SKD1 AAA ATPase-Dependent Endosomal Transport is Involved in Autolysosome Formation

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ABSTRACT. Mouse SKD1 AAA ATPase is involved in the sorting and transport from endosomes; cells overexpressing a dominant-negative mutant, SKD1E235Q, were defective in endosomal transport to both the plasma membrane and lysosomes (Yoshimori et al., 2000). In the present study, we demonstrated that overexpression of SKD1E235Q using an adenovirus delivery system caused a defect in autophagy-dependent bulk protein degradation. Morphological observations suggested that this inhibition of autophagy results from an impairment of autolysosome formation. SKD1E235Q overexpression also inhibited transport from endosomes to autophagosomes, an event normally occurring prior to fusion with lysosomes. These results indicate that SKD1-dependent endosomal membrane trafficking is required for formation of autolysosomes.

Key words: endocytosis/autophagy/membrane traffic/adenovirus-mediated expression system

Cells take up extracellular macromolecules into early endosomes by endocytosis. The cargo is then either sorted to late endosome-lysosomes or recycled back to the plasma membrane (Corvera et al., 1999; Gruenberg and Maxfield, 1995; Lemmon and Traub, 2000; Mellman, 1996; Mukherjee et al., 1997; Mullins and Bonifacino, 2001). Recent studies on endocytosis have unravelled molecular events involved both in forming transport vesicles at the plasma membrane and targeting and fusing these vesicles to early endosomes. The mechanisms underlying post-endocytic membrane traffics via endosomes, however, remain cryptic. SKD1, a mammalian homologue of yeast Vps4, is involved in endosomal function and morphology (Bishop and Woodman, 2000; Yoshimori et al., 2000). Both SKD1 and Vps4 belong to the AAA (ATPases associated with a variety of cellular activities) protein family. Although the members of this family have diverse functions, these proteins share the common features of homo-oligomerization (often as hexamers) and chaperone-like activity (Beyer, 1997; Dalal and Hanson, 2001; Ogura and Wilkinson, 2001; Patel and Latterich, 1998). Overexpression of a dominant-negative SKD1 mutant (SKD1E235Q), which is expected not to hydrolyse ATP, induced exaggerated multivesicular structures, termed E235Q-compartments (Yoshimori et al., 2000). Expression of this dominant-negative protein also inhibited the recycling of surface receptors to the plasma membrane and the transport of ligands and cargo from endosomes to lysosomes. Transferrin receptor (TfR), internalized dextran and horseradish peroxidase, and epidermal growth factor accumulated in E235Q-compartments, heavily decorated with SKD1E235Q. These results suggest that SKD1 is necessary for membrane trafficking originating from endosomes.

Autophagy allows the bulk degradation of cytoplasmic constituents in lysosomes (Dunn, 1994; Klionsky and Ohsumi, 1999; Seglen and Bohley, 1992). Autophagy is in-
duced by nutrient limitation or hormones, such as glucagon (Mortimore and Pöösö, 1987; Seglen and Bohley, 1992). Primarily, cup-shaped isolation membranes enclose cytoplasmic components, including organelles such as mitochondria. Sealing of these isolation membranes results in the formation of double membrane-bounded autophagosomes, also called initial autophagic vacuoles (AVi). The Apg12-Apg5 protein conjugation system participates in the formation of autophagosomes, along with the action of LC3 (Kabeya et al., 2000; Mizushima et al., 2001). Eventually, autolysosomes, also known as degrading AV (AVd), are generated by fusion of the autophagosomal outer membrane with lysosomes. Lysosomal hydrolases then degrade the inner membranes and cytoplasmic contents.

We previously showed that yeast Csc1, isolated in a screen for autophagy-related genes, is identical to VPS4 (Shirahama et al., 1997). Starvation-induced autophagy is impaired in null mutants of Vps4/Csc1; a specific point mutation within the gene (csc1-1) confers a gain-of-function phenotype. The mechanism by which Vps4/Csc1 influences autophagy, however, remains unknown.

To test the involvement of SKD1 in mammalian autophagy, we examined the effects of a dominant-negative SKD1 mutant on autophagolysis. Overexpression of SKD1<sup>E235Q</sup> led to a block in the autophagic pathway prior to autophagosome-lysosome fusion, ceasing autophagic membrane degradation. We demonstrated that the membrane trafficking from endosomes to autophagosomes was also inhibited in dominant-negative transfectants. These data suggest that SKD1-dependent endosomal membrane trafficking is required for the late stages of the autophagic pathway and is critical in autolysosome formation.

**Materials and Methods**

**Plasmid and adenovirus preparation**

An EcoRI fragment of approximately 1.3-kb SKD1<sup>E235Q</sup> was amplified by PCR using the SK5-primer (5'-GTTGAGATTTTCACTGCTTAGCCATCACAAACC-3') and SKD1-3rc primer (5'-AAACGGATCCCTTGCTGTTAGCC-3'). This fragment was subcloned into the EcoRI site of pEGFP-C2 (Clontech Laboratories, Inc., Palo Alto, CA). The 3x Myc epitope fragment was inserted between the GFP and SKD1<sup>E235Q</sup> genes to generate pEGFP-MSKD1<sup>E235Q</sup>. The GFP-Myc-SKD1<sup>E235Q</sup> fragment from pEGFP-MSKD1<sup>E235Q</sup> was inserted into the SpeI cloning site of the pAxCALNLw reporter plasmid (pAxCALS KD<sup>E235Q</sup>). We then generated a recombinant adenovirus (AxCALS KD<sup>E235Q</sup>) using a Cre/loxP adenovirus expression system, according to the manufacturer's instructions (TaKaRa, Otsu, Japan). Cre recombinase-producing viruses (AxCANCre) were also generated from the pAxCANCre plasmid. Plasmids encoding SKD1 and SKD1<sup>E235Q</sup> were previously described (Yoshimori et al., 2000). pGFP-LC3, an expression plasmid encoding the GFP-LC3 fusion protein, was previously described (Kabeya et al., 2000).

**Cell culture and infection**

Media and reagents for cell culture were purchased from Life Technologies (Grand Island, NY). HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 5 U/ml penicillin, and 50 μg/ml streptomycin. HeLa cells were co-infected with AxCALS KD<sup>E235Q</sup> at a multiplicity of infection (MOI) of 3440 and AxCANCre at a MOI of 150 to generate the overexpression of SKD1<sup>E235Q</sup>. Cells were examined 17 hours after infection. For serum and amino-acid starvation conditions, cells were incubated in Hanks' Balanced Salt Solution containing 10 mM HEPES, pH 7.5 for 2 hours. 100 nM Bafilomycin A<sub>1</sub> (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added where indicated. HeLa cells were transfected with the above expression plasmids using FuGENE-6 reagent to achieve the overexpression of GFP-LC3, SKD1 or SKD1<sup>E235Q</sup> (Roche Molecular Biochemicals, Tokyo, Japan).

**Immunofluorescence microscopy**

Immunofluorescence microscopy was performed as previously described (Yoshimori et al., 2000). To visualize cell-surface TfR expression, HeLa cells, grown on cover slips, were incubated with a monoclonal antibody specific for TfR (Yoshimori et al., 1988) for 1 hour at 4°C, as previously described (Yoshimori et al., 2000). To examine intracellular TfR distribution, samples were fixed, permeabilized, and incubated for 1 hour with the antibody against TfR. To detect lysosomes, cells were stained with 100 nM Lysotracker Red (Molecular Probes, Eugene, OR) for 2 hours. These cells were then fixed and stained with mouse monoclonal antibody against the lysosome-associated membrane protein 1 (Lamp-1). Both the anti-TfR and anti-Lamp-1 antibodies were visualized using an Alexa-660-conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR). To allow the simultaneous detection of SKD1 and LBPA, transfected cells were cultured under the indicated conditions, fixed, and stained with rabbit polyclonal anti-SKD1 antibody and mouse monoclonal anti-lysobisphosphatidic acid (LBPA) antibody (Kobayashi et al., 1998), following an incubation with secondary antibodies. All samples were visualized under a LSM510 fluorescent laser scanning confocal microscope (Carl Zeiss, Inc., Thornwood, NY), as described (Yoshimori et al., 2000). Rhodamine-conjugated goat anti-mouse antibody (Bio-source, Camarillo, CA) and Alexa-660-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR) were utilized as secondary antibodies.

**Bulk protein degradation assay**

The degradation of endogenous long-lived proteins was measured as previously described (Mizushima et al., 2001). Briefly, SKD1<sup>E235Q</sup> was expressed in HeLa cells by co-infected with AxCALS KD<sup>E235Q</sup> and AxCANCre. Transfectants were then incubated with 1.5 μCi/ml L-[<sup>14</sup>C]-valine (Moravek Biochemicals Inc., Brea, CA) for 17 hours. After washing, cells were incubated with either 10% FCS/DMEM or Hanks' solution containing 0.1% BSA and 10 mM cold valine in the presence or absence of bafilomycin A<sub>1</sub>. The
subsequent release of L-[14C]-valine was measured as previously described (Mizushima et al., 2001).

**Electron microscopy**

Conventional electron microscopy was performed as described previously (Yoshimori et al., 2000). The area of sections of autophagosomes and autolysosomes was measured on the electron micrographs by NIH image software, as previously described (Kabeya et al., 2000). Autophagosomes and autolysosomes were defined as previously (Liou et al., 1997); autophagosomes were compartments bounded by double or multilayered membranes containing unaltered cytoplasmic constituents inside, whereas autolysosomes are enclosed by a single distinctive membrane and contain components at various stages of degradation.

**Preparation of cell lysates and immunoblotting**

Cell lysates were prepared following lysis with 1% Triton X-100 in PBS supplemented with protease inhibitors. Immunoblotting was performed as previously described (Yoshimori et al., 2000).

**RT-PCR for endogenous LC3**

Total RNA from HeLa cells was prepared using Isogen (Nippon Gene, Toyama, Japan). First-strand cDNA was synthesized by incubating 10 µg of the purified RNA with Moloney murine leukemia virus (MMLV) reverse transcriptase at 37°C and oligo-dT primer (Stratagene, La Jolla, CA). The resulting cDNA was then subjected to PCR using primers hLC3-5 (5'-ATGCCGTCGAAGAAGACCTT-3') and hLC3-3 (5'-TTACACTGACAATTTCCACC-3').

**Results**

**Establishment of adenovirus-mediated SKD1E235Q expression system**

We established an adenovirus-mediated transfection system (Sato et al., 1998) to overexpress SKD1E235Q with high efficiency (Fig. 1 A). We utilized a Cre/loxP inducible adenosivirus system, because constitutive expression of SKD1E235Q is toxic for HEK 293 cells, in which recombinant adenosviruses are multiplied. Cleavage of the stuffer fragment, containing a polyadenylation signal between the two-loxP sites, by Cre recombinase induces the expression of GFP-Myc-SKD1E235Q. When HeLa cells were co-infected with SKD1E235Q-producing (AxCALSKD1E235Q) and Cre recombinase-producing viruses (AxCANCRe), almost 100% of the cell population overexpressed GFP-Myc-SKD1E235Q. GFP-Myc-SKD1E235Q exhibited a perinuclear punctate distribution in aberrant endocytic E235Q-compartment (Yoshimori et al., 2000) (Fig. 1 B, a). To examine the dominant-negative effect of SKD1E235Q following adenovirus-mediated overexpression, we observed the disappearance of cell-surface TIR in all cells (Fig. 1 B, b). TIR was instead detected in internal E235Q-compartment (Fig. 1 B, h), ruling out that the virus-mediated overexpression of GFP-SKD1E235Q exerts receptor stability. These data confirm that the adenovirus-mediated expression of SKD1E235Q exerts a dominant-negative effect; the high efficiency of this expression system allows its use not only for morphological but also biochemical analyses.
Autophagic degradation of long-lived proteins is impaired in HeLa cells overexpressing SKD1^{E235Q}

We used this adenovirus overexpression system to examine the effect of SKD1^{E235Q} on autophagic protein degradation. The degradation of long-lived proteins occurs mainly by autophagy (Henell et al., 1987). HeLa cells were labelled for 17 h with L-[14C]-valine in the presence of the recombinant adenoviruses. The degradation of long-lived proteins was determined by measuring L-[14C]-valine release. In cells infected with only AxCALSKD^{EQ}, bulk degradation was accelerated by culturing without amino acids and serum (starvation condition). Bafilomycin A1, an inhibitor of V-type proton ATPase (Fig. 2, column 5 versus 6), suppresses lysosomal protein degradation by elevating the lumenal pH. Under starvation conditions, bafilomycin A1 inhibited bulk degradation, indicating that drug-treated and -untreated conditions differ substantially in the quantity of lysosomal degradation. Under nutrient-containing conditions, lysosomal degradation was at a very low level, as drug treatment only slightly suppressed degradation (column 2). Lysosomal degradation was almost abolished in cells overexpressing SKD1^{E235Q} following co-infection of AxCALSKD^{EQ} and AxCANCre (column 7); the uptake of labelled-amino acids, however, was unaffected (see Fig. 2 legend). The inhibitory effects of SKD1^{E235Q} overexpression and bafilomycin A1 were not additive (column 8), suggesting that autophagic degradation is severely suppressed by SKD1^{E235Q} overexpression.

Overexpression of SKD1^{E235Q} causes accumulation of autophagosomes, but formation of only a few autolysosomes

We then analyzed cells overexpressing SKD1^{E235Q} by electron microscopy. Under nutrient-containing conditions, uninfected cells or cells infected with only AxCALSKD^{EQ} contained few autophagosomes (Fig. 3, A and C). Following a 2-hour-starvation, approximately 0.3% of control cell cytoplasm was occupied by autophagic vacuoles, half of which were autolysosome in nature (Fig. 3 C, column 2; for morphological criteria of autophagosomes and autolysosomes, see Materials and Methods). In contrast, accumulation of autophagosomes was observed in cells overexpressing SKD1^{E235Q} even under nutrient-containing conditions (Fig. 3 B and C, column 4, closed bar). Cells overexpressing SKD1^{E235Q} formed autolysosomes under half of those in starved cells (Fig. 3 C, column 4, open bar), despite the fact that the number of autophagosomes was 2.7 times as many as those in starved cells. This result suggests that the dominant-negative mutant blocks the transition of autophagosomes to autolysosomes.

We previously reported that rat LC3 has two molecular forms, LC3-I (18 kDa) and LC3-II (16 kDa). The latter is specifically associated with autophagic vacuoles, while the former remains in the cytoplasm (Kabeya et al., 2000). LC3-II is formed from LC3-I in a post-translational manner. Levels of LC3-I correlate well with autophagosome numbers. Overexpression of SKD1^{E235Q} significantly increases the amounts of LC3-II (Fig. 3 D), confirming the accumulation of autophagosomes.

Although the accumulation of autophagosomes appeared to result from the inhibition of autolysosome formation, it is possible that an increased induction of autophagosome formation may account for increases in autophagosome numbers. The mRNA expression levels of LC3 increased during amino acid starvation, indicating that the induction of autophagy is accompanied by increases in LC3 synthesis (Fig. 3 E). LC3 mRNA expression increased following overexpression of SKD1^{E235Q} (Fig. 3 E), suggesting the induction of autophagosome formation. We assume, however, that this induction is a secondary effect of the blockade of the late step of autophagy. Because the treatment of unstarved cells with bafilomycin A1 also induced LC3 expression and increased the LC3-II protein levels (Fig. 3 D and E). It is known that the drug inhibits autophagosome-lysosome fusion (Yamamoto et al., 1998). Therefore, autophagosome formation may be accelerated by a feedback loop sensing

![Fig. 2. Starvation-induced protein degradation is impaired in the SKD1^{E235Q} transfectants. HeLa cells were incubated for 17 hours with 1.5 µCi/ml of L-[14C]-valine during infection with either AxCALSKD^{EQ} and AxCANCre or AxCALSKD^{EQ} alone. We then measured the degradation of endogenous long-lived proteins during a 2-hour-incubation in complete 10% FCS/DMEM (nutrient) or Hanks’ medium (starvation) as described in the Materials and Methods. Each bar represents the mean ± s.d. of triplicates from representative experiments.](image-url)
Fig. 3. Autophagosomes accumulated, but few autolysosomes are formed, in the cells overexpressing SKD1E235Q. (A and B) HeLa cells infected with AxCALSKD\textsuperscript{E235Q} alone (A) or co-infected with AxCALSKD\textsuperscript{E235Q} and AxCANC\textsubscript{Re} (B) were cultured for 17 hours in complete medium. Cells were then fixed and observed by electron microscopy. Bar, 1 \(\mu\)m. (C) Morphometric analysis of HeLa cells infected with the indicated viruses. Uninfected control cells were subjected to 2 h-starvation in the absence of virus. The ratio of the total area of autophagosomes (closed columns; AP) and autolysosomes (open columns; AL) to the total cytoplasmic area is indicated. (D) HeLa cells infected as indicated were cultured in nutrient-rich medium in the absence (lane 1 and 4) or presence of 100 nM bafilomycin A\textsubscript{1} for 4 hours (lane 3), or Hanks' solution alone for 2 hours (lane 2). After incubation, cells were lysed and analysed by immunoblotting using an anti-LC3 (upper) or an anti-SKD1 antibody (lower). Asterisks indicate the degradation products of GFP-Myc-SKD1\textsuperscript{E235Q}. (E) Expression levels of endogenous LC3 (top panel) were examined in HeLa cells after a 2 hour incubation under starvation conditions (lane 2), under nutrient conditions in the absence (lane 1 and 5) or presence of 100 nM bafilomycin A\textsubscript{1} (4 hours) (lane 3 and 6), or in SKD1\textsuperscript{E235Q}-overexpressing cells under nutrient conditions (lane 4 and 7). Exp-1 and Exp-2 are represented as independent experiments. Expression of \(\beta\)-actin (bottom panel) served as an internal control for RNA integrity.
the autophagic degradation.

Altogether, the data suggest that, in the cells overexpressing SKD1\textsuperscript{E235Q}, maturation to autolysosome is primarily affected; the induction of autophagosome formation occurs as a secondary effect. Both effects result in the accumulation of autophagosomes. The defect in autophagic degradation may result from the inhibition of autolysosome generation.

**Lysosomes are still present in cells overexpressing SKD1\textsuperscript{E235Q}**

The observed inhibition of autolysosome formation may result from the disappearance of the fusion partners, lysosomes, from cells overexpressing SKD1\textsuperscript{E235Q}, possibly occurring through an inhibition of membrane supply. In cells infected with AxCALSKD\textsuperscript{E235Q} alone, the staining patterns of Lamp-1, a lysosomal membrane protein, and Lysotracker Red, a marker of acidic compartments, overlapped well (Fig. 4, b–d), in structures considered to be lysosomes. In cells infected with both AxCALSKD\textsuperscript{E235Q} and AxCANCre, Lamp-1 and Lysotracker Red-positive structures, distinct from the E235Q-compartments, were observed in similar numbers, although their distribution was altered from a juxtanuclear to a peripheral region (Fig. 4, e–h). Therefore, the overexpression of SKD1\textsuperscript{E235Q} does not result in the disappearance of lysosomes at 17 hours after infection, the time point used in our experiments.

**Late endosome-specific lipid is not transported to autophagosomal membranes in cells overexpressing SKD1\textsuperscript{E235Q}**

Endosomes or endosome-derived vesicles have been suggested to fuse with autophagosomes prior to autophagosome-lysosome fusion (Dunn, 1994; Fengsrud et al., 2000; Liou et al., 1997; Seglen and Bohley, 1992; Tooze et al., 1990). If endosomes provide autophagosomes with putative components necessary for autolysosome formation by di-

![Fig. 4](image-url) Lysosomes are still present in the cells overexpressing the SKD1\textsuperscript{E235Q}. HeLa cells infected with AxCALSKD\textsuperscript{E235Q} alone (a–d) or co-infected with AxCALSKD\textsuperscript{E235Q} and AxCANCre (e–h) were incubated for 2 hours with Lysotracker Red. The cells were then fixed, stained with an anti-Lamp-1 antibody, and analysed by confocal microscopy. a, c, and green in d and h, GFP-SKD1\textsuperscript{E235Q}; b, f, and red in d and h, Lysotracker Red; c, g, and blue in d and h, Lamp-1. Co-localization of GFP-SKD1\textsuperscript{E235Q}, Lysotracker Red, and Lamp-1 is indicated as white. Purple indicates the co-localization of Lysotracker Red and Lamp-1. Bar, 20 µm.
Role of SKD1 in Autophagy

Rect-fusion or by vesicular transport, SKD1 may be involved in autophagy by controlling membrane trafficking between endosomes and autophagosomes. This hypothesis would explain the inability of autolysosome formation in the cells overexpressing SKD1E235Q. To examine this possibility, we analyzed the distribution of lysobisphosphatidic acid (LBPA), a lipid localizing in late endosomes (Kobayashi et al., 1998), in cells overexpressing SKD1E235Q. We detected LBPA in autophagic vacuoles by immunoelectron microscopy, confirming the convergence of the endocytic and the autophagic pathways (Yamamoto A, Tsuneoka M, Mizushima N, Yoshimori T, and Kobayashi T, unpublished results). We also demonstrated the existence of LBPA on autophagosomes by simultaneous visualization of LBPA and LC3 by immunofluorescence microscopy. HeLa cells were co-transfected with GFP-LC3 and wild-type SKD1 and cultured under starvation conditions in the presence of 100 nM bafilomycin A1 for 2 hours in order to accumulate autophagosomes. In these cells, GFP-LC3 and LBPA co-localized well (Fig. 5, d and e, yellow). In contrast, LBPA was no longer distributed in the GFP-LC3-localized autophagosomes accumulated by overexpression of the dominant-negative, SKD1E235Q (Fig. 5, i). Most of the LBPA accumulated in the E235Q-compartments (Fig. 5, j), as suggested by the previous finding that a variety of endosomal markers was trapped in these compartments (Yoshimori et al., 2000). Consequently, the overexpression of SKD1E235Q inhibits the delivery of LBPA from late endosomes to autophagosomes. As SKD1E235Q is located in a compartment distinct from that of GFP-LC3, it is unlikely that the mutant SKD1 acts directly on autophagosomes (Fig. 5, k).

Discussion

Overexpression of the dominant-negative SKD1 caused an almost complete inhibition of autophagic degradation. Electron microscopic and LC3 immunoblot analysis of cells overexpressing SKD1E235Q demonstrated the accumulation of autophagosomes. Although the enhanced expression of LC3 mRNA indicated the induction of autophagy, the reduction in the number of autolysosomes from that seen in starvation-induced autophagy suggests that autophagosome accumulation results from both the induction of autophagosome formation and the prevention of maturation to autolysosomes. The former appears to follow from the latter, as the same phenomenon was observed following bafilomycin A1 inhibition of autophagosome-lysosome fusion (Yamamoto et al., 1998). These results suggest that the SKD1 function is required for autolysosome formation, prerequisite for autophagic degradation.

SKD1 is indirectly involved in maturation of autophagosome to autolysosome, since SKD1E235Q was not associated with autophagosomes. If the overexpression of SKD1E235Q caused the loss of lysosomes, autophagosomes would be unable to convert to autolysosomes. In the cells overexpressing SKD1E235Q, the observation, however, of a number of compartments stained with both anti-Lamp-1 antibody and Lysotracker, distinct from the SKD1E235Q-positive aberrant endosomes, ruled out this possibility. Thus, although overexpression of SKD1E235Q impairs transport from endosomes to lysosomes (Yoshimori et al., 2000), lysosomes remained at the time point analyzed. These lysosomes may have formed prior to the expression of SKD1E235Q, as they accumulated dextran loaded prior to infection (data not shown). When given after transfection, dextran was trapped in the E235Q-compartments and not transported to lysosomes (Yoshimori et al., 2000). Therefore, it is unlikely that these pre-existing lysosomes specifically lost their fusion machinery.

An alternative possible mechanism suggests that autophagosomes cannot become competent for fusion with lysosomes in cells overexpressing SKD1E235Q. Earlier studies suggested that autophagosomes fuse with both early and late endosomes to form intermediate autophagic vacuoles (Avi/d) (Dunn, 1990; Tooze et al., 1990), called amphisomes (Berg et al., 1998; Fengsrud et al., 2000; Seglen and Bohley, 1992). These structures were morphologically identified as autophagic vacuoles containing endocytosed markers but few lysosomal proteins, such as cathepsin D (Dunn, 1990; Liou et al., 1997; Tooze et al., 1990) and Lgp120 (Dunn, 1990). These amphisomes are acidic compartments (Seglen and Bohley, 1992), indicating the delivery of a proton pump to autophagosomes. We hypothesized that the putative component(s) required for fusion with lysosomes are supplied by the endosome-to-autophagosome membrane traffic; SKD1 is necessary for the transport. To examine this possibility, we analyzed LBPA, a lipid specifically found in late endosomes, as a cargo marker for endosome-to-autophagosome transport (Yamamoto A, Tsuneoka M, Mizushima N, Yoshimori T, and Kobayashi T, unpublished results). We demonstrated that the overexpression of SKD1E235Q reduced the number of LBPA-positive autophagosomes; an accumulation of this molecule in E235Q-compartments prevailed instead. Hence, LBPA transport from late endosomes to autophagosomes may be dependent on SKD1 function. This transport pathway may also deliver some putative component(s) necessary for fusion with lysosomes, for example, soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE), specific for this step. It is also possible that in the cells expressing SKD1E235Q autophagosomes could not meet lysosomes, because they lack some factors necessary for moving along cytoskeleton. Further analyses are required to determine a precise role of SKD1 in autolysosome formation.

This study established an adenovirus-mediated expression system to allow the transient overexpression of SKD1E235Q in most cells. This highly efficient system allowed us to biochemically quantify effects of SKD1E235Q and will be a very useful tool in the future examination of the SKD1 function.
Fig. 5. LBPA is not co-localized with LC3 in cells overexpressing the SKD1E235Q. HeLa cells co-transfected with GFP-LC3 and SKD1 were incubated for 2 hours under starvation conditions in the presence of 100 nM bafilomycin A1 (left panel). Cells co-transfected with GFP-LC3 and SKD1E235Q were incubated for 17 hours in complete medium (right panel). Cells were then fixed, permeabilized, and incubated with antibodies specific for SKD1 (a, f and blue in j and k) and LBPA (b, g, and red in d, e, i and j). Bound antibodies were visualized by Alexa-660-conjugated goat anti-rabbit antibody (blue) or Rhodamine-conjugated goat anti-mouse antibody (red). The panels of c, h, and green in d, e, i and k are images detailing GFP-LC3 distribution. Merged samples double-stained with GFP-LC3 and LBPA are shown in d, e, and k. Magnification of the merged images (d, e, and i–k) was highlighted by the surround-ed area in b, c, and f–h. Yellow indicates the co-localization of GFP-LC3 in green and LBPA in red (d and e). Purple details the co-localization between SKD1E235Q in blue and LBPA (j). Bar, a–c and f–h, 20 µm; d, e, and i–k, 10 µm.
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