Propeptide of Aminopeptidase 1 Protein Mediates Aggregation and Vesicle Formation in Cytoplasm-to-Vacuole Targeting Pathway

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Background: Aminopeptidase 1 (Ape1) aggregates and is sequestered by autophagic proteins into a vesicle for vacuolar transport.

Results: The propeptide of Ape1 binds prApe1 and Atg19. Atg19 and Atg11 binding is not sufficient for vacuolar transport.

Conclusion: Aggregate shape is important in vesicle formation. Ape1 dodecamerization may cluster propeptides, causing aggregation.

Significance: These results provide a better understanding of protein aggregation and autophagic vesicle formation.

Misfolded protein aggregation causes disease and aging; autophagy counteracts this by eliminating damaged components, enabling cells to survive starvation. The cytoplasm-to-vacuole targeting pathway in yeast encompasses the aggregation of the premature form of aminopeptidase 1 (prApe1) in cytosol and its sequestration by autophagic proteins into a vesicle for vacuolar transport. We show that the propeptide of Ape1 is important for aggregation and vesicle formation and that it is sufficient for binding to prApe1 and Atg19. Defective aggregation disrupts vacuolar transport, suggesting that aggregate shape is important in vesicle formation, whereas Atg19 binding is not sufficient for vacuolar transport. Aggregation involves hydrophobicity, whereas Atg19 binding requires additional electrostatic interactions. Ape1 dodecamerization may cluster propeptides into trimeric structures, with sufficient affinity to form propeptide hexamers by binding to other dodecamers, causing aggregation. We show that Ape1 aggregates bind Atg19 and Atg8 in vitro; this could be used as a scaffold for an in vitro assay of autophagosome formation to elucidate the mechanisms of autophagy.

Autophagy enables cells to eliminate and recycle damaged components and to survive when starved of vital nutrients (1). During autophagy, cellular components are surrounded by membrane to form an autophagosome for subsequent degradation (2). The majority of autophagic proteins and their function were first identified in yeast (3). Although autophagy is universal and highly conserved, at the same time, because the autophagic machinery is very adaptable, there are many different types of selective autophagy.

Importantly, because the different modes of selective autophagy represent adaptations of the overall mechanism of macroautophagy, relying on many of the same proteins, any insight into a specific mode of selective autophagy can be extrapolated to other pathways (4, 5). Selective autophagy generally requires autophagic receptors and adaptors to target specific cargo. For example, in Saccharomyces cerevisiae, the Atg19 receptor and its parologue Atg34 specifically target vacuolar proteases; meanwhile, in mammalian cells, the Nix receptor is required for mitophagy, and the p62/SQSTM1 and NDP52, and optineurin (11–18). The mode of interaction between some autophagic receptors and adaptors with Atg8, or its mammalian homologue LC3, is conserved. Atg19, Atg34, p62, NBR1, and Nix all have WXXL motifs for binding Atg8, binding by forming an intermolecular parallel β-sheet (8–10, 19).

Selective autophagy also plays a role in the clearance of defective aggregates that cause neurodegenerative diseases and also in cancer prevention by the removal of damaged mitochondria that produce toxic reactive oxygen species through mitophagy (20–25). There is already interest in cancer drugs that target autophagy; silencing of autophagic modulators has been shown to sensitize cancer cells to different therapies (26–28). Furthermore, in fly and mouse models for Huntington disease and spinocerebellar ataxia, up-regulation of autophagy led to an increase in clearance of aggregates and to a reduction of symptoms (29–31).

The cytoplasm-to-vacuole targeting (Cvt) pathway transports several vacuolar proteases that were synthesized in cytosol to the vacuole via selective autophagy. It has so far only been found in yeast, specifically in S. cerevisiae and Pichia pastoris (23, 32). The Cvt pathway has been of further interest because it targets protein aggregates and packages them inside autophagic vesicles. The protein aggregates are constituted by the vacuolar protease aminopeptidase 1 (Ape1), which is synthesized in cytosol and forms tetrahedral dodecamers (33, 34). Increasing...
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Protein concentrations cause dodecamers to disassemble in a stepwise manner, suggesting that during dodecamer assembly, firstly monomers bind and form symmetrical dimers; these trimerize to form hexamers, and then two hexamers bind to form the final dodecamer (35). Its homologue, a *Borrelia burgdorferi* aminopeptidase (Protein Data Bank (PDB) ID: 1Y7e), forms a similar dodecameric structure. Mutants defective in dodecamerization are enzymatically inactive, and thus dodecamerization is critical for functionality (36).

The Cvt pathway is unique from other forms of vesicular traffic because cytosolic Ape1 concentrates itself by forming a single aggregate that recruits its receptor Atg19 and directs vesicle formation (37–42). Atg19 recruits the adaptor protein Atg11 and the membrane-anchored protein Atg8. Atg11 in turn binds Atg1, Atg17, and Atg20, all of which are part of the vesicle-forming machinery (43, 44). Atg8 plays a key role; it is a lipidated protein that tethers autophagic membrane to form the double membrane vesicle (45). Atg19 also binds α-mannosidase 1 (Ams1) for vacuolar transport via the Cvt pathway and may recruit proteins targeted for degradation to autophagosomes (46, 47). For delivery of Ape1 to the lumen of the vacuole, the outer membrane of the vesicle fuses with the vacuole, whereas the inner lipid membrane is degraded by Atg15 to release the cargo. Recently, it was found that the Cvt pathway transports two additional proteins: the vacuolar aminopeptidase 4 (Ape4) and the cytosolic cysteine protease Lap3 (48, 49). Ape4, similarly to Ape1 and Ams1, binds Atg19 for transport to the vacuole and is a vacuolar protease. Lap3 is cytosolic and is only spatially associated with the Cvt complex; the purpose of its transport appears to be for its selective degradation.

Ape1 is synthesized as a precursor (prApe1) with an N-terminal propeptide of 45 amino acids (50, 51). The propeptide directs transport to the vacuole via binding to Atg19 (42). It is predicted to form a helix-turn-helix, of which the N-terminal helix is amphipathic (52). Once in the vacuole, the propeptide is cleaved to generate the mature form, mApe1. Although many precursor proteins are inactive, prApe1 may be enzymatically active (36, 53). Aggregation may facilitate vesicle formation and help inhibit prApe1 proteolytic activity. In comparison with the *B. burgdorferi* aminopeptidase, which does not form aggregates, prApe1 has additional amino acids that are predicted to be positioned on the dodecamer surface: 45 on its N termini that constitute the propeptide and 10 on its C termini. We propose that the propeptide of Ape1 directs aggregation and Cvt vesicle formation and that this involves hydrophobic interactions, where an amphipathic α helix on the propeptide enables dimerization of prApe1 proteins. The mechanism of Atg19 binding to the propeptide differs from that of prApe1 aggregation, enabling Atg19 to localize to the surface of aggregates, acting as an adaptor for vesicle formation.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmid Construction**—Standard methods were used for cell growth, manipulation, and cloning procedures. The yeast strains used in this study were SEY6210 (MATa/MATα leu2-3,112/leu2-3,112 ura3-52/ura3-52 his3-D200/ his3-D200 trp1-D901/trp1-D901 ade2/ADE2 suc2-D9/suc2-D9 GAL/GAL LYS2/lys2-801), BY4742 (MATα/α his3Δ1/Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ ura3Δ0) and P69-4A (MATα trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ LYS::GAL1-HIS3 GAL2-ADE2. GAL2 met2::GAL7-lacZ). Rosetta *Escherichia coli* were used for protein purification.

**Density Glycerol Gradient**—200 μl of cell lysates were loaded on a 5.5-ml 20–50% glycerol step gradient prepared in 20 mM K-Pipes, pH 6.8, 1 mM PMSF. The gradients were spun in an ultracentrifuge (model Optima LE-80K; Beckman) for 5 h at 55,000 RPM, at 4 °C, using a SW55Ti rotor (Beckman). Thirteen fractions were collected from the top of the gradient and TCA-precipitated.

**Pulldown Assay**—Rosetta propeptide-His6 were collected by centrifugation, lysed using lysozyme 1 mg/ml in lysis buffer (50 mM sodium phosphate, 300 mM sodium chloride, 3 mM imidazole, pH 8), and incubated at 4 °C for 30 min. Lysates were incubated with nickel-nitrilotriacetic acid-agarose beads (Qiagen) overnight at 4 °C. Yeast lysates were incubated with propeptide-His6-agarose beads for 2 h at 4 °C.

**DSP Cross-linking and Propeptide Cleavage with PrtK**—Di-thiobis succinimidyl propionate (DSP) was used to cross-link proteins following the manufacturer’s instructions (Pierce). DSP was dissolved in dimethyl sulfoxide (DMSO) at a 25 mM final concentration, immediately added to cell lysate to a final 2 mM concentration, and incubated for 2 h in ice. Proteinase K (PrtK) was added to a final 50 μg/ml concentration and incubated for 30 min at 4 °C, unless a different time interval is stated.

**RESULTS**

*prApe1 Aggregates Are Stable in Vitro*—In atg19Δ cells, RFP-prApe1 aggregates are visible as a single large red puncta in cytosol (Fig. 1A). However, when cells were lysed in a 10 mM Tris, pH 7, buffer, aggregates disassembled in vitro. To identify a buffer in which aggregates would be stable in vitro, atg19Δ RFP-prApe1 cells were lysed in buffers with different compositions. RFP-prApe1 aggregates are stable in vitro in 0.5 mM sodium chloride buffers (Fig. 1A).

**Hydrophobicity Is Important in Aggregation**—The Hofmeister series classifies ions in their ability to change the arrangement of water molecules and influence the solubility of nonpolar molecules (54–57). Early members of the series decrease the solubility of nonpolar molecules (54–57). Early members of the series decrease the hydrophobic interactions; potassium iodide, potassium phosphate, sodium phosphate, and sodium fluoride) stabilized aggregates, whereas salts predicted to decrease hydrophobic interactions (potassium iodide, sodium chloride, and calcium chloride) caused aggregates to disassemble (Fig. 1B). Thus hydrophobicity is involved in prApe1 aggregation. To identify the best buffers for stabilizing aggregates in vitro, the stability of aggregates was tested using different concentrations of four of the buffers that stabilize aggregates (Fig. 1C). 0.2 mM potassium phosphate and 0.5 mM sodium chloride buffers effectively stabilize aggregates. A 0.2 or
0.25 M potassium phosphate buffer was used for future experiments because the intracellular concentrations of sodium and chloride are typically maintained very low in cells for osmoregulation (61).

Furthermore, our methodology and choice of a 0.2–0.25 M potassium phosphate buffer are physiologically relevant because previous studies using S. cerevisiae have measured the cytosolic concentration of potassium and free phosphate to be between 290 and 340 mM for potassium and between 10 and 75 mM for free phosphate (62–68). Similar studies show that the concentration of additional free ions is very low: 5 mM for sulfate, 0.05–0.5 mM for calcium, 0.1–1 mM for magnesium, about 20 mM for sodium, and about 0.1 mM for chloride (62–65, 69–74). It must also be noted that during preclearing, a very low centrifugal speed was used, 1,500 × g RCF for 1 min, to ensure that aggregates were not lost in pellet fractions; consequently, most of the cytosolic components would still be present during the in vitro studies. In addition, because a high density of cells was used during lysis, whole lysates consisting of about one-third of cells and two-thirds of buffer and cytosolic components would only be diluted by about a 1:3 ratio. Lastly, our results from studies using sodium chloride are valid because aggregate stability in 0.5 M sodium chloride is similar as when using 0.2 M potassium phosphate (see Fig. 5B).

Effects of Amino Acids, Sugars, and Detergents on Aggregate Stability—Sugars stabilized aggregates, possibly by increasing viscosity and by changing the molecular structure through their hydroxyl groups (Fig. 1D). We tested a nonpolar (alanine), polar (serine), and basic (arginine) amino acid. Only arginine stabilized aggregates in vitro. If hydrophobicity is involved in prApe1 aggregation, nonpolar amino acids may interfere with aggregation by blocking hydrophobic regions on the protein. Basic or acidic amino acids may act as salts and potentiate hydrophobic interactions. Consistent with this, the basic amino...
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Acid arginine stabilized prApe1 aggregates. Detergents help solubilize membranes and reduce nonspecific interactions, so we tested whether detergents disrupt aggregate stability (Fig. 1D). atg19Δ cells were lysed in a 0.25 M potassium phosphate buffer, and different detergents were added up to 1% concentration. Tween 20 did not disrupt prApe1 aggregation.

Propeptide Is Required for Aggregation—Hydrophobicity is important for prApe1 aggregation; hence if the propeptide forms an amphipathic structure, its hydrophobic region could contribute to aggregation. Consistent with this, the propeptide is cleaved by proteinase B to form mApe1, which does not aggregate in the vacuole (36, 50, 53). To investigate whether the propeptide is required for aggregation, a GFP-tagged construct of Ape1 that lacked the propeptide (ape1Δ2–45-GFP) was made. The cellular localization of prApe1Δ2–45-GFP in ape1Δ cells was compared with that of full-length, GFP-tagged Ape1 (prApe1-GFP). ape1Δ ape1-GFP cells show small punctate structures and green vacuoles, suggesting that prApe1-GFP aggregates and goes to the vacuole (Fig. 2A). ape1Δ ape1Δ2–45-GFP cells instead show a diffuse green cytosolic signal and no vacuolar localization. The lack of prApe1Δ2–45-GFP aggregates suggests that the propeptide is required for dodecamerization or aggregation.

4th N-terminal Amino Acid Is Required for Aggregation—To identify which regions of the propeptide are required for aggregation, prApe1 lacking 2, 3, 4, 5, 7, and 11 N-terminal amino acids and tagged with GFP was expressed in ape1Δ cells (Fig. 2A). Their localization was compared with that of prApe1-GFP and prApe1Δ2–45-GFP. prApe1Δ2-GFP forms a single aggregate in cytosol similar to prApe1-GFP and is effectively transported to the vacuole. prApe1Δ2–3-GFP has a block in vacuolar transport but still forms single aggregates. prApe1Δ2–4–GFP, prApe1Δ2–5–GFP, and prApe1Δ2–7–GFP form multiple small aggregates, suggesting that aggregation is defective. prApe1Δ2–11–GFP is similar to prApe1Δ2–45-GFP, showing a diffuse cytosolic GFP signal that suggests a complete block in aggregation.

5th N-terminal Amino Acid Is Required for Atg19 Binding—A two-hybrid assay was used to verify propeptide binding to Atg19, using PJ69-4A ape1Δ atg19Δ cells with AD-Atg19 and BD-prApe1 with N-terminal deletions (Fig. 3B). BD-prApe1Δ2 and BD-prApe1Δ2–4 bind AD-Atg19 similarly to full-length BD-prApe1. When more than 5 N-terminal amino acids are deleted from prApe1, Atg19 binding is blocked, suggesting that the 5th amino acid is required for Atg19 binding. Consistent with this, in ape1Δ GFP-atg19Δ cells with N-terminal deletions in RFP-prApe1 and prApe1, RFP-prApe1Δ2–4 co-localizes with GFP-Atg19, suggesting that they still bind; however, RFP-prApe1Δ2–5 does not effectively co-localize with GFP-Atg19, showing that their binding is defective (Fig. 3A). However, prApe1Δ2–4–GFP is not effectively transported to the vacuole, although the two-hybrid assay and GFP-Atg19 co-localization with RFP-prApe1Δ2–5 suggest that Atg19 does bind prApe1Δ2–4–GFP. This is consistent with the lack of mApe1 in ape1Δ ape1Δ2–4 cells (see Fig. 5C). In contrast, wild type, ape1Δ ape1Δ2, and ape1Δ ape1Δ2–3 cells have mApe1. Because prApe1Δ2–4–GFP can still bind to Atg19, perhaps the impaired vacuolar transport results from defective aggregation.

FIGURE 2. Propeptide is required for aggregation and vacuolar transport, not for dodecamerization. A, the 4th N-terminal amino acid in the propeptide is required for aggregation and vacuolar transport. ape1Δ cells with prApe1-GFP that have N-terminal deletions are shown. Punctate structures are aggregates. Multiple, small punctae or a diffuse cytosolic GFP signal are due to defective aggregation. DIC, differential interference contrast. B, prApe1Δ2–45-GFP is incorporated into prApe1 aggregates and transported to the vacuole. Wild type cells with full-length prApe1 and either prApe1-GFP or prApe1Δ2–45-GFP are shown. C, the propeptide is not required for dodecamerization. ape1Δ, Wild type, atg19Δ, or atg19 ape1Δ2–45-GFP cells were lysed and loaded onto a step glycerol gradient. Albumin, ferritin, and thyroglobulin were used as a ladder. Ape1, prApe1, mApe1, and Ape1Δ2–45 were all present in similar fractions as thyroglobulin. Fractions were analyzed by Western blot using an antibody against Ape1.
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because there are multiple small aggregates in cytosol instead of the single large aggregate formed by full-length prApe1-GFP (Fig. 2A); therefore aggregate shape is important for Cvt vesicle formation.

Propeptide Is Not Required for Dodecamerization—Although wild type cells with both full-length prApe1 and prApe1Δ2–45-GFP show a diffuse cytosolic localization of GFP due to the defective aggregation of prApe1Δ-GFP (Fig. 2B), there are also green punctate structures and green vacuoles, suggesting that prApe1Δ2–45-GFP can be incorporated into prApe1 aggregates and transported to the vacuole. mApe1 forms dodecamers but does not form aggregates in the vacuole, suggesting that the propeptide is not required for dodecamerization, only for aggregation (33). To investigate whether prApe1Δ2–45 forms dodecamers, we used ape1Δ, wild type, atg19Δ, and atg19Δ ape1Δ ape1ΔΔ2–45 cell lysates and estimated the molecular weight of prApe1, mApe1, and prApe1Δ2–45 in native conditions using a glycerol density gradient (Fig. 2C). prApe1, mApe1, and prApe1Δ2–45 are present in similar fractions as thyroglobulin, which has a molecular mass of 670 kDa, suggesting that they form a dodecamer; the molecular mass of an individual prApe1 is 61 kDa, and the molecular mass of Ape1 and mApe1 is 56 kDa. Therefore the propeptide is not required for dodecamerization.

prApe1 Forms Aggregates in Vitro—We verified that soluble prApe1 can form aggregates in vitro. atg19Δ ape1Δ-GFP and atg19Δ RFP-Ape1 were lysed using a 2 mM potassium phosphate buffer. Lysates were mixed, and no aggregates were visible, suggesting that prApe1 was soluble. To cause aggregation, potassium phosphate was added to a final concentration of 0.2 M. We verified the formation of red and green punctae constituted by prApe1-GFP and RFP-prApe1, showing that soluble prApe1 aggregates in vitro (Fig. 4A).

Propeptide Is Required for Aggregation in Vitro—We investigated whether in vitro aggregation is also directed by the propeptide. atg19Δ RFP-ape1 lysate was mixed with atg19Δ ape1Δ ape1Δ ape1ΔΔ2–45-GFP lysate, using 0.2 M potassium phosphate buffer. The green prApe1-GFP aggregates bound to the surface of the larger prApe1/RFP-prApe1 aggregates, forming large red puncta with green rings on the surface (Fig. 4B). However, when Ape1Δ2–45-GFP was present instead, no green punctae nor green rings formed on the surface of prApe1/RFP-prApe1 aggregates, suggesting that the propeptide is required for aggregation in vitro. prApe1 forms aggregates of a similar spherical structure both in vivo and in vitro, suggesting that aggregation occurs by a similar mechanism and is not due to nonspecific binding. To further verify that the propeptide is required for aggregation, differential centrifugation of cell lysates was used to separate aggregates from soluble protein (pellet and supernatant fractions, respectively). Wild type, atg19Δ, or atg19Δ ape1ΔΔ2–45 cells were lysed in a 0 or 0.5 M sodium chloride buffer, and differential centrifugation sepa-

FIGURE 3. Mechanism of Atg19 binding differs from aggregation mediated by propeptide. A, RFP-prApe1Δ2–5 does not interact with GFP-Atg19. GFP-atg19 cells with prApe1 and prApe1-RFP, both with N-terminal deletions, are shown. B, two-hybrid assay showing that the 5th N-terminal amino acid of Ape1 is required for Atg19 binding. P69-4A ape1Δ atg19Δ cells where used to test the interaction of AD-Atg19 and BD-prApe1 with N-terminal deletions. BD-prApe1Δ2–5 does not interact with AD-Atg19. C, the propeptide binds Atg19 in vivo. A two-hybrid assay was performed with P69-4A atg19Δ ape1ΔΔ2–45 cells. AD-Atg19 interacts with BD-prApe1 but not with BD-Ape1Δ2–45. BD-propeptide binds to AD-Atg19. D, Atg19 binds to the aggregate surface. atg11Δ GFP-atg19 RFP-ape1 cells are shown. RFP-prApe1 forms a single aggregate and GFP-Atg19 binds to the surface, forming red punctae with a green ring around them. E, the propeptide is required for Atg19 binding to the aggregate surface. atg19Δ RFP-ape1 lysate was treated with DSP, or with DSP and PrtK, and then mixed with ape1/atg8Δ GFP-atg19 lysate. DSP cross-links aggregates, and PrtK cleaves propeptides. GFP-Atg19 bound to the surface of aggregates from untreated and DSP-treated lysate, but not to those treated with DSP and PrtK.
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FIGURE 4. Propeptide is required for aggregation in vitro. A, prApe1 forms aggregates. agt19Δ cells with RFP-prApe1 or prApe1-GFP were lysed in 2 mM potassium phosphate buffer. Lysates were mixed, and potassium phosphate was added to a final concentration of 200 mM. Aggregates were assembled and constituted by RFP-prApe1, prApe1-GFP, and prApe1. DIC, differential interference contrast. B, prApe1Δ2–45-GFP does not bind RFP-prApe1/prApe1 aggregates. Atg19Δ, prApe1, atg19Δ prApe1-GFP, and atg19Δ prApe1Δ2–45-GFP cells were lysed in a 0.2 M potassium phosphate buffer. RFP-prApe1 lysate was mixed with the prApe1-GFP or prApe1Δ2–45-GFP lysate. prApe1-GFP binds the surface of RFP-prApe1/prApe1 aggregates, whereas Ape1Δ2–45-GFP does not. C, mApe1 and prApe1Δ2–45 do not form aggregates. Wild type, atg19Δ, or atg19Δ ape1Δ2–45 cells were lysed in a 0 or 0.5 M sodium chloride buffer. Only prApe1 in 0.5 M sodium chloride forms stable aggregates in vitro and is in the pellet fraction, whereas mApe1 and prApe1Δ2–45 are in the supernatant (Sup). D, cleavage of propeptides disassembles aggregates. Atg19Δ cells were lysed in a 0.5 M sodium chloride buffer. 30 µg/ml of PrtK was added for different time intervals. mApe1 is mostly in the supernatant fraction, showing that cleavage of the propeptide causes aggregates to disassemble. C and D, centrifugation separated aggregates from soluble protein. Fractions were resolved by Western blot with an antibody against Ape1.

rated aggregates from soluble protein. Only prApe1 in 0.5 M sodium chloride is in the pellet fraction, suggesting that buffers that potentiate hydrophobic interactions are required for aggregate stability. Meanwhile, mApe1 is in the supernatant, showing that it does not aggregate because the propeptide is required.

Propeptide Cleavage Disassembles Aggregates—Although cytosolic prApe1 forms aggregates, vacuolar mApe1 does not. This suggests that vacuolar proteolytic cleavage of the propeptide causes aggregates to disassemble. To test this, the stability of aggregates after propeptide cleavage by PrtK was studied. PrtK was added for different time periods to a final 50 µg/ml concentration to atg19Δ cell lysates. Differential centrifugation separated aggregates from soluble protein (Fig. 4C). Between 2 and 5 min after the addition of PrtK, most of the propeptides had been cleaved, and most Ape1 was disassembled and present in the supernatant fraction. Cleavage of the propeptide by PrtK leads to disassembly of aggregates in vitro, suggesting that vacuolar removal of the propeptide by protease B disassembles aggregates.

Propeptide Binds prApe1—Comparison of the S. cerevisiae Ape1 dodecamer with its B. burgdorferi homologue suggests that the propeptide may protrude from the surface of the dodecamer, consistently with its role in aggregation and the fact that it is readily cleaved by proteinase B and PrtK (11). Propeptides protruding from the dodecamer surface could readily bind to other dodecamers to form aggregates. To investigate whether the propeptide can bind prApe1 independently, binding of the propeptide to prApe1 and mApe1 was tested in vitro. We made a construct of the propeptide consisting of the 45 N-terminal amino acids of Ape1, with a His6 tag (propeptide-His6), and expressed it in E. coli cells. Cells were lysed, and the propeptides were bound to Ni2+ -coated agarose beads. Binding of the propeptide to prApe1 and mApe1 from wild type yeast lysate was then tested (Fig. 4A). The propeptide binds prApe1 but not mApe1, suggesting that during aggregation, propeptides from separate dodecamers interact with each other, dimerizing. Binding only occurred in a 0.5 M sodium chloride buffer, suggesting that hydrophobicity directs propeptide binding to prApe1. The propeptide is hypothesized to have an N-terminal amphipathic helix, and thus the hydrophobic region of the amphipathic helix may direct aggregation (52).

High Propeptide Concentrations Disassemble Aggregates—To further verify that the propeptide can independently bind to prApe1, we tested whether binding of the propeptide to prApe1 would physically interfere with aggregation. atg19Δ cells were lysed in 0.2 M potassium phosphate buffer, and different concentrations of propeptide-His6 added. Differential centrifugation was used to identify aggregate from soluble protein. The greater the concentration of propeptide, the less prApe1 found in the pellet fraction, suggesting that the propeptide binds Ape1 and physically interferes with aggregation (Fig. 5B). The high concentration of propeptide-His6 required to disassemble aggregates suggests that propeptide binding to prApe1 is weak and may require previous dimerization of dodecamer propeptides during dodecamer formation.

Propeptide Binds Atg19—The propeptide is required for binding to Atg19 and may be sufficient for targeting GFP to the vacuole (42, 52). This suggests that the propeptide may bind Atg19 independently. To verify this, we tested binding of propeptide-His6 to Atg19 isolated from prApe1Δ cell lysate in vitro (Fig. 5B), using Ni2+ and propeptide-His6-coated Sepharose beads. In addition, to test whether hydrophobicity also directed Atg19 binding to the propeptide, we used a 0 or 0.5 M sodium chloride buffer (Fig. 5C). In contrast to prApe1 binding, Atg19 bound propeptide-His6 in a 0 and 0.5 M sodium chloride buffer, suggesting that Atg19 binding does not rely on hydrophobicity but requires additional electrostatic interactions. A two-hybrid assay was used, using PJ69-4A ape1Δ atg19Δ cells, in which BD propeptide bound to AD-Atg19, showing that the propeptide interacts with Atg19 in vivo (Fig. 3C).

4th N-terminal Amino Acid Is Required for Propeptide Binding to prApe1—To verify that the mechanism of propeptide binding to prApe1 is similar to prApe1 aggregation, we verified that the same N-terminal deletions that disrupt aggregation
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Also interfere with propeptide binding. atg19Δ ape1Δ cells expressing prApe1 with 2, 3, 4, 5, and 11 N-terminal deletions were lysed in a 0.2 m potassium phosphate buffer (Fig. 5D). Lysates were mixed with Sepharose beads coated with Ni⁺ and propeptide-His₆. Consistently with studies showing that prApe1Δ2-GFP and prApe1Δ2–3–GFP form a similar aggregate in cytosol as in full-length prApe1-GFP (Fig. 2A), prApe1Δ2 and prApe1Δ2–3 bind propeptide-His₆ (Fig. 5D). Even more, although prApe1Δ2–4–GFP, prApe1Δ2–5–GFP, and prApe1Δ2–11–GFP aggregate defectively or not at all (Fig. 2A), prApe1Δ2–4, prApe1Δ2–5, and prApe1Δ2–11 do not bind propeptide-His₆ (Fig. 5D). This suggests that propeptide-His₆ binding to prApe1 uses a similar mechanism as prApe1 aggregation, requiring the 4th N-terminal amino acid.

Atg11 Binds to Surface of Aggregates—Atg11 binds Ape1 and directs vesicle formation (42, 52). To verify this, we looked at the localization of Atg11 in vivo. We overexpressed prApe1 to make larger aggregates and better observe Atg11 binding. Atg11 was N-terminally tagged with GFP (GFP-Atg19) and expressed in atg11A RFP-ape1 cells. We show that Atg11 binds to the surface of large red prApe1 aggregates, forming a green ring around them (Fig. 3D).

Atg11 Binding to Aggregate Surface Requires Propeptide—To test whether Atg11 binding to the aggregate surface is dependent on the propeptide, we isolated large prApe1 aggregates with or without surface propeptide and looked at Atg11-GFP localization (Fig. 5B). For this, atg19Δ GFP-ape1 lysate was treated with just DSP or with DSP and PrtK and then mixed with ape1Δ atg8Δ GFP-ape1 lysate, and a 0.2 m potassium phosphate buffer was used. Lysates were cross-linked with DSP so that aggregates did not disassemble while PrtK cleaved the propeptide from their surface. GFP-Atg11 bound to the surface of aggregates from untreated and DSP-treated lysate. However, GFP-Atg19 did not bind aggregates treated with both DSP and PrtK. These results suggest that the propeptide is required for Atg11 binding to the aggregate surface. Although prApe1-GFP and RFP-prApe1 form homogeneous red and green aggregates in vitro (Fig. 4A), instead GFP-Atg19 localizes on the RFP-prApe1/prApe1 aggregate surface, as in vivo (Fig. 3E); thus the mechanism of Atg19 and prApe1 binding is different from aggregation.

Atg11 Binds Defective Aggregates—Although Atg11 could still bind to defective aggregates, its localization was verified in WT cells expressing GFP N-terminally tagged atg11Δ 950 and also prApe1 and RFP-prApe1, both with N-terminal deletions: Δ2, Δ2–3, Δ2–4, and Δ2–9 (Fig. 6A). Atg11Δ 950 was used instead of full-length Atg11 because, when fused with GFP, it shows better binding than the full-length Atg11, perhaps because the size of GFP interferes with protein–protein interactions. GFP-Atg11Δ 950 bound aggregates even when they were defective due to expression of prApe1Δ2–4. This suggests that proper aggregation is not required for the receptor Atg11 to recruit the adaptor Atg11. Hence it may be defective membrane recruitment that is interfering with vesicle formation during vacuolar transport.

Atg8 Binding Is Stable in Vitro—Atg8 binds Ape1, perhaps helping tether the complex to autophagic membrane for vesicle formation (45). atg1Δ or atg19Δ cells, expressing GFP-Ape1 and GFP-Atg8ΔR and overexpressing Ape1, were lysed in a 50 mM potassium phosphate buffer (Fig. 6B). During Atg8 processing, the C-terminal arginine is cleaved by Atg4, so to ensure proper processing of Atg8 when different autophagic deficient strains were screened, Atg8ΔR was used. GFP-Atg8ΔR localizes to the surface of aggregates even after cell lysis, showing that binding is stable in vitro (Fig. 6B).

DISCUSSION

Propeptide Is Required for Aggregation and Atg19 Binding—Ape1Δ2–45 dodecamerization explains how Ape1Δ2–45-GFP can be incorporated into prApe1 aggregates in wild type cells (Fig. 2B). Also, after differential centrifugation, prApe1Δ2–45 and mApe1 are present in the supernatant fraction constituted of soluble protein, whereas prApe1 is in the pellet fraction constituted of aggregates (Fig. 4C). Dodecamers are very stable structures that resist the mechanical stress of density glycerol gradients and require high protein concentrations for disassembly, suggesting that strong protein interactions are involved. In contrast, the propeptide is required for aggregation, both in vivo and in vitro. Ape1Δ2–45-GFP in ape1Δ cells shows no aggregation, with a diffuse GFP cytosolic signal, and Ape1Δ2–45-GFP does not bind RFP-prApe1/prApe1 aggregates in vitro (Fig. 4B). Consistently,
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aggregates disassemble after propeptide cleavage by PrtK, suggesting that aggregates disassemble in the vacuole due to cleavage of the propeptide by proteinase B and that propeptides are accessible for protein interactions and are localized on the surface of dodecamers (Fig. 4D). The prompt disassembly of aggregates after propeptide cleavage suggests that binding between dodecamers is weak and requires a high prApe1 concentration for equilibrium to be surpassed and aggregation to occur. A low binding affinity of dodecamers would facilitate aggregate disassembly once in the vacuole for rapid accessibility to substrate.

The propeptide can independently bind to prApe1 but not to mApe1 because propeptide-His6-coated Sepharose beads bound prApe1 and not mApe1, suggesting that propeptides from separate dodecamers interact directly, forming dimers/trimers during aggregation (Fig. 5A). However, prApe1 or propeptide-His6-coated Sepharose beads did not bind a construct of the propeptide tagged with HA, whereas preliminary studies using nuclear magnetic resonance (NMR) suggest that the propeptide aggregates at high concentrations (data not shown). Furthermore, a very high concentration of the propeptide was required to disassemble aggregates in vitro (Fig. 5B). This suggests that the interaction between single propeptides is weak. However, several propeptides clustering closely together, as would occur on the surface of agarose beads coated with propeptide-His6, may form a more stable structure. Dodecamerization may cluster propeptides together, forming propeptide dimers/trimers on dodecamer surfaces, which may in turn bind with higher affinity to propeptides on separate dodecamers.

Consistently, the structure of *S. cerevisiae* prApe1 dodecamers, based on the structure of its homologue in *B. burgdorferi*, predicts that in a dodecamer, the way prApe1 proteins are arranged forms clusters of three propeptides on each of the four faces of the tetrameric dodecamer, forming propeptide trimers (Fig. 7). Consequently, during aggregation, the propeptide trimer on one side of the dodecamer could bind to a propeptide trimer of another dodecamer. Hence aggregation would be caused by two propeptide trimers coming together to form propeptide hexamers. However, aggregates constituted by a mix of prApe1 and Ape1Δ2–45 can form, suggesting that not all 12 propeptides of a dodecamer are required for aggregation (Fig. 2B). Binding between some sides of each dodecamer may be sufficient to form stable aggregates. After all, once prApe1 begins to aggregate, dodecamers in the interior of the spherical aggregate would be completely surrounded and bound to other dodecamers, making the aggregate very stable even if the dodecamer-dodecamer interaction is weak. This model of aggregation could explain the presence of a single aggregate per cell because as aggregates grow, they become more stable (Fig. 2A). Small aggregates would readily disassemble, whereas those incorporated into a larger, single aggregate do not. This is consistent with RFP-prApe1/prApe1 aggregation in vitro, in which shortly after cell lysis, numerous small aggregates are visible, but over time, a smaller number of much larger aggregates are present (Figs. 3E and 4A and B).

Further studies showed that deletion of the 4 N-terminal amino acids disrupts aggregation, with prApe1Δ2–4-GFP in *ape1Δ* cells forming multiple small aggregates instead of a single large aggregate like in full-length prApe1-GFP, prApe1Δ2-GFP, and prApe1Δ2–3-GFP (Fig. 2A). Consistently, prApe1Δ2–4 does not bind propeptide-His6-coated Sepharose beads, whereas prApe1, prApe1Δ2, and prApe1Δ2–3 do (Fig. 5D). Similarly, other studies show that mApe1 does not form aggregates in the vacuole (36, 50, 53). The propeptide is predicted to form a helix-turn-helix, constituted by an N-terminal amphipathic helix (52). Under this scenario, the 4th N-terminal amino acid of the amphipathic helix disrupts aggregation, perhaps by changing the positioning of the N-terminal helix or by disrupting its helical structure (Figs. 2A and 5D).

The current model for the Cvt pathway is that Atg19 binds prApe1 aggregates in cytosol and recruits autophagy proteins...
for Cvt vesicle formation and vacuolar transport (33, 40). The propeptide is required and sufficient for prApe1 binding to Atg19 (Figs. 2, 3, and 5). Although prApe1-GFP is transported to the vacuole in ape1Δ cells, prApe1Δ2–45-GFP is not, and it remains in cytosol (Fig. 2A). Similarly, although GFP-Atg19 binds RFP-prApe1/prApe1 aggregates in vitro, GFP-Atg19 does not bind DSP cross-linked aggregates that have their propeptides cleaved off using PrtK (Fig. 3E). Meanwhile, Atg19 binding to propeptide-His6-coated Sepharose beads shows that the propeptide is sufficient for Atg19 binding (Fig. 5C). Consistently, in two-hybrid assays, AD-Atg19 binds BD-prApe1 and BD-propeptide, but not BD-prApe1Δ2–45 (Fig. 3C).

Ape1 forms tetrahedral dodecamers, which further bind to form spherical aggregates in vivo and in vitro (Figs. 1A and 2, A and B). Computer models show that tetrahedrons optimally pack into icosahedral structures, which are regular polyhedrons with 20 identical, triangular faces (75). Ape1 aggregates may be constituted by icosahedral structures, constituted of 20 tetrahedral dodecamers (Fig. 7, lower panel). This would enable each dodecamer to contact four similar dodecamers, in which each side would bind to another dodecamer, generating a very stable structure. Icosahedrons have a spherical-like shape, and the further addition of dodecamers to the icosahedral surface would continue to generate a regular, optimally packed, spherical structure. Icosahedral structures are common in viral coats and some enzyme complexes (76–81). It may be that polyhedral enzymatic complexes are more common than we think but go unnoticed by standard molecular biology techniques (82). One of the benefits of icosahedral shapes is that they provide a good enclosure for protecting viral DNA/RNA or for preventing inappropriate substrates from reaching an enzymatic active site (83). Although clathrin does not form icosahedral structures, nevertheless it is a good example of how spherical polyhedra are used as structural components, in this case forming vesicles for cellular transport (84). Similarly, prApe1 icosahedral structures could prevent binding to the wrong substrate while in cytosol and help form a structural scaffold for Cvt vesicle formation. Better understanding of how Ape1 assembles...
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into regular polyhedra could advance the nanoscale compartmentalization of drugs for better delivery (85, 86).

Mechanism of prApe1 Aggregation Differs from Atg19 Recruitment—Although the propeptide is sufficient for prApe1 and Atg19 binding, there are differences in how these proteins interact. Firstly, although propeptide-His₆ binding to prApe1 requires a 0.5 M sodium chloride or 0.2 M potassium phosphate buffer, binding to Atg19 does not (Fig. 5, A and C). Aggregates are more stable in the presence of ions that, based on the Hofmeister series, potentiate hydrophobicity (54). Consistently, propeptide-His₆ binds prApe1 in a 0.5 M but not in a 0 M sodium chloride buffer. This suggests that hydrophobicity is important in prApe1 binding to the propeptide and in aggregation. It is conceivable that propeptides have an N-terminal amphipathic helix, as predicted, and that nonpolar regions of propeptides are pushed together by hydrophobicity, forming propeptide trimers or hexamers and causing aggregation. However, propeptide-His₆ binds Atg19 in 0 M sodium chloride buffer, suggesting that Atg19 binding is less dependent on hydrophobicity and involves additional electrostatic interactions, perhaps on different regions on the propeptide.

Another difference between aggregation and Atg19 recruitment is illustrated by which amino acids are required for propeptide binding to prApe1 or Atg19. The binding of propeptide-His₆ to prApe1 requires the 4th N-terminal amino acid, whereas Atg19 binding does not because AD-Atg19 still binds BD-prApe1Δ2–4 (Fig. 5D). However, AD-Atg19 does not bind BD-prApe1Δ2–5, nor does GFP-Atg19 effectively bind RFP-prApe1Δ2–5, showing that removal of the 5th N-terminal amino acid disrupts Atg19 binding (Fig. 3, A and B). Interestingly, although AD-Atg19 can bind BD-prApe1Δ2–4, prApe1Δ2–4 and prApe1Δ2–4–GFP are not effectively transported to the vacuole. This is not due to lack of Atg11 binding because GFP-Atg11Δ2–950 still co-localizes with defective prApe1Δ2–4 aggregates (Fig. 6A). Furthermore, ape1Δ cells with prApe1Δ2–4 have no mApe1, suggesting that it is not transported to the vacuole for maturation by the Cvt pathway (Fig. 5D). Meanwhile, prApe1Δ2–4–GFP in ape1Δ cells is not localized to the vacuole but instead in cytosol (Fig. 2A). Lack of vacuolar transport of prApe1Δ2–4 is likely due to defective aggregation interfering with Cvt vesicle formation. For vacuolar transport, it is not sufficient for dodecamers to clump together and bind Atg19 and the adaptor Atg11; in addition, they must form a regular structured aggregate, with a specific shape and positioning of dodecamers that facilitate binding of autophagic membrane for vesicle formation.

The localization of Atg19 on aggregates further suggests that the binding mechanism is different because Atg19 localizes to the surface of aggregates instead of being homogeneously incorporated (Fig. 3, D and E). Although the mechanisms of Atg19 binding differ from that of prApe1 aggregation, nevertheless Atg19 and prApe1 may compete for an overlapping binding site, which prApe1 binds more strongly than Atg19; consequently, Atg19 does not block aggregation. Its interaction with the propeptide must be dynamic, and hence Atg19 binds and dissociates, enabling additional dodecamers to bind the propeptides and gradually enlarge the aggregate. This would explain why prApe1 continues to aggregate even when Atg19 appears to bind throughout its surface. However, at very high Atg19 concentrations that surpass the equilibrium of Atg19 and Ape1 binding to the propeptide, Atg19 would probably disrupt aggregation by physically interfering with dodecamer binding to the propeptides, causing aggregates to disassemble.

Atg19 localization at the aggregate surface is consistent with its role as a receptor of prApe1 that initiates Cvt vesicle formation. Entire aggregates are not transported intact to the vacuole because in atg15Δ cells, in which the inner membrane of the Cvt vesicles remains intact inside the vacuole, several small prApe1-GFP aggregates moving rapidly by Brownian motion are visible inside the vacuole. Most likely, after Atg19 binding, a portion of the aggregate is broken off, with the aid of autophagic proteins, during membrane recruitment. The formation of the Cvt vesicle itself may mechanically break or pinch off a portion of aggregates, perhaps in the form of an icosahedron or a similar structure, for vacuolar transport. Alternatively, as prApe1 is incorporated into aggregates, the concentration of soluble prApe1 in cytosol decreases below equilibrium, facilitating Atg19 binding and membrane recruitment.

Atg19, or another autophagic protein, may function as a new type of adaptor or coat-like protein. The formation of icosahedral or spherical aggregates would eliminate the need for additional proteins for bending autophagic membrane around the cargo. A similar mechanism may be involved in other forms of selective autophagy in mammals, in which large cytosolic structures, such as bacteria, viruses, defective aggregates, or organelles, are targeted by autophagic receptors and adaptors for degradation (6–18). For Cvt vesicle formation, Atg19 binding to aggregates must become temporarily stable to recruit autophagic proteins and membrane for vesicle formation. Atg19 binding may also be stabilized by some of the Atg proteins, such as Atg11 and Atg8, which are recruited early during the Cvt pathway. Consistently, Atg8 localizes to the surface of aggregates, even in vitro (Fig. 6B). However, unlike Atg19, Atg8 does not uniformly bind throughout the surface of aggregates. Perhaps only Atg8 associated with membrane can stably bind to Atg19 on aggregates, or autophagic proteins localized irregularly on the aggregate surface mediate Atg8 binding.

Atg34, the parologue of Atg19, similarly binds to Ams1, Atg8, and Atg11; it helps transport Ams1 to the vacuole via autophagy during starvation (87). Because Atg34 recruits Ams1 to autophagosomes instead of initiating Cvt vesicle formation, it does not rely on Ape1 aggregation for Ams1 vacuolar transport. In stark contrast, during vegetative growth, Atg19 transport, and consequently that of its additional cargo Ams1 and Ape4, is dependent on Ape1 (49, 88). Although Ams1 oligomerizes and Ape4 forms dodecamers in cytosol similarly to Ape1, they have not been shown to form aggregates, and just as Atg19 and Atg11 binding to defective aggregates is not sufficient for vacuolar transport, similarly Atg19 binding to Ams1 and Ape4 is also not sufficient for Cvt vesicle completion (38, 49, 88). Consequently, proper prApe1 aggregation is critical for transport of additional Cvt cargo. Furthermore, although Atg19 and its parologue Atg34 both link cargo to autophagic proteins, Atg19 has an additional role by being part of specialized machinery
for the bending of membrane around the Cvt complex for vesicle formation.

prApe1 aggregation, as well as stable binding to Atg19 and Atg8, takes place in vitro (Figs. 1A, 2 and C, 3E, 4A, and 6B). The prApe1, Atg19, and Atg8 complex could be used as a scaffold for an in vitro assay of autophagic vesicle formation. Atg8 binding suggests that autophagic membrane may be recruited to autophagosomes in vitro. An in vitro assay of autophagic vesicle formation would help us elucidate the mechanism of membrane recruitment and fusion.

A propeptide that is only 45 amino acids long causes aggregation and recruitment of a receptor, facilitating vesicle formation and very likely inhibiting prApe1 proteolytic activity in cytosol. Although Ape1 has homologues with similar proteolytic functions and dodecamer structures, its propeptide has large repercussions on its quaternary structure, localization, and function.

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