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Regulation of Beclin 1 in autophagy

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Abstract

Class III phosphatidylinositol 3-kinase (PI3KC3) plays a pleiotropic role in autophagy and protein sorting pathways. The human core complex of PI3KC3 consists of three major components including PI3KC3/hVps34, p150 and Beclin 1. How the specificity of PI3KC3 complex is derived towards autophagy is not clear. Utilizing a sequential affinity purification coupled with mass spectrometry approach, we have successfully purified a human Beclin 1 complex and cloned a novel protein we called Barkor (Beclin 1-associated autophagy-related key regulator). The function of Barkor in autophagy has been manifested in several assays, including stress-induced LC3 lipidation, autophagosome formation and *Salmonella typhimurium* amplification. Mechanistically, Barkor competes with UV radiation resistance associated gene product (UVRAG) for interaction with Beclin 1, and orients Beclin 1 to autophagosomes. Barkor shares considerable sequence homology with Atg14 in yeast, representing an evolutionary conserved autophagy specific regulatory step in early autophagosome formation.

Keywords

Beclin 1; Atg14; autophagy; UVRAG; phosphatidylinositol 3-kinase; autophagosome; PI3KC3; cancer

Beclin 1 sits at the core of autophagy regulation.[1] Genetic analysis has revealed its functions in tumor suppression, life-span extension, cell death regulation, embryo development, immune defense, as well as preventing neurodegeneration and heart diseases.[2] Beclin 1 is a major component in class III phosphatidylinositol 3-kinase (PI3KC3), which catalyzes the phosphorylation of the 3 position hydroxyl group of the inositol ring of phosphatidylinositol to produce phosphatidylinositol-3-phosphate (PI3P).[3] Among three classes of PI3Ks, PI3KC3 is the only one highly conserved from yeast to humans; it consists of a core complex with PI3KC3 (Vps34), p150 (Vps15) and Beclin 1 (Vps30/Atg6). PI3KC3 plays a pleiotropic role in autophagy and vacuolar protein sorting (Vps). How the specificity of this complex differentiates among different cellular processes is still not clear in mammals but there are some clues in yeast. In yeast, Atg14 and Vps38 mediate different complex formation, subcellular localization and lipid phosphorylation of the core PI3K kinase that functions in autophagy and Vps.[4] Although several positive and negative regulators of Beclin1 and the PI3KC3 complex have been identified in mammals,[5–9] functional orthologs of Atg14 and Vps38 in human were not identified until two recent publications,[10,11] which suggests an evolutionarily conserved regulatory pathway in human cells from eukaryotic ancestors.

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Purification of the Human Beclin 1/PI3KC3 Complex

It is generally believed that the ability of a protein to carry out its designed function depends on its ability to interact with other proteins. In turn, the partner a protein interacts with is a key component that describes the function of any protein. We assume the same principle also applies to Beclin 1, so we purified and identified a Beclin 1 complex in human cells through a tandem affinity purification coupled with mass spectrometry (MS) approach.

Beclin 1 was first cloned by Dr. Beth Levine's group through a yeast two-hybrid screening due to its interaction with Bcl-2.[12] Similar yeast two-hybrid screenings also identified Ambra1 and nPist as Beclin 1-associating proteins.[8,9] Since yeast two-hybrid only produces binary interactions, and this method favors transient rather than stable interactions, these interactions may not necessarily give a comprehensive picture of the Beclin 1 complex composition. An immunoprecipitation-based assay coupled with MS should provide more information about the architecture of the Beclin 1/PI3KC3 complex. An affinity purification/MS-based study was used to identify UVRAG as a Beclin 1-interacting protein, but the bait was viral Bcl-2 rather than Beclin 1, per se.[7] Therefore, we are the first group to purify and identify the Beclin 1 complex from human cells through an affinity purification/MS approach, and this approach gave rise to a highly specific and clearly present Beclin 1 complex that contains seven stoichiometric components (Fig. 1).

In our tandem affinity purified Beclin 1 complex, there are three highly conserved components, including Beclin 1 (bait), PI3KC3 and p150. These three components likely compose the core PI3KC3 complex. A known Beclin 1 interacting protein, UVRAG,[7] was also identified. Most interestingly, we identified three novel components in the Beclin 1 complex that we named as Barkor, Baron (p120) and p40 respectively. Barkor (KIAA0831, named as "*B*eclin 1 associated *a*utophagy *r*elated *k*ey regulat*or*") clearly has a function in autophagy as discussed below.[10] Baron (named as "*B*eclin 1 *a*ssociated *R*UN d*o*mai*n* containing protein," also called p120 based on its molecular weight) is another protein, which contains a RUN domain. The RUN domain is used by a group of proteins that interact with GTPase,[13] therefore this protein most likely participates in recruiting a novel or known GTPase to participate in Beclin 1-mediated membrane metabolism. Its function in autophagy is currently being investigated intensively. The third protein (named as p40 based on its molecular weight) contains a coiled-coil domain; so far the function of this protein in autophagy is still not clear. Beclin 1 complex formation did not alter upon autophagic stress (Fig. 1), suggesting that other activating mechanisms like post-translational modifications might be involved in a stress response. Elucidating the function and regulation of individual components will be required in order to reconstitute PI3KC3 activity on autophagosomes, and such activity could be crucial for subsequent membrane extension and protein complex recruitment.

Several known interacting proteins did not show up in the complex, for example, Bcl-2. Bcl-2 directly interacts with Beclin 1 and negatively regulates its activity.[5] It is likely that Bcl-2 exists in the complex as a substoichiometric component, since we can only detect it by western blot analysis but not by MS, and it does not show up on silver stained gels.[9] Consistently, it has been reported that cellular Bcl-2 has a much lower binding affinity with Beclin 1 compared to viral Bcl-2.[14] We did not detect other known interacting proteins such as Bif-1,[6] Ambra1,[8] and nPist,[9] in our tandem affinity purification through mass spectrometry identification. It is likely these interactions are substoichiometric, transient, or tissue-specific.

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Human Barkor and UVRAG could be Functional Orthologs of Yeast Atg14 and Vps38

Based on our and Itakura's observations,[10,11] it is reasonable to speculate that Barkor and UVRAG act as functional mammalian orthologs of yeast Atg14 and Vps38: Barkor and UVRAG form two distinct subcomplexes with Beclin1 and PI3KC3,[10,11] and these two complexes are mutually exclusive through competitive binding to Beclin 1's coiled-coil domain.[10] Barkor is required for autophagy and localizes to autophagosomes,[10,11] whereas UVRAG localizes on endosomes and is required for endosomal trafficking.[15] Most importantly, Barkor recruits Beclin 1 from the trans Golgi network (TGN) to autophagosomes, [10] whereas Atg14 is required for Atg6 recruitment to phagophore assembly sites.[4] All these activities point to Barkor as an autophagy-targeting factor similar to the role of Atg14 for Atg6; UVRAG is likely an endosome-targeting factor for Beclin 1, although more evidence is still needed to prove this point (Fig. 2).

Nevertheless, there are several discrepancies in this functional ortholog hypothesis that should not be neglected. First, the homology between Atg14 and Barkor, or UVRAG and Vps38 is relatively low (about 10%–15% identity and 30%–40% similarity).[10,11] It has been shown that UVRAG is also essential for autophagosome formation,[7] although this requirement has been challenged by Itakura et al.[11] It is possible that Barkor and UVRAG function in a sequential order to participate in autophagosome formation and autophagosome-lysosome fusion. Since Barkor cannot complement a yeast Atg14 autophagy-deficient phenotype,[11] we prefer our name of Barkor to Atg14 referring to its interaction with Beclin 1 and its critical function in autophagy in human cells.

The Apical Protein-Membrane Interaction

The identification of Barkor could lead to a new direction in biochemical interaction between proteins and autophagosome membranes. PI3KC3 is critical in the early stage of autophagosome formation to nucleate the phagophore. Such activity must be regulated in mammalian cells since Beclin 1 normally resides predominantly on the TGN membrane.[10, 16] A transition of Beclin 1, and presumably PI3KC3, from the TGN to the phagophore (the precursor of the autophagosome) is highly dependent on Barkor and its interaction with Beclin 1.[10] This observation leads us to speculate that Barkor could be the initiating protein factor to bind to the phagophore.

From sequence analysis, UVRAG has a putative Ca^{2+}-dependent phospholipid binding C2 domain that mediates its interaction with endosome membranes.[15] Whether Barkor can directly interact with the autophagosome membrane, likely through a PI3P-independent manner, is still an open question. At least so far, no potential membrane binding sequence has been identified. Identification of such a targeting sequence, specifically targeting to autophagosomes, if one exists, will be of great significance in understanding the biogenesis of this unique vesicle.

Barkor and Human Cancers

Beclin 1 has been implicated in many pathogenesis processes including various forms of cancer.[2] Monoallelic *beclin 1* deletions have been found in up to 75% of ovarian, 50% of breast and 40% of prostate cancers.[17] Heterozygous *beclin* $1^{+/-}$ mice have reduced autophagy activity and increased incidence of spontaneous tumors.[18,19] UVRAG is also monoallelically deleted in a high percentage of colon cancers.[20] Barkor/KIAA0831 maps to the 14q22.3 chromosome locus, a region that is frequently lost in multiple human cancers.[21–24] Interestingly, low expression of Barkor/KIAA0831 is associated with poor survival of glioma patients.[25] All these observations implicate the connection between the Beclin 1-mediated pathway and

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human malignancies. One interesting question is whether the autophagy activity of this pathway contributes to cancers. So far among *Atg* genes, very few of them have shown tumor suppressor properties.[2] It is important to sort out whether Beclin 1 functions as a tumor suppressor through its autophagy function. Identification of Barkor as an autophagic targeting factor will help to clarify this question, especially if we can investigate the tumorigenesis phenotype of Barkor knockout mice and compare them side-by-side with UVRAG and Beclin 1 knockout mice.

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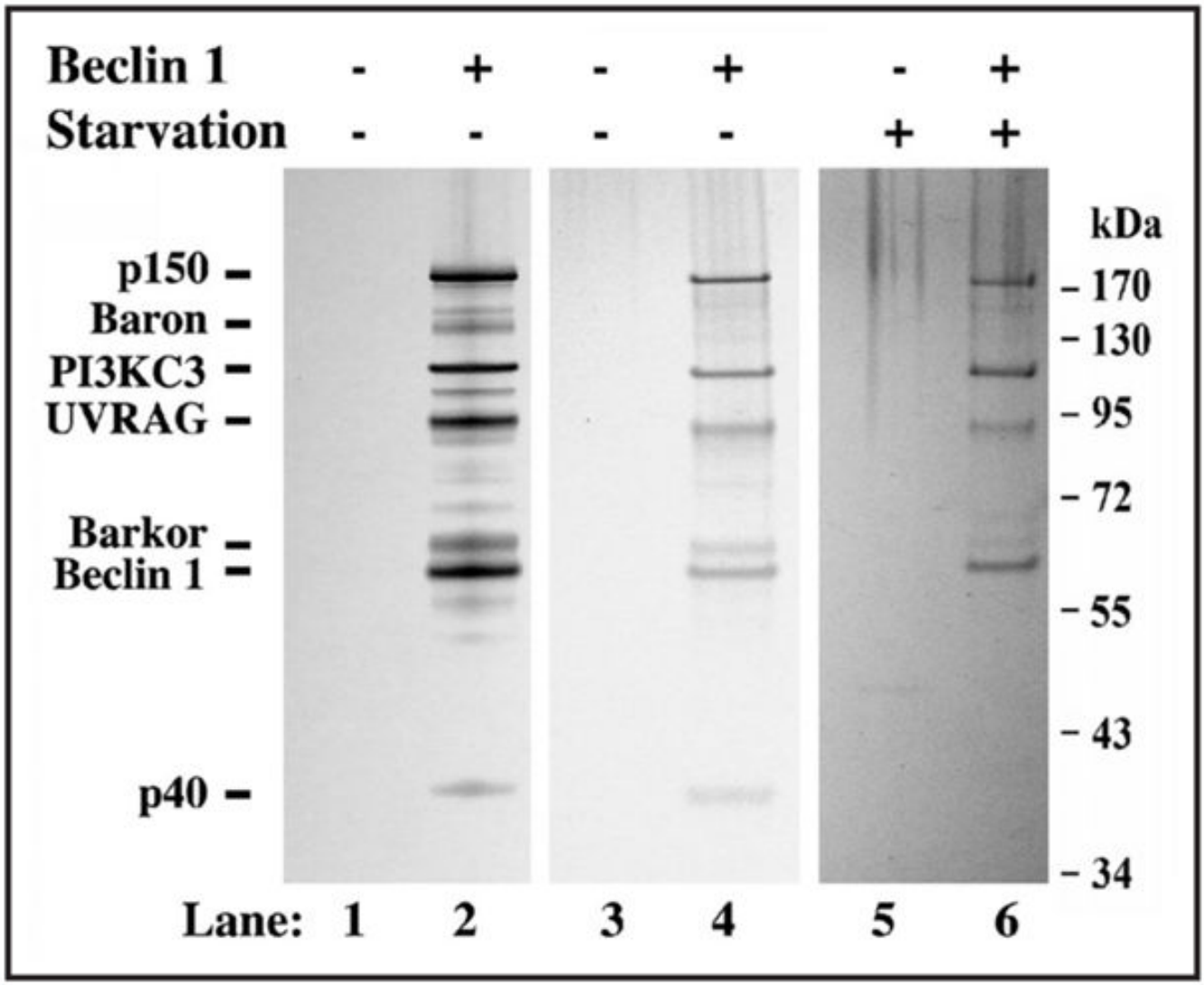

Figure 1.

Purification of Beclin 1 complex in human cells. 293T (lanes 1 and 2) or U_2OS cells (lanes 3–6) expressing ZZ-Beclin 1-FLAG were starved or mock-treated, and cells were lysed and subjected to sequential affinity purification through IgG beads (bind to ZZ) and M2 beads (bind to FLAG). The resulting complexes were resolved by 4%–12% gradient SDS-PAGE and silver staining. The Beclin 1 complex was analyzed by mass spectrometry and identified proteins were marked.

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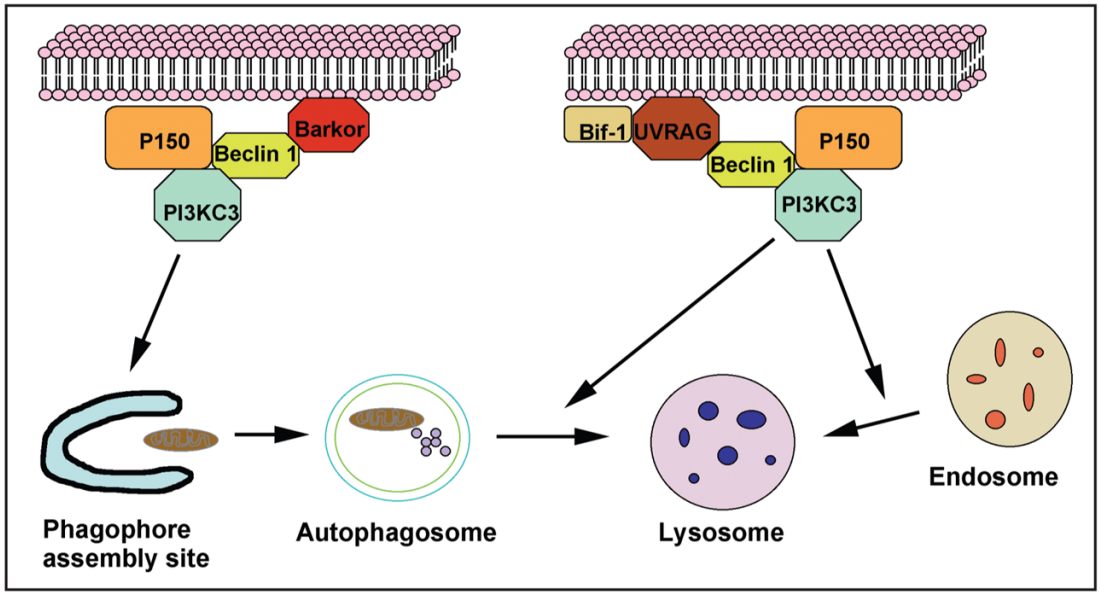

Figure 2.
A working model for the human Beclin 1/PI3KC3 complex. At least two subcomplexes p150-PI3K-Beclin 1-Barkor and p150-PI3K-Beclin 1-UVRAG coexist in mammalian cells. The complex containing Barkor is specific for autophagosome assembly, whereas the UVRAG complex is required for autophagosome maturation and endosome/lysosome fusion.