Autophagosome targeting and membrane curvature sensing by Barkor/Atg14(L)

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The class III phosphatidylinositol 3-kinase (PI3KC3) is crucial for autophagosome biogenesis. It has been long speculated to nucleate the autophagosome membrane, but the biochemical mechanism of such nucleation activity remains unsolved. We recently identified Barkor/Atg14(L) as the targeting factor for PI3KC3 to autophagosome membrane. Here, we show that we have characterized the region of Barkor/Atg14(L) required for autophagosome targeting and identified the BATS [Barkor/Atg14(L) autophagosome targeting sequence] domain at the carboxyl terminus of Barkor. Bioinformatics and mutagenesis analyses revealed that the BATS domain binds to autophagosome membrane via the hydrophobic surface of an intrinsic amphipathic alpha helix. BATS puncta overlap with Atg16 and LC3, and partially with DFCP1, in a stress-inducible manner. Ectopically expressed BATS accumulates on highly curved tubules that likely represent intermediate autophagic structures. PI3KC3 recruitment and autophagy stimulation by Barkor/Atg14(L) require the BATS domain. Furthermore, our biochemical analyses indicate that the BATS domain directly binds to the membrane, and it favors membrane composed of phosphatidylinositol 3-phosphate [PtdIns(3)P] and phosphatidylinositol 4,5-biphosphate [PtdIns(4,5)P2]. By binding preferentially to curved membranes incorporated with PtdIns(3)P but not PtdIns(4,5)P2, the BATS domain is capable of sensing membrane curvature. Thus, we propose a novel model of PI3KC3 autophagosome membrane nucleation in which its autophagosome-specific adaptor, Barkor, accumulates on highly curved PtdIns(3)P enriched autophagic membrane via its BATS domain to sense and maintain membrane curvature.

Autophagy is characterized by the formation of double-membrane vesicles called autophagosomes that engulf cytoplasm, organelles, protein aggregates, and microorganisms and deliver these components to the lysosome for degradation (1). Dysfunction of autophagy has been implicated in multiple human diseases including cancers, neurodegenerative diseases, diabetes, and infectious diseases (2, 3). The autophagy pathway is highly conserved from yeast to humans; at least 34 autophagy-related proteins have been identified (4–7). Among these autophagy proteins, the class III phosphatidylinositol 3-kinase (PI3KC3) complex composed of Vps34, p150/Vps15, and Beclin 1/Atg6 has an evolutionary conserved role in autophagosome formation (8). It has been long speculated that PI3KC3 nucleates the initiating autophagosome membrane (also called phagophore or the isolation membrane) (9). However, direct biochemical evidence to support the nucleation activity of PI3KC3 is still missing.

One major barrier to understanding the function of PI3KC3 in autophagosome biogenesis is the pleiotropy of this lipid kinase in multiple membrane trafficking pathways (8, 9). By generation of phosphatidylinositol 3-phosphate [PtdIns(3)P], an event that might ignite a cascade of signaling pathways, PI3KC3 is required for autophagy and endocytosis via assembly of distinct protein complexes (10–16). The recent identification of an autophagic specific adaptor, Barkor/Atg14(L), of PI3KC3 paved the way for its specific role on autophagosome membrane (10–15). Barkor/Atg14(L) mainly localizes to initiating autophagosome membrane, evidenced by colocalization with both the early autophagosome marker Atg16 and the omegasome marker DFCP1 (17). Omegasome is a potential precursor of autophagosome originated from endoplasmic reticulum (ER) (18). The N-terminal cysteine repeats target Barkor/Atg14(L) to ER, and such distribution is crucial for its function in autophagy activation (17). However, the ER targeting of Barkor/Atg14(L) is neither induced by stress nor inhibited by a dominant negative Ulk1/Atg1 mutant (17). The biochemical mechanism underlying the stress-inducible Barkor/Atg14(L) accumulation on autophagic membrane remains unknown. In general, membrane-associated proteins not only bridge the interplay between lipids and proteins; they are also involved in generating, sensing, and stabilizing local regions of membrane curvature. Many proteins or protein complexes are known to be crucial for membrane deformation, including protein coat complexes (clathrin, COPI, and COPII) or Bin-Amphiphysin-Rvs (BAR) domain containing proteins (19–26). These proteins that modulate membrane structures often associate with amphipathic alpha helices either by inserting small hydrophobic patch of amphipathic alpha helices into the membrane leaflet, or by mounting the membrane surface to the intrinsically curved protein scaffolds. In addition to the hydrophobic interactions, membrane-associated proteins are also often bind to the lipid headgroups (26). Phosphoinositides (PtdIns) are particularly important because their headgroups are easily modified (27). For example, PtdIns(4,5)P2 is important for the budding of clathrin-coated vesicles and the fusion of vacuoles (28–31), whereas PtdIns(3) P is required for the invagination of vesicles into late endosomes and autophagosome biogenesis (8, 9, 13, 32).

Barkor/Atg14(L) function is highly specific to autophagosome formation because of its localization on the phagophore (10–14). In this study, we investigated the autophagosomal targeting of Barkor/Atg14(L) through deletion mutagenesis. We identified a previously uncharacterized Barkor/Atg14(L) autophagosomal targeting sequence (BATS) domain in Barkor/Atg14(L) that specifically recognizes early autophagic structures. The BATS domain is required for Beclin 1 recruitment and autophagy activation. Within this domain, the hydrophobic patch of an amphipathic alpha helix directly interacts with membrane. In addition, we found that BATS preferentially bound to more highly curved membranes in a PtdIns(3)P dependent manner. Hence, we propose a biochemical mechanism of PI3KC3 autophagosome membrane nucleation through autophagosome binding and membrane curvature sensing of Barkor/Atg14(L).
Results

Identification of the Barkor/Atg14(L) Autophagosome Targeting Sequence (BATS). Barkor/Atg14(L) was recently identified as a stoichiometric subunit of PI3KC3 (10–12, 14) that specifically localizes to initiating autophagosomes and targets PI3KC3 to autophagosome (10). It was found that N-terminal cysteine repeats of Barkor/Atg14(L) is essential for ER localization and autophagy function (16). However, the Barkor/Atg14(L) ER membrane association is neither induced by starvation nor blocked by Ulk1 dominant negative mutant (16), suggesting there is another stress-inducible membrane association mechanism for Barkor/Atg14(L). We performed an in-depth deletion mutagenesis analysis to search for protein sequence that is required for autophagosome targeting. To better visualize autophagic vacuoles, chloroquine (CQ), a drug that blocks autophagy flow by raising the pH of endo/lysosomes (33), was used to treat U2-OS cells stably expressing Myc-tagged LC3. In these cells, we co-expressed serial deletion mutants of Barkor/Atg14(L) tagged with humanized Renilla reniformis green fluorescence protein (hrGFP) and tested their colocalization with LC3 upon CQ treatment (Fig. L4 and Fig. S1). By this way, we mapped the autophagosome membrane association domain to the C terminus of Barkor/Atg14(L). We found that the extreme C terminus, the last 80 amino acids of Barkor, is sufficient for autophagosomes targeting (Fig. 1A and B and Fig. S1). Consequently, we determined that the final 80 amino acids in the C-terminus of Barkor/Atg14(L) define a minimum required region for autophagosome binding and named this region BATS for Barkor/Atg14(L) autophagosome targeting sequence. BATS puncta not only accumulated upon CQ treatment, but also strongly induced by Rapamycin and starvation treatments (Fig. 1B and Fig. S2). To consolidate the requirement for the carboxyl terminal of Barkor/Atg14(L) in autophagosome targeting, we generated mutants missing various lengths of amino acids from C terminus of Barkor. Deletion of last 10 amino acids from full-length Barkor/Atg14(L) is sufficient to abolish the cytosolic puncta and colocalization with LC3 (Fig. 1 A and B). This demonstrates the essential role for these amino acids in autophagosome association.

To test whether Barkor/Atg14(L) membrane association is necessary for its autophagy function, we expressed the targeting deficient Barkor/Atg14(L) Δ10aa mutant in U2-OS cells. This mutant only lacks the final 10 amino acids on the C terminus required for autophagosome targeting; it is still capable of interacting with Beclin 1. We speculated that if Barkor/Atg14(L) membrane association is required for autophagy, BarkorΔ10aa mutant might be unable to stimulate autophagy as wild-type Barkor. Electron microscopy allows the direct analysis of autophagosome formation. Autophagic vacuoles (AVs) can be captured by a high-resolution transmission electron microscope. Barkor/Atg14(L) overexpression leads to increased number and size of AVs in U2-OS cells (Fig. 1C) (10). However, in U2-OS cells overexpressing the Barkor/Atg14(L) Δ10aa mutant, the promotion effect of Barkor/Atg14(L) on autophagosome formation is abolished (Fig. 1 C and D). The autophagy flux was also determined in Barkor/Atg14(L) wild-type and Δ10aa mutant expressing cells. LC3-II conversion is compromised in both normal or Bafilomycin-treated cells expressing Δ10aa mutant compared to the cells expressing Barkor/Atg14(L) wild-type cells (Fig. 1E). These combined results suggest that Barkor/Atg14(L) autophagosome association via its carboxyl terminus is required for autophagosome formation.

BATS Associates with Autophagosome Membrane via the Hydrophobic Side of an Amphipathic Alpha Helix. The final 80 amino acids of the C terminus of Barkor/Atg14(L) define a minimum required region for autophagosome binding (Fig. 1L). Through bioinformatics analysis, we determined specific characteristics of BATS that aid in its ability to function as a targeting sequence. The BATS domain is highly conserved in vertebrates (Fig. 2A). Secondary structural analysis revealed that 19 amino acids of BATS form a classical amphipathic alpha helical wheel with hydrophobic and hydrophilic residues that align on opposite sides of the helix (Fig. 2B). This arrangement may allow the hydrophobic patch of the BATS domain to shallowly embed into the lipid bilayer. To investigate the hydrophobic interaction between BATS amphipathic alpha helix and the lipid bilayer, we replaced three hydrophobic residues, tryptophan (W), phenylalanine (F), and tyrosine (Y), with arginine (R) residues. As expected, the expression of this WYFR mutant lacked BATS puncta (Fig. 2C). Alternatively, the mutation of two hydrophilic residues (K486A, R492A) on the polar side has no effect on BATS puncta formation (Fig. 2C), suggesting that the hydrophobic interaction between BATS and lipid bilayer is critical for membrane association.

We have previously shown that recruitment of Beclin 1 to autophagosomes by Barkor/Atg14(L) requires its coiled-coil domain (CCD) (10). If BATS is sufficient for membrane targeting, we anticipate that CCD tethered to BATS might be sufficient to direct Beclin 1 to autophagosomes. To test this hypothesis, we generated a chimeric protein consisting only of a CCD and BATS domain (CCD) (10). If BATS is sufficient for membrane targeting, we anticipated that CCD tethered to BATS might be sufficient to direct Beclin 1 to autophagosomes. To test this hypothesis, we generated a chimeric protein consisting only of a CCD and BATS domain (CCD) (10). If BATS is sufficient for membrane targeting, we anticipated that CCD tethered to BATS might be sufficient to direct Beclin 1 to autophagosomes. To test this hypothesis, we generated a chimeric protein consisting only of a CCD and BATS domain (CCD) (10). If BATS is sufficient for membrane targeting, we anticipated that CCD tethered to BATS might be sufficient to direct Beclin 1 to autophagosomes. To test this hypothesis, we generated a chimeric protein consisting only of a CCD and BATS domain (CCD) (10). If BATS is sufficient for membrane targeting, we anticipated that CCD tethered to BATS might be sufficient to direct Beclin 1 to autophagosomes. To test this hypothesis, we generated a chimeric protein consisting only of a CCD and BATS domain (CCD) (10). If BATS is sufficient for membrane targeting, we anticipated that CCD tethered to BATS might be sufficient to direct Beclin 1 to autophagosomes. To test this hypothesis, we generated a chimeric protein consisting only of a CCD and BATS domain (CCD) (10). If BATS is sufficient for membrane targeting, we anticipated that CCD tethered to BATS might be sufficient to direct Beclin 1 to autophagosomes. To test this hypothesis, we generated a chimeric protein consisting only of a CCD and BATS domain (CCD) (10). If BATS is sufficient for membrane targeting, we anticipated that CCD tethered to BATS might be sufficient to direct Beclin 1 to autophagosomes. To test this hypothesis, we generated a chimeric protein consisting only of a CCD and BATS domain (CCD) (10). If BATS is sufficient for membrane targeting, we anticipated that CCD tethered to BATS might be sufficient to direct Beclin 1 to autophagosomes.
along the time course (0–30 min) mark one representative tubule after starvation. (C) U2OS cells were transfected with hrGFP-BATS together with Myc-Atg16. Forty-eight hours after transfection, cells were either treated with EBSS for 1 h or with 200 μM CQ for 2 h; Myc-Atg16 was stained with Rhodamine Red antibody and detected by laser confocal microscope. (D) U2OS cells expressing GFP-DFCP1 were captured after starvation or Rapamycin treatment (Fig. 3E). Images were captured immediately after the addition of EBSS medium (C) or at 30-min time points (D) (also see Movie S1). Arrows mark tubules. (E) Arrows along the time course (0–30 min) mark one representative tubule after starvation. (F) U2OS cells expressing GFP-BATS and Tomato-LC3 were captured after addition of EBSS medium; both channels were captured every 2 s. Two representative images were presented (also see Movie S2). An arrow marks a tubule.

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Fig. 4. Membrane binding activity of the BATS domain. (A) GST-tagged constructs of wild-type BATS, BATS WFYm mutant, endophilin 1, and PH domain of PLCδ were recombinantly expressed and purified from E. coli. Full-length His-tagged Barkor/Atg14(L) was expressed and purified from baculovirus-infected insect cells (Fig. 4A). Recombinant endophilin 1 and PH domain of PLCδ were also purified from E. coli. These proteins were incubated with liposome prepared from Folch fractions of brain lipid extracts in a cosedimentation assay (Fig. 4B). The BATS domain alone does not precipitate. Instead, the BATS domain coprecipitated with liposome (Fig. 4B), indicating that BATS directly binds to the lipid bilayer. In contrast, the BATS WFYm mutant with the hydrophobic residues changed to hydrophilic amino acids failed to precipitate with liposome (Fig. 4B). Full-length Barkor/Atg14 (L) purified from insect cells is also capable of liposome binding (Fig. 4B). We failed to purify Barkor/Atg14(L) with C terminus 10 amino acids deletion from insect cells because of its instability. However, we speculate that multiple membrane binding domains may exist along Barkor/Atg14(L). For example, its N terminus is derived liposomes with different sizes (100–800 nm) were incubated with recombinant BATS in a cosedimentation assay. S denotes supernatant and P denotes pellet. The binding efficiency is calculated as bound proteins (P) versus input (P + S). (C) Recombinant wild-type BATS, BATS WFYm mutant and the PH domain of PLCδ were incubated with liposomes incorporated with different forms of phosphoinositides and tested for their liposome binding in a cosedimentation assay. S denotes supernatant and P denotes pellet. (D). Cells expressing GFP-BATS and Myc-LC3 were left untreated, treated with Rapamycin alone or in combination with 3-Methyladenine (3-MA), and observed under the fluorescence microscope. Dots per cells were calculated in at least 50 cells from three independent experiments.

Membrane curvature sensing by BATS. The time-lapse video microscopy analysis suggests that BATS might bind to the curved membrane. The amphipathic alpha helix of the BATS domain is not highly charged but serine/threonine enriched at its polar side (Fig. 2B), resembling the BAR domains that serve as sensors of membrane curvature (26, 36–38). We speculated that the BATS domain might serve as a sensor of curvature, binding more tightly to curved, as opposed to flat, membranes. We tested this hypothesis using liposomes of different intrinsic curvatures. For this purpose, we generated liposomes with different sizes made from the Folch fractions of the brain lipid extracts. Recombinant BATS...
domain binds preferentially to the highly curved membranes of small liposomes rather than to the relatively flat membranes of large liposomes (Fig. 5A). The quantity of liposomes was normalized by Texas-Red-labeled PE incorporated into liposomes (Fig. 5B). The sensitivity of the BATS domain to the vesicle size and therefore to the extent of curvature confirms that it is a membrane curvature sensor.

To investigate the role that PtdIns(3)P plays in BATS membrane curvature sensing, we incubated reconstituting BATS with two different sizes of liposomes (100 and 800 nm) composed of different amounts of PtdIns(3)P (0.2, 0.6, 2, and 6%). The membrane binding of BATS to liposomes with 0.2 and 0.6% PtdIns(3)P incorporated is barely detectable, regardless of the size of liposomes. When the concentration of PtdIns(3)P of liposomes is increased to 2%, there is a clear preference for BATS to interact with 100-nm liposomes rather than 800-nm liposomes (Fig. 5C). Further increasing PtdIns(3)P to 6% in liposomes induced strong interaction between BATS and liposomes, and no obvious difference of BATS binding to 100-nm or 800-nm liposomes was observed (Fig. 5C). These data indicate that BATS senses membrane curvature in a PtdIns(3)P-dependent manner, and local high concentration of PtdIns(3)P is enough to override the curvature sensing activity of BATS. Interestingly, BATS could only sense the membrane curvature of liposomes incorporated with PtdIns(3)P but not PtdIns(4,5)P (Fig. 5D).

Discussion

In this study, we identified a membrane association BATS domain of Barkor/Atg14(L) that specifically targets Barkor/Atg14(L) to highly curved PtdIns(3)P enriched early autophagic membrane. The BATS domain is essential for the function of Barkor/Atg14(L) in autophagy activation. We have also determined that the BATS domain utilizes the hydrophobic residues within the amphiphatic alpha helix to interact with membrane. BATS localizes to Atg16 and LC3 positive autophagic membrane, and also partially colocalizes with DFCP-1 marked omegasome. In vitro, the BATS domain preferentially binds to the highly curved membrane in a PtdIns(3)P-dependent manner. These data indicate that Barkor/Atg14(L) is concentrated on the curved autophagic membrane enriched in PtdIns(3)P via the BATS domain in a stress-inducible manner.

It is reported that Barkor/Atg14(L) targets to ER membrane through its N-terminus cysteine repeats. Upon autophagic stress, Barkor/Atg14(L) recruits Beclin 1-Vps34-p150 complex to ER to produce PtdIns(3)P on local ER membrane and likely cradles the curvature sensor. Whether the BATS domain and Barkor/Atg14(L) tend to serve as membrane curvature sensor. In this study, we demonstrate that Barkor/Atg14(L) functions as a membrane curvature sensor. Whether the BATS domain and Barkor/Atg14(L) can deform membrane by itself or in cooperation with other P13K3 subunits has yet to be investigated. The driving and sensing of autophagosome membrane curvature could be coordinated with protein scaffolds and lipid modification. One possible protein scaffold is the Atg5/Atg12/Atg16 complex, which recruits LC3/Atg8 and its conjugation enzymes to the nucleating autophagosome membrane. Both Atg5/Atg12/Atg16 and LC3 recruitment and conjugation are critical for the elongation of the initiating membrane. PtdIns(3)P generation by the PI3K kinase is also indispensable for membrane formation, which is required for the orientation of the Atg5/Atg12/Atg16 complex to autophagosome membrane rather than other types of membrane (39).

Identification of the BATS domain as a sensor of initial membrane curvature should provide a biochemical platform to test the necessity of different proteins or nonprotein factors for reconstitution of autophagosome biogenesis in the near future.

Material and Methods

All Barkor/Atg14(L) mutants were cloned into the Bgl II and EcoR I sites on hrGFP-N1 and a 3XFlag tag was inserted between the Sal I and BamH I sites. hrGFP-BATS triple R mutation (W484R, F485R, Y488R) was cloned from full-length Barkor using the following primers: P1: GCGAATTCGctggagaatcagatcacgagcttt acgccgggagagcg and P2: GCGAATTCGaatggtgtccagtgcgagcttt acgccgggagagcg. See SI Materials and Methods for other details.

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