The N-terminus and Phe52 residue of LC3 recruit p62/SQSTM1 into autophagosomes

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Summary
LC3 belongs to a novel ubiquitin-like protein family that is involved in different intracellular trafficking processes, including autophagy. All members of this family share a unique three-dimensional structure composed of a C-terminal ubiquitin core and two N-terminal α-helices. Here, we focus on the specific contribution of these regions to autophagy induced by amino acid deprivation. We show that the ubiquitin core by itself is sufficient for LC3 processing through the conjugation machinery and for its consequent targeting to the autophagosomal membrane. The N-terminal region was found to be important for interaction between LC3 and p62/SQSTM1 (hereafter termed p62). This interaction is dependent on the first 10 amino acids of LC3 and on specific residues located within the ubiquitin core. Knockdown of LC3 isoforms and overexpression of LC3 mutants that fail to interact with p62 blocked the incorporation of p62 into autophagosomes. The accumulation of p62 was accompanied by elevated levels of polyubiquitylated detergent-insoluble structures. p62, however, is not required for LC3 lipidation, autophagosome formation and targeting to lysosomes. Our results support the proposal that LC3 is responsible for recruiting p62 into autophagosomes, a process mediated by phenylalanine 52, located within the ubiquitin core, and the N-terminal region of the protein.

Key words: Autophagy, LC3, p62

Introduction
Autophagy is an intracellular trafficking pathway that mediates delivery of cytoplasmic constituents (proteins and organelles) for bulk degradation within the lysosome/vacuole compartments. This pathway, initiated predominantly in response to nutrient starvation, is involved in diverse physiological functions, such as cell development, programmed cell death, cancer, pathogen infection and degradation of ubiquitylated protein aggregates formed in many pathological conditions (Boland and Nixon, 2006; Huang and Klionsky, 2007). During autophagy, cytoplasmic protein aggregates are randomly sequestered into an autophagosome, a double-membrane vesicle, that fuses with the lysosome/vacuole, releasing a single-membrane vesicle (autophagic body) into this compartment to generate an autolysosome. Finally, lysosomal hydrolases degrade the autophagosome inner membrane and its sequestered contents (autophagic body).

Two conserved ubiquitin-like (UBL) conjugation systems, Atg12 and Atg8, are essential for early stages of autophagosome biogenesis in yeast and mammals (Ohsumi, 2001). Atg8 is first cleaved by Atg4, a specific cysteine protease, thereby exposing a C-terminal glycine residue. This allows Atg8 to become conjugated to phosphatidylethanolamine (PE), a unique modification not found in other UBL proteins. The Atg8-PE conjugate plays a crucial role in subsequent dynamic rearrangement of membranes, leading to the formation of an autophagosome (Abeliovich et al., 2000; Kirisako et al., 2000; Lang et al., 1998; Mizushima et al., 2001). Atg8 shows a high level of sequence identity with a family of UBL mammalian proteins, including MAP1-LC3 (LC3, official symbol MLP3B), GATE-16 and GABARAP (Mann and Hammarskja, 1994; Sagiv et al., 2000; Wang et al., 1999). The modification of LC3 at its C-terminus, similarly to that of Atg8, is an essential step in the formation of autophagosomes in mammals (Tanida et al., 2004). All members of the Atg8 family share a similar structure made of a ubiquitin-like core decorated with two unique N-terminal α-helices. It has been recently found (Nakatogawa et al., 2007) that Atg8 serves as a membrane-tethering and a hemifusion factor during the expansion of autophagosomal membranes and that truncation of its N-terminus impairs this activity. However, the exact role of mammalian Atg8, especially that of its different regions, has not yet been fully determined. Recent reports showed that LC3 directly interacts with sequestosome-1 (SQSTM; also known as p62) (Bjorkoy et al., 2005; Pankiv et al., 2007), a protein involved in cell signaling, receptor internalization and protein turnover (Moscat et al., 2007; Seibenhener et al., 2007), via a newly identified LC3-interacting region (LIR), located between its ZZ and ubiquitin-associated (UBA) domains. However, the region of LC3 involved in this interaction is not known. p62 binds polyubiquitin via the UBA domain and is commonly present in protein inclusions associated with neurodegenerative diseases (Babu et al., 2005; Seibenhener et al., 2004; Zatloukal et al., 2002). Thus, p62 interaction with LC3 is required for the degradation of polyubiquitylated protein aggregates by autophagy (Bjorkoy et al., 2005). The question remains, however, whether this interaction is essential for autophagy or merely required for delivery of polyubiquitylated protein aggregates for lysosomal degradation.

In the present study, we determined the minimal structure required for conjugation and the consequent targeting of LC3 to the autophagosomal membrane. We found that LC3 N-terminal α-helix together with a specific site located within the ubiquitin core...
of the protein are involved in the interaction with p62 for the consequential delivery of this protein to lysosomes for degradation. However, p62 is not required for either LC3 lipidation or for autophagosome formation and targeting to the lysosomes. Moreover, total autophagy-mediated protein degradation was not affected by p62 knockdown. This study provides molecular information regarding the cargo-recruiting activity of LC3, which involves the conserved residue Phe52, together with the first ten amino acids of the protein.

Results

The ubiquitin core of LC3 is sufficient for its lipidation and targeting to autophagosomes

A key step in the autophagic process is the conjugation of Atg8s, a novel UBL protein family, to the autophagosomal membrane. The mammalian homologues of Atg8 include GATE-16, GABARAP and LC3, all sharing a similar structure composed of a ubiquitin core decorated by two N-terminal α-helices (Coyle et al., 2002; Knight et al., 2002; Paz et al., 2000; Sugawara et al., 2003). To determine the specific contribution of these protein regions to the autophagic process, Chinese hamster ovary (CHO) cells, previously characterized for their autophagic activity (Fass et al., 2006), were stably transfected with GFP fused to either wild-type LC3, LC3\[^N10\], LC3\[^N28\] or LC3\[^N40\], where \[^N\] indicates the truncation of the N-terminal region (Fig. 1A; the truncated regions are marked in red). PyMol molecular visualization system was used for presentation of 1UGM:A file from RCSB PDB (Sugawara et al., 2004). LC3\[^N28\] contains the ubiquitin core alone. (B) CHO cells stably expressing GFP-LC3, GFP-LC3\[^N10\], GFP-LC3\[^N28\] or GFP-LC3\[^N40\] were cultured for 2 hours in αMEM (control) or EBSS (starvation) medium, fixed and analyzed by fluorescence microscopy. (C) Stably expressing CHO cells were starved in the presence of 100 nM bafilomycin A (Baf A), lysed with RIPA extraction buffer and cell lysates were analyzed by immunoblotting using anti-GFP antibodies (right). Asterisk labels the lipidated form (LC3-II). Quantification of GFP-LC3 lipidation (left) was performed as described in Materials and Methods. Data are means ± s.d. (D) Left, extracts of GFP-LC3 and GFP-LC3\[^N28\] expressing cells were subjected to western blot analysis using anti-GFP or anti-LC3 antibodies. Middle panels, cells expressing GFP-LC3\[^N28\] were incubated in αMEM or EBSS medium in the presence or absence of Baf A, fixed and immunostained with anti-LC3 antibodies. Colocalization was analyzed by confocal microscopy. The boxed areas shown are enlarged on the right. Right, colocalization analysis of GFP-LC3\[^N28\] or GFP-LC3\[^N28\] G120A with endogenous LC3 in starved cells was performed using the JACoP plugin in ImageJ (NIH Image) as described in the Materials and Methods. (E) Cells expressing GFP-LC3, GFP-LC3\[^N28\] or GFP-LC3\[^N40\] were incubated for 2 hours under starvation conditions in the presence of 100 nM Baf A. Lysosomes were labeled with monoclonal antibodies against LAMP-1 and colocalization was analyzed by confocal microscopy (left panels). The areas shown in white boxes are enlarged on the right. Colocalization analysis of GFP-LC3\[^WT\], GFP-LC3\[^N28\] or GFP-LC3\[^N40\] with LAMP-1-labeled lysosomes (right) was performed as described in the Materials and Methods. Scale bars: 5 μm.
LC3-II). To this end, the formation of the lipidosomal form was induced in cells by amino acid starvation, and its degradation was blocked by bafilomycin A, a known lysosomal inhibitor. This agent was previously shown (Yoshimori et al., 1991) to block lysosomal acidification, leading to the accumulation of autolysosomes (Fass et al., 2006; Mousavi et al., 2001) and autophagosomes-associated LC3 (LC3-II). The cells were lysed and cell extracts were subjected to western blot analysis with anti-LC3 antibodies. As shown in Fig. 2D, a ~60 kDa band was predominantly precipitated in cells expressing GFP-LC3, whereas no effect was observed in cells expressing GFP-LC3

LC3 specifically interacts with p62 in an N-terminus-dependent manner. (A) CHO cells stably expressing GFP-LC3 or GFP-LC3

To study the role of LC3 N-terminus we searched for proteins that interact specifically with the full-length LC3 but not with the truncated protein lacking the two N-terminal α-helices (LC3-N10). For that purpose, cells expressing GFP-LC3 or GFP-LC3

LC3 specifically interacts with p62 in an N-terminus- and Phe52-dependent manner

To narrow down the specific region of LC3 that is crucial for interaction with p62, we performed a series of experiments using different LC3 constructs. We found that the first 10 amino acids of LC3 are required for this interaction. For that purpose, we tested several constructs expressing different parts of LC3, and found that the construct expressing the first 10 amino acids of LC3 specifically interacted with p62. This finding suggests that the interaction between LC3 and p62 is dependent on the presence of the N-terminal α-helix of LC3.

To further examine whether GFP-LC3-N10-labeled autophagosomes are delivered to lysosomes by treating starved cells with vacuolar H+ ATPase inhibitor Baf A. As shown in Fig. 1E, cells treated with Baf A accumulated GFP-LC3-N10-labeled vesicles within LAMP-1-labeled lysosomes (autolysosomes), similarly to the full-length protein (GFP-LC3), whereas no effect was observed in cells expressing GFP-LC3-N40. These findings indicate that GFP-LC3-N10 localizes to LC3-labeled autophagosomes that are properly targeted and fuse with lysosomes.

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These proteins to PE (Kabeya et al., 2004). As depicted in Fig. 3B, similar amounts of p62 protein coprecipitated with both wild-type and the mutants (LC3F52A, LC3AN10, LC3AN28, LC3G120A) were transiently expressed at high levels in HeLa cells. We next determined whether these proteins acquired appropriate subcellular localization by utilizing antibodies directed against different markers of the autophagic pathway. As shown in Fig. 4A, all mutant proteins (excluding LC3G120A) were partially colocalized with Atg16, indicating that these proteins are properly localized to the phagophore or isolation membranes. To test whether the LC3-labeled autophagosomes are efficiently delivered to lysosomes, HeLa cells transiently expressing these proteins were starved in the presence of Baf A, stained with LAMP-1 and analyzed by confocal microscopy. Quantitative colocalization analysis revealed that delivery of mutants LC3F52A, LC3AN10 and LC3AN28 into the lysosomes is similar to that of the wild-type protein (Fig. 4B). The various LC3 mutants can be divided into two types based on their interaction with p62: mutants LC3F52A, LC3AN10 and LC3AN28, which do not bind p62 but are efficiently conjugated to the autophagosomal membrane, and LC3G120A, which binds p62 with similar affinity as the wild-type protein but does not undergo conjugation to autophagosomal membrane. Hence, when the effect of these mutants on p62 delivery into lysosomes was analyzed in cells starved in the presence of Baf A, two patterns of p62 localization were detected. As depicted in Fig. 4C, expression of wild-type GFP-LC3 did not affect the level of p62 accumulation in lysosomes (appears as small vesicles), and most p62 vesicles fused to GFP were overexpressed in cells, fewer vesicles with p62 were observed and only partial colocalization with different LC3 proteins was detected. By contrast, when LC3G120A, the mutant that actively prevents incorporation of p62 into autophagosomes, was overexpressed in vivo, p62 accumulated in several large structures, often associated with GFP-LC3G120A (Fig. 4C, left panels). Notably, when LC3G120A was expressed at high levels, it often colocalized with p62 to separate large structures, which may represent protein aggregates (data not shown). Consistently, extensive quantitative analysis showed that although colocalization of LC3AN10, LC3AN28 and LC3F52A with p62 was significantly inhibited compared to wild-type LC3, overexpression of LC3G120A resulted in strong inhibition of p62 delivery into lysosomes (Fig. 4C, right). To carry out biochemical analysis on the effect of these mutants on p62 degradation, HeLa cells expressing these proteins were starved in the absence or presence of Baf A and the level of p62 was determined by western blot using specific anti-p62 antibodies. The degradation of p62 following overexpression of different LC3 mutants was estimated as the difference between its level in the absence or presence of Baf A and calculated relative to its degradation in cells transfected with the wild-type protein. As depicted in Fig. 4D, overexpression of all LC3 mutants (LC3F52A,
Fig. 4. Overexpression of LC3 mutants inhibits incorporation of p62 into autophagosomes and its degradation within lysosomes. (A) HeLa cells were transfected with GFP fused to LC3 WT or to its mutants and after 24 hours were cultured for 3 hours in EBSS medium, fixed, immunostained with anti-Atg16 antibodies and analyzed by confocal microscopy. The boxed areas are enlarged on the right. (B) Cells transfected as in A were starved in the presence of 0.1 μM Baf A, fixed, immunostained with monoclonal anti-LAMP-1 antibodies and analyzed by confocal microscopy. The areas shown in white boxes are enlarged on the right. Colocalization analysis of GFP fused proteins with LAMP-1 (right panel) was performed as described in the Materials and Methods. (C) Cells transfected as in A were starved in the presence of 0.1 μM Baf A, fixed, immunostained with monoclonal anti-p62 antibodies and analyzed by confocal microscopy. The boxed areas are enlarged on the right. Arrows indicate nontransfected cells, arrowheads indicate cells overexpressing GFP-LC3 proteins. Quantitative colocalization analysis of GFP fused proteins with p62 (right panel) was performed as described in the Materials and Methods. Mean ± s.d. of five independent experiments is presented below. ** P < 0.001. (D) Cells transfected as in A were starved in the presence or absence of 0.1 μM Baf A, lysed with RIPA extraction buffer and analyzed by western blot with anti-p62, anti-actin and anti-GFP antibodies (left panel). Quantification of relative p62 degradation (right panel) was calculated as described in Materials and Methods, and mean ± s.d. of four independent experiments is presented below. * P < 0.05. (E) The rate of degradation of long-lived proteins was measured in HeLa cells incubated in either αMEM (control) or EBSS (starvation) medium, 24 hours following transfection of GFP-fused proteins. Values expressing the percentage of cellular proteins degraded in 4 hours are represented as the means ± s.d. of six determinations. Scale bars: 5 μm.
LC3\textsuperscript{ΔN10}, LC3\textsuperscript{ΔN28} and LC3\textsuperscript{G120A} led to a significant inhibition in p62 degradation compared with wild-type protein. These results are consistent with the notion that LC3 delivers p62 to autophagosomes for lysosomal degradation.

We next examined the effect of these LC3 mutants on autophagy-mediated protein degradation. To this end, cells transfected with different GFP-fused proteins were prelabeled with \([^{14}\text{C}]\text{valine for 16 hours, and starvation-induced protein degradation was measured in these cells. As depicted in Fig. 4E, overexpression of different LC3 mutants had no effect on protein degradation activity compared with cells expressing the wild-type protein, suggesting that these mutants are not dominant-negative with respect to the overall autophagic process. In summary, although overexpression of LC3 mutants resulted in inhibition in p62 degradation (Fig. 4C,D), no defect was observed in autophagic activity of these cells (Fig. 4E), implying that association of p62 with autophagosomes is not essential for autophagy.

To directly determine the involvement of LC3 in the delivery of p62 into autophagosomes, LC3 was efficiently knocked down (Fig. 5A) with a specific pool of siRNA against all three LC3 isoforms (for details see Materials and Methods) and the effect on p62 degradation and localization was examined. Transfected cells were starved in the absence or presence of 0.1 \(\mu\text{M Baf A}, lysed with RIPA extraction buffer and analyzed by western blot with anti-p62, anti-actin and anti-LC3 antibodies. The asterisk indicates a nonspecific band. Arrows indicate LC3-I and LC3-II. (B) Quantification of relative p62 degradation (left panel) or relative p62 level (right panel) was performed as described in the Materials and Methods. Data are means ± s.d. of five independent experiments. The cells were transfected as in A, starved for 3 hours in the absence (C) or presence (D) of 0.1 \(\mu\text{M Baf A, immunostained with anti-p62 and anti-LC3 antibodies, and analyzed using confocal microscopy. Scale bars: 5 \(\mu\text{m.}}\)
LC3 recruits p62 into autophagosomes

Knockdown of LC3 leads to the accumulation of polyubiquitylated aggregates

P62 has been recently reported to serve as an adaptor molecule, mediating degradation of polyubiquitylated protein aggregates through the autophagic machinery (Bjorkoy et al., 2005). We therefore examined whether impaired degradation of p62 affected the level of polyubiquitylated proteins. To this end, LC3-knockdown cells were starved and polyubiquitylated proteins were detected either by western blot analysis or by confocal microscopy. (C) Cells were transfected as in A, starved for 3 hours in the presence of Baf A, then treated for 20 minutes with 1% Triton X-100 (right panel), fixed and stained with anti-LC3, anti-ubiquitin (UB) and anti-p62 antibodies. Scale bars: 5 μm. (D) The cells were transfected as in A, starved for 3 hours, lysed with RIPA extraction buffer, the lysates were centrifuged for 30 minutes at 350,000 g. The supernatant (soluble, left) and pellet (insoluble, right) fractions were analyzed by western blot with anti-p62, anti-actin and anti-ubiquitin antibodies.

Knockdown of LC3 leads to the accumulation of detergent-insoluble p62 and polyubiquitylated proteins. (A) HeLa cells were transfected with either nontargeting siRNA (control siRNA), or with a pool of LC3 siRNAs (A, B and C isoforms) using DharmaFect reagent. After 72 hours, the cells were incubated in EBSS medium, lysed with RIPA extraction buffer and analyzed by western blot with anti-p62, anti-actin and anti-ubiquitin antibodies (left panel). Quantification of relative ubiquitin level (right panel) was performed as described in Materials and Methods. Mean ± s.d. of three independent experiments is presented below. (B) The cells were treated as in A, fixed, immunostained using anti-LC3, anti-ubiquitin (UB) and anti-p62 antibodies, and analyzed using confocal microscopy. (C) Cells were transfected as in A, starved for 3 hours in the presence of Baf A, then treated for 20 minutes with 1% Triton X-100 (right panel), fixed and stained with anti-LC3, anti-ubiquitin (UB) and anti-p62 antibodies. Scale bars: 5 μm. (D) The cells were transfected as in A, starved for 3 hours, lysed with RIPA extraction buffer, the lysates were centrifuged for 30 minutes at 350,000 g. The supernatant (soluble, left) and pellet (insoluble, right) fractions were analyzed by western blot with anti-p62, anti-actin and anti-ubiquitin antibodies.

P62 is not essential for starvation-induced autophagy

To further test the involvement of p62 in the autophagic process, the protein was knocked down by RNAi and autophagy was examined by several assays: LC3 lipidation, LC3 delivery to the lysosomes and starvation-induced protein degradation. p62 was effectively knocked down by the specific siRNA (Fig. 7A, and B lower panel); however, no effect on LC3 lipidation either under control or starvation conditions was detected (Fig. 7A, and 7B upper panel). Next, we show that the formation of LC3-labeled autophagosomes and their delivery to lysosomes are not affected...
Fig. 7. p62 is not an essential factor in starvation-induced autophagy. (A) HeLa cells were transfected with either transfection reagent (mock), non-targeting siRNA (control siRNA), or p62 siRNA using DharmaFect reagent. After 48 hours, the cells were incubated under control or starvation conditions in the absence or presence of 0.1 μM Baf A, lysed using RIPA extraction buffer and analyzed by western blot with anti-p62, anti-actin and anti-LC3 antibodies. (B) Quantification of LC3 lipidation and the level of p62 protein were performed as described in Materials and Methods. (C) The cells were transfected as in A, starved for 3 hours in the presence of 0.1 μM Baf A, immunostained with anti-p62 and anti-LC3 antibodies, and analyzed using confocal microscopy. Scale bar: 10 μm. (D) The cells were transfected as in A, starved for 3 hours in the presence of 0.1 μM Baf A, immunostained with anti-LC3 and anti-LAMP-1 antibodies, and analyzed using confocal microscopy (left panels). Scale bar: 10 μm. Quantitative colocalization analysis of endogenous LC3 with LAMP-1 (right) was performed as described in the Materials and Methods. Data are means ± s.d. of three independent experiments. (E) Following 48 hours of transfection, the rate of degradation of long-lived proteins was measured in siRNA-transfected cells incubated in either αMEM or EBSS medium, in the absence or presence of 3-MA (10 mM) or Baf A (0.1 μM). Values express the percentage of cellular proteins degraded in 4 hours represented as the mean ± s.d. of six determinations.
Discussion

Atg8 is a key autophagic factor involved in the early stages of autophagosome biogenesis in yeast. This protein belongs to a UBL protein family, conserved from yeast to mammals, whose members share a similar structure composed of a ubiquitin-like core and two unique α-helices attached at their N-termini. Among their other functions, all Atg8 homologues are involved in autophagy whereas their active form is conjugated to phosphatidylethanolamine (PE) of the autophagosomal membrane. Atg8 lipidation is a crucial step for dynamic membrane rearrangement during autophagosome formation and therefore, this process is well controlled by multiple autophagic factors (Meijer and Codogno, 2006; Ohsumi, 2001; Scherz-Shouval et al., 2007). Here we determined the minimal structural requirement for this conjugation process. We found for LC3 – a member of the UBL protein family – that the ubiquitin core alone is sufficient and essential for both C-terminal processing and appropriate targeting to bona fide autophagosomes. The role of the unique N-terminal region flanking the ubiquitin core of Atg8 and its homologues with respect to autophagy remains unclear. By examining the activity of truncated proteins we show that the first N-terminal α-helix of mammalian Atg8 is involved in the interaction with p62, a protein recently reported to mediate the delivery of polyubiquitylated proteins for autophagic degradation. Moreover, specific residues (Phe52-Leu53) located at a distinct site within the ubiquitin core of LC3 are also involved in this interaction. Knockdown of LC3 or overexpression of LC3 mutants that fail to interact with p62 significantly reduced the incorporation of p62 into autophagosomes, indicating that LC3 is responsible for delivery of this protein into lysosomes for degradation. P62, however, affected neither LC3 lipidation nor autophagosome formation and targeting into lysosomes, implying that this protein acts to deliver polyubiquitylated aggregates for lysosomal degradation but is not essential for the autophagic process.

All Atg8 family members belong to a widespread family of UBL proteins, which includes SUMO, ISG15, Nedd8 and Atg12, all of which function as critical regulators of many cellular processes (Kerscher et al., 2006; Kirkin and Dikic, 2007). In spite of their low sequence homology, they share the same three-dimensional structure (ubiquitin-like fold) and all use a C-terminal glycine as the site of substrate conjugation. Here we show that the LC3 ubiquitin core is sufficient for its conjugation to PE, indicating that this structural element contains all the information necessary for recognition by a priming enzyme (Atg4) and by the conjugation machinery. Moreover, this scaffold is also sufficient for the appropriate targeting to a membrane of bona fide autophagosomes. It has been recently reported that a point mutation in the ubiquitin core of both yeast and mammalian Atg8 affected their C-terminal processing and the consequent conjugation to lipid (Amar et al., 2006; Fass et al., 2007). Consistent with this finding, we showed here that the truncation of this conserved ubiquitin core affects all these processes, indicating that the intact ubiquitin fold serves as a structural recognition module for interacting proteins. A recent report, however, showed that appropriate conjugation to a target membrane (lipidation) is not sufficient for full function of Atg8 (Amar et al., 2006). Also, Ohsumi and colleagues have recently shown that following conjugation to PE, Atg8 might act in membrane tethering and hemifusion, two essential processes for the expansion of the autophagosomal membrane (Nakatogawa et al., 2007). This study also identified multiple residues within the ubiquitin core that are essential for these activities of the protein. Although similar in structure, all UBL proteins are involved in different cellular functions. To better understand the function of mammalian Atg8, it is important to identify their specific interacting partners and the way in which such an interaction takes place. Here we identified a specific residue within the ubiquitin core of mammalian Atg8, which is essential for binding to p62. It has been recently demonstrated that Ile44 in ubiquitin is involved in the interaction with many different effector molecules (Seet et al., 2006). Based on our findings, we propose that Phe52 of LC3 (and its equivalents in other members of the Atg8 family) has a similar role in binding effectors.

Unlike other UBL molecules, Atg8 family members contain two unique α-helices at their N-termini. Here we addressed the role of this region in LC3 function. Several reports propose that the N-terminus of both GABARAP (Coyle et al., 2002; Wang and Olsen, 2000) and LC3 (Kouno et al., 2005) contains a tubulin-binding domain, which is required for interaction with tubulin or microtubules. Nevertheless, the implication of this interaction in autophagy remains unclear. Crystal structure analysis of GABARAP revealed two possible conformational forms of this protein (‘open’ and ‘closed’), which differ mainly in the orientation of the first α-helix (Coyle et al., 2002). It was also suggested that the lipidation of Atg8 induces a conformational change in its N-terminal region (Ichimura et al., 2004), implying that not only conjugation to a lipid but also a consequential conformational change in the N-terminal region may contribute to the function of Atg8. By utilizing an in vitro system for Atg8 lipidation, it has been shown that Atg8 conjugated to PE mediates tethering between adjacent membranes and stimulates membrane hemifusion, whereas truncation of the N-terminal region resulted in a significant reduction in these activities (Nakatogawa et al., 2007). Here we propose a novel role for this region by showing that the N-terminal α-helices, particularly the first 10 amino acids of LC3, are essential for interaction with p62. Most importantly, this interaction also depends on residues Phe52-Leu53 located at the ubiquitin core (equivalent to residues 49 and 50 of the yeast Atg8), implying that these two regions are involved in this interaction. Residue Phe52 appears essential for the interaction of LC3 and P62 whereas mutation of Leu53 led only to a partial defect in binding, which may result from an effect of such mutation on its neighboring residues. Since p62 binding was not dependent on LC3 C-terminal processing, we suggest that p62-LC3 interaction requires the N-terminal region, but not LC3 conformational change following lipidation. However, the significance of this interaction in the autophagic process is still unexplained. By utilizing different ways to examine autophagy, we demonstrated that p62 is not required for total autophagic activity, consistently with a recent report by Komatsu and co-workers who demonstrated that the starvation-induced autophagy was similar in control and p62-deficient hepatocytes (Komatsu et al., 2007). However, by knockdown of LC3 or expression of its mutants that fail to bind p62, we showed that LC3 is essential for p62 recruitment into autophagosomes and the consequential degradation within lysosomes. Notably, when p62 incorporation into autophagosomes was inhibited, this protein accumulated with polyubiquitylated proteins.
proteins in detergent-insoluble structures. These results further support the notion that p62 acts as an adaptor, linking between polyubiquitylated protein aggregates and the autophagic machinery (Bjorkoy et al., 2005; Pankiv et al., 2007). Thus, autophagy, essentially a nonselective process operating under starvation conditions, mediates targeting of specific cargo molecules for selective degradation.

Materials and Methods
Antibodies and reagents
Minimal essential medium (oMEM), Earle’s balanced salt solution (EBSS), valine-free oMEM medium and fetal calf serum (FCS) were obtained from Biological Industries (Beit Haemek Laboratories, Israel). Bafilomycin A1 (Baf A) was provided by LC Laboratories, 3-methyladenine (3-MA) was obtained from Sigma-Aldrich and L-[U-14C]-valine was obtained from Amersham Pharmacia Biotech. The following antibodies were used: mouse monoclonal and rabbit polyclonal anti-p62 antibodies (Santa Cruz); anti-actin monoclonal antibodies (Sigma); mouse monoclonal anti-ubiquitin antibodies (Covance); mouse monoclonal anti-LAMP-1 (Developmental Studies Hybridoma Bank, University of Iowa); mouse monoclonal anti-green fluorescence protein (GFP) (BabCo); rabbit polyclonal anti-GFP (Molecular Probes); rhodamine-conjugated goat anti-mouse IgG; FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories); and horseradish peroxidase (HRP)-coupled goat antibody against mouse IgG (Bio-Rad). Anti-LC3 antibody was produced as previously described (Fass et al., 2006).

DNA construction
pEGFP-LC3 vector encoding human LC3 fused with GFP was prepared as previously described (Fass et al., 2006). The point mutation for glycine to alanine at position 53 of LC3 was created by PCR-based site-directed mutagenesis using LC3 sense primer (5’-ACGACATTTCATGGCAGCTGCTGTCCGTA-3’) and LC3 antisense primer (5’-TGACTAACTTACGTCTCCATTTGCCGTTCGTA-3’). The point mutation for phenylalanine to alanine at position 52 of LC3 was created by PCR-based site-directed mutagenesis using LC3 sense primer (5’-GGATGACAAAAAGTCCCTGCTACGATGTC-3’) and LC3 antisense primer (5’-GGTCAGTACAGCAGCCCTTCATTTGC-3’). The point mutation for leucine to alanine at position 53 of LC3 was created by PCR-based site-directed mutagenesis using LC3 sense primer (5’-GGATGAAACAAAAGTCCCTGCTACGATGTC-3’) and LC3 antisense primer (5’-GGATGACAAAAAGTCCCTGCTACGATGTC-3’). The truncation of first 10 amino acids (LC3Δ1-10) was created by PCR-based site-directed mutagenesis using LC3 sense primer (5’-CACCGTACGGACATCGCAGCTGCTTCGTA-3’) and LC3 antisense primer (5’-GGATGAAACAAAAGTCCCTGCTACGATGTC-3’). The truncation of the first 24 amino acids (LC3Δ1-24) was created by PCR-based site-directed mutagenesis using LC3 sense primer (5’-AACACTTGCGACACAAATCTCCGTTGATATAGACG-3’) and LC3 antisense primer (5’-GGATGAAACAAAAGTCCCTGCTACGATGTC-3’).

Cell culture and transfection
HeLa and CHO cells were grown on oMEM medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (Sigma) at 37°C in 5% CO2. CHO cells were transfected with 5 μg DNA/10 cm plate using Lipofectamine (4 μl/1 μg DNA). Stable clones of GFP-LC3 transfected CHO cells were selected in 1 mg/ml geneticin (G418). Subconfluent HeLa cells were transfected with 10 μg DNA/10 cm plate using standard calcium phosphate technique for oversexpression of GFP-LC3 and its mutants, or with DharmaFect 1 (Dharmacon, Lafayette, CO) for siRNA transfection. DNA SMARTools (50 μM), consisting of four RNA duplexes targeting p62 (catalogue number M-010230-00), or LC3 and non-targeting siRNAs control (catalogue number D-001206-14), were purchased from Dharmacon. For LC3, 50 nM of each LC3A (M-013579-00), LC3B (M-025600-00) and LC3C (M-032399-01) RNAs were targeted, bringing the total number of transfected duplexes to 12. Cells were grown to densities of 5×10^5 per 4 cm dish in 2 ml oMEM without antibiotics, and transfected with DharmaFect according to the manufacturer’s instructions. Experiments with p62 siRNA were performed 48 hours after transfection and with LC3 siRNA, 72 hours after transfection. To obtain starvation conditions, cells were washed three times with PBS and incubated in EBSS medium at 37°C for 1 hour. This led to 50% lysosomal degradation, cells were then starved in the presence of 100 nM Baf A. Total cell extracts were made using RIPA extraction buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 1.5 mM MgCl2, 1% DOC, 1% Triton X-100) with protease inhibitor mixture (Sigma).

Co-immunoprecipitation
For immunoprecipitation experiments, cells were lysed 24 hours after transfection using RIPA extraction buffer with protease inhibitor mixture. Cells were centrifuged at 150,000 g and supernatant was taken for immunoprecipitation experiment. GFP-fused proteins were immunoprecipitated from total cellular extracts (500 μg) using rabbit polyclonal anti-GFP antibody (Molecular Probes) and subjected to western blot analysis.

Quantification analysis
Results from separate experiments (a minimum of three) were analyzed using ImageQuant image program and quantified as follows: the percentage of p62 degradation was calculated as the difference in total p62 (normalized to actin as loading control) in the presence or absence of Baf A. The largest number obtained was set to 100% and the relative degradation in cells undergoing other treatments was calculated accordingly. The relative level of p62 or ubiquitin proteins was calculated as difference in total p62/ubiquitin (normalized to actin as loading control) in LC3 siRNA transfected cells compared with non-targeting siRNA-transfected cells. The number obtained from non-targeting siRNA transfected cells was set to one and the relative protein level in LC3 siRNA-transfected cells was calculated accordingly. For the LC3 lipidation analysis, the fraction of lipidated LC3 out of the total LC3 was calculated for each treatment in each experiment. The largest value obtained in each experiment was set to 100% and the relative lipidation in other treatments was calculated accordingly.

Fluorescence microscopy
HeLa cells were plated on sterile coverslips (15 mm diameter) and cultured under the conditions indicated, fixed with cold methanol for 5 minutes at −20°C and permeabilized by quick washing with cold acetone. Cells were blocked by incubation with 10% FCS in PBS for 30 minutes at room temperature, followed by incubation for 1 hour with the primary antibody. Cells were then incubated with the secondary antibody for 30 minutes. For fluorescence imaging, a Nikon Eclipse TE300 microscope equipped with a GFP filter (HQ FITC 41001) and Hamamatsu digital camera C4742-95 were used. A fluorescence filter was used to observe Rhodamine (543 nm, mirror, emission 560-600 nm), and GFP/FITC (excitation 488, dichroic 560 nm, emission 505-525 nm). Confocal images were taken by a FV500 laser-scanning confocal microscope equipped with a PLAPO 60× 1.4 NA oil-immersion lens and Fluoview software (Olympus).

Colocalization analysis
The quantitative colocalization analysis was performed using JACoP (Just Another Co-localization Plugin) plugin in ImageJ program (NIH Image). For analysis, images of approximately 50-60 cells were taken in each experiment and 3-5 experiments were analyzed, bringing the total number of cells to 150-300 per determination. The value shown represents Pearson’s coefficient (Manders et al., 1992). A linear equation describing the relationship between the intensities in two images is calculated by linear regression. The Pearson’s coefficient provides an estimate of the goodness of this approximation. Its value can range from 1 to −1, with 1 representing complete positive correlation and −1 a negative correlation; zero represents no correlation.

Degradation of long-lived proteins
Cells were transfected as described earlier and grown to 70-80% confluence in 12-well plates (35 mm). Cells were then labeled for 16 hours in medium containing [14C]valine (0.5 mCi/ml) and 5% FCS in valine-free oMEM. After three rinses with PBS, cells were incubated in valine-free oMEM containing 0.1% BSA and 10 mM cold valine. When required, 100 nM bafilomycin A or 10 mM 3-MA was added. After the 3 hour incubation, the medium was replaced with either oMEM or EBSS medium also containing 0.1% BSA and 10 mM cold valine, and cells were incubated for an additional 4 hours. The medium was precipitated in 10% TCA, and TCA-soluble radioactivity was measured. Total cell radioactivity was measured after incubation with 0.1 M NaOH. [14C]-valine release was calculated as a percentage of the radioactivity in the TCA-soluble supernatant relative to total cell radioactivity.

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