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Review Article

Membrane recruitment of autophagy proteins in selective autophagy

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Autophagy is a stress response that is upregulated in response to signals such as starvation, growth factor deprivation, endoplasmic reticulum stress, and pathogen infection. Defects in this pathway are the underlying cause of a number of diseases, including metabolic aberrations, infectious diseases, and cancer, which are closely related to hepatic disorders. To date, more than 30 human ATG (autophagy) genes have been reported to regulate autophagosome formation. In this review, we summarize the current understanding of how ATG proteins behave during autophagosome formation in both non-selective and selective autophagy.

Key words: autophagy, autophagy proteins, LC3 recruitment, salmonella autophagy

INTRODUCTION

AUTOPHAGY IS A process of self-degradation of cellular components in which double-membrane autophagosomes sequester organelles or portions of cytosol and fuse with lysosomes so that the contents can be digested by hydrolases.[1,2] By degradation of cellular components, autophagy supplies energy so that cells can survive under starvation conditions, or regulates the balance between biogenesis and degradation of cellular structures. Autophagy also plays a role in the elimination of unwanted substances from the cytoplasm, including invading bacteria or viruses, or misfolded or aggregated proteins. Therefore, autophagy plays critical roles in growth, survival, and differentiation in eukaryotes, and defects in autophagy are associated with numerous human diseases.[1,2]

There are two different autophagic pathways, a starvation-induced non-selective pathway and a target-specific selective pathway.[2] The non-selective pathway is thought to play a role in supplying energy by degrading proteins, and the selective pathway may function to eliminate unwanted substrates from the cytoplasm. In mammalian cells, the membrane dynamics of autophagosome formation in both pathways are thought to be driven by common ATG (autophagy) protein factors that were originally identified by yeast genetic analyses.[2] The molecular mechanisms of ATG-mediated autophagosome formation have been analyzed using the yeast system, and it has generally been assumed that the mammalian ATG proteins function similarly to their counterparts in the yeast system.[1,2]

Recently, however, our study of autophagy of invading bacteria revealed that the mammalian autophagy system is partially different from the yeast system.[3] In this review, we summarize the current understanding of how the mammalian ATG proteins behave during autophagosome formations, especially focusing on the LC3 proteins, well known markers of autophagosomes in mammalian cells.

AUTOPHAGY IN THE LIVER

A PHYSIOLOGICAL FUNCTION of autophagy under starvation conditions in liver tissue has been reported. Mice with deficient autophagy specifically in the liver die more quickly than wild type (WT) mice in starvation conditions due to low levels of gluconeogenesis,[4] which result from lack of energy that is usually supplied by autophagy. This indicates that autophagy plays a critical function in hepatic cells in energy

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homeostasis under starvation conditions. On the other hand, a function of autophagy under steady state conditions has also been reported. Liver-specific autophagy-deficient mice show dramatic enlargement of the liver, and present with adenoma.[4] The liver tissue of these mice shows an accumulation of inclusion bodies similar to the aggregates frequently identified in human hepatocellular carcinomas (HCC). These results suggest that basal autophagy plays an indispensable role in cellular homeostasis by clearing inclusion bodies and protein aggregates. Similar results have been reported using mice with systemic or chimeric deficiency of autophagy.[5] Interestingly, tumor formation in these mice has been observed only in the liver tissue, suggesting that autophagy has a critical function in suppressing spontaneous tumorigenesis, especially in the liver.

Another aspect of the involvement of autophagy in liver disease is hepatitis C virus (HCV) infection. There are more than 180 million patients in the world with chronic HCV infection, and about 75% of HCC is caused by HCV infection.[6] Recently, several independent groups reported the function of autophagy in HCV life cycle and pathogenesis. Common observations from these reports indicate that autophagosome formation is significantly upregulated in HCV-infected cells.[7–11] Interestingly, this upregulation is dependent on the cellular unfolded protein response (UPR), which is usually induced by endoplasmic reticulum (ER) stress. HCV infection efficiently activates the UPR, and induced autophagy is attenuated by depleting the factors involved in the UPR.[11] Furthermore, several groups reported that induction of autophagy suppressed the innate immune response and resulted in the upregulation of viral replication.[12–14] Though the detailed mechanism is still unclear, autophagy may be a therapeutic target for controlling HCV infection.

PROTEIN FACTORS INVOLVED IN AUTOPHAGOSOME FORMATION

GENES INVOLVED IN autophagosome formation have been identified by two different analyses in yeast. Treatment of yeast with vacuolar proteinase inhibitors leads to the massive accumulation of "autophagic bodies," which are derived from cytoplasmic contents sequestered by autophagy.[15] In cells lacking autophagy, however, autophagic bodies are never detected in the vacuole.[15] In the early 1990s, Yoshinori Ohsumi's group performed an extensive genetic screen against a yeast single-gene-deletion library to search for mutant cells that had empty vacuoles even in the presence of proteinase inhibitors.[16] At almost the same time, Daniel Klionsky's group performed a yeast genetic analysis focusing on the Cvt (cytoplasm-to-vacuole targeting) pathway, which mediates the biosynthetic transport of the vacuolar protein aminopeptidase 1 (Ape1) from the cytoplasm to the vacuole.[17] Interestingly, by screening for deficiencies in the Cvt pathway, they also isolated genes involved in autophagy.[18] From these screens, 18 genes were identified as core ATG genes; some of these are conserved in mammalian cells. The ATG gene products are currently thought to be involved in membrane dynamics during autophagosome formation. A series of biochemical analyses subsequently classified the 18 *ATG* proteins into six functional subgroups: (i) the ULK1 kinase and its regulatory complex; (ii) the ATG14L complex containing PI3K (the phosphoinositide 3-kinase complex); (iii) the WIPI-ATG2, PI3P (Phosphatidylinositol 3-phosphate) binding complex; (iv) the ATG9 subfamily; (v) the ATG12 UBL (ubiquitin-like) conjugation system; and (vi) the LC3 UBL and PE (phosphatidylethanolamine) conjugation system. These six functional complexes are thought to be involved in membrane extension, curvature formation, fusion, and fission during autophagosome formation. Detailed information regarding the function of each sub-complex is provided in several other nice reviews; therefore, we will only summarize briefly below.

The ULK1/2 kinase complex is an approximately 3 MDa complex, which includes the ULK1/2 kinase, ATG13, FIP200, and ATG101.[19–22] In both yeast and mammalian cells, starvation induced autophagy is under the control of the Tor (target of rapamycin) kinase.[19] In nutrient-rich conditions, the ULK complex is phosphorylated by mTOR complex 1 (mTORC1); however, in starvation conditions, nutrient sensor signals downregulate mTORC1 activity and the ULK1 complex is dephosphorylated. This dephosphorylation activates ULK1 and triggers activation of the autophagic pathway.

The ATG14L complex consists of four subunits, VPS34, VPS15, Beclin1, and ATG14L.[23,24] VPS34 is the PI3-kinase subunit, and generates phosphatidylinositol 3-phosphate at local membrane sites. PI3P plays an important role in the assembly of downstream effector molecules such as the WIPI family and DFCP1.[25–28] PI3-kinase activity is essential for autophagosome formation, as small molecule inhibitors like 3-methyladenine and wortmannin block the autophagic pathway.[29] The ATG14L subunit functions to recruit downstream ATG proteins to the ER membrane;[30] however, the molecular

mechanism by which ATG14L itself is recruited to the membrane remains unknown.

WIPI–ATG2 complexes are downstream effectors of PI3P, and are recruited to PI3P-enriched sites by direct binding to PI3P.[26] These complexes consists of two sub-families, the WIPI family and the ATG2 family. To date, four WIPI sub-family proteins (WIPI-1 to –4)[26,31] and two ATG2 sub-family proteins (ATG2A and ATG2B) have been identified in humans, and all WIPI proteins can bind to both ATG2A and ATG2B (Morita *et al.*, unpubl. data, 2011), resulting in eight different combinations. WIPI proteins have WD40 repeat domains that can bind to both PI3P and ubiquitin. The ATG2 proteins weigh 180 kDa, but no obvious protein motifs have been identified, and their function is unclear.

ATG9 is a six-transmembrane protein. In yeast, ATG9 mainly localizes at small cytoplasmic dot-like structures that are recruited to the autophagosome initiation site (termed the PAS, pre-autophagosomal membrane/phagofore assembly site; see below) under starvation conditions.[32] Cytoplasmic ATG9 may exist on small membrane vesicles, and the fusion of ATG9-positive vesicles under starvation conditions may be related to the extension of the isolation membrane.[33] In mammals, two ATG9 family proteins (ATG9A and ATG9B) have been identified and mainly localize at the Golgi apparatus; the meaning of the Golgi localization is unknown.[33,34] The localization of yeast ATG9 to the PAS is dependent on the ATG1 complex, and ATG9 directly interacts with ATG17, one of the subunits of the yeast ATG1 complex. However, ATG9 localization is not affected by deficiencies of the ATG12 or ATG8 conjugation systems (see below).[35] Therefore, it is thought that ATG9 functions just downstream of the ATG1 complex, but upstream of the ATG12 and ATG8 conjugation systems (Fig. 1).

Among the ATG proteins, two different families of ubiquitin-like (UBL) proteins are involved in autophagosome formation: ATG12 and LC3 family proteins.[36,37] Almost half of all ATG proteins are involved in the conjugation systems for these UBLs. ATG12 is conjugated to the amide moiety of lysine 130 of ATG5 via an isopeptide bond. This reaction is catalyzed by two enzymes, ATG7 and ATG10, which correspond to the E1 and E2 enzymes of the ubiquitin conjugation system. ATG12–ATG5 forms a stable complex with ATG16L1 or

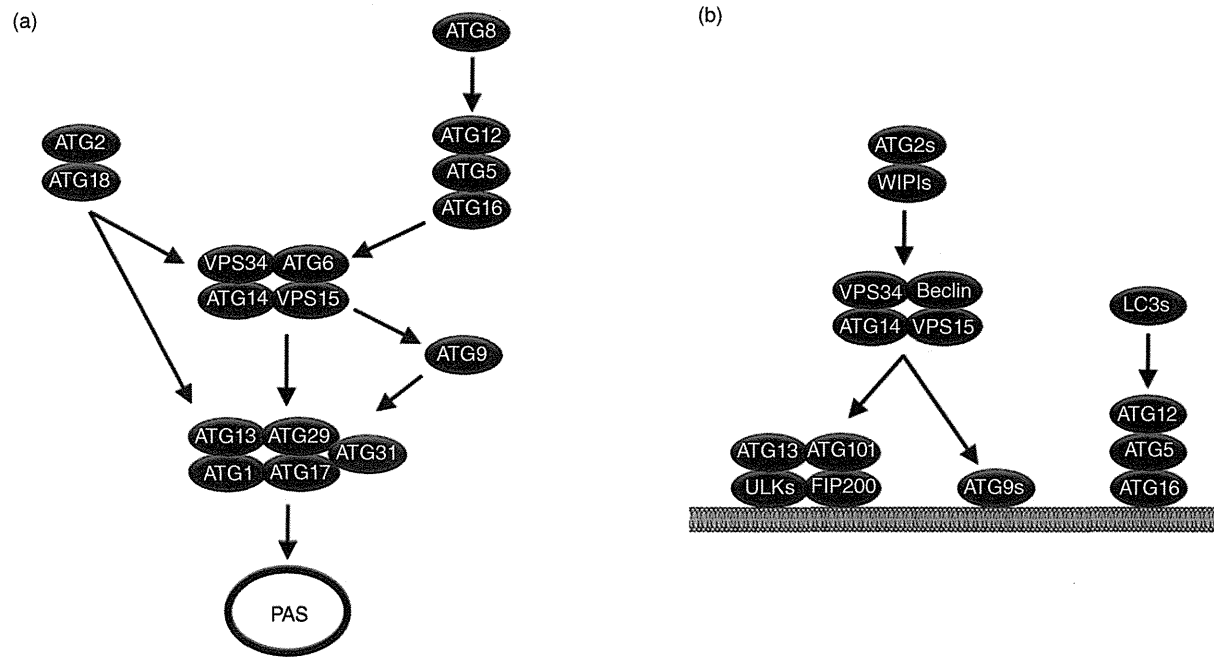

Figure 1 Hierarchical relationships among the autophagy (ATG) proteins during recruitment. When upstream ATG protein function is deficient, the localization of downstream ATG proteins is lost. These schemas indicate the localization of ATG proteins to the pre-autophagosomal membrane/phagofore assembly site (PAS) during starvation-induced autophagy in yeast (a), or to the membrane in autophagy of invading bacteria in mammalian cells (b).

ATG16L2, and functions in ATG8–PE conjugation.[38] LC3s, the mammalian homologs of yeast ATG8, consist of multiple protein families (LC3A, LC3B, LC3C, GABARAP, GATE-16, ATG8L) in the human genome.[39] All of these proteins are processed at their C-terminal residues by specific cysteine proteinases, ATG4s, that are encoded by four different genes (ATG4A-D) in humans.[40] Subsequently, PE is conjugated to the exposed C-terminal glycine residue of LC3 by the catalytic enzymes ATG7 and ATG3, which correspond to the E1 and E2 enzymes of the ubiquitin conjugation system, and LC3-PE is anchored to the membrane.[37,39] The ATG12–ATG5–ATG16L complex is thought to recruit PE conjugated LC3 to the site of autophagosome formation.[41] Lipidated LC3s are the final products of the ATG reaction cascade, and LC3 puncta formation is widely used as a marker for autophagosome formation.[29]

SEQUENTIAL MEMBRANE RECRUITMENT OF ATG COMPLEXES

THE ORDER OF recruitment of the ATG complexes to the site of autophagosome formation has been analyzed in the yeast system.[35] In yeast, all of the ATG proteins localize at a peri-vacuolar dot structure termed the PAS, which is thought to be the point of origin of the autophagosome. The detailed characteristics of the PAS have not yet been elucidated.[42] Importantly, the PAS localization of each ATG protein is dependent on another ATG protein. These observations allow us to determine hierarchical relationships among the ATG proteins during autophagosome formation (summarized in Figure 1 left panel). [2,35] For example, in cells lacking the ATG1 complex, almost all of the other ATG proteins lose PAS localization, suggesting that the ATG1 complex plays an important role in the initiation of assembly of the ATG proteins at the PAS. On the other hand, lack of ATG8 does not affect the PAS localization of any other ATG protein, indicating that ATG8 may function at the downstream end of this pathway. Similar types of experiments in mammalian cells have shown that ATG proteins form dot structures close to the ER membrane under starvation conditions. Though there is no evidence that these structures correspond to the yeast PAS, the hierarchical relationships found in yeast seem to be conserved in mammalian cells.

However, several differences in the relationships of the ATG complexes have been identified during the process of selective autophagy in mammalian cells. Recently, our group performed a systematic analysis of ATG complex recruitment to the membrane during autophagy of bacteria.[3] *Salmonella enterica* serovar typhimurium is a Gram-negative bacterium that can invade epithelial cells in a single membrane organelle called a Salmonella-containing vacuole (SCV).[43,44] Invading bacteria can be surrounded by autophagosomes in a selective process and killed by lysosomal hydrolases; thus, autophagy functions to protect host cells from pathogenic bacteria as an intracellular innate immune system.[45] During this process, almost all of the ATG proteins are recruited to the membrane around the invading bacterium. We examined the localization of all ATG complexes to the bacterial membrane in a series of MEF (mouse embryonic fibroblasts) cells derived from several different ATG knockout mice, and showed that the hierarchical relationships of the ATG complexes during membrane recruitment are different from those during starvation-induced autophagy in yeast cells (summarized in Figure 1 right panel).[3]

THE LC3-POSITIVE COMPARTMENT DOES NOT ALWAYS REPRESENT THE ISOLATION MEMBRANE/AUTOPHAGOSOME

ONE OF THE important observations from our study is that three ATG complexes (the ATG1, ATG9, and ATG16L complexes) are recruited to the membrane independently.[3] Interestingly, LC3 proteins are recruited to the invading bacteria even in the absence of ATG1, ATG9L1, or ATG14L, indicating that LC3s can be recruited to the membrane without autophagosome formation. These results are quite different from the model of PAS recruitment in yeast. We do not understand why ATG complexes show such different behavior in each system; however, it may be explained by the differences between the selective and non-selective pathways.

Though the canonical relationships among ATG complexes cannot be adapted to autophagy of bacteria, a similar phenomenon has been reported in the yeast system. A systematic analysis of ATG8–PE conjugation (ATG8 was named Aut7p in this paper) reactions in ATG-deficient cells showed that ATG8–PE conjugation is blocked only in ATG3-, ATG4-, ATG5-, ATG7-, ATG10-, ATG12-, and ATG16-deficient cells, but not in ATG1-, ATG2-, ATG6-, ATG9-, ATG13-, ATG14-, and ATG17-deficient cells.[35] The latter mutants lack functional autophagy, but show the same ATG8–PE levels as WT cells, indicating that ATG8–PE formation does not always correlate with autophagy function.

Thus, although LC3–PE formation can be a good marker of autophagosome formation, LC3–PE

conjugation can be induced independent of autophagosome formation in some contexts, and the LC3 positive compartment does not always represent isolation membranes.

LC3 IS NOT REQUIRED FOR FORMATION OF DOUBLE-MEMBRANE STRUCTURES OR TARGET RECOGNITION DURING AUTOPHAGY OF BACTERIA

IN ADDITION TO the results discussed above, we observed that cytoplasmic invading bacteria were positive for ATG5 and were surrounded by double-membrane structures even in ATG3 knockout cells, which are deficient for the LC3–PE conjugation reaction.[3] These results indicate that neither LC3–PE conjugation nor LC3–PE recruitment to the membrane are required for the formation and elongation of the isolation membrane during autophagy of bacteria.

Additional study is needed to determine whether this phenotype occurs commonly in non-selective autophagy; however, similar results have been reported from the study of starvation-induced autophagy in mammalian cells. In ATG3 knockout cells or catalytic domain-inactive ATG4B mutant-expressing cells, double-membrane structures were induced efficiently under starvation conditions despite a complete block of LC3–PE conjugation.[46,47] These results suggest that LC3–PE conjugation may not be required for the formation of the isolation membrane in both non-selective and selective autophagy. Interestingly, the majority of isolation membranes accumulated in ATG3-deficient or ATG4B mutant-expressing cells appear to be unclosed and incomplete autophagosomes,[46,47] suggesting that LC3 functions in the final step of autophagosome formation, by closing the double-membrane structure to separate the internal vesicle from the outer membrane.

Recently, an adaptor protein model has been proposed for target recognition during selective autophagy. This model is based on the idea that adapter proteins, such as p62, NBR1, NDP52, and OPTN, recognize both ubiquitinated targets and LC3 proteins, and function to bridge the targets and LC3 proteins on the isolation membrane.[48–52] This model indicates that LC3 proteins have essential roles in target recognition during selective autophagy. However, the fact that LC3 is not necessary for the formation of isolation membranes during sequestration of target proteins indicates that the adaptor protein model is not an essential pathway in selective autophagy.

How does the isolation membrane first recognize the target protein in selective autophagy? We cannot fully explain this yet, but upstream ATG complexes, such as ATG1, ATG9, and ATG14L complexes, may be involved prior to LC3 recruitment, because the deficiencies in these complexes are critical in the formation of isolation membranes and elimination of invading bacteria from the cell.[3]

Recently, we performed extensive yeast two-hybrid and IP-mass spec analyses to generate a direct interaction network for the mammalian ATG complexes and identified several new protein–protein interactions that may link the entire protein network together. This effort has led to a new model for understanding the entire molecular machinery of the mammalian autophagic pathway.

CONCLUSION

THERE ARE MANY remaining questions regarding the molecular mechanisms of mammalian selective autophagy, including (i) how the autophagy recognizes unwanted substrates that have accumulated in the cytoplasm? (ii) How does each ATG complex physically interact with the other complexes, and function in the membrane dynamics of autophagosome formation? (iii) What is the function of LC3? The molecular mechanisms of mammalian autophagy are currently thought to be similar to the yeast system. However, our recent study of autophagy of invading bacteria has demonstrated that the hierarchical relationships in membrane recruitment of ATG complexes during mammalian selective autophagy is not identical to that of recruitment to the yeast PAS. Further analysis is needed to more fully understand the pathway of mammalian selective autophagy.

LC3-PE formation has been widely used as a marker of autophagosome formation, and has contributed to our understanding of the physiological roles of autophagy in various biological processes. However, our recent findings show that LC3 puncta formation is induced independently of autophagosome formation in some contexts. This alerts us that monitoring LC3 behavior is not enough to judge the induction of autophagy.

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MINIREVIEW

Differential requirements of mammalian ESCRTs in multivesicular body formation, virus budding and cell division

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The endosomal sorting complexes required for transport (ESCRTs) mediate membrane fission from the cytoplasmic face of the bud neck. ESCRTs were originally identified as factors involved in multivesicular body vesicle biogenesis in yeast but have since been shown to function in other membrane fission events in mammalian cells, including enveloped virus budding and the abscission step of cytokinesis. Several recent studies have revealed that not all ESCRT factors are required for each of these biological processes, and this review summarizes our current understanding of the different requirements for ESCRT factors in these three different ESCRT-mediated mammalian membrane fission processes.

Introduction

Integral membrane proteins, such as receptors and ion channels, are ultimately degraded within lysosomes. During this process, cargos destined for degradation are removed from limiting endosomal membranes by sorting into intralumenal vesicles (ILVs) that bud into the interior of multivesicular bodies (MVBs)/late endosomes. Screening from a yeast single deletion mutant library identified a set of approximately 20 proteins that drive formation of ILVs at the MVB [1,2]. These proteins form multiprotein complexes named endosomal sorting complexes required for transport (ESCRTs) [3]. These factors are well conserved in higher eukaryotes and are encoded by multiple genes; the number of factors thus far identified has expanded to more than 30 in humans [1,2]. The role of the ESCRT machinery in higher eukaryotic cells extends to other membrane fission events, including enveloped viral budding and abscission of cytokinesis. All of these events require topologically similar membrane transformations that require membrane constriction and fission from the cytosolic face of the bud neck [1,2,4].

ESCRTs can be classified into six mono- or hetero-oligomeric complexes (ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III, ALIX and VPS4). These complexes are

Abbreviations
AAA-ATPase, ATPase associated with a variety of cellular activities; CHMP, charged MVB protein; EGFR, endothelial growth factor receptor; ESCRT, endosomal sorting complex required for transport; ILV, intralumenal vesicle; MIT, microtubule interacting and transport; MVB, multivesicular body; PRR, proline-rich region; RNAi, RNA interference.

recruited sequentially to membranes and function in protein sorting, membrane remodeling and fission. The role and action of the ESCRTs in membrane dynamics has been proposed from both *in vivo* and *in vitro* study of yeast systems [5–7]. At the first step, early-acting ESCRTs such as ESCRT-I and ESCRT-II interact with upstream recruiting factors, such as ESCRT-0, and these factors have an important role in the concentration of cargo proteins and assist in the deformation of membranes. These early-acting factors function to recruit late-acting ESCRTs, subunits of the ESCRT-III complex, which assemble like filaments within the necks of membrane tubules and mediate membrane fission. ESCRT-III assemblies, in turn, recruit VPS4 ATPases, which use the energy of ATP hydrolysis to disassemble the ESCRT complexes.

ESCRTs have been considered to employ similar mechanisms for their roles in each of the three biological processes mentioned above: MVB vesicle formation, virus budding, and cytokinesis. However, several recent studies have suggested that only subsets of ESCRTs are required to complete membrane fission in some specific pathway. Therefore, this review will describe the recent findings on the differential requirements of mammalian ESCRTs in the three biological processes of MVB vesicle formation, virus budding, and cytokinesis.

ESCRT recruitment

The ESCRT pathway must function at different cellular membranes, which implies that there must be membrane-specific adaptor complexes that recruit the pathway to the proper membrane at the proper time. The HIV-1 structural protein Gag contains two distinct late domains that are essential for the detachment of budding particles from the cell surface. These late domains recruit cellular ESCRTs that complete the budding process. The P(T/S)AP late domain recruits ESCRT-I via its interaction with the TSG101 subunit, and the YP(X)nL late domain recruits ALIX [8,9]. Both ESCRT-I and ALIX (called early-acting ESCRTs) in turn recruit downstream ESCRT-III (a late-acting ESCRT) that facilitates membrane fission and virus release. These late domains are identified in several different viral structural proteins, and the ESCRT pathway is thought to be a common machinery for the budding process in several different enveloped RNA viruses [4].

The P(T/S)AP late domain was also identified in the cellular protein HRS, which forms a stable complex with STAM1 or STAM2. The PSAP motif in HRS has been shown to recruit ESCRT-I through its direct interaction with the TSG101 subunit, and this interaction is critical for cargo protein degradation at the lysosome [10–13]. Thus, HRS has been considered to be a cellular counterpart of Gag and to function as an ESCRT recruiter for the MVB pathway. The P(T/S)AP late domain has also been identified in two other endosomal proteins, GGA3 and TOM1L1 [14,15]. The primary structures of GGA3 and TOM1L1 are similar to that of HRS: both contain VHS (Vps27, His, STAM) and GAT (GGA and TOM) domains, directly interact with TSG101 via its P(T/S)AP motifs and function as ESCRT-I recruiters like HRS. So far, there is no strong evidence of functional differences among these endosomal proteins, but it is very likely that the proteins recognize different types of cargo proteins to deliver to the lysosome by using the same ESCRT machinery.

In the case of cytokinesis, CEP55 functions as an ESCRT recruiter. CEP55 was originally identified as a protein localized at a centrosome during interface and at a Fleming body (center of the midbody) during cytokinesis, and CEP55 is considered to have a role in the coordination of mitosis and cytokinesis [16]. In screens for binding partners of early-acting ESCRTs, CEP55 was identified as a direct binding partner for both TSG101 (ESCRT-I) and ALIX [17]. The CEP55 binding region of both TSG101 and ALIX is located in the proline-rich region (PRR), and CEP55 binds both proteins in a similar fashion [18]. So far, no CEP55 paralogs have been identified, but it would not be surprising if an alternative factor was found to function in cytokinesis because the effect of RNA interference (RNAi) depletion is seen only in limited types of cultured cells.

Thus, three different sets of ESCRT recruiting factors function in each of the three ESCRT-mediated biological processes, either by recognizing target proteins or by recruiting soluble downstream factors to the site of the budding neck (Fig. 1).

ESCRT-I

Soluble ESCRT-I complexes contain single copies of each of the four different subunit types (TSG101, VPS28, VPS37 and MVB12) [19,20]. In humans, multiple VPS37 (A–D) and MVB12 (A and B) paralogs can combine to produce up to eight different ESCRT-I variants [19]. RNAi depletion of TSG101, a unique ESCRT-I subunit, inhibits all ESCRT-mediated biological processes, indicating its essential roles in all pathways [8,12,17,21]. However, several studies have suggested that the eight different ESCRT-I variants are functionally diverse. Simultaneous RNAi depletion

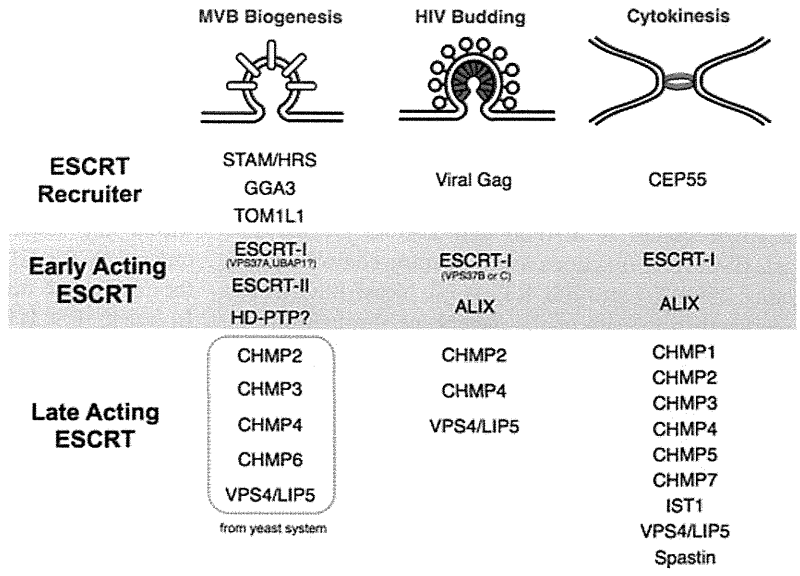

Fig. 1. Factor requirements for ESCRT-mediated biological processes. The list indicates proteins that are known to be involved in MVB biogenesis, virus budding and cytokinesis. The list of late-acting ESCRTs for MVB biogenesis was derived from analyses in yeast, because of the lack of systematic analyses of the effects of their RNAi depletion in mammalian cells.

of VPS37B and VPS37C dramatically reduces virus budding, although individual RNAi depletion of VPS37B or VPS37C has no effects on such budding [22]. These results suggest that virus budding requires at least VPS37B- or VPS37C-containing complexes, and these complexes can function redundantly. On the other hand, single depletion of VPS37A is enough to inhibit ligand-dependent endothelial growth factor receptor (EGFR) degradation [23], suggesting that only VPS37A-containing complexes have an essential role in MVB vesicle formation. Similar results have been reported for MVB12 subunits. Co-depletion, but not single depletion, of MVB12A and MBV12B inhibits infectivity of released HIV [19], while single depletion of MVB12A is enough to inhibit ligand-dependent EGFR degradation [24], suggesting that both MVB12A and MVB12B function in virus release but MVB12A plays the more critical role in MVB vesicle formation. These results suggest that ESCRT-I variants that contain different subunits of VPS37 or MVB12 may function in specific ESCRT-mediated processes, although systematic RNAi depletion analysis for all ESCRT-I subunits will be needed to completely clarify this matter. Little is known about the functional differences of ESCRT-I variants in cytokinesis, because the effect of RNAi depletion is very modest and it is very difficult to judge whether each ESCRT-I variant is involved in cytokinesis (Fig. 1).

Recently, another subunit, named UBAP1, was identified as a novel ESCRT-I subunit [25,26]. This subunit contains the UBA domain, which is also found in TSG101, and an MVB12-related sequence element. Interestingly, UBAP1 was found only in the VPS37A-containing complex and was shown to function in MVB formation but not in cytokinesis [25].

We are still lacking much information on the biological/biochemical characteristics of the eight different ESCRT-I variants, i.e. the expression patterns (tissue distributions), specific binding partners, membrane binding affinities, effects on the stability of the ESCRT-I complexes etc. This information would help to reveal how the different ESCRT-I variants function in each specific ESCRT-mediated pathway.

ALIX

ALIX is recruited to the site of virus budding through the direct interaction of its V domain with the YP(X)nL late domain of viral Gag [9] or to the site of cytokinesis through direct interaction of its PRR with CEP55 [17,21]. In both cases, the N-terminal Bro domain of ALIX binds to CHMP4 proteins, and ALIX serves as an adaptor that recruits CHMP4/ESCRT-III proteins to function at distinct biological membranes [17,27]. ALIX is also known to interact with TSG101 (ESCRT-I) directly or indirectly [28,29]. However, ESCRT-I and ALIX can function independently, at least in some contexts. ALIX is known to be activated through a series of conformational changes and dimerization, and functions to nucleate the assembly of two strands of CHMP4 that form filaments within the necks.

So far, there is no clear evidence that ALIX is involved in MVB vesicle formation. However, another Bro domain protein, HD-PTP, was reported to have a specific function in MVB vesicle formation but not in

cytokinesis (Fig. 1) [30]. These results suggest that the different Bro domain proteins may have specific functions in the different biological processes.

ESCRT-II

The ESCRT-II complex contains two copies of EAP20 and single copies of EAP30 and EAP45 [31,32]. In yeast, ESCRT-II functions as an adaptor complex connecting ESCRT-I and ESCRT-III [1]. Yeast ESCRT-II is recruited to the site of the membrane through direct interaction with ESCRT-I [33]. The NZF domain of Vps36 (human EAP45) interacts with Vps28 of ESCRT-I [34]. Then, ESCRT-II recruits two strands of ESCRT-III through direct interaction of Vps25 (human EAP20) with Vps20 (human CHMP6) [35]. In human cells, however, ESCRT-II is recruited to the site of the membrane in a different way, because the NZF domain is missing in EAP45. Several studies have shown a direct interaction between human ESCRT-I and ESCRT-II [36,37], but this interaction has not yet been fully characterized. ESCRT-II seems to be involved in MVB vesicle formation in human cells, because RNAi depletion of EAP45 blocks ligand-dependent EGFR degradation [36,38]. However, RNAi depletion of EAP45 does not have any effect on HIV budding or cytokinesis [36], suggesting that ESCRT-II has a specific function in MVB vesicle formation in human cells (Fig. 1).

Table 1. ESCRT factors. The names of complexes and their components in the yeast *Saccharomyces cerevisiae* and in humans are shown.

Complex	Yeast proteins	Mammalian proteins
ESCRT-0	Vps27	Hrs
	Hse1	STAM1, 2
ESCRT-I	Vps23	TSG101
	Vps28	VPS28
	Vps37	VPS37A, B, C, D
	Mvb12	MVB12A, B
ESCRT-II	Vps22	EAP30
	Vps25	EAP20
	Vps36	EAP45
ESCRT-III	Vps2	CHMP2A, B
	Vps24	CHMP3
	Vps20	CHMP6
	Snf7	CHMP4A, B, C
	Vps60	CHMP5
	Did2	CHMP1A, B
	–	CHMP7
	Ist1	IST1
Others	Vps4	VPS4A, B
	Vta1	LIP5
	Bro1	ALIX, HD-PTP
	Doa4	UBPY, AMSH

ESCRT-III

ESCRT-III subunits play a critical role in membrane fission reactions by assembling into helical filaments within the membrane neck structure. These higher order ESCRT-III assemblies are thought to constrict the membrane neck, ultimately leading to fission [6,7]. In yeast, ESCRT-III consists of four core subunits, Vps20, Snf7, Vps24 and Vps2, and three regulatory subunits, Did2, Vps60 and Ist1. All seven proteins seem to work together in MVB biogenesis, although deficiencies in the regulatory subunits produce only weak phenotypes [35,39,40]. A mechanistic model of the action of ESCRT-III in membrane fission has been proposed based on investigation in a yeast system both *in vivo* and *in vitro* [5–7]. First, two Vps20 subunits, which are recruited by ESCRT-II, appear to nucleate the assembly of two spiraling Snf7 filaments within the necks. Next, these filaments are capped by Vps24 and Vps2. Vps24 has the essential function of bridging Snf7 and Vps2. Finally, Vps2 recruits Vps4, and then the ESCRT factors are disassembled by its ATPase activity.

In humans, ESCRT-III proteins are called CHMPs (charged MVB proteins). The number of CHMP/ESCRT-III proteins has expanded from seven in yeast to 12 in humans (Table 1), including three isoforms of CHMP4 (yeast Snf7) and two each of CHMP1 (yeast Did2) and CHMP2 (yeast Vps2), and an additional family member, CHMP7, not found in yeast [2]. Due to the multiplicity of CHMP proteins in human cells, the overexpression of wild-type or truncated forms of CHMPs has frequently been utilized to examine the function of ESCRT-III. We recently performed systematic RNAi depletion analyses to determine the requirements for each of the CHMP proteins for both HIV budding and cytokinesis [41,42]. In these screens, we found that depletion of any of the 12 human CHMP proteins, with the possible exception of CHMP6, inhibited cytokinesis, and in most cases also gave rise to earlier defects in mitosis [42,43]. These results suggest that nearly all CHMP proteins play significant functional roles in cytokinesis and other stages of cell division. On the other hand, virus release was profoundly inhibited only when either CHMP2 or CHMP4 family members were co-depleted, and virus budding proceeded efficiently in the absence of CHMP1, CHMP3, CHMP5–7 and IST1 proteins [41,43]. These results indicate that efficient HIV-1 budding requires only a subset of CHMP proteins,

including at least one member of both the CHMP2 and CHMP4 protein families, and that different members within each of these two families can function redundantly.

In these screens, we found that two core ESCRT-III subunits, CHMP6 and CHMP3, are dispensable for HIV budding, although their homologs play essential roles in yeast MVB biogenesis. These results indicate that the connections between different ESCRT factors probably differ between yeast and man. For example, the yeast CHMP3 homolog (Vps24p) creates an essential bridge between CHMP2 (Vps2p) and CHMP4 (Snf7p), whereas mammalian CHMP4 proteins interact directly with CHMP2 [41]. This interaction appears to alleviate a strict requirement for CHMP3 in HIV-1 budding (Fig. 1).

It has generally been assumed that the different human ESCRT-III proteins and their isoforms function similarly in MVB vesicle formation to their counterparts in the yeast system [36,44]. However, systematic RNAi depletion experiments of CHMP proteins have not yet been performed, due at least in part to the absence of a facile, sensitive assay for measuring mammalian MVB vesicle formation.

VPS4

VPS4 enzymes belong to the AAA-ATPase (ATPases associated with a variety of cellular activities) protein family. The VPS4 proteins function to disassemble ESCRT-III polymers and thereby recycle them to participate in multiple rounds of membrane fission [1,2]. VPS4 binds LIP5, which serves as an activator of VPS4 assembly and ATPase activities [45,46]. VPS4 enzymes are evolutionally conserved, and there is a single Vps protein in yeast (Vps4p) but two VPS4 proteins in humans (VPS4A and VPS4B). VPS4 enzymes all contain an N-terminal microtubule interacting and transport (MIT) domain connected by a linker to a canonical ATPase cassette [47,48]. The VPS4 MIT domains bind to sequence elements known as MIMs (MIT interacting motifs), located at or near the C-termini of ESCRT-III proteins [49,50]. MIT–MIM interactions link the VPS4 proteins and their ESCRT-III substrates and are required to release ESCRT-III polymers from the membrane.

In HIV-1 budding, depletion of either VPS4A or VPS4B alone has only a modest (~50%) inhibitory effect, whereas co-depletion of both VPS4A and VPS4B dramatically inhibits budding (> 95%) [44,51]. This synergistic inhibition indicates that VPS4A and VPS4B function redundantly in HIV budding (Fig. 1).

Co-depletion of VPS4A and VPS4B also inhibits ligand-dependent EGFR degradation [51], although the effects of depleting each VPS4 isoform individually are not yet known. However, single depletions of either VPS4A or VPS4B have quite a dramatic inhibitory effect on cytokinesis/cell division [42], suggesting that each of the VPS4 isoforms performs a non-redundant function in this pathway (Fig. 1).

Another MIT domain containing AAA-ATPase, Spastin, has been reported to interact with CHMP1 and function in cytokinesis [52,53]. The function of Spastin has been implicated in cytoskeletal rearrangement and dynamics, via microtubule severing. It is not surprising if Spastin may specifically function in cytokinesis, by coordinating both microtubule severing and membrane abscission at the midbody.

Other MIT domain proteins

In addition to VPS4 and Spastin, several MIT-domain-containing proteins have been reported to be involved in the ESCRT pathways. Two ubiquitin hydrolases, UBPY and AMSH, previously identified as binding partners for the STAM-HRS complex [54,55], have recently been shown to possess MIT domains and to use them to interact with specific ESCRT-III proteins [56–58]. Both UBPY and AMSH have deubiquitinating enzyme activities and are thought to modulate the modification levels of ubiquitylated target proteins within ESCRT pathways [59–62].

Another MIT domain protein is calpain-7, one of the cysteine proteases of the calpain superfamily. The MIT domain of calpain-7 associates with CHMP1 and IST1 [63]. However, the role of calpain-7 in cytokinesis is not fully understood. The yeast ortholog of calpain-7, Rim13, regulates proteolytic activation of the transcription factor Rim101, a pH-responsive zinc finger transcription factor [64,65]. Interestingly, several biochemical and genetic experiments have revealed that Rim101 activation is dependent on ESCRT factors [66]. It is therefore conceivable that calpain-7 functions in activation of unknown membrane associated transcription factors that may be activated by the ESCRT pathway in mammalian cells [67].

Conclusion

Comparative studies of ESCRT function during MVB biogenesis, enveloped virus budding and cytokinesis have begun to elucidate the complexity of the ESCRT pathway. However, additional functional studies are required to elucidate the exact roles of different ESCRT factors and their selective requirements for

different biological processes. The ESCRT pathway has been linked to several important classes of human diseases, including tumorigenesis, neurodegeneration and infectious disease, and an improved understanding of the differential requirements for ESCRT factors may therefore assist in the development of therapeutic agents with minimum adverse effects.

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