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Beclin 1 Self-Association Is Independent of Autophagy Induction by Amino Acid Deprivation and Rapamycin Treatment

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ABSTRACT

Autophagy, a process of self-digestion of cellular constituents, regulates the balance between protein synthesis and protein degradation. Beclin 1 represents an important component of the autophagic machinery. It interacts with proteins that positively regulate autophagy, such as Vps34, UVRAG, and Ambra1, as well as with anti-apoptotic proteins such as Bcl-2 via its BH3-like domain to negatively regulate autophagy. Thus, Beclin 1 interactions with several proteins may regulate autophagy. To identify novel Beclin 1 interacting proteins, we utilized a GST-Beclin 1 fusion protein. Using mass spectroscopic analysis, we identified Beclin 1 as a protein that interacts with GST-Beclin 1. Further examination by cross linking and co-immunoprecipitation experiments confirmed that Beclin 1 self-interacts and that the coiled coil and the N-terminal region of Beclin 1 contribute to its oligomerization. Importantly, overexpression of vps34, UVRAG, or Bcl-x_L, had no effect on Beclin 1 self-interaction. Moreover, this self-interaction was independent of autophagy induction by amino acid deprivation or rapamycin treatment. These results suggest that full-length Beclin 1 is a stable oligomer under various conditions. Such an oligomer may provide a platform for further protein–protein interactions. J. Cell. Biochem. 110: 1262–1271, 2010.

KEY WORDS: AUTOPHAGY; BECLIN 1; UVRAG; vps34; OLIGOMERIZATION

Autophagy is the major cellular mechanism for degradation and recycling of long-lived proteins and cytoplasmic organelles (Gozuacik and Kimchi, 2004; Levine and Klionsky, 2004). It contributes to the maintenance of cellular homeostasis and thereby plays essential roles in normal cell growth and development (Kelekar, 2005). The autophagic process can also lead to programmed cell death (PCD) mechanism known as type II PCD (Bursch, 2004). The deregulation of autophagy is associated with several human diseases including cancer, cardiomyopathy, muscular disorders, and neurodegenerative diseases (Yuan et al., 2003; Gozuacik and Kimchi, 2004; Shintani and Klionsky, 2004; Erlich et al., 2006, 2007a).

Beclin 1 (or Atg6 in yeast) is an evolutionary conserved protein that promotes nutrient deprivation-induced autophagy (Liang et al., 1999). Beclin 1 was first identified as a Bcl-2 interacting protein (Liang et al., 1998). It is a 60-kDa protein that contains a myosin-like coiled-coil region (amino acids 144–269) and is highly conserved between different species (Liang et al., 1998). The Bcl-2 binding domain is located at the N-terminus of the protein and includes residues 88–150, from which residues 108–127 create a putative BH3 domain (Erlich et al., 2007b; Maiuri et al., 2007; Oberstein et al., 2007). The BH3 domain is the minimal element necessary for interaction with anti-apoptotic Bcl-2 proteins (Oberstein et al., 2007). Beclin 1 also contains a short leucine-rich amino acid

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PI3K, phosphoinositide 3-kinase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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sequence that is responsible for efficient nuclear export signal (NES) (Liang et al., 2001). The Beclin 1 NES is localized to amino acids 180–189. Mutations of the Beclin 1 NES interfere with its ability to promote nutrient deprivation-induced autophagy and its tumor suppression ability (Liang et al., 2001). Another region of the human Beclin 1 is the highly evolutionary conserved domain (ECD) that includes amino acids 244–337 and is responsible for Beclin 1 interaction with Vps34/PI3KcIII (Furuya et al., 2005). The ECD is essential for Beclin 1 ability to mediate autophagy and to inhibit cell tumorigenicity (Furuya et al., 2005).

Recent studies, including our own, proposed that Beclin 1 contains a putative BH3-like domain which is necessary for its interaction with the anti-apoptotic Bcl-2 family proteins (Erlich et al., 2007b; Maiuri et al., 2007; Oberstein et al., 2007). Our recent results reveal that while Beclin 1 binds, with different efficiency, several anti-apoptotic Bcl-2 proteins, it does not interact directly with the pro-apoptotic Bcl-2 family proteins. The interaction between Beclin 1 and the anti-apoptotic proteins is inhibited by tBid and Bad but not by Bak and Bax (Erlich et al., 2007b).

Beclin 1 is important for localization of autophagic proteins (Apg) to a pre-autophagosomal structure (Kihara et al., 2001; Cao and Klionsky, 2007). Beclin 1-mediated autophagy depends on its interaction with Vps34/PI3KcIII (Furuya et al., 2005). It also interacts with the anti-apoptotic proteins Bcl-2, Bcl-x_L Bcl-w and to a lesser extent with Mcl-1, through the BH3 domain in the Bcl-2-binding region (amino acids 108–127) (Liang et al., 1998; Erlich et al., 2007b). The later interaction inhibits autophagy and fails to induce apoptosis. It was recently shown that Beclin 1 interacts with two other proteins, UVRAG and Ambra1 and that these interactions positively regulate autophagy (Liang et al., 2006; Maria Fimia et al., 2007). Two novel Beclin 1 interacting proteins, Atg14L and Rubicon were recently identified (Matsunaga et al., 2009a,b; Zhong et al., 2009a,b). It was demonstrated that Atg14L and Rubicon play opposing roles in autophagy regulation by forming distinct complexes with Beclin 1 (Matsunaga et al., 2009a,b; Zhong et al., 2009a,b). Thus, Beclin 1 interactions with several proteins may positively and negatively regulate autophagy. During the search for new Beclin 1 interacting proteins, we identified Beclin 1 self-interaction. The coiled-coil domain and the N-terminal portion of Beclin 1 mediate this self-interaction. Notably, this Beclin 1 self-interaction is not affected by amino acid deprivation or rapamycin-induced autophagy or by vps34, Bcl-x_L, or UVRAG overexpression.

MATERIALS AND METHODS

MATERIALS AND BUFFERS

Polyclonal rabbit anti-Beclin 1, anti-Flag and monoclonal mouse anti-GFP (B-2) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were from Sigma. HNTG buffer contained 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol. Solubilization buffer contained 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 1.5 mM $MgCl_2$, 10% glycerol, 2 mM sodium vanadate, 1 mM phenylmethylsulfonylfluoride, 10 mg/ml aprotinin, and 10 mg/ml leupeptin. Pull-down assay buffer contained: CHAPS 50 mM Tris–HCl (pH 7.6), 20 mM $MgCl_2$, 150 mM NaCl, 1 mM DTT, 2 mM sodium vanadate, 1 mM phenylmethylsulfonylfluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 0.5% NP 40, 5 mg/ml pepstatine, and 1 mM benzamidine. Binding buffer contained 50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 100 mM NaCl, 0.5 mM DTT, and 0.5 mg/ml BSA.

CELL LINES

COS-7, HEK293, and U87 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum. Cells were transfected using DNA–calcium phosphate precipitation. Cell lysates were prepared 48 h following transfection as described.

DNA CONSTRUCTS

The coding sequence of Beclin-pEGFP N2 served as template for PCR amplification of the various Beclin 1 domains. At the 5′ end of each fragment *Kpn*I restriction site was added and in the 3′ end a *Bam*H1 restriction site was added by using the following primers: N-ter fragment: 5′-GCGGTACCCGCCACCATGGAAGGGTCTAAGA-CGT-3′ and 5′-CCGGATCCCATCTGTGCATTCCTCACAGA-3′. Coiled-coil fragment: 5′-GCGGTACCCGCCACCATGCTTTTAGACCAGCTG-GACAC-3′ and 5′-CCGGATCCCGACGTTGGTTTTCTTCAG-3′. C-ter fragment: 5′-GCGGTACCCGCCACCATGTTTAATGCAACCTTCCAC-AT-3′ and 5′-CCGGATCCCTCATTTGTTATAAAATTGTGAG-3′. The PCR products *Kpn*I and *Bam*H1 fragments were cloned into Beclin 1-pEGFP N2 vector that was digested with *Kpn*I and *Bam*H1. To delete the coiled-coil domain (amino acids 144–270) two additional primers were used: 5′-GATACTCTTTTTAATGCAACCTTCCACATC-3′ and 5′-TGCATTAAAAAGAGTATCTGTGCATTCCTC-3′ (Beclin 1 accession number: MN_003766). pEGFP-human UVRAG plasmid (Itakura et al., 2008) was kindly provided by Prof. N. Mizushima (Tokyo, Japan).

LYSATE PREPARATION, IMMUNOPRECIPITATION, AND IMMUNOBLOTTING

Cells were exposed to the indicated stimuli. After treatment, cells were solubilized in lysis buffer. Lysates were cleared by centrifugation. For direct electrophoretic analysis, boiling gel sample buffer was added to cell lysates. For other experiments, antibodies were first coupled to anti-mouse IgG agarose (for monoclonal antibodies) or protein A-sepharose (for polyclonal antibodies) for 1 h at RT. Then the proteins in the lysate supernatants were immunoprecipitated with aliquots of the beads–antibody complexes for 2 h at 4°C. The immunoprecipitates were washed three times with HNTG, resolved by SDS–polyacrylamide gel electrophoresis (PAGE) through 7.5% gels and electrophoretically transferred to nitrocellulose membrane. Membranes were blocked for 1 h in TBST buffer (0.02 M Tris–HCl pH 7.5, 0.15 M NaCl, and 0.05% Tween 20) containing 6% milk, blotted with 1 μg/ml primary antibodies for 2 h, followed by 0.5 μg/ml secondary antibody linked to horseradish peroxidase. Immunoreactive bands were detected with the enhanced chemiluminescence reagent (Amersham Corp, Buckinghamshire, UK).

CROSS-LINKING

Cross-linking experiments were performed by addition of 2 mM bis (sulfosuccinimidyl) suberate (BS^3), to the lysis buffer for 20 min on

ice. The chemical cross linking reaction was stopped by adding 50 mM glycine and the samples were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Citri et al., 2003).

GST PULL-DOWN ASSAY

A GST-Beclin 1 column was generated by absorbing 20 ml of GST-Beclin 1-producing *E. coli* lysate (resulting from a 1-L culture) to 1 ml of glutathione–sepharose (Sigma). A similar column was prepared from GST-producing *E. coli*. Cell lysate was prepared and a total of 1 mg protein was loaded on GST or GST-Beclin 1 columns (in the presence of 0.5 ml CHAPS buffer) for 2 h at 4°C. After the incubation the beads were washed three times with binding buffer. The GST and GST-Beclin 1 bound proteins were eluted in boiling sample buffer and resolved by SDS–PAGE through 7.5% gels and either electrophoretically transferred to nitrocellulose membrane, or protein bands on the gel were visualized by Coomassie Blue staining. In the latter case, a 60 kDa band uniquely bound to GST-Beclin 1 was further analyzed by mass spectrometry using an API QSTAR™ Pulsar Hybrid LC/MS/MS System.

RESULTS

BECLIN 1 SELF-INTERACTION

Affinity chromatography of U87 cell extracts on a GST-Beclin 1 affinity matrix revealed a major Beclin 1-binding band at a molecular mass of 60 kDa. Mass spectrometry analysis indicated that the 60-kDa band is Beclin 1 (Fig. 1A). The identification of the 60-kDa protein as Beclin 1 was confirmed by immunoblotting. A monoclonal anti-GFP antibody, revealed a major 90-kDa band in the GST-Beclin 1-bound material of COS-7 cells expressing pEGFP-Beclin 1 cDNA (Fig. 1B). This band was not present in eluates from the control GST matrix. To further confirm this self-interaction, cells were co-transfected with Flag-Beclin 1 and Beclin 1-pEGFP or with pEGFP as a control. As a positive control cells were co-transfected with Flag-Bcl-x_L and Beclin 1-pEGFP. Cell lysates were immunoprecipitated with monoclonal anti-Flag antibodies and blotted with monoclonal antibodies to GFP. As shown in Figure 2A, GFP-Beclin 1 co-immunoprecipitated with Flag-Beclin 1 indicating that Beclin 1 self-interacts. Next, we examined whether the GFP tagging location on Beclin 1 (N-terminus or C-terminus) affects its self-interaction. To this end, cells were co-transfected with Flag-Beclin 1 and either Beclin 1-pEGFPN2 or pEGFPC3-Beclin 1 or pEGFP. Cell lysates were subjected to co-immunoprecipitation with monoclonal anti-Flag antibodies, followed by immunoblotting with anti-GFP antibodies. As shown in Figure 2B, the GFP tag location does not affect Beclin 1 ability to self-interact.

IDENTIFICATION OF BECLIN 1 DOMAINS THAT REGULATE ITS SELF-INTERACTION

In order to identify Beclin 1 domains involved in Beclin 1 self-interaction, we generated three expression vectors of different

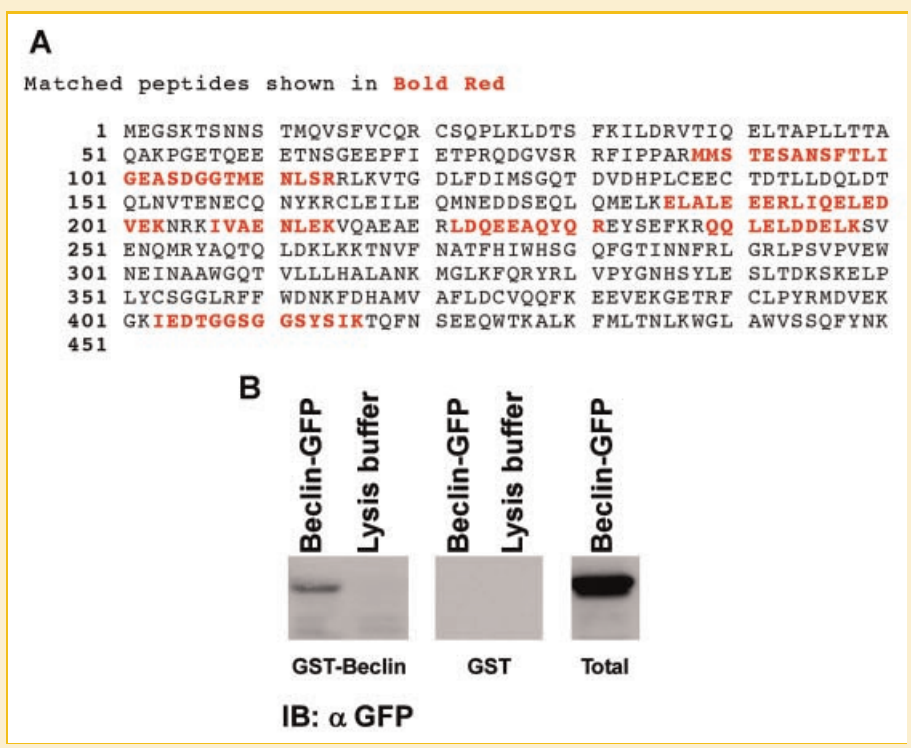

Fig. 1. Beclin 1 binds Beclin 1. A 60 kDa protein was identified on SDS gel electrophoresis of Coomassie blue–stained proteins isolated from U87 cell extracts on GST–Beclin 1 agarose affinity matrix (not shown). A: Mass spectroscopy analysis identified Beclin 1 as this 60 kDa specific band. The sequence identity is marked in red. B: Lysates from HEK293 cells transfected with Beclin 1–pEGFP expression vectors were used for the pull-down assay. Immunoblotting of eluates from GST Beclin 1 and GST control affinity matrices with a monoclonal mouse anti–GFP antibody is presented.

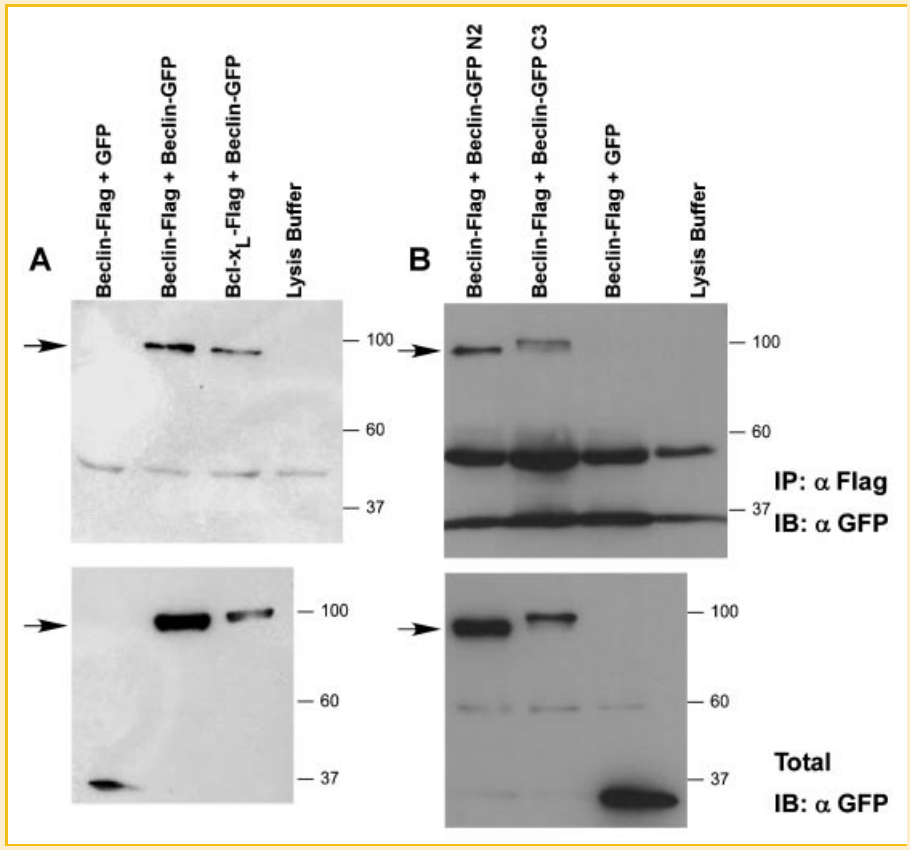

Fig. 2. Beclin 1 self-interacts. A: HEK293 cells were co-transfected with Flag-Beclin 1 expression vector and either pEGFP or Beclin 1-pEGFP expression vectors. As a control cells were transfected with Flag-Bcl-x_L and Beclin 1-pEGFP expression vectors. Forty-eight hours following transfection total cell lysates were prepared and subjected to immunoprecipitation using immobilized monoclonal anti-Flag antibodies. Proteins retained on the beads were resolved on SDS–PAGE and then processed for Western blot using anti-GFP monoclonal antibodies. As a control (lower panel), total cell lysates were reacted with anti-GFP antibodies. The results shown are from a representative experiment (one of at least three independent experiments). B: HEK293 cells were co-transfected with Flag-Beclin 1 and either Beclin 1-pEGFPN2 or pEGFPC3-Beclin 1 or pEGFP expression vectors. Forty-eight hours following transfection total cell lysates were processed as in (A) above. As a control, total cell lysates were reacted with monoclonal anti-GFP antibodies. The results shown are from a representative experiment.

Beclin 1 domains fused to pEGFP; N-ter containing amino acids 1–142, coiled-coil (CC) containing amino acids 144–269, C-ter containing amino acids 270–448. The vectors were termed: N-ter-pEGFP, CC-pEGFP, and C-ter-pEGFP, respectively (Fig. 3A). As shown in Figure 3B, cells transiently transfected with the indicated expression vectors, express the mutants in addition to the endogenous wild-type Beclin 1 proteins. Next, we determined which of the Beclin 1 domains mediates the self-interaction. For this purpose, we used the chemical cross linker bis(sulfosuccinimidyl)-suberate (BS^3) and examined which of the various Beclin 1 fragments oligomerize. First, cells were transfected with Flag-Beclin 1 expression vector. Cell lysates were prepared and incubated with or without BS^3 reagent and the resulting cell lysates were subjected to SDS–PAGE. Immunoblot analysis with anti-Flag monoclonal antibodies (Fig. 3C) showed that the full-length Beclin 1 can form a high molecular weight complex of ~190 kDa (that may represent a trimeric complex). Next, cells were transfected with either Beclin 1-pEGFP, N-ter-pEGFP, CC-pEGFP, or C-ter-pEGFP expression vectors. Cell lysates were prepared and incubated with or without BS^3 reagent and the resulting cell lysates were subjected to SDS–PAGE. Immunoblot analysis with anti-GFP monoclonal antibodies shows that the full-length Beclin 1 as well as the CC domain can form high molecular weight complexes. A weaker signal was also obtained using the N-terminal fragment of Beclin 1, indicating that the N-terminal region may also contribute to Beclin 1 oligomerization. These results indicate that the full-length Beclin 1 can form oligomers (with itself or with other proteins) and that the coiled-coil region may be the major region responsible for this interaction.

In order to further examine which of the Beclin 1 domains is responsible for its self-interaction, cells were co-transfected with Flag-Beclin 1 and either Beclin 1-pEGFP or N-ter-pEGFP or CC-pEGFP. Cell lysates were immunoprecipitated with anti-Flag monoclonal antibodies and immunoblotted with monoclonal anti-GFP and polyclonal anti-Beclin 1 antibodies. As shown in Figure 4A, the N-ter and CC fragments co-immunoprecipitated with the full-length Beclin 1. These results further support our previous results showing that the coiled-coil region is responsible for Beclin 1 self-interaction while the N-terminal region may contribute to this interaction.

Recent studies and structural modeling discovered that Beclin 1 contains a putative BH3-like domain (in the N-terminal region)

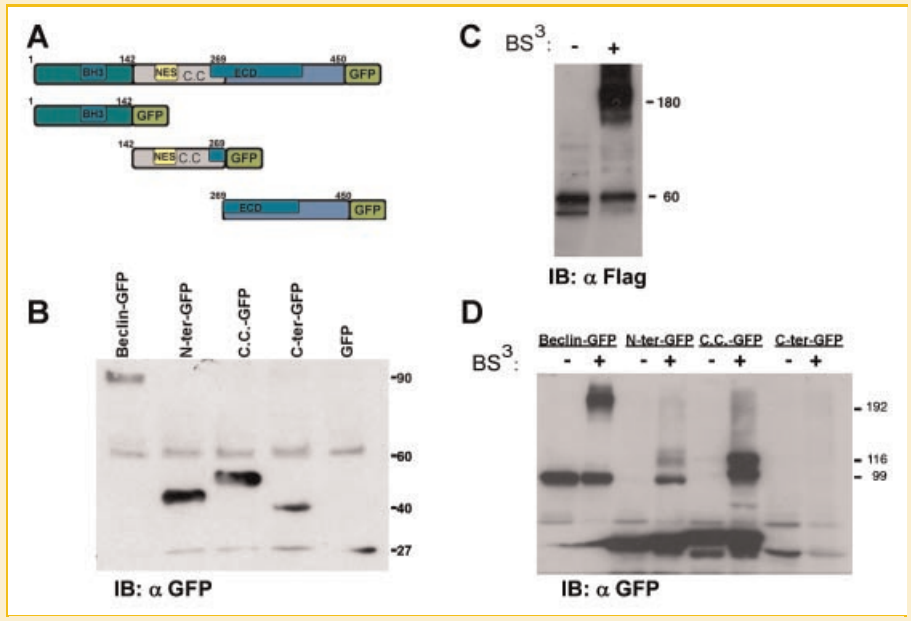

Fig. 3. Beclin 1 domains: the CC fragment and the N-terminal fragment self-interact. A: A schematic presentation of the expression vectors: full-length Beclin 1-GFP and three Beclin 1 domains fused to pEGFP: N-terminal (N-ter-GFP), coiled-coil (CC-GFP), and C-terminal (C-ter-GFP). B: HEK293 cells were transfected with Beclin 1-pEGFP, N-ter-pEGFP, CC-pEGFP, C-ter-pEGFP, and pEGFP expression vectors. Forty-eight hours following transfection total cell lysates were resolved by SDS–PAGE and then processed for Western blot using monoclonal anti-GFP antibodies. C,D: HEK293 cells were transfected with Flag-Beclin 1, Beclin 1-pEGFP, N-ter-pEGFP, CC-pEGFP, and C-ter-pEGFP expression vectors. Forty-eight hours following transfection, cell lysates were crosslinked with 2 mM bis(sulfosuccinimidyl)-suberate(BS3) and samples were resolved by SDS–PAGE. Blots were reacted with monoclonal anti-Flag (C) or anti-GFP (D) antibodies.

which mediates its interaction with the anti-apoptotic Bcl-2 family of proteins (Erlich et al., 2007b; Oberstein et al., 2007). In order to investigate the importance of the BH3 domain of Beclin 1 for its self-interaction we used the following BH3 mutants: single point mutants, L116A; M109A; a double point mutation R114A and R115A; deletion ΔGD, a deletion of both G120 and D121; and ΔBcl_2, a deletion of the Bcl_2-binding domain (amino acids 79–151). Cells were co-tranfected with Flag-Beclin 1 and either pEGFP-Beclin 1 or the above-mentioned pEGFP-Beclin 1 mutants. As shown in Figure 4B, the Beclin 1 BH3 mutants co-immunoprecipitated with wild-type Flag-Beclin 1 indicating that these mutations have no effect on Beclin 1 self-interaction. We therefore concluded that the Bcl-2 binding domain and the BH3 domain of Beclin 1 are not necessary for Beclin 1 self-interaction.

Since the N-ter-Beclin 1 co-immunoprecipitated with full-length Beclin 1 and mutation in the BH3 domain, part of the N-ter region, did not affect Beclin 1 self-interaction, we decided to use other approaches to determine which domain is responsible for Beclin 1 self-interaction. For this purpose, we constructed a Beclin 1 mutant with deletion of the coiled-coil domain (△CC) and used the GST-Beclin 1 pull-down assay. Cells were transfected with Beclin 1-pEGFP, N-ter-pEGFP, CC-pEGFP, △CC-pEGFP, and pEGFP-nucleolin (as a control) expression vectors. Cell lysates were incubated with immobilized GST or GST-Beclin 1 agarose beads; the bound proteins were analyzed by immunoblot with anti-GFP monoclonal antibodies. As shown in Figure 4C, full-length Beclin 1-pEGFP interacts with GST-Beclin 1. Furthermore, the CC domain of Beclin 1, the N-ter domain and the ΔCC mutant all interact with GST-Beclin 1. As controls we used GST beads which did not precipitate GFP-fused proteins (data not shown) and pEGFP-nucleolin protein that did not interact with GST-Beclin 1. Taken together the results indicate that the CC domain is sufficient for Beclin 1 self-interaction. However, the N-terminal region may also contribute to Beclin 1 self-interaction.

Beclin 1 interacts with several proteins including PI3KcIII/vps34 protein. Autophagy mediated by PI3K depends on interaction of the latter with Beclin 1 (Kihara et al., 2001). To determine the effect of vps34 on Beclin 1 self-interaction, we performed a GST-Beclin 1 pull-down assay using lysates of cells overexpressing increasing amounts of GFP-vps34 and a constant amount of GFP-Beclin 1. As shown in Figure 5A in cells overexpressing increasing amounts of GFP-vps34, the levels of GFP-vps34 pulled down by GST-Beclin 1 increased, whereas, the levels of GFP-Beclin 1 pulled down by GST-Beclin 1 was not affected, indicating that the levels of vps34 may not affect Beclin 1 self-interaction. Moreover, to further evaluate the effect of vps34 on Beclin 1 self-interaction we generated stable cell lines overexpressing Flag-tagged Beclin 1 and GFP-Beclin 1. These cells were then transiently transfected with GFP-vps34 expression vector, and lysates were tested for the self-association of Beclin 1 by immunoprecipitation with anti-Flag antibodies. As shown in Figure 5B, overexpression of GFP-vps34 had no effect on the levels of GFP-Beclin 1 co-immunoprecipitated with Flag-Beclin 1, although GFP-vps34 was also co-immunoprecipitated with Flag-Beclin 1.

The effect of UVRAG and Bcl-x_L on Beclin 1 self-association was determined in a similar set of experiments (Fig. 6). Both UVRAG and Bcl-x_L were co-immunoprecipitated with Flag-Beclin 1, and in both cases the self-association of Beclin 1 was not affected.

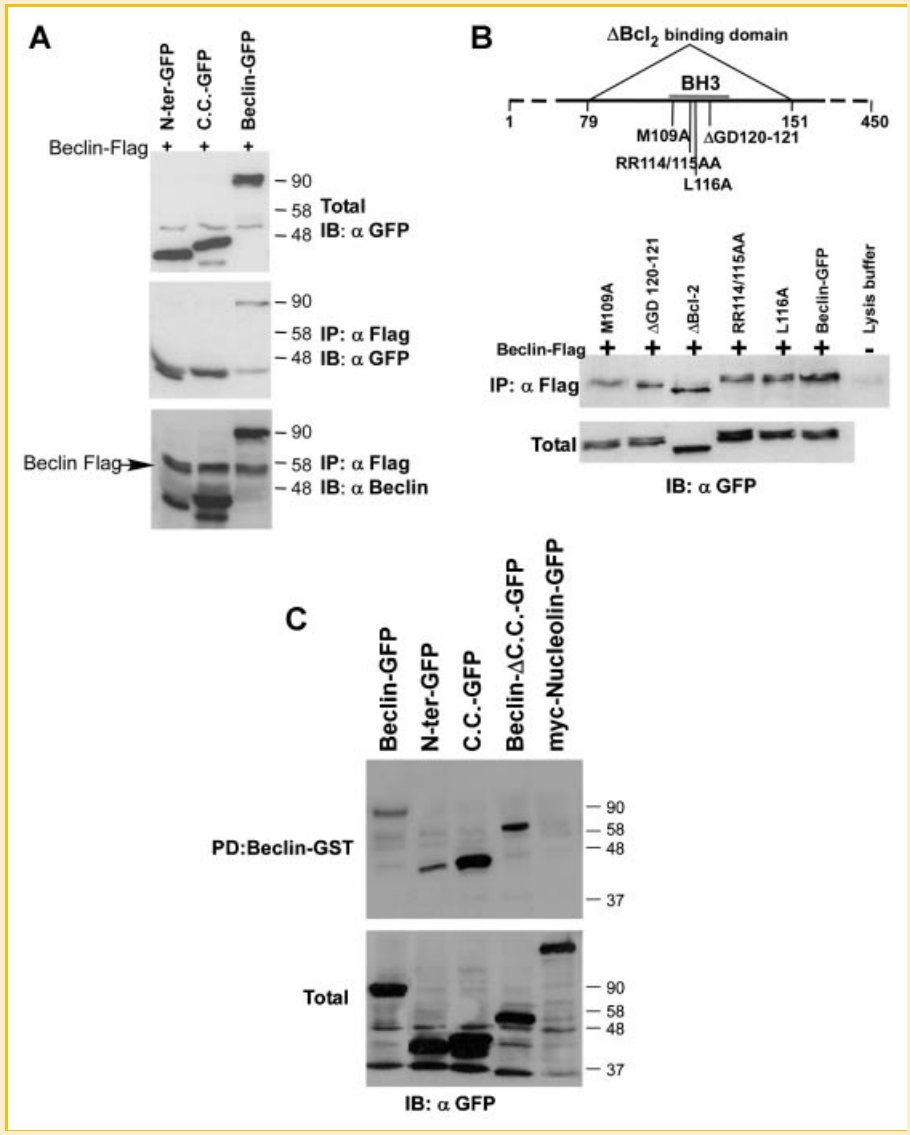

Fig. 4. Beclin 1 interacting domains. A: Beclin 1 co-immunoprecipitates with N-ter and CC fragments. HEK293 cells were transfected with Flag-Beclin 1 and either Beclin 1-pEGFP or N-ter-pEGFP or CC-pEGFP. Forty-eight hours following transfection cell lysates were subjected to immunoprecipitation using monoclonal anti-Flag antibodies and blotted with anti-GFP antibodies or with anti-Beclin 1 antibodies as indicated. As control, total cell lysates blots were reacted with monoclonal anti-GFP antibodies (upper panel). B: Mutations in Beclin 1 BH3 domain do not inhibit the interaction with wild-type Beclin 1. HEK293 cells were transfected with Flag-Beclin 1 and either pEGFP-Beclin 1 or pEGFP-M109A or pEGFP-△GD or pEGFP-△Bcl2 (deletion of amino acids 79-151) or pEGFP-RR114-115AA or pEGFP-L116A mutants. Forty-eight hours following transfection cell lysates were subjected to immunoprecipitation using monoclonal anti-Flag antibodies and blotted with anti-GFP antibodies. As control, total cell lysates blots were reacted with monoclonal anti-GFP antibodies. C: The coiled-coil domain and the N-ter domain mediate Beclin 1 self-interaction. HEK293 cells were transfected with Beclin 1-pEGFP, N-ter-pEGFP, CC-pEGFP, and ΔCC-pEGFP expression vectors. Forty-eight hours following transfection cell lysates were prepared using CHAPS buffer and then incubated with immobilized GST-Beclin 1 or GST. As a control, lysates of cells expressing myc-pEGF-N2-nucleolin were also incubated with immobilized GST-Beclin 1 or GST. Proteins retained on the beads were resolved by SDS–PAGE and then processed for Western blot using anti-GFP monoclonal antibodies (upper panels). Total cell lysate served as loading control (lower panel).

To evaluate the effect of autophagy induction on Beclin 1 self-interaction we have used the stable cell lines overexpressing Flag-tagged Beclin 1 and GFP-Beclin 1. As shown in Figure 7A, 2 and 4 h amino acid starvation induced autophagy as determined by the increased levels of LC3 II. Under these conditions no significant change was observed in the levels of GFP-Beclin 1 co-immunoprecipitated with Flag-Beclin 1. Similarly, autophagy was induced by rapamycin treatment (Fig. 7B). As shown, rapamycin induced autophagy as determined by increased LC3 II levels. However, the co-immunoprecipitation of GFP-Beclin 1 with Flag-Beclin 1 remained un-changed. These results may suggest that level of Beclin 1 self-interaction remains unchanged during amino acid deprivation- or rapamycin-induced autophagy.

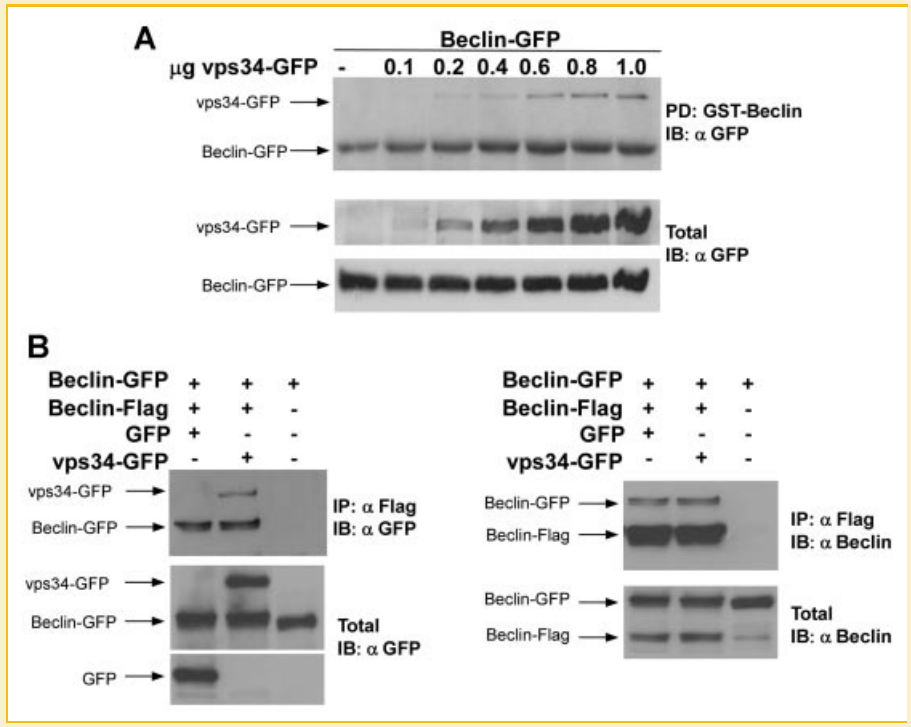

Fig. 5. Vps34 does not affect Beclin 1 self-interaction. A: HEK293 cells were transfected with Beclin 1-pEGFP (0.4 μg) and increasing concentration of GFP-Vps34 expression vector (0–1 μg) as indicated. Forty-eight hours following transfection cell lysates were prepared using CHAPS buffer and then incubated with immobilized GST-Beclin 1 or GST (not shown). Proteins retained on the beads were resolved by SDS–PAGE and then processed for Western blot using anti-GFP monoclonal antibodies (upper panel). Total cell lysates served as loading control (lower panel). B: HEK293 cells stably overexpressing GFP-Beclin 1 and Flag-Beclin 1 were transfected with pEGFP-vps34 or pEGFP empty vector. Forty-eight hours following transfection cell lysates were subjected to immunoprecipitation using monoclonal anti-Flag antibodies and blotted with anti GFP (left panels) or anti-Beclin 1 antibodies (right panels). Self-association of Beclin-1 is expressed by the co-immunoprecipitation of GFP-Beclin 1 with Flag-Beclin 1. Lysates from HEK293 cells stably expressing only GFP-Beclin 1 served as a negative control for the specific binding to the beads. As control, total cell lysates blots were reacted with monoclonal anti-GFP antibodies or anti Beclin 1 antibodies as indicated.

DISCUSSION

Autophagy is an evolutionary conserved process that regulates the balance between synthesis and degradation of cellular components (Reggiori and Klionsky, 2005). The process has an important housekeeping role as it promotes protein turnover and removes damaged proteins and organelles (Liang et al., 2006). Moreover, the autophagic process is activated during development, under stress conditions, and it was associated with several human diseases including cancer and neurodegenerative disorders (Huang and Strasser, 2000; Kelekar, 2005). Thus, it is critical to understand mechanisms regulating autophagy.

Beclin 1 is a 60 kDa protein originally identified as a Bcl-2 interacting protein (Zeng et al., 2006). It contain a putative BH3 domain through which it binds to anti-apoptotic Bcl-2 family proteins (Erlich et al., 2007b), a myosin-like coiled-coil region that binds UVRAG protein (Liang et al., 2006) and an ECD that is required for PI3KcIII/Vps34 binding (Liang et al., 1998, 2001; Furuya et al., 2005). Beclin 1 functions in autophagy in a wide range of species, as part of the PI3KcIII/Vps34 complex which participates in vesicle nucleation (Klionsky, 2005). By using a GST-Beclin 1 pull-down assay and mass spectrometry, we identified Beclin 1–Beclin 1 interaction. To verify Beclin 1 self-interaction, we have used Beclin 1 tagged with two different tags and co-IP experiments. We demonstrated that Beclin 1-pEGFP co-immunoprecipitated with Flag-Beclin 1 in similar manner to Flag-Bcl-x_L. These results strongly suggest that Beclin 1 self-interacts.

In order to identify Beclin 1 self-interacting domains, we generated expression vectors of Beclin 1 fragments that correspond to its three main functional domains: the N-ter that includes the Bcl-2 binding domain (Liang et al., 1998), the coiled-coil domain which in general is known to be a universal oligomerization domain (Hodges, 1996), previously shown to be involved in Beclin 1/UVRAG interaction (Liang et al., 2006) and the C-ter domain that includes part of the ECD region which was previously recognized as an essential region for binding PI3KcIII/Vps34 (Furuya et al., 2005). We have used a chemical cross-linker to examine which of the fragments can oligomerize. Our results indicate that the CC and the N-ter fragments contribute to Beclin 1 self-interaction, generating higher molecular weight size complexes. Further examination of the domains responsible for Beclin 1 oligomerization, using a co-immunoprecipitation assay, indeed demonstrated that both the N-ter and the CC fragments can bind to full-length Beclin 1 but with different efficiency; the N-ter binds less effectively than the CC, indicating that the N-ter domain may also contribute to Beclin 1 self-interaction.

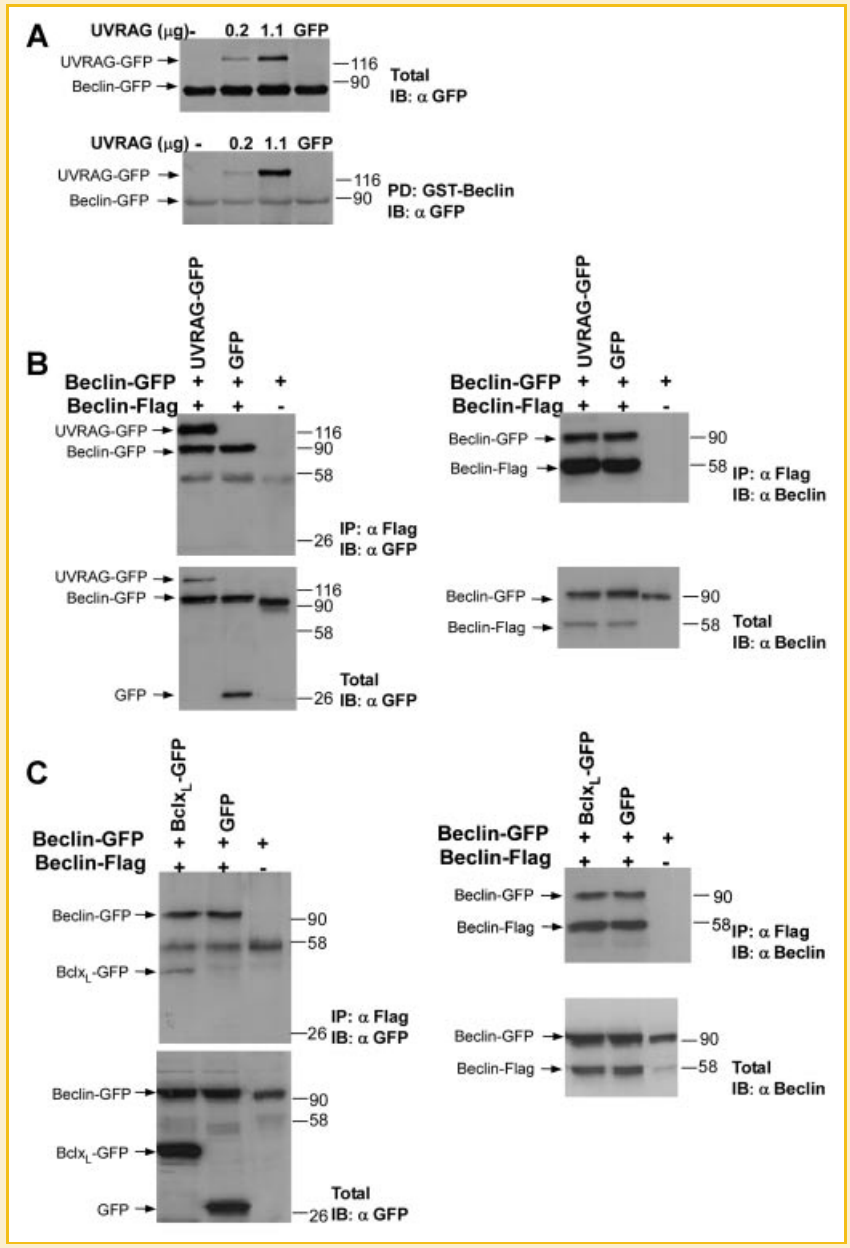

Fig. 6. UVRAG and $Bclx_L$ do not affect Beclin 1 self-interaction. A: HEK293 cells stably expressing Beclin 1 were transfected with GFP-UVRAG expression vector (0–1.1 μg) as indicated. Forty-eight hours following transfection cell lysates were prepared using CHAPS buffer and then incubated with immobilized GST-Beclin 1 or GST (not shown). Proteins retained on the beads were resolved by SDS–PAGE and then processed for Western blot using anti-GFP monoclonal antibodies (lower panel). Total cell lysate served as loading control (upper panel). B,C: HEK293 cells stably overexpressing GFP-Beclin 1 and Flag-Beclin 1 were transfected with pEGFP-UVRAG (B) or pEGFP-$Bclx_L$ (C) or pEGFP empty vector. Forty-eight hours following transfection cell lysates were subjected to immunoprecipitation using monoclonal anti-Flag antibodies and blotted with anti-GFP (left panels) or anti-Beclin 1 antibodies (right panels). Self-association of Beclin-1 is expressed by the co-immunoprecipitation of GFP-Beclin 1 with Flag-Beclin 1. Lysates from HEK293 cells stably expressing only GFP-Beclin 1 served as a negative control for the specific binding to the beads. As control, total cell lysates blots were reacted with monoclonal anti-GFP antibodies or anti Beclin 1 antibodies as indicated.

It has been well documented that members of the Bcl-2 family proteins, as well as death promoting proteins, form homo/heterodimers (Hanada et al., 1995; Hsu et al., 1997; Conus et al., 2000; Shohat et al., 2002; Moldoveanu et al., 2006). We assumed that the oligomerization of Beclin 1 might regulate autophagy. Since the N-ter domain includes a Bcl-2 binding domain with the putative BH3 region that was recently found to be essential for Beclin 1 interaction with the Bcl-2 anti-apoptotic family members (Erlich et al., 2007b; Oberstein et al., 2007), and previous studies have shown that binding of Bcl-2 to Beclin 1 inhibits autophagy (Pattingre et al., 2005), we determined whether the Beclin 1 BH3 domain is also essential for Beclin 1 self-interaction. Our results using the various Bcl-2 binding domain mutants strongly suggest that the Bcl-2 binding domain is not important for Beclin 1

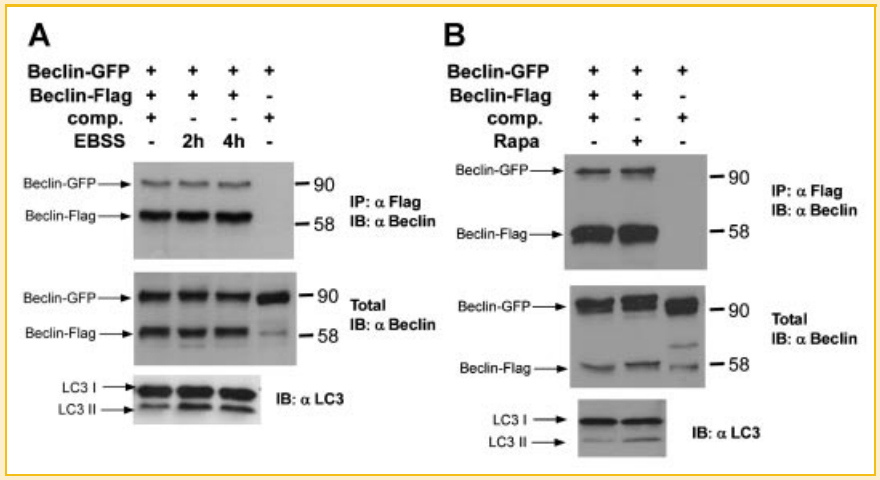

Fig. 7. Autophagy induction does not affect Beclin 1 self-interaction. Autophagy was induced in HEK293 cells stably overexpressing GFP-Beclin 1 and Flag-Beclin 1 either by amino acids deprivation (EBSS) for 2 and 4 h (A) or by Rapamycin treatment (200 ng/ml) for 4 h (Rapa) (B). Control cells in both experiments were grown in complete medium (DMEM containing 10% FCS) (comp). Cell lysates were subjected to immunoprecipitation using monoclonal anti-Flag antibodies and blotted with anti-Beclin 1 antibodies. Self-association of Beclin 1 is expressed by the co-immunoprecipitation of GFP-Beclin 1 with Flag-Beclin 1. Lysates from HEK293 cells stably expressing only GFP-Beclin 1 served as a negative control for the specific binding to the beads. As control, blots of total cell lysates were reacted with monoclonal anti-Beclin 1 antibodies. Autophagy was verified by the accumulation of LC3 II using anti-LC3 antibodies.

oligomerization. Moreover overexpression of Bcl-x_L did not affect Beclin 1 self-interaction (Fig. 6C). These results indicate that the Bcl-2 binding domain is not essential for Beclin 1 self-interaction.

Further support for the involvement of the CC domain and the N-ter region in Beclin 1 oligomerization came from a GST-Beclin 1 pull-down assay shown in Figure 4C, in which the CC fragment, the N-ter fragment and ΔCC mutant were pulled down with GST-Beclin 1. The coiled-coil motif is a well-known protein–protein interaction motif, often mediating self-interactions (Hodges, 1996). Scores of proteins oligomerize through their CC domain (Zhang and Epstein, 2003; Dubin et al., 2004). To date, there are three known proteins that bind Beclin 1 through its CC domain: nPIST, Ambra1, and UVRAG (Yue et al., 2002; Liang et al., 2006; Fimia et al., 2007). We found that the CC domain is important for Beclin 1 self-interaction. However how the N-ter contributes to the oligomerization remains to be studied.

The interaction between Beclin 1 and PI3KcIII/Vps34 is known to be important for autophagy (Tassa et al., 2003) since this interaction is essential for vesicle nucleation and protein recruitment (Klionsky, 2005). To examine the effect of vps34 on Beclin 1 self-interaction we used GST-Beclin pull-down and co-immunoprecipitation assays. Our results demonstrate that Beclin 1 self-interaction is not affected by the vps34 levels, indicating that upon Beclin 1/vps34 interaction Beclin 1 self-interaction is not disturbed. Moreover, overexpression of UVRAG had no effect on Beclin 1 self-interaction (Fig. 6A,B). In addition our study examined the state of Beclin 1 self-interaction under conditions of autophagy induction. Autophagy was induced by amino acid deprivation or rapamycin treatment and confirmed by measuring the levels of lipidated LC3 (LC3 II) (Fig. 7). Under these conditions, Beclin 1 self-interaction was not affected suggesting that Beclin 1 self-interaction is independent of autophagy activation.

Several recent reports have relevance for our findings. The first study demonstrated that Beclin 1 forms a homo-oligomer which is disrupted partly by viral Bcl-2 (M11) (Ku et al., 2008). Second study shows that Beclin 1 forms a dimer in solution via its coiled-coil domain (Noble et al., 2008). We found that full-length Beclin 1 self-interacts in live cells. This self-interaction is independent of vps34, Bcl-xL, and UVRAG overexpression and their association with Beclin 1. Importantly, Beclin 1 forms a stable oligomer under normal and autophagy conditions. Such an oligomer may provide a platform for further protein–protein interactions.

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