Insights into autophagosome biogenesis from structural and biochemical analyses of the ATG2A-WIPI4 complex

Saikat Chowdhury a,1, Chinatsu Otomo a,1, Alexander Leitner b, Kazuto Ohashi d, Ruedi Aebersold b, c, Gabriel C. Lander a,3, and Takanori Otomo a,3

aDepartment of Integrative Structural and Computational Biology, The Scripps Research Institute, La Jolla, CA 92037; bDepartment of Biology, Institute of Molecular Systems Biology, Eidgenössische Technische Hochschule Zürich, 8093 Zurich, Switzerland; and cFaculty of Science, University of Zurich, 8093 Zurich, Switzerland

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Autophagy is a nonselective process that removes cytoplasmic contents for delivery to lysosomes. Expansion of the precursor membrane phagophore requires autophagy-related 2 (ATG2), which localizes to the PI3P-enriched ER-phagophore junction. We combined single-particle electron microscopy, chemical cross-linking coupled with mass spectrometry, and biochemical analyses to characterize human ATG2A in complex with the PI3P effector WIPI4. ATG2A is a rod-shaped protein that can bridge neighboring vesicles through interactions at each of its tips. WIPI4 binds to one of the tips, enabling the ATG2A-WIPI4 complex to tether a PI3P-containing vesicle to another PI3P-free vesicle. These data suggest that the ATG2A-WIPI4 complex mediates ER-phagophore association and/or tethers vesicles to the ER-phagophore junction, establishing the required organization for phagophore expansion via the transfer of lipid molecules from the ER and/or the vesicles to the phagophore.

Significance

Autophagosomes are double-membrane organelles that form de novo adjacent to the endoplasmic reticulum (ER) and package cytoplasmic contents for delivery to lysosomes. Expansion of the precursor membrane phagophore requires autophagy-related 2 (ATG2), which localizes to the PI3P-enriched ER-phagophore junction. We combined single-particle electron microscopy, chemical cross-linking coupled with mass spectrometry, and biochemical analyses to characterize human ATG2A in complex with the PI3P effector WIPI4. ATG2A is a rod-shaped protein that can bridge neighboring vesicles through interactions at each of its tips. WIPI4 binds to one of the tips, enabling the ATG2A-WIPI4 complex to tether a PI3P-containing vesicle to another PI3P-free vesicle. These data suggest that the ATG2A-WIPI4 complex mediates ER-phagophore association and/or tethers vesicles to the ER-phagophore junction, establishing the required organization for phagophore expansion via the transfer of lipid molecules from the ER and/or the vesicles to the phagophore.
small immature phagophores and small autophagosome-like vesicles that are distant from the ER (20).

Although the precise function of ATG2 is unknown, previous studies in yeast suggest that ATG2 may be a peripheral membrane protein (21, 22) that binds directly to membranes (10, 23). In accordance with this described affinity for membranes, mammalian ATG2A/B also localizes to lipid droplets (LDs) and thereby regulates their size (13, 24) as well as localizing at the ER-phagophore junction. These observations collectively suggest that ATG2 may directly mediate a membrane reorganization process, although functional studies have not yielded results to support such claims.

The sequences of ATG2 proteins span ~1,600-2,300 residues across eukaryotes and contain evolutionarily conserved regions at the N and C termini as well as in the middle of the polypeptide. These domains have been assigned in the Pfam database (25) to the Chorein_N (ID: PF12624), ATG_C (ID: PF09333), and ATG2_CAD (ID: PF13329) families, respectively (Fig. 1 A). Chorein_N and ATG_C share sequence similarity with the N and C termini of VPS13 (24, 26), a paralog of VPS13A/Chorein (27). The 200 N-terminal residues containing Chorein_N and the ATG_C region of ATG2A have been reported to be required for the localization of ATG2A to autophagosome-forming sites and LDs, respectively (28). ATG2_CAD contains a highly conserved cysteine-alanine-aspartic acid triad, but its role in autophagosome formation is unknown. In addition, a short region (residues 1,723-1,829) preceding ATG_C is also conserved but is not registered in the Pfam database. This short region is required for the localization of ATG2A to both phagophores and LDs and in isolation localizes to LDs (13, 28). We hereafter refer to this region as the “C-terminal localization region” (CLR). The CLR has been predicted to contain an amphipathic α-helix, indicative of association with membranes (28). Apart from these domains, the regions flanked by Chorein_N and ATG2_CAD and by ATG2_CAD and ATG2_C in yeast ATG2 were reported to share similarities with the mitochondrial protein FMP27, whose function is unknown, and with the Golgi-localized protein of maize AP1, which has been suggested to be involved in membrane trafficking, respectively (Fig. 1 A) (23, 29). A fragment containing the APT1 region of yeast ATG2 was shown to interact with membranes containing phosphatidylinositol phosphates, including PI3P (23). However, whether these similarities also apply to higher eukaryotic species is unclear.

To better understand the role of ATG2, we characterized human ATG2A in complex with WIPI4 using structural and biochemical methods. Using EM and chemical cross-linking coupled with mass spectrometry (CXL-MS), we show that ATG2A has an elongated structure with a WIPI4-binding site at one tip (end). We determined that ATG2A is a bipartite membrane-binding protein that bridges two membranes through interactions at each tip of its elongated structure. Furthermore, we demonstrate that the ATG2A-WIPI4 complex can mediate asymmetric tethering between liposomes with and without PI3P. Placed in the context of

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Fig. 1. Structural analyses of the human ATG2A-WIPI4 complex and the ScATG2-ATG18 complex by negative-stain EM. (A) Diagram of the primary structure of ATG2. The lengths of human ATG2A/B and ScATG2 are indicated. The regions conserved among all species are indicated by ovals with solid outlines. The similarities suggested in ScATG2 to FMP27 and APT1 proteins are indicated as ovals with dashed outlines. (B) Affinity capture experiment with ATG2A immobilized on the beads and WIPI4 in solution. (C) Superpose 6 size-exclusion chromatography profile of the mixture of ATG2A and an excess amount of WIPI4. (D and E) 2D class averages of the ATG2A-WIPI4 complex (D) and ATG2A alone (E). (F and G) Reconstructed 3D structures of the ATG2A-WIPI4 complex (F) and ATG2A alone (G). (H) 2D class averages of the ScATG2-ATG18 complex. Green asterisks in 2D class averages in D and H indicate the locations of WIPI4 and ATG18, respectively.

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the current literature, our findings indicate that the ATG2-WIPI4 complex tethers the PI3P-enriched omegasome to a neighboring membrane(s), such as the ER, phagophore, and other vesicles that may be recruited as a membrane source.

Results

Reconstitution and Overall Structure of the Human ATG2A-WIPI4 Complex. To enable the structural characterization of ATG2 and investigate its interactions with WIPI4/ATG18, we expressed and purified human ATG2A and WIPI4, a pair of proteins that have been reported to interact strongly (12–14), from baculovirus-infected insect cells. The binding was confirmed by an affinity capture experiment in which WIPI4 bound to beads preimmobilized with ATG2A but not to beads lacking ATG2A (Fig. 1B). Furthermore, during size-exclusion chromatography the mixture of ATG2A and WIPI4 comigrated as a single peak (Fig. 1C), thus supporting their ability to form a stable complex. Negative-stain EM studies with the purified ATG2A-WIPI4 complex showed that the particles were monodisperse and homogeneous in size and shape (SI Appendix, Fig. S1). The 2D class averages of the stained particles revealed that the ATG2A-WIPI4 complex is composed of a rod-shaped protein associated with a small, distinct, bead-like feature at one end of the molecule (Fig. 1D). The structural details visible in the 2D averages suggest that the rod-shaped portion of the images corresponds to the multidomain protein ATG2A. The bead-shaped feature can be provisionally attributed to WIPI4 since the overall shape and size is consistent with its predicted β-propeller fold (30–32). Comparison of these class averages with 2D averages of ATG2A alone supports this proposed organization (Fig. 1E) and establishes that ATG2A and WIPI4 form a 1:1 stoichiometric complex upon reconstitution.

3D reconstructions of the ATG2A-WIPI4 complex and free ATG2A further support the 2D analyses, resolving a rod-shaped ATG2A about ~200 Å in length with a width of ~30 Å. One end of the rod is hook-shaped with a cleft in the middle (Fig. 1F and G). The WIPI4 density exhibited characteristics consistent with a β-propeller and directly contacts ATG2A through a thin density (Fig. 1F). This contact likely serves as a hinge through which WIPI4 can adopt a range of orientations relative to ATG2A, as observed in both 2D analyses (Movie S1) and 3D reconstructions (SI Appendix, Fig. S1). Collectively, these results establish the overall structure of ATG2A in complex with WIPI4. WIPI4 is flexibly associated with ATG2A, inducing no significant conformational change in ATG2A.

The Overall Shape Is Conserved in the Yeast ATG2-ATG18 Complex. The significance of the interactions between mammalian WIPIs and ATG2A/B has not been thoroughly studied. Much of our knowledge regarding this interaction comes from studies of the Saccharomyces cerevisiae (Sc) ATG2-ATG18 complex. Thus, we investigated whether the structural organization of the human ATG2A-WIPI4 complex described above is conserved in the yeast complex. ScATG2 is smaller than mammalian ATG2A (Fig. L4) and appears to bind ATG18 weakly (SI Appendix, Fig. S1), which makes EM studies of the yeast complex more challenging than studies of its human counterpart. Nevertheless, we obtained 2D class averages of the ATG2-ATG18 complex (Fig. 1H), which show an elongated object with a bead-like density at one end, very similar to the human complex. These results confirm that the overall structure of the ATG2-ATG18 complex is evolutionarily conserved from yeast to human and indicate that functional studies in yeast are relevant in the context of structural work with the human version.

Identification of the WIPI4-Binding Site and Insights into the Chain Topology of ATG2A. We used an integrative approach to gain further structural information about the ATG2A-WIPI4 complex.

First, we sought to establish a coarse-grained chain trace of ATG2A and identify the sites of interaction between WIPI4 and ATG2A by CXL-MS. However, there were some foreseeable technical obstacles in performing a CXL-MS analysis of the ATG2A-WIPI4 complex. For example, the overall 3D organization of the ATG2A-WIPI4 complex is composed of a rod-shaped ATG2A and the small binding interface between ATG2A and WIPI4, would limit the number of residue pairs that can be cross-linked. Furthermore, the protein complex was prone to aggregation at higher concentrations, limiting the highest protein concentration achievable without introducing aggregation to a moderate level for a CXL-MS analysis. Therefore, to maximize the number of the cross-linked pairs, we performed two cross-linking reactions: a standard amine-coupling reaction with disuccinimidyl suberate (DSS), which cross-links pairs of lysines up to ~30 Å apart (33), and another reaction with the coupling reagent 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinum chloride (DMTMM) and a linker, pemalic acid dihydrazide (PDH), which cross-links pairs of acidic and lysine residues with zero-length (ZL) or two acidic residues per PDH (34). We used relatively low concentrations of the cross-linkers to suppress nonspecific intermolecular cross-linking that would cause protein aggregation (Methods). Despite these technical challenges, mass spectrometry of these samples successfully identified 20 cross-linked peptide fragments overall (Fig. 2A and SI Appendix, Table S1).

One cross-link of DSS and one cross-link of PDH were identified within WIPI4, and both are consistent with the WIPI4 homology structure model (SI Appendix, Fig. S2), validating our experiments. Three cross-links were intermolecular between ATG2A and WIPI4: one DSS cross-link between Lys1539 of ATG2A and Lys89 on blade 2 of WIPI4 and two ZL cross-links between Asp1376 or Glu1378 of ATG2A and Lys134 on blade 3 of WIPI4 (SI Appendix, Fig. S2). These data agree with previous work reporting that yeast Atg18 interacts with ScATG2 through blade 2 and loop 2, which connects blades 2 and 3 (30, 35), and with another study showing that a truncation construct (residues 1–1561) of ATG2A containing the WIPI4 cross-linked residues is able to bind to ATG18 in a yeast two-hybrid assay (36), suggesting evolutionary conservation of this interaction mode. Furthermore, these data also suggest that the WIPI4-bound tip comprises amino acids that are located in a central region of the primary structure. Fifteen cross-links were collected within ATG2A: 11 short- to midrange (11–93 residues) DSS/PDH/ZL cross-links, which are indicative of locally folded subdomains (Fig. 2A), and five long-range (293–1,160 residues) DSS cross-links between the residues in ATG_C and Lys720/725/1359. SDS/PAGE analysis of the DSS cross-linking reveals an intense band at ~250 kDa (SI Appendix, Fig. S3). Because the mass of the ATG2A-WIPI4 complex is ~250 kDa, and the ~250 kDa band is the single major band, it seems likely that the five long-range DSS cross-links are intramolecular (SI Appendix, Fig. S3), indicating that the folded ATG2A polypeptide adopts a nonlinear chain topology. However, a faint smear in the ~500- to 600-kDa range is also observable, which may result from two cross-linked ATG2A-WIPI4 complexes, raising the possibility that the long-range cross-links could be inter-ATG2A molecules. Attempts to identify cross-links from in-gel digestion of the monomeric band were unsuccessful, probably due to limited recovery of cross-linked peptides from the gel, preventing us from drawing an unambiguous conclusion.

To map the conserved regions of ATG2A as well as the WIPI4-interacting site, a 42-kDa maltose-binding protein (MBP) was fused to ATG2A at the N terminus and separately inserted into the ATG2_CAD (after residue 1224) (Fig. 2A). 2D image analyses of negatively stained samples revealed that the MBP fused to the N terminus localized to the tip opposite the WIPI4-bound tip of the elongated ATG2A (Fig. 2B). The MBP inserted into the ATG2_CAD localized to the same tip that binds WIPI4.
Our identification of the CAD region is (fraction#) that we attribute to MBP. However, in both constructs this MBP density was observed adjacent to both tips of ATG2A-WIPI4 (Fig. 2B). These data do not allow unambiguous localization of the C-terminal regions but rather suggest that the C terminus of ATG2A is flexible with respect to the rest of the molecule. In the CXL-MS experiments, three lysine residues in ATG2_C cross-linked to two residues in the middle of the ATG2A sequence (720/725 and 1539) (Fig. 2A). If these cross-links are indeed intramolecular, then this also supports the notion that the C terminus of ATG2 is flexible and can reach the WIPI4-bound tip. Given that our EM structural analysis revealed that WIPI4 was flexibly attached to ATG2A and that β-propellers often bind to peptides, we hypothesized that the WIPI4 binding site is in a flexible linear region of ATG2A. To test this hypothesis, we generated a fragment of ATG2A (residues 1358–1404) containing two of the intermolecularly cross-linked residues (Asp1375 and Glu1378) as a fusion to the B1 domain of streptococcal protein G (GB1) protein. The GB1-fused peptide coimmunoprecipitated with WIPI4 in a size-exclusion chromatography column and eluted earlier than WIPI4 alone (Fig. 2C). A control using only GB1 and WIPI4 shows no comigration, demonstrating that this linear region is indeed capable of binding to WIPI4.
Based on these results, we fit a WIPI4 homology model into the 3D EM density with blade 2 facing ATG2A (30, 35) and the membrane-binding surface of WIPI4, including the two PI3P recognition sites (blades 5 and 6) (30–32), on the opposite side of ATG2A (Fig. 2D).

**ATG2A Associates with Membranes Through Its Tips.** Next, we sought to characterize membrane binding by ATG2A by performing a liposome flotation assay using a Nycodenz density gradient (37). Small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs) were prepared by sonication and extrusion methods, respectively. These vesicles were mixed with ATG2A in the presence of Nycodenz, floated to the top of a gradient by centrifugation, and subsequently collected and further analyzed. ATG2A rose to the top of the gradient only in the presence of liposomes (Fig. 3A), confirming direct membrane binding. The recovery of ATG2A proteins was substantially higher with SUVs than with LUVs [14- to 32-fold with 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) vesicles], suggesting that ATG2A prefers binding to highly curved membranes. Additionally, incorporation of negatively charged lipid (1,2-dioleoyl-sn-glycero-3-phospho-L-serine; DOPS) into the liposomes increased ATG2A–liposome association by 1.3- to ninefold, suggesting some electrostatic contribution to this interaction.

To better understand how ATG2A associates with membranes, we performed negative-stain single-particle EM analysis on the ATG2A-WIPI4 complex bound to SUVs. Because ATG2A is a thin, somewhat featureless rod, we foresaw difficulties in clearly visualizing such proteins on large membrane surfaces. Therefore, we added WIPI4 to serve as a molecular marker, allowing us to determine unambiguously the orientation of ATG2A bound to the liposomes. We used SUVs composed of DOPC and DOPS, but not PI3P, to avoid any effects introduced by the WIPI4–PI3P interaction. Because SUVs produced by sonication were highly heterogeneous in size, we generated more homogenous SUVs using a dialysis methodology (38). We performed a flotation assay with WIPI4 and these SUVs and confirmed that WIPI4 does not bind to these SUVs (SI Appendix, Fig. S4). In the raw micrographs of the ATG2A-WIPI4-SUV complex (Fig. 3B), we observed elongated objects resembling ATG2A associated with either one or two liposomes as well as clustered liposomes. 2D analyses focused on the elongated object produced averages containing features consistent with the previously observed ATG2A-WIPI4 complex, including the characteristic hook and cleft (Fig. 3C). The 2D classes could be categorized into three major structural classes of the protein–SUV complexes. In the first class, WIPI4 and the CAD tip of ATG2A are bound to the membrane, with the long axis of ATG2A aligned roughly orthogonal to the membrane, positioning the N tip away from the membrane. In the second class, ATG2A is bound to the membrane through the N tip, with the

![Fig. 3. Interaction between ATG2A and liposomes and its visualization by EM. (A) Liposome flotation assay with 50 nM ATG2A. The liposomes composed of 99% DOPC and 1% 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD) (indicated as “−PS”) or 74% DOPC, 25% DOPS, and 1% DiD (+PS) were prepared by sonication (SUVs) or extrusion (LUVs) using 100- or 400-nm filters. The inputs (4%) and the top layers after centrifugation (24%) were loaded onto SDS/PAGE. The percentage of ATG2A recovered in each of the top fractions was quantified and is shown below the gel image. (B) Micrographs of the negative-stained ATG2A-WIPI4-SUV complex. Colored arrowheads and arrows denote an elongated object that emanates perpendicularly (blue arrowheads) or tangentially (yellow arrowheads) from an SUV or is tethering two SUVs (green arrows). (C) 2D class averages of the ATG2A-WIPI4-SUV complex shown without (Upper) and with (Lower) a manually placed 3D model of ATG2A (shown in yellow). The green dot marks the WIPI4 density.](http://www.pnas.org/cgi/doi/10.1073/pnas.1811874115)
ATG2A Tethers SUVs. To confirm the membrane-tethering activity of ATG2A, we examined the effect of the presence of ATG2A on the size distribution of liposomes using dynamic light scattering (DLS). The DLS profile of the SUVs shifted markedly to larger sizes upon incubation with ATG2A (Fig. 4A), whereas those of the LUVs (100 and 400 nm) did not change (Fig. 4B and C). To confirm that the increase in the SUV particle size was due to liposome clustering mediated by the protein, we added proteinase K to the final sample of the ATG2A-SUV mixture and monitored its effect. Upon incubation, the observed particle size decreased to its original dimensions (Fig. 4D and E), demonstrating that homotypic tethering mediated by ATG2A resulted in the clustering.

We also performed a fluorescence-based liposome-tethering assay, in which biotin-incorporated liposomes were mixed with liposomes containing fluorescent lipids and separated by streptavidin beads (39). The fluorescence intensity of the beads reports the degree of tethering occurring between these two types of liposomes. The results show that the fluorescence signals of the liposomes, regardless of size, were increased by the presence of ATG2A (Fig. 4F), but the difference between the signals in the presence or absence of ATG2A was approximately five times larger in the SUVs than in the LUVs. These data suggest that ATG2A is capable of tethering liposomes, with a preference for SUVs. Taken together, the results from flotation assays, DLS, and fluorescence measurements establish that ATG2A can tether small liposomes (i.e., membranes with high curvature).

The CLR Fragment Binds to Membranes in an Amphipathic α-Helical Conformation. The observation that the CLR in isolation localizes to LDs (13) raises the possibility that the CLR is a membrane-binding domain, as many proteins localize to LDs through direct interaction with the lipid monolayer surface (40). To further characterize the role of CLR in membrane binding, we generated a CLR fragment as an MBP fusion construct, since MBP was required to maintain the solubility of the CLR fragment. In a liposome flotation assay, MBP-CLR was recoverable in the top fraction only in the presence of liposomes, whereas MBP alone was not detected in the top fraction, demonstrating direct membrane binding by the CLR (Fig. 6A). MBP-CLR did not exhibit any preferences for membrane curvature, as it associated with both SUVs and LUVs.

Because LD-localized proteins often interact with the LD membrane through their amphipathic α-helices (40), we sought to examine the secondary structure of the CLR. We purified a CLR-SUV complex by removing the MBP tag from the complex.
SUV-bound MBP-CLR by proteolytic cleavage, followed by liposome flotation (Fig. 6B). The CD spectrum of the purified CLR-SUV complex shows a profile typical of an α-helix, with local minima at 208 and 220 nm (Fig. 6C). The helical content predicted from the CD spectrum is 61%. A secondary structure prediction suggests that the CLR may contain three α-helical regions, and helical wheel drawings of these regions show that all three (referred to as “H1,” “H2,” and “H3”) may form amphipathic α-helices (Fig. 6D). To determine which region is responsible for membrane binding, we generated each region as an MBP fusion and tested its membrane-binding ability. The results of the flotation assay revealed that H2 and H3, but not H1, bind to LUVs consisting of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS). H3

Fig. 5. Tethering of PI3P-containing LUVs by the ATG2-WIP14 complex. (A–C) DLS profiles of LUVs (100 nm) consisting of 75% DOPC, 15% DOPS, and 10% PI3P in the absence (A–C: cyan) or the presence of 200 nM WIP14 (A), 200 nM ATG2A (B), or both proteins (C). (D) Auto-scaled autocorrelation functions of the four DLS measurements. (E) Fluorescence-based liposome tethering assay. The higher the fluorescence, more associations there are between the liposomes with and without PI3P. LUVs consisting of 73.3% DOPC, 15% DOPS, 10% PI3P, 0.2% biotinylated lipids, and 1.5% rhodamine-PE were mixed with LUVs consisting of 73% DOPC, 25% DOPS, and 2% 1,1-Dioctadecyl-3,3,3,3′-tetramethylindodicarboxyanine perchlorate in the presence of the indicated proteins. For each experiment, the average of three repeats is shown; whiskers indicate the SD.

Fig. 6. Characterization of the CLR. (A) Liposome flotation assay with 100 nM MBP-fused ATG2A CLR (residues 1723–1819) or MBP alone (control). The liposomes used are indicated as in Fig. 3A. The inputs (4%) and the top layers after the centrifugation (25%) were loaded onto SDS/PAGE. The percentage of MBP-CLR recovered in each of the top fractions was quantified and is shown below the gel image. (B) Preparation of the CLR-LUV complex by liposome flotation. The input containing MBP-CLR and SUVs (75% DOPC and 25% DOPS) was mixed with tobacco etch virus (TEV) to cleave off MBP, and the resulting CLR-LUV complex was isolated in the top fraction after centrifugation. (C) The CD spectrum of the CLR-LUV complex. (D) Predicated α-helical regions in the CLR. A multiple sequence alignment (MSA) of the CLR of Homo sapiens (Hs) ATG2A, HaATG2B, Dro sophila melanogaster (Dm) ATG2, Schizosaccharomyces pombe (Sp) ATG2, and ScATG2 was generated by ClustalW. Secondary structure predictions were obtained using jPred (57) and PSIPred (58) servers. The three fragments generated for flotation assays (H1: residues 1721–1739; H2: residues 1751–1774; H3: residues 1777–1819) are shown as a cartoon drawing generated by the HelixQuest server (59). The asterisks shown above the MSA and in the cartoon drawings indicate the residues mutated to aspartic acid. (E) Liposome flotation assay with 1 μM MBP-fused CLR fragments and LUVs prepared by extruding a lipid mixture consisting of 74% POPC, 25% POPS, and 1% 1,1-Dioctadecyl-3,3,3,3′-tetramethylindodicarboxcyanine perchlorate through a 100-nm membrane.
appears to have a higher affinity for membranes than H2, based on its higher recovery. We then replaced four residues in the hydrophobic side of H2 and eight residues in the hydrophobic side of H3 with aspartic acids. These mutations abolished the membrane-binding capability of each fragment (Fig. 6E), supporting the likelihood that H2 and H3 bind to membranes via an amphipathic α-helix.

The CLR Is Not Responsible for Membrane Tethering by ATG2A. To determine whether the membrane-binding property of the CLR plays a role in membrane tethering, we incorporated all the mutations described above (a total of 12 mutations to aspartic acid) into the full-length ATG2A protein and characterized this mutant (ATG2A_{12xD}). We tested membrane binding by flotation assay using liposomes containing PO lipids (POPC and POPS), which were prepared by sonication or extrusion with a 30- or 100-nm filter to eliminate potential artifacts (nonspecific binding) caused by DO lipids and sonication. ATG2A bound to all types of liposomes tested but exhibited higher affinity for smaller liposomes (Fig. 7A). These results with DO lipids are consistent with those observed for wild-type ATG2A. The higher preference for sonicated liposomes over the 30-nm liposomes suggests that ATG2A binds to membrane surfaces with local defects rather than sensing overall membrane curvature, as sonication introduces surface defects (37). Our results with ATG2A_{12xD} were very similar to those with the wild type, indicating that these mutated residues are not essential for membrane binding. We then performed membrane-tethering assays with ATG2A_{12xD} and found that these mutations do not affect tethering activity. That ATG2A_{12xD} clusters SUVs and also mediates the PI3P- and WIPI4-dependent homotypic and heterotypic tethering as efficiently as the wild type (Fig. 7B–G) leads us to conclude that the CLR is not involved in membrane tethering.

Discussion

Recent related studies by Zheng et al. (41) on the rat ATG2B-WIPI4 complex described the overall shape of the ATG2B-WIPI4 complex by negative-stain EM as well as PI3P-independent membrane binding by ATG2B. They also identified the WIPI4-binding site of ATG2B by a combination of CXL-MS and mutagenesis. Our findings are in agreement with this published work, and their mutational studies reinforce our identification of the WIPI4-binding site on ATG2A. Here, however, we structurally and biochemically demonstrate that ATG2A is capable of tethering membranes, which provides valuable insights into autophagosome biogenesis. Gómez-Sánchez et al. (42) also recently characterized the Sc:ATG2 protein and its interaction with ATG18 and Sc:ATG9. Their discovery that Sc:ATG2 binds to membranes by recognizing surface defects is in agreement with our observation that ATG2A binds most strongly with sonicated liposomes. Additionally, their conclusion that Sc:ATG2 is a mediator of ER-phagophore association complements our structural and biochemical data that demonstrate membrane tethering by the ATG2A-WIPI4 complex.

The membrane organization of the ER-phagophore junction, a site intrinsically tied to the omegasome, is poorly understood due to its highly complex and dynamic nature. Thus, predicting the precise location of ATG2A within this junction is a challenge. In Fig. 8 we illustrate possible pairings of membranes that may be tethered by the ATG2A-WIPI4 complex at the ER-phagophore junction. The PI3P-enriched omegasome has been suggested to be a cluster of tubular membranes with a diameter of ~30 nm (9), similar to the diameters of the SUVs used in this study (Fig. 8A). Our 2D averages of the ATG2A-WIPI4-SUV complex show that the CAD tip and WIPI4 can simultaneously contact the same membrane (Fig. 8B). Thus, it is most logical to assume that the ATG2A-WIPI4 complex associates directly with the omegasome through the CAD tip as well as through WIPI4 (Fig. 8B). With the CAD tip attached to the omegasome, the N tip could bind to either the ER or the phagophore edge, resulting in a tethering of the omegasome to the ER and/or the phagophore. Alternatively, the N tip may bind to membrane vesicles, such as ATG9 vesicles or COP11 vesicles, since ATG9 has been reported to interact with ATG9 and SEC23 (a component of COP11 vesicles) (18, 21, 42). These vesicles have been proposed to transform into early phagophores and also may serve as membrane sources for phagophore expansion (6, 43–47). Therefore, tethering of these membrane vesicles to the

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**Fig. 7.** The CLR is not involved in membrane tethering. (A) Flotation assay with the ATG2A_{12xD} mutant and liposomes composed of 74% POPC, 25% POPS, and 1% 1,1′-Dioctadecyl-3,3,3,3′-tetramethylindodicarbocyanine perchlorate. The liposomes were prepared by sonication or extrusion using 30- or 100-nm filters. The result of SDS/PAGE is shown as in Figs. 3A and 6A. (B and C) DLS homotypic membrane-tethering assay with ATG2A_{12xD}. The DLS profiles (B) and the autocorrelation functions (C) of the 75%POPC/25%DOPS SUVs in the absence and presence of ATG2A_{12xD} are shown. (D) Fluorescence homotypic membrane assay with ATG2A wild-type and ATG2A_{12xD} performed and presented as in Fig. 4F. (E and F) DLS homotypic membrane-tethering assay with ATG2A_{12xD}. The DLS profiles (E) and the autocorrelation functions (F) are shown. (G) Fluorescence heterotypic tethering assay with ATG2A_{12xD} performed as shown in Fig. 5E. The experiments with the wild-type ATG2A repeat the data shown in Fig. 5E but were performed at the same time as the experiments with mutants. Although the fluorescence values are different from those in Fig. 5E, the results from both experiments with the wild type are consistent with each other.
deletions at these regions has also proven extremely difficult. Such issues have prevented further investigation into the role that these regions play in the membrane-tethering process. On a related note, we also attempted to characterize two other previously studied ATG2A constructs having a deletion of the CLR or ATG_C (13, 28) but again failed to obtain sufficient amounts of protein to perform in vitro assays. Although the challenges that have been met during the preparation of these truncated proteins may be attributed to suboptimal constructs or expression and purification conditions, we cannot rule out the possibility that the regions targeted for deletion are integral to the structural stability of the protein. Therefore, site-directed mutagenesis, rather than truncations, would serve as a better strategy for probing the molecular mechanics of membrane tethering, but such studies would require an accurate atomic model of the complexes. While we are unable to provide direct demonstrations of membrane interaction by each tip in isolation, the following evidence supports our conclusions.

First, the N terminus of VPS13 has been shown to interact with membranes containing negatively charged lipids (49). Thus, the N terminus of ATG2, which shares sequence similarity with VPS13’s N terminus, could also be a membrane-interacting domain. Second, the APT1 domain located between ATG9_CAD and the CLR in ScATG2 has been shown to interact with PI3P-containing membranes (23). Although recent reports (41, 42) and the data we presented here show that ATG2A(B) bind membranes irrespective of the presence of PI3P, it is still possible that the same region in human ATG2A is responsible for membrane interaction. In that case, the residues in this region, rather than those of ATG9_CAD, may form the CAD tip.

In this work, we focused on characterizing another conserved region, the CLR. Our data show that the CLR fragment binds to membranes through its two amphipathic α-helices, which is consistent with the LD localization of the CLR fragment (13). However, the mutations in this region that disrupt membrane interaction of the fragment did not affect the membrane-tethering activity of the full-length protein (Fig. 7). Recently, a similar set of mutations has been shown to reduce cellular autophagic activity (28). We confirmed that this mutant protein, referred to as “AH-E” (28), is also active in membrane tethering in vitro (SI Appendix, Fig. S5). Thus, the CLR is likely to possess another role essential for phagophore expansion. The membrane-tethering activity of ATG2A described in our work cannot alone explain how the phagophore expansion, a process that must involve either lipid transfer or new lipid synthesis, would occur. We speculate that the biochemical function of the CLR may hold the key to this long-standing mystery in autophagosome biogenesis.

Some ATG factors, such as the ATG1 kinase complex, the ATG12-ATG5-ATG16 complex, and ATG8-phosphatidylethanolamine (PE) conjugate, have been reported to mediate membrane tethering in vitro (39, 50, 51). While these factors are distinct in their molecular organization, they all use protein self-oligomerization to tether neighboring membranes: The ATG1 and ATG12-ATG5-ATG16 complexes both self-dimerize so that two molecules of their membrane-interacting subunits (ATG1 and ATG5, respectively) can associate independently with two separate membranes to tether two vesicles (39, 50, 52). ATG8 is associated with a membrane via its covalent linkage to a PE molecule in the membrane, and multimerization of ATG8 molecules on separate vesicles leads to clustering of the vesicles (51). In contrast, ATG2A has a capacity of bridging two membranes without the requirement for self-oligomerization. ATG2A is rather similar to multisubunit tethering complexes, such as the Dsl1, HOPS, COG, and TRAPP complex, all of which tether two membranes through the tips of their elongated shapes (53). The roles of the

Fig. 8. Proposed models of the ER-phagophore/isolation membrane association mediated by the ATG2-WIPI/ATG18 complex. (A) Illustration of the ER-phagophore junction based on current knowledge from cell biological studies. Each gray line represents a lipid bilayer. (B) Structural model of the ATG2-WIPI/ATG18 complex tethering the omegasome to its neighboring membranes (ER, phagophore edge, ATG9 vesicle, or COPII vesicle). The dark red color of ATG2 represents conserved regions as in Fig. 2A. The WIPI/ATG18-binding region of ATG2 is represented as a black line emanating from the middle region of ATG2 to indicate the flexible attachment of WIPI/ATG18.
variety of autophagic membrane tetherers in autophagosome biogenesis must be different from one other, as they function at different steps. In the earliest step of biogenesis, the ATG1 kinase complex multitimerizes ATG9 vesicles, triggering nucleation of a phagophore. The membrane tethering by the ATG12-ATG5-ATG16 complex, which is the E3-ligase–like factor for ATG8–PE conjugation, and tethering by ATG8–PE have also been suggested to occur during the nucleation step (44, 50, 51). There is also evidence that ATG8–PE is involved in the final step of phagophore membrane closure (54, 55). ATG2A, which is required for phagophore expansion during the intermediate steps of the autophagosome formation, may be an important collaborator of these other membrane tetherers and requires future study.

Materials and Methods

Experimental procedures for protein expression and purification, affinity capture binding assays of the ATG2-WIPI4 and ATG2–ATG18 complexes, reconstitution of the ATG2A-WIPI4-SUV complex for negative-stain EM analysis, in vivo autophagy, liposome flotation assay, CXL-MS analysis, DLS analysis, fluorescence liposome tethering assays, and CD spectroscopy are described in SI Appendix, Supplementary Materials and Methods.

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