Structure of the Novel C-terminal Domain of Vacuolar Protein Sorting 30/Autophagy-related Protein 6 and Its Specific Role in Autophagy*

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Vaccular protein sorting 30 (Vps30)/autophagy-related protein 6 (Atg6) is a common component of two distinct phosphatidylinositol 3-kinase complexes. In complex I, Atg14 links Vps30 to Vps34 lipid kinase and exerts its specific role in autophagy, whereas in complex II, Vps38 links Vps30 to Vps34 and plays a crucial role in vacuolar protein sorting. However, the molecular role of Vps30 in each pathway remains unclear. Here, we report the crystal structure of the carboxyl-terminal domain of Vps30. The structure is a novel globular fold comprised of three β-sheet-α-helix repeats. Truncation analyses showed that the domain is dispensable for the construction of both complexes, but is specifically required for autophagy through the targeting of complex I to the pre-autophagosomal structure. Thus, the domain is named the BARA (BAR and autophagy-specific) domain. On the other hand, the N-terminal region of Vps30 was shown to be specifically required for vacuolar protein sorting. These structural and functional investigations of Vps30 domains, which are also conserved in the mammalian ortholog, Beclin 1, will form the basis for studying the molecular functions of this protein family in various biological processes.

Vaccular protein sorting 30 (Vps30)/autophagy-related protein 6 (Atg6) was originally identified by yeast genetic screening as an essential factor for targeting the vacuolar hydrolase, carboxypeptidase Y (CPY), to the vacuole (4). At that time, yeast genetic screening also identified Vps30 as an essential factor for autophagy (2, 3), a bulk degradation pathway, in which a double membrane-bound structure, an autophagosome, sequesters a portion of the cytoplasm and delivers it to the vacuole for degradation (4).

In yeast, two distinct phosphatidylinositol 3-kinase (PI 3-kinase) complexes (complexes I and II) have been reported (5), and share the following three proteins between them: Vps34, the sole PI 3-kinase in yeast, Vps15, a putative protein kinase that activates Vps34 (6, 7), and Vps30. In addition to these three common components, Atg14 and Vps38 are incorporated into complexes I and II, respectively, and contribute to the complex construction by linking the Vps34-Vps15 complex to Vps30 (5). Autophagy, as well as most yeast genes responsible for autophagy, is conserved in higher eukaryotes including mammals. In mammals, Beclin 1, the orthologue of yeast Vps30, is known to be a key regulator of mammalian autophagy (8, 9). Beclin 1 forms a complex with mammalian Vps34 (10), and recent identification of mammalian Atg14 (also called as Atg14L or Barkor) showed that Beclin 1 forms a PI 3-kinase complex whose composition is similar to that of the yeast PI 3-kinase complex I (11–14), although Beclin 1 interacts directly with Vps34 (13, 15). Beclin 1 also forms several distinct PI 3-kinase complexes, some of which contain UVRAG, a putative Vps38 orthologue (11–14), and appears to regulate various membrane trafficking events.

In addition to its complex construction, yeast Atg14 was shown to be crucial for targeting of the PI 3-kinase complex I to the pre-autophagosomal structure (PAS), a perivacuolar structure where most of the Atg proteins colocalize and contribute to autophagosome formation (16–18). Similarly, mammalian Atg14 was shown to target the mammalian PI 3-kinase complex I to a specific site in the endoplasmic reticulum, termed an omegasome, which is a putative site of autophagosome formation domain; PAS, pre-autophagosomal structure; ECD, evolutionarily conserved domain; prApe1, proform of Ape1; mApe1, mature Ape1.
tion in mammals (19, 20). Vps38 was shown to be crucial for targeting of the PI 3-kinase complex II to endosomes in yeast (18). In mammals, UVRAG localizes at endosomes, suggesting that it functions similarly with Vps38 (11). Phosphatidylinositol 3-phosphates produced at autophagic membranes and endosomes by PI 3-kinase complexes recruit effector proteins to regulate each pathway (21–25). Although elucidation of the molecular roles of PI 3-kinase complexes and their components other than Vps30/Beclin 1 is underway, the molecular roles of Vps30/Beclin 1 remain poorly understood.

Thus far, structural study on Vps30/Beclin 1 family proteins has been limited; such research has been restricted to the Bcl-2 homology 3 (BH3) domain (~20-residue fragment) of Beclin 1 as a complex with Bcl-2 family proteins (26–29). A coiled-coil domain (CCD) has been predicted to be located at the central region of Vps30/Beclin 1 family proteins, and Beclin 1 CCD was shown to mediate interactions with various target proteins including Atg14 and UVRAG (12–15). Residues 244–337 of Vps30/Beclin 1 remain poorly understood. Despite the important roles of these conserved domains, their structural information has been totally lacking.

Here, we report the crystal structure of the C-terminal region of Vps30, which overlaps the C-terminal portion of the CCD and the extreme C-terminal region of Vps30. The structure is a novel globular fold comprising three β-sheet-α-helix repeats. In vivo studies have shown that the region is required for autophagy but not for vacuolar protein sorting. Thus, this region is named the β-α repeated, autophagy-specific (BARA) domain. Further analyses demonstrated that BARA is dispensable for the construction of PI 3-kinase complexes, but is crucial for the targeting of complex I to the PAS.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The genes encoding residues 1–557 (full-length), 1–319 (ΔBARA), 187–319 (CCD), and 309–557 of *Saccharomyces cerevisiae* Vps30 were amplified by polymerase chain reaction and cloned into pGEX6p-1 (GE Healthcare), whereas the gene encoding residues 73–123 of *S. cerevisiae* Atg14 was cloned into pET-28a (+) (Novagen) into which the glutathione S-transferase (GST) gene had already been inserted. To construct *Escherichia coli* co-expression plasmids encoding VPS30(187–319) and ATG14(73–123), the VPS30 gene was amplified by PCR and cloned into pET-11a (Novagen). Next, a fragment consisting of a ribosome binding site, GST gene, and the ATG14 gene derived from pET28a (+)-GST-ATG14 was ligated downstream of the VPS30 gene. The constructs were transformed into *E. coli* BL21(DE3) and expressed as a GST-fused protein. After cell lysis, GST-fused proteins were purified by affinity chromatography using a glutathione-Sepharose 4B (GS4B) column (GE Healthcare). In the case of GST-fused Vps30 proteins, GST was excised from the proteins with PreScission protease (GE Healthcare). Vps30(309–557) was then applied to a Resource S cation-exchange column (GE Healthcare) equilibrated with 20 mM HEPES, pH 6.8, and eluted by a linear gradient from 0 to 1 M NaCl in 20 mM HEPES, pH 6.8. Vps30(1–319) and Vps30(187–319) were again applied to a GS4B column after exchanging the solvent with phosphate-buffered saline using a HiPrep Desalting column (GE Healthcare). In the case of the co-expression experiments of Atg14 and Vps30, GST was excised from GS4B-purified Atg14(73–123) with PreScission protease and the sample was then again applied to a GS4B column after exchanging the solvent with phosphate-buffered saline using the HiPrep desalting column.

**Limited Proteolysis for Crystallization**—Purified Vps30(309–557) was incubated with trypsin (enzyme/substrate ratio: 1/300) for 2 h at 25 °C in 20 mM HEPES, pH 6.8, 200 mM NaCl. After addition of phenylmethylsulfonyl fluoride, the trimmed C-terminal domain of Vps30 was purified using a Superdex 75 gel filtration column (GE Healthcare) and concentrated to 4 mg/ml in 20 mM HEPES, pH 6.8, 500 mM NaCl. The concentrated sample was used for crystallization. MALDI-TOF mass analyses revealed that the 11 residues of the N-terminal (309–319) and 18 residues of the C-terminal (540–557) were removed from the sample.

**X-ray Crystallography**—Crystallization was performed by the sitting drop vapor diffusion method at 20 °C. A 2.0-μl protein solution was mixed with a 0.5 μl of reservoir solution consisting of 0.1 M sodium acetate, pH 4.5, and 2.4 M sodium acetate. For data collection, crystals were soaked in the reservoir solution supplemented with 20% glycerol, flash-cooled, and kept in a stream of nitrogen gas at ~178 °C during data collection. Diffraction data of both native and selenomethionine-labeled crystals were collected on an ADSC Quantum 315 charge-coupled device detector using beamline BL-5A (KEK, Japan). Diffraction data were processed using the HKL2000 program suite (31). The initial phasing was performed by the single-wavelength anomalous dispersion method using peak data of the selenomethionine-labeled crystal. After identifying four selenium sites and calculating initial phases using the SOLVE program (32), density modification was performed using the RESOLVE program (33). Model building was performed manually using the COOT program (34), and crystallographic refinement was performed using the Crystallography and NMR system software (35). Data collection, phasing, and refinement statistics are summarized in Table 1.

**Cell Strains and Media**—We utilized standard methods for yeast manipulation (36, 37). Yeast cells used in this study are listed on Table 2. Yeast cells were incubated in YEPD (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% D-glucose) or S.D. (0.17% yeast nitrogen base without amino acids or ammonium sulfate, 0.5% ammonium sulfate, and 2% D-glucose) + CA (0.5% casamino acid) medium containing appropriate amino acids.

**Plasmid Construction for Yeast Experiments**—pRS316-based plasmids for Atg14-HA-GFP and Vps38-HA-GFP were generated as reported previously (18). To construct yeast expression plasmids encoding VPS30 tagged with 3× myc (pRS314-VPS30-myc), the 3× myc sequence was amplified by PCR and inserted into pRS314–VPS30 (18). Deletion mutants of VPS30 were produced by inverse PCR of pRS314–VPS30-myc. To construct multicopy plasmids overexpressing the variants, the
After digesting pTK2672 using AvrII, it was transcribed into pRS303 using BamHI, resulting in pRS303-mRFP-APE1. All the constructs were sequenced to confirm their identities. HA-GFP were cultured in S.D. Vps30 mutants (pRS424) and either Atg14-HA-GFP or Vps38-myc were suspended in Z buffer (50 mM Tris-HCl, pH 7.5, 1.2M sorbitol, 1% yeast extract, 2% polypeptone, 1% glucose) containing 0.1 mg/ml of Zymolyase 100T (Seikagaku Corp.) and cultured in S.D. for 2 h. After washing the cell pellets with distilled water, cells were suspended in Z buffer, Vps30 WT, Vps30BARA, or Vps30CCD were added to the beads and further incubated at 4 °C or 10 min. After washing the beads three times with phosphate-buffered saline, proteins were then eluted with 10 mM glutathione in 50 mM Tris-HCl buffer, pH 8.0.

In Vivo Assays—To quantify the autophagic activity, we employed an Pho8Δ60 alkaline phosphatase assay as described previously (39). A CPY sorting assay was performed with the method modified from a previous report (40). Briefly, mid-log phase yeast cells (A600 = 1) were inoculated in S.D. + CA + Ura + Ade medium containing 50 mM KPO4, pH 5.7, for 2 h. The harvested cells and supernatant media were subjected to trichloroacetic acid (TCA) precipitation. Samples (I: intracellular, E: extracellular, corresponding to 0.3 A600 unit; E: extracellular, corresponding to 1.5 A600 unit) were subjected to Western blotting using anti-CPY antibody (1:5000; Lab stock), or anti-Pgk1 antibody (1:10000; Molecular Probes) as a control of cytosolic protein.

Fluorescent Microscopic Observation—The intracellular localization of mRFP-Ape1, Vps30-GFP, Atg14-GFP, or Vps38-GFP fusion proteins were visualized using inverted fluorescent microscopes (IX-71; Olympus, Tokyo, Japan), equipped with an EM-CCD digital camera (ImagEM; Hamamatsu Photonics). Images were acquired using Aquacosmos 2.6 software (Hamamatsu Photonics) and processed using Photoshop software (Adobe Systems). To observe PAS, yeast cells were finally treated with 0.2 μg/ml of rapamycin (Sigma) for 1 h to induce autophagy.

RESULTS

Structure of Vps30BARA—S. cerevisiae Vps30 consists of 557 amino acids, and a coiled-coil motif was predicted in its central region (residues 187–319; supplemental Fig. S1). However, structural information on the C-terminal region of the Vps30 family proteins has not yet been reported. The C-terminal region (residues 320–539) of Vps30 was obtained by limited data to an x-ray crystallography. The structure was refined against 2.3 Å data to an R-factor of 0.209 and a free R-factor of 0.243 (Table 1). The region corresponding to amino acids 320–539 of Vps30 was modeled, but three loop regions (residues 397–411, 422–432, and 461–487) were omitted from the model because they lacked defined electron density. The structure is comprised of three α-helices (H1, H2, and H3) and three β-sheets (S1, S2, and S3) with an approximate 3-fold symmetry (Fig. 1A). S1, S2, and S3 contain the 113.97 residues, and represent the BAR domain of Vps30.

Table 1: Data collection, phasing, and refinement statistics

- **Phasing statistics**
  - Resolution range (Å): 50–2.3 / 50–2.5
  - Compleness (%): 99.9 (100.0) / 100.0 (100.0)
  - Mean figure of merit: 0.27 / 0.32

- **Refinement statistics**
  - Resolution range (Å): 50–2.3 / 50–2.5
  - No. of protein atoms: 1,312 / 1,432
  - No. of water molecules: 42 / 41
  - Mean r.m.s. deviation from ideality: 0.006 / 0.006
  - Angles (°): 1.13 / 1.15

**TABLE 1**

| Strain   | Genotype         | Source       |
|----------|------------------|--------------|
| SEY6210  | MATαΔtdc1-3,112 ura3-52 his3Δ200 trplΔ961 lys2-801 suc2-2-A9 | Lab stock    |
| KY135    | SEY6210 vps30ΔΔ1LEU12 | 48           |
| KOY192   | SEY6210 pho8Δ60:kanMX | 48           |
| TKY1290  | SEY6210 VPS38-yeGFP:kanMX | Lab stock |
| TKY1307  | TKY1290 vps30Δ::3phNT1 | This study |
| TKY1308  | KOY192 vps30Δ::3phNT1 | This study |
| TKY1647  | KVY135 mRFP-APE1:His3 | This study |
| TKY1675  | TKY1647 ATG14–2xGFP::kanMX | This study |

Sacl/Sall fragment derived from these plasmids was ligated into the SacI/Sall site of pRS424. To construct plasmids to express GFP-tagged Vps30 truncates in yeast, a linker sequence containing Nhel and NotI sites was inserted into the site between the myc tag sequence and the terminal codon of pRS314-EGFP fragments. Then, the Vps30 BARA plasmids. Then, the EGFP fragment was inserted into yeast cells. The EGFP fragment was inserted into this site, resulting in pRS314-EGFP plasmids. All the constructs were sequenced to confirm their identities. To visualize PAS, mRFP-APE1 was inserted into yeast cells. The mRFP-APE1 fragment from pPS128 (38) was inserted into pRS303 using BamHI resulting in pRS303-mRFP-APE1 (pTK2672). After digesting pTK2672 using AvrII, it was transformed into KVY135, resulting in TKY1647 yeast cells.

Coimmunoprecipitation—Vps30Δ cells (KVY135) expressing Vps30 mutants (pRS424) and either Atg14-HA-GFP or Vps38-HA-GFP were cultured in S.D. + CA + Ade medium and harvested. After washing the cell pellets with distilled water, cells were suspended in Z buffer (50 mM Tris-HCl, pH 7.5, 1.2 M sorbitol, 1% yeast extract, 2% polypeptone, 1% glucose) containing 0.1 mg/ml of Zymolyase 100T (Seikagaku Corp.) and cultured at 30 °C for 20 min. Spheroplast cells were harvested by centrifugation at 500 x g for 5 min. After washing with Z buffer, the spheroplasts were suspended in lysis buffer (phosphate-buffered saline, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5% Tween 20, 1 mM PMSF, 20 μg/ml of leupeptin, 20 μg/ml of benzamidin, 10 μg/ml of pepstatin, and 40 μg/ml of aprotinin) and incubated at 4 °C for 5 min. Samples were then centrifuged at 15,000 x g for 10 min. Supernatants were incubated with anti-c-Myc antibody 9E10 (Covance) at 4 °C for 1 h. After the addition of protein G-Sepharose 4 beads, samples were incubated for an additional hour at 4 °C. Beads were then washed three times with lysis buffer. Bound proteins were eluted with SDS sample buffer and separated by SDS-PAGE. Protein bands were detected by Western blotting using either anti-c-Myc 9E10 (Santa Cruz Biotechnology), anti-HA (Abcam), or anti-Vps34 antibodies.
S3 are three-stranded, anti-parallel β-sheets resembling each other. The helices and the sheets are aligned in the order: S1-H1-S2-H2-S3-H3 from the N terminus to the C terminus (Fig. 1B). Three subdomains (S1-H1, S2-H2, and S3-H3) resemble each other and can be superimposed on each other with root mean square differences of 1.5–2.4 Å for main chain atoms (Fig.
Therefore, the subdomains are named repeat 1, repeat 2, and repeat 3, respectively. The hydrophobic residues in H1, H2, and H3 interact with each other (Fig. 1D, left) to form an inter-subdomain helix bundle. In addition, the hydrophobic residues in S1, S2, and S3 interact with the hydrophobic grooves formed between H1-H3, H1-H2, and H2-H3, respectively (Fig. 1D, right), stabilizing the helix bundle. These inter-subdomain interactions make the three subdomains into a stable globular fold.

Sequence alignment of Vps30 with its higher eukaryote homologues (Beclin 1) shows that the C-terminal region is highly conserved among them (Fig. 2B). Beclin 1 ECD corresponds to residues 297–390 of Vps30, which comprise the C-terminal portion of the predicted coiled-coil, repeat 1, and the N-terminal portion of repeat 2. The region C-terminal to the ECD is also conserved, especially the residues constituting the secondary structures of repeat 3 (Fig. 1D, right). Most of the hydrophobic residues contributing to the interaction among the three subdomains in Fig. 1D are conserved (Fig. 2B). These observations suggest that the three-repeat architecture of the C-terminal region of Vps30 is conserved among Vps30/Beclin 1 homologues.

Crystal Structure of Vps30 BARA

Previous study showed that the ECD of Beclin 1 is crucial for mammalian autophagy (30). The ECD corresponds to the C-terminal portion of CCD and the N-terminal portion of BARA so that deletion of the ECD could influence both CCD and BARA structures. Therefore, we propose that the residues constituting BARA should be treated as a structural and functional unit when studying the function of Vps30/Beclin 1 domains. Consequently, Vps30 can be structurally divided into three regions: N-terminal domain that is structurally uncharacterized (NTD, residues 1–186), CCD (residues 187–319), and BARA (residues 320–557) (Fig. 2A).

Vps30BARA Is Required for Autophagy—Because Vps30 BARA (Vps30BARA) is conserved among Vps30/Beclin 1 homologues, it is speculated that Vps30BARA plays important roles in the functioning of Vps30. To investigate the contribution of Vps30BARA on autophagy, cells expressing truncated forms of Vps30 were subjected to the Pho8 assay, a method commonly used for the assessment of autophagic activity (42, 43). This method utilizes a genetically engineered cytosolic form of an alkaline phosphatase, Pho8 (Pho8WT), which is delivered into the vacuole exclusively by autophagy, and activated. Thus, the autophagic activity correlates well with the phosphatase activity. As shown in Fig. 3A, wild-type cells as well as vps30Δ cells expressing wild-type Vps30 (Vps30WT cells) showed increased phosphatase activity in response to starvation conditions for 4 h, whereas vps30Δ cells showed no increase in the activity. Compared with these cells, Vps30BARA or Vps30BARA cells showed only a slight increase in activity.
pathway under nutrient-rich conditions and by autophagy in response to starvation conditions or rapamycin treatment. In the vacuole, prApe1 is processed into a mature form (mApe1), which can be monitored by Western blotting for Ape1. Therefore, the mApe1:prApe1 ratio observed in this assay reflects the progress of autophagy. As shown in Fig. 3B, Vps30WT and Vps30CCD+BARA cells showed a strong mApe1 band and a weak prApe1 band in response to rapamycin treatment, whereas vps30Δ cells and Vps30BARA cells showed no mApe1 band and a strong prApe1 band on the blot. These results are well consistent with those obtained by the Pho8Δ60 assay, showing that Vps30NTD is dispensable and Vps30BARA is insufficient for autophagy. Vps30BARA cells showed strong mApe1 and prApe1 bands. Because monitoring Ape1 maturation is a much more sensitive method to detect autophagic activity compared with the Pho8Δ60 assay, this partial progression of Ape1 maturation may reflect the weak but remaining autophagic activity in these cells and suggest that Vps30BARA is important for efficient autophagy.

**Vps30BARA Is Dispensable for Vacuolar Protein Sorting**—In addition to autophagy, Vps30 is also involved in the vacuolar protein sorting pathway. The contribution of Vps30BARA on vacuolar protein sorting was studied by a carboxypeptidase Y (CPY) sorting assay, which observes the secretion of a vacuolar hydrolase CPY to the extracellular space from cells defective in Golgi apparatus-endosome transport, a critical step in CPY sorting that requires PI 3-kinase complex II. It has been reported that vps30Δ and vps38Δ cells but not atg14Δ cells show missorting of CPY, resulting in the secretion of the Golgi-derived p2 form of CPY (p2-CPY) (2, 22). We first confirmed that p2-CPY was secreted in vps30Δ cells, whereas in Vps30WT cells, p2-CPY was scarcely secreted and mCPY was accumulated in the vacuole (Fig. 3C, lanes 1–4). In Vps30BARA cells, most CPY were detected as mCPY in the intracellular extracts, and p2-CPY was scarcely detected in the extracellular extracts as in Vps30WT cells (Fig. 3C, lanes 3–6). In contrast, in Vps30CCD+BARA cells, significant amounts of p2-CPY were detected in the extracellular extracts and mCPY was only weakly detected in the intracellular extracts as in vps30Δ cells (Fig. 3C, lanes 1, 2, 7, and 8). These results suggest that Vps30NTD but not Vps30BARA is required for vacuolar protein sorting.

**Vps30BARA Is Not Required for Interaction with Atg14 or Vps38**—Vps30 interacts directly with Atg14 and forms the autophagy-specific PI 3-kinase complex I. Because Vps30BARA has been shown as dispensable for autophagy, we next examined whether Vps30BARA is responsible for the interaction with Atg14 by coimmunoprecipitation experiments. Myc-tagged Vps30WT and three truncated forms of Vps30, Vps30BARA, Vps30CCD+BARA, and Vps30CCD−BARA, were co-expressed with Atg14 fused to a 3X hemagglutinin (HA) tag and green fluorescent protein (GFP; Atg14-HA-GFP) in vps30Δ cells, and Myc-tagged Vps30s were pulled down with anti-Myc antibody and protein G-Sepharose beads. As shown in Fig. 4, Vps30WT, Vps30BARA, and Vps30CCD+BARA but not Vps30CCD−BARA interacted with Atg14. These results suggest that Vps30BARA is dispensable for the interaction with Atg14. Similarly, immunoprecipitation experiments using vps30Δ cells co-expressing...
truncated mutants of Vps30 and Vps38-HA-GFP showed that Vps30BARA is dispensable for the interaction with Vps38. Vps34 was coimmunoprecipitated with Vps30 WT and truncated forms of Vps30 except for Vps30BARA, which is consistent with the previous report that Vps30 interacts with Vps34 through either Atg14 or Vps38 (5).

Vps30BARA Is Required for Targeting of PI 3-Kinase Complex I to PAS—Vps30BARA is crucial for autophagy; nevertheless, Vps30BARA was shown to be dispensable for the construction of PI 3-kinase complex I. What then is the function of Vps30BARA in autophagy? To examine the role of Vps30BARA in PAS targeting of PI 3-kinase complex I, we first monitored the localization of GFP-tagged Vps30 truncates in cells expressing mRFP-tagged Ape1 as a PAS marker (Fig. 5).

In vps30Δ cells expressing Vps30WT-GFP (Vps30WT-GFP cells), GFP signals were observed as dots, some of which were merged with those of mRFP-Ape1 in response to rapamycin treatment. This result suggests that some populations of Vps30 localize to the PAS and others to endosomes as reported previously (18). Similar co-localization with mRFP-Ape1 was observed for Vps30CCD/H11001BARA-GFP although the number of total dots was decreased. This result suggests that Vps30CCD/H11001BARA localizes to the PAS, whereas its localization to endosomes is impaired. In Vps30BARA-GFP cells, the signals were diffused in the cytoplasm and not merged with the dots of mRFP-Ape1. In the latter two cells, the signals of mRFP-Ape1 were strong, suggesting that autophagy is inactive and mRFP-Ape1 accumulated in the cytosol. All these results are consistent with the activities of Vps30 truncates in autophagy and CPY sorting (Fig. 3).

We next monitored the localization of GFP-tagged Atg14 in cells expressing Vps30 truncates and mRFP-tagged Ape1 (Fig. 5B). In Vps30WT cells, the signals of Atg14-GFP were merged with those of mRFP-Ape1 in response to rapamycin treatment. In Vps30CCD/H11001BARA cells, the signals of Atg14-GFP were observed as dots, some of which were merged with those of mRFP-Ape1. In Vps30BARA cells, the signals of Atg14-GFP were also observed as dots, but less frequently, and they were seldom merged with those of mRFP-Ape1 as in the case in vps30Δ cells.
containing a control vector. These results are well consistent with the localization of Vps30 trun- cates, strongly suggesting that Vps30BARA is required for efficient PAS targeting of PI 3-kinase complex I.

Characterization of Functions of Vps30CCD and Vps30NTD—At this point, the autophagy-specific function of Vps30BARA has been established. But what then is the function of the other two domains, Vps30CCD and Vps30NTD? PAS targeting of PI 3-kinase complex I requires not only Vps30BARA but also Vps30CCD (Fig. 5) and Atg14 (18). Immunoprecipitation experiments suggest that Vps30CCD is crucial for the interaction with Atg14 (Fig. 4). These data suggest that one function of Vps30CCD is to link Vps30BARA and Atg14 together for PAS targeting of complex I. We subsequently studied the direct interaction between Vps30 and Atg14 using recombinant proteins. Three coiled-coil motifs (I, II, and III) were predicted in the N-terminal domains: NTD, CCD, and BARA (Fig. 1). Because the repeat architecture, so it is named BARA (Fig. 1). Because the residues constituting the secondary structural elements are highly conserved among Vps30/Beclin 1 family proteins (Fig. 2B), the domain appears to be structurally and functionally conserved among them. Based on structural information and sequence alignment, we divided Vps30/Beclin1 into three domains: NTD, CCD, and BARA.

Based on this domain definition, we established the functions of NTD, CCD, and BARA of Vps30. They are summarized in Fig. 8. Vps30BARA is dispensable for the interaction with both Atg14 and Vps38 and for CPY sorting, but is crucial for autophagy through the targeting of the PI 3-kinase complex I to the PAS. On the other hand, Vps30NTD is necessary for the interaction with Vps38 but not with Atg14, and is crucial for CPY sorting but not for autophagy. Vps30CCD is crucial for the interaction with Atg14 and presumably with Vps38 too, and thus appears to be essential for both autophagy and CPY sorting. During truncation analysis, we succeeded in obtaining Vps30 mutants whose activity is restricted to either autophagy

FIGURE 6. Vps30CCD directly interacts with Atg14CCII. A, in vitro pulldown assay between GST-Atg14CCII and Vps30 mutants. Asterisks indicate degraded products of GST-Atg14CCII and Vps30CCD. B, purification of GST-Atg14CCII coexpressed with Vps30CCD in E. coli. After purification with GS4B beads (lane 1), GST was excised from Atg14CCII with PreScission protease (lane 2) and free GST was removed from the sample using GS4B beads (lane 3). The asterisk indicates a degradation product of Vps30CCD. Protein bands in A and B were stained with Coomassie Brilliant Blue.

DISCUSSION

Thus far, structural information of the Vps30/Beclin 1 family proteins has been limited. Here, we succeeded in determining the structure of the C-terminal region of Vps30 by x-ray crystallography. The structure shows a unique β-sheet-α-helix repeat architecture, so it is named BARA (Fig. 1). Because the residues constituting the secondary structural elements are highly conserved among Vps30/Beclin 1 family proteins (Fig. 2B), the domain appears to be structurally and functionally conserved among them. Based on structural information and sequence alignment, we divided Vps30/Beclin1 into three domains: NTD, CCD, and BARA.

Based on this domain definition, we established the functions of NTD, CCD, and BARA of Vps30. They are summarized in Fig. 8. Vps30BARA is dispensable for the interaction with both Atg14 and Vps38 and for CPY sorting, but is crucial for autophagy through the targeting of the PI 3-kinase complex I to the PAS. On the other hand, Vps30NTD is necessary for the interaction with Vps38 but not with Atg14, and is crucial for CPY sorting but not for autophagy. Vps30CCD is crucial for the interaction with Atg14 and presumably with Vps38 too, and thus appears to be essential for both autophagy and CPY sorting. During truncation analysis, we succeeded in obtaining Vps30 mutants whose activity is restricted to either autophagy...
Because targeting of Vps30 to the PAS requires Atg14 (18), it is suggested that the Vps30-Atg14 complex is targeted to the PAS interdependently, for which Vps30<sup>CCD+BARA</sup> plays a crucial role. How then is the Vps30-Atg14 complex targeted to the PAS? Systematic and quantitative analysis of the PAS localization of Atg proteins using fluorescence microscopy showed that the Vps30-Atg14 complex localizes to the PAS depending on the presence of Atg9, Atg13, or Atg17 (44). Therefore, the simplest idea is that the Vps30-Atg14 complex localizes to the PAS through direct interaction with these proteins. Alternatively, it is also possible that the Vps30-Atg14 complex interacts with some unidentified factor(s) on the PAS. In either case, Vps30<sup>BARA</sup> may be directly involved in such interactions together with Atg14. It is not clear whether Vps30<sup>CCD</sup> is also directly involved in such interactions or whether its role is restricted to linking Vps30<sup>BARA</sup> and Atg14. Conserved residues are clustered on one surface of Vps30<sup>BARA</sup> (supplemental Fig. S2, left). Because this surface contains the N terminus, it may form a continuous surface with the Vps30<sup>CCD</sup>-Atg14 complex. We speculate that such a conserved continuous surface might be responsible for recognizing conserved factor(s) such as Atg9 and Atg13, through which the PI 3-kinase complex I might be targeted to the PAS. Likewise, PI 3-kinase complex II might be targeted to endosomes through the interaction between the Vps30<sup>BARA</sup>-Vps38 complex and some factor(s) on endosomes. Very recently, crystal structures of the CCD and the C-terminal regions of Beclin 1 have been reported (45, 46), which show that the C-terminal region has a globular fold similar to Vps30 BARA and Beclin 1 ECD is a part of CCD and BARA. It was also reported that Beclin 1 BARA possesses an aromatic finger (Phe-Phe-Trp sequence) in the loop region, which mediates direct association with lipids, especially cardiolipin, and this lipid-binding ability is important for mammalian autophagy (45). However, sequence alignment shows that the aromatic finger is not conserved in Vps30 (Fig. 2B), and thus the PAS-targeting function of Vps30 BARA could not be attributed to lipid binding. Structural studies on the Vps30-Atg14 and Vps30-Vps38 complexes and identification of their interacting partner(s) are required for revealing the molecular mechanism of proper targeting of PI 3-kinase complexes.

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