cells were used to detect intermediate sub-complexes of the retromer directly with knockdown of their binding partners (Fig. 3). N-terminal tagging of VPS35 does not interfere with the protein function in protein–protein interaction studies described previously [20], while there are a few reports about tagged VPS26A and VPS29. To overcome the steric hindrances by N-terminal or C-terminal FLAG-tag for protein–protein interactions, we used both N-terminal and C-terminal tagged VPS29 and VPS26A in this study. Although the expression levels of mCherry-VPS35 were also reduced by VPS29 knockdown, VPS26A knockdown did not have an obvious effect on mCherry-VPS35 (Fig. 3a). Similar to mCherry-VPS35 expression, VPS29 knockdown reduced co-immunoprecipitation of mCherry-VPS35 and VPS26A, whereas VPS26A knockdown did not influence co-immunoprecipitation of mCherry-VPS35 and VPS29. In addition, Parkinson’s disease linked mutation (D620N) of VPS35 did not show any difference with wild-type interaction with VPS29 or VPS26A in VPS26A knockdown or VPS29 knockdown cells, respectively. These results are...
consistent with previous report [27]. However, expression of C-terminal FLAG-tagged VPS29 and N-terminal FLAG-tagged VPS26A were much higher than N-terminal FLAG-VPS29 and C-terminal FLAG-VPS26A (Fig. 3b and c). The levels of immunoprecipitated binding partners were comparable with each other in scramble siRNA transfected cells. Therefore, expression levels of N-terminal FLAG-VPS29 and C-terminal FLAG-VPS26A seemed to be efficient to interact with endogenous binding partners, and both N- and C-terminal FLAG-tag did not cause steric hindrances for retromer formation. In addition, levels of both FLAG-VPS29 and FLAG-VPS26A were not influenced by knockdown of their binding partners. However, VPS29 knockdown significantly decreased VPS35 levels in FLAG-VPS26A immunoprecipitants (Fig. 3c), while VPS26A knockdown had no effect on VPS35 levels in FLAG-VPS29 immunoprecipitants (Fig. 3b). These results suggest that VPS35 forms two different intermediate dimers with VPS29 or VPS26, and the levels of VPS26A-VPS35 dimers are easily affected by VPS29 expression than VPS29–VPS35 is by VPS26A expression.

3.4. VPS26–VPS35 sub-complex is easily ubiquitylated in vivo

VPS26 and VPS35 are degraded by proteasomes in VPS29 knockdown cells (Fig. 2c). It is possible that the interaction with VPS29 determines the stability of the other two subunits. To confirm the hypothesis, we investigated ubiquitylation levels of mCherry-tagged VPS35 immunoprecipitants in VPS29 or VPS26A

Fig. 3. VPS29 and VPS35 forms stable intermediate sub-complex without VPS26A expression. (a–c) Western blotting of immunoprecipitants from cells expressing retromer components. mCherry-VPS35, mCherry-VPS35 D620N mutant (a), N- or C-terminal FLAG-VPS29 (b), or N- or C-terminal FLAG-VPS26 (c) were expressed in cells knocked down with siRNA for VPS35, VPS29, or VPS26A. Cell lysates were applied to immunoprecipitation with anti-RFP or anti-FLAG.

VPS26A knockdown had no effect on VPS35 levels in FLAG-VPS29 immunoprecipitants (Fig. 3b). These results suggest that VPS35 forms two different intermediate dimers with VPS29 or VPS26, and the levels of VPS26A-VPS35 dimers are easily affected by VPS29 expression than VPS29–VPS35 is by VPS26A expression.

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VPS26 and VPS35 are degraded by proteasomes in VPS29 knockdown cells (Fig. 2c). It is possible that the interaction with VPS29 determines the stability of the other two subunits. To confirm the hypothesis, we investigated ubiquitylation levels of mCherry-tagged VPS35 immunoprecipitants in VPS29 or VPS26A
knockdown cells (Fig. 4a and b). There were no differences in polyubiquitylation of mCherry-VPS35 immunoprecipitants between all conditions in the absence of MG-132. However, more polyubiquitylation of mCherry–VPS35 immunoprecipitant was detected in immunoprecipitants from VPS29 knockdown cells than the others in the presence of MG-132. These results indicate that VPS26–VPS35 sub-complex can be easily ubiquitylated and degraded by proteasome compared with the VPS29–VPS35 dimer or VPS26–VPS29–VPS35 trimer. VPS29 acts as a scaffold protein in cargo-recognition complex assembly and localizes to endosomes by binding with VPS35 [9,10]. In addition, SNX1 interacts with a groove on VPS29 on the face distal to VPS35, and with two different sites on the N- and C-terminal of VPS35 [12]. Therefore, interaction between VPS29 and VPS35 is intrinsically important for retromer assembly. However, mutation of VPS35 at arginine 107 in the PRRYL motif, which is the VPS26 binding site, results in loss of co-localization with Rab7 [15]. As Rab7 plays a role for retromer recruitment to endosome, interaction between VPS26 and VPS35 is important for the localization of retromer on endosomes. It is possible that the VPS26–VPS35 sub-complex is able to compete with the VPS26–VPS29–VPS35 trimer in localization on endosomes via interaction with Rab7. To avoid the competition, in the absence of VPS29 interaction, the VPS26–VPS35 sub-complex may be immediately removed by the ubiquitin–proteasome system. Therefore, even if the efficiency of VPS29–VPS35 and VPS26–VPS35 sub-complex formation is comparable in vitro [26], the VPS29–VPS35 sub-complex can be regarded as an initial sub-complex in retromer complex assembly in vivo. Together, VPS29–VPS35 sub-complex formation is thought to be the initial step of retromer assembly process in vivo, and the loss of VPS29 or VPS35 reduces the stability of the other components of the cargo-recognition complex of retromer.

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