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Atg8, a Ubiquitin-like Protein Required for Autophagosome Formation, Mediates Membrane Tethering and Hemifusion

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SUMMARY

Autophagy involves de novo formation of double membrane-bound structures called autophagosomes, which engulf material to be degraded in lytic compartments. Atg8 is a ubiquitin-like protein required for this process in *Saccharomyces cerevisiae* that can be conjugated to the lipid phosphatidylethanolamine by a ubiquitin-like system. Here, we show using an in vitro system that Atg8 mediates the tethering and hemifusion of membranes, which are evoked by the lipidation of the protein and reversibly modulated by the deconjugation enzyme Atg4. Mutational analyses suggest that membrane tethering and hemifusion observed in vitro represent an authentic function of Atg8 in autophagosome formation in vivo. In addition, electron microscopic analyses indicate that these functions of Atg8 are involved in the expansion of autophagosomal membranes. Our results provide further insights into the mechanisms underlying the unique membrane dynamics of autophagy and also indicate the functional versatility of ubiquitin-like proteins.

INTRODUCTION

Autophagy is an evolutionally conserved protein degradation pathway in eukaryotes that is essential for cell survival under nutrient-limiting conditions (Levine and Klionsky, 2004). In addition, recent studies have revealed a wide variety of physiological roles for autophagy (Mizushima, 2005) as well as its relevance to diseases (Cuervo, 2004).

During autophagy, cup-shaped, single membrane-bound structures called isolation membranes appear and expand, which results in the sequestration of a portion of the cytosol and often organelles. Eventually, spherical, double membrane-bound structures called autophagosomes are formed (Baba et al., 1994), and then delivered to and fused with lysosomes or vacuoles to allow their contents to be degraded. Studies in *S. cerevisiae* have identified 18 *ATG* genes required for autophagosome formation, most of which are also found in higher eukaryotes (Levine and Klionsky, 2004). Recent studies have shown that Atg proteins constitute five functional groups: (i) the Atg1 protein kinase complex, (ii) the Atg14-containing phosphatidylinositol-3 kinase complex, (iii) the Atg12-Atg5 protein conjugation system, (iv) the Atg8 lipid conjugation system, and (v) the Atg9 membrane protein recycling system (Yorimitsu and Klionsky, 2005). The mechanisms by which these units act collaboratively with lipid molecules to form the autophagosomes, however, are still poorly understood.

Atg8 is one of two ubiquitin-like proteins required for autophagosome formation (Mizushima et al., 1998; Ichimura et al., 2000). Because it has been shown that Atg8 and its homologs (LC3 in mammals) localize on the isolation membranes and the autophagosomes, these proteins have been used in various studies as reliable markers for the induction and progression of autophagy (Kirisako et al., 1999; Kabeya et al., 2000; Yoshimoto et al., 2004). In *S. cerevisiae*, Atg8 is synthesized with an arginine residue at the C terminus, which is immediately removed by the cysteine protease Atg4 (Kirisako et al., 2000). The resulting $Atg8^{G116}$ protein has a glycine residue at the new C terminus and can serve as substrate in a ubiquitin-like conjugation reaction catalyzed by Atg7 and Atg3, which correspond to the E1 and E2 enzymes of the ubiquitination system, respectively (Ichimura et al., 2000). Remarkably, unlike other ubiquitin-like conjugation systems, Atg8 is conjugated to the lipid phosphatidylethanolamine (PE), thereby Atg8 is anchored to membranes (Ichimura et al., 2000; Kirisako et al., 2000). Immunoelectron microscopy revealed that Atg8, probably as a PE-conjugated form (Atg8-PE), is predominantly localized on the isolation membranes rather than on the complete autophagosomes (Kirisako et al., 1999), suggesting that Atg8-PE plays a pivotal role in the process of autophagosome formation. The precise function of Atg8-PE, however, has remained unknown.

The conjugation of Atg8 to PE is reversible; Atg4 also functions as a deconjugation enzyme, resulting in the

release of Atg8 from the membrane (Kirisako et al., 2000). This reaction is thought to be important for the regulation of the function of Atg8 and/or the recycling of Atg8 after it has fulfilled its role in autophagosome formation.

We reconstituted the Atg8-PE conjugation reaction in vitro with purified components (Ichimura et al., 2004). Here, we show using this system that Atg8 mediates the tethering and hemifusion of liposomes in response to the conjugation with PE. These phenomena observed in vitro are suggested to reflect a bona fide in vivo function of Atg8 in the expansion of the isolation membrane. Based on mutational analyses and structural information, the mechanisms of Atg8-mediated membrane tethering and hemifusion as well as its regulation are discussed. This study sheds light on the molecular basis of unconventional membrane dynamics during autophagy, which is governed by the Atg proteins.

RESULTS

Lipidation of Atg8 Causes Clustering of Liposomes In Vitro

As reported previously (Ichimura et al., 2004), when purified $Atg8^{G116}$ (hereafter, referred to as Atg8), Atg7, and Atg3 were incubated with liposomes containing PE in the presence of ATP, Atg8-PE was efficiently formed (Figure 1A, lanes 1–6). Intriguingly, the reaction mixture became turbid during the incubation (Figure 1B), which under a light microscope, was found to be a result of gradually forming aggregates (Figure 1C). Both the degree of turbidity and the size of the aggregates appeared to correlate with the amount of Atg8-PE produced in the mixture. Size-distribution analyses using dynamic light scattering (DLS) clearly showed that the aggregates formed in an Atg8-PE dose-dependent manner (Figure 1D). These aggregates disappeared when the samples were treated with the detergent CHAPS (Figure 1E, +CHAPS). In addition, if a small amount of PE modified with the fluorescent dye 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD) was included in the liposome preparation, the aggregates became uniformly fluorescent (Figure 1E, NBD-PE). These results suggest that the aggregates generated during the production of Atg8-PE were clusters of liposomes.

When the proteins were denatured with urea, the clusters of liposomes dissociated, although Atg8 remained conjugated to PE (Figure 1E, +urea and Figure 1F, lane 2), indicating that the liposomes aggregated due to some function of the Atg8 protein rather than an artifact caused by Atg8-PE as the lipid with the extraordinarily large head group. When the aggregates were sedimented by centrifugation, Atg8-PE co-precipitated with the liposomes (Figure 1G, lane 2), whereas Atg7, Atg3, and unconjugated Atg8 did not (Figure 1G, lane 3). The sedimented liposomes containing Atg8-PE remained clustered even if they were briefly sonicated (Figure 1H, ppt.). These results suggested that Atg8-PE molecules function to tether together membranes to which they are anchored.

Atg8-PE Also Mediates Liposome Fusion

We also examined if membrane fusion occurred between the liposomes connected by Atg8-PE. To this end, we took advantage of a well-characterized lipid mixing assay (Struck et al., 1981). This method is based on energy transfer from NBD to lissamine rhodamine B (Rho), each of which is conjugated to PE. Because the amino group of the ethanolamine moiety is modified with the dyes, these lipids cannot be conjugated with Atg8. If both of the conjugated dyes are present at appropriate concentrations in the same liposome, the fluorescence of NBD is effectively quenched by Rho (Figure 2A, compare columns 1 and 4). If a ''NBD+Rho'' liposome is fused with a ''nonlabeled'' liposome, which results in an increase of the average distance between the two dyes on the membrane, the NBD fluorescence will be dequenched. A mixture of the nonlabeled and NBD+Rho liposomes were subjected to the conjugation reaction. The resulting liposome clusters were dissociated by proteinase K treatment, followed by fluorescence measurements. Remarkably, a significant ATP-dependent increase of the fluorescence was observed (ATP is required for the production of Atg8-PE; Figure 2B, column 6). This increased fluorescence was not observed with samples of nonlabeled liposomes alone, NBD+Rho liposomes alone, or a mixture of nonlabeled liposomes and liposomes containing NBD-PE but not Rho-PE (Figure 2B, columns 1-3). These results suggest that membrane fusion occurred between the liposomes tethered together by Atg8-PE. The increased fluorescence was only observed if the reaction mixture was treated with proteinase K (Figure 2B, columns 4 and 6). This appeared to be due to the presence of Atg7 and/or Atg3 rather than Atg8 or some effect of the clustering, because the NBD fluorescence was not increased by the addition of Atg4 (Figure 2B, column 5), which detached Atg8 from the membranes and dissociated the clusters of liposomes (see below). Instead, decreasing the concentrations of the conjugation enzymes allowed the dequenching of the NBD fluorescence to be detected without proteinase K digestion (Figure 2B, column 7).

The fusion of the liposomes was examined with various amounts of Atg8 (Figure 2C). The level of fusion increased Atg8 dose-dependently and reached maximum at 2 μM (Figure 2C). In contrast, a larger amount of Atg8 produced an inhibitory effect (data not shown). This suggested that formation of the large aggregates resulted from excessive tethering by Atg8-PE, which no longer lead to fusion.

We also carried out time-course experiments to roughly estimate the fusion rate using the lower concentrations of the conjugation enzymes (Figure 2D), which eliminated the need for the proteinase K treatment (Figure 2B). It should be noted that the incubation time includes the times required for the formation of Atg8-PE and the subsequent tethering and fusion reactions. Under these conditions, the band of Atg8-PE could be seen on an SDS-PAGE gel after a 10 min incubation, and the reaction was completed within 30 min (Figure S1 in the Supplemental Data available with this article online). It appeared that