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Alix differs from ESCRT proteins in the control of autophagy

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ABSTRACT

Alix/AIP1 is a cytosolic protein that regulates cell death through mechanisms that remain unclear. Alix binds to two protein members of the so-called Endosomal Sorting Complex Required for Transport (ESCRT), which facilitates membrane fission events during multivesicular endosome formation, enveloped virus budding and cytokinesis. Alix itself has been suggested to participate in these cellular events and is thus often considered to function in the ESCRT pathway. ESCRT proteins were recently implicated in autophagy, a process involved in bulk degradation of cytoplasmic constituents in lysosomes, which can also participate in cell death. In this study, we shown that, unlike ESCRT proteins, Alix is not involved in autophagy. These results strongly suggest that the capacity of several mutants of Alix to block both caspase-dependent and independent cell death does not relate to their capacity to modulate autophagy. Furthermore, they reinforce the conclusion of other studies demonstrating that the role of Alix is different from that of classical ESCRT proteins.

Alix/AIP1 is a cytoplasmic protein which was first characterised as a partner of ALG-2 [1,2], a small Ca^{2+} binding protein involved in apoptosis [3]. Alix overexpression, *in vivo* as well as *in vitro*, is sufficient to activate caspases and thereby neuronal death, while some deletion mutants of the protein block both caspase-dependent and independent neuronal death [4,5]. Alix is a known partner of ESCRT proteins [6,7] (endosomal sorting complexes required for transport), which are central elements for the making of multivesicular bodies (MVBs), late endosome intermediates containing intralumenal vesicules (ILVs) that collect receptors and other membrane proteins endocytosed from the cell surface [8].

Alix interacts with two proteins of the ESCRT complex, Tsg101 [6] and CHMP-4B [7], lysobisphosphatidic acid (LBPA), a phospholipid involved in intralumenal vesiculation of endosomes [9], as well as with other regulators of endocytosis (endophilins [10] and CIN85 [11]). Like ESCRT proteins, Alix is also required for the budding of enveloped viruses [12] and for the abscission reactions that complete mammalian cell division [13,14]. Even if Alix has been proposed to regulate membrane budding and abscission in MVBs, its precise role remains largely unclear as neither we nor other laboratories have found any striking effect of the protein on endocytosis and degradation of EGF receptors (J. Faure and coll, unpublished observations, [15–18]).

Macroautophagy, hereafter referred to as autophagy, is the major pathway for degradation of long-lived proteins and the only known pathway for elimination of organelles. Depending on the stimulus, autophagy leads to cellular destruction or, on the contrary, to survival by supplying nutrients from cell material degradation [19,20]. Formation of double-membrane vacuoles, referred to as autophagosomes, allows the engulfment of material which is then degraded in lysosomes. In higher eukaryotes, autophagosomes first fuse with endosomes, thereby giving rise to amphisomes which in turn fuse with lysosomes [21]. This may explain why proteins involved in the biogenesis of endosomes, like ESCRTs [22], the ATPase SKD1 [23], the small GTPases Rab7 [24] and Rab5 [25], and Hrs [26], all have an effect on the maturation of autophagosomes. Given the fact that Alix is somehow involved in cell death which is associated with autophagy, we have challenged the hypothesis that it may control autophagy in a similar way to ESCRT proteins. Surprisingly, we could not find any effect of Alix mutants or of Alix knock-down on several aspects of autophagy induced by amino acid depletion, thereby strongly suggesting that

Abbreviations: ALG-2, apoptosis linked gene-2; Alix, ALG-2 interacting protein X; ESCRT, endosomal sorting complex required for transport; GFP, green fluorescent protein; Hrs, hepatocyte growth factor regulated tyrosine kinase substrate; ILV, intralumenal vesicles; LC3, microtubule-associated protein 1 light chain 3; MVB, multivesicular body; Vps, vacuolar protein sorting.

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Alix does not directly participate in this process and therefore differs from classical ESCRT proteins.

Experimental procedures

Plasmids. Expression plasmids encoding GFP-SKD1(E235Q), GFP-LC3 (pEGFP LC3), and RFP-LC3 were kindly provided by T. Yoshimori (National Institute of Genetics, Mishima, Japan). Alix and Alix mutants expression vectors have been described in Chatellard-Causse et al., 2002 [10].

Construction of Alix sh RNA. For silencing Alix expression, a DNA insert encoding an RNA hairpin was cloned downstream of the human H1 promoter in the pSuperGFP vector (Oligoengine, Seattle, WA, USA). Forward and reverse sequences for the Alix shRNA construct were: 5′-GATCCCCGCCGCTGGTGAAGTTCATCTTCAAGAGAGA TGAACTTCACCAGCGGCTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAA AGCCGCTGGTGAAGTTCATCTCTCTTGAAGATGAACTTCACCAGCGGC GGG-3′, respectively (Invitrogen). The annealed oligonucleotides were ligated into the BglII–HindIII cleavage site within the pSuperGFP vector.

Cells culture and antibodies. Baby Hamster Kidney cells (BHK-21) were used as described in Ref. [27]. Rabbit anti-Alix as been previously described [10], mouse anti-Tsg101 was from Abcam, mouse anti-actin was from Chemicon, mouse anti Flag was from Sigma–Aldrich, mouse anti-GFP was from Clontec and rabbit anti-LC3 was a kind gift from Priault M., (IBGC, UMR 5095, Bordeaux).

pSuper expressing BHK-21 cells. BHK-21 cells were transfected with pSuper/shAlix plasmid or pSuperGFP vector as control by using JetSi transfection (Polyplus tranfection). Transfected cells were selected by adding G418 (800 μg/ml) to the culture medium. After 15 days, clones were isolated and screened for the best reduction in Alix expression. Permanent cell lines (PSuper/shAlix and control) were maintained with G418 (800 μg/ml).

Transient cell transfections of BHK-21 cells. Plasmids encoding the different constructions were transfected using the JetPEI method. Cells were used 36 h following transfection.

Determination of cell viability. Cells were stained with Hoechst 33342 as described previously [4]. Cell viability was scored on the basis of nuclear morphology; condensed or fragmented nuclei were taken to indicate cell death.

In some cases, MTT (tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, 0.5 mg/ml) was added to cells and incubated for 30 min in the dark at 37 °C. The cells were rinsed and dissolved in 500 μl DMSO per well. Absorbance was measured at 540 nm.

Immunofluorescence. BHK-21 cells grown on glass coverslips were fixed in 4% paraformaldehyde in PBS for 20 min at 4 °C, permeabilized with 0.02% saponin, and immunostained as described in [4]. The primary antibody was revealed with anti-IgG coupled

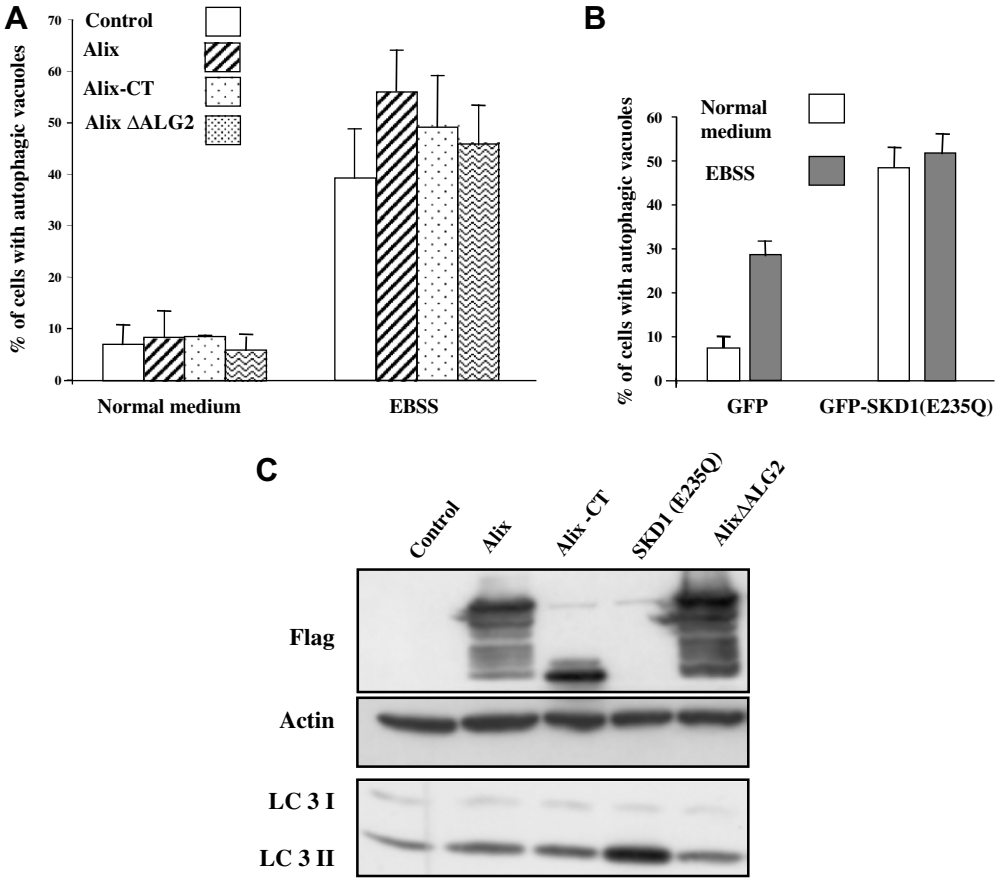

Fig. 1. Alix expression is not associated with the formation of autophagic vacuoles in BHK cells. (A) BHK cells were co-transfected with LC3-GFP and Flag tagged Alix plasmids. Cells were incubated for 4 h in normal medium or medium depleted of amino acids (EBSS condition) before labelling with a monoclonal anti-FLAG antibody. The percentage of autophagic cells represents the ratio between the number of cells expressing an autophagic pattern (vesicular labelling of LC3-GFP) and the total number of transfected cells. For each experiment, 200–300 transfected cells were counted per condition in 10 random fields in each of three different wells. Each experiment was performed three times. (B) BHK cells were co-transfected with LC3-RFP and GFP or GFP-SKD1(E235Q) plasmids. The percentage of autophagic cells was calculated as described above. (C) BHK cells transfected with different plasmids and incubated 4 h in normal or medium depleted of amino acids (EBSS). Conversion of LC3-I to LC3-II was analysed by immunoblotting using an anti-LC3 antibody. FLAG antibody was used to visualise expression of different Alix proteins. GFP-SKD1(E235Q) expression was visualized using an anti-GFP antibody (not shown). This Western blot is representative of three independent experiments.

to fluorochrome Alexa 594 or Alexa 488 (Molecular Probes). Image analysis was performed using Metamorph™ software (Universal Imaging Corp.).

Western blot analysis. Western blot analysis were performed as described [10].

Measurement of the degradation of long-lived proteins. Protein degradation was determined as previously described in Ref. [28]. Briefly, cells were incubated for 18 h at 37 °C with 0.2 μCi/ml L-[^{14}C] valine. After three washes with PBS, cells were incubated 1 h in fresh medium supplemented with 10 mM cold valine. Autophagic degradation was induced by incubating cells in EBSS medium (lacking amino acids and foetal calf serum) supplemented with 10 mM cold valine throughout the chase period (4 h). Following the 4 h chase, proteins of the media and of the cell cultures were precipitated with trichloroacetic acid (10% at 4 °C). Radioactivity was determined by liquid scintillation counting. Percentage of protein degradation represents the ratio of acid-soluble radioactivity recovered from both cells and medium to the radioactivity contained in the precipitated proteins from both cells and medium.

Statistical analysis. Statistical analysis was performed using Student's *t* test. $p < 0.05$ was considered statistically significant.

Results

Alix is not involved in the making of autophagic vacuoles

In BHK-21 cells, like in many others, autophagy can be induced by depleting amino acids from the culture medium. The first steps of autophagy were recorded by using the microtubule-associated protein-1 light chain 3 (LC3) to label autophagic membranes to which it binds selectively throughout the process of autophagy. As expected, the percentage of cells transfected with LC3-GFP displaying a vacuolar staining was less than 10% under normal conditions and rose to nearly 40%, 4 h after amino acid depletion (Fig. 1A). We tested the effect of expressing Alix or Alix mutants on autophagosomes: Alix-CT, which binds to Tsg101 of ESCRT-I but lacks the N terminal binding domain to SNF7/CHMP-4B of ESCRT-III, is thought to inhibit the ESCRT pathway. Alix ΔALG-2 lacks the ALG-2 binding site and is capable of blocking cell death *in vivo*. None of the overexpressed Alix proteins impaired cell viability (data not shown). In normal or amino acid-depleted cultures, none of the Alix proteins changed significantly the number of cells with vacuolated LC3-GFP staining (Fig. 1A). In contrast, a significant increase in the number of vacuoles stained with LC3-RFP was observed in normal or amino acid-depleted cells transfected with an ATPase dead version of SKD1 (SKD1(E235Q)), (Fig. 1B). This mutant impairs the dissociation of the ESCRT complex from endosomes and thereby MVB formation. Furthermore, the level of the 16 kD lipidated, autophagosome-bound form of LC3 (LC3-II), detected by Western blot analysis, was visibly increased compared to the 18 kD cytosolic form in cells expressing SKD1(E235Q). In contrast, there was no change in the level of LC3-II in cells expressing wt. or mutated Alix, cultured in normal (not shown) or amino acid-depleted medium (Fig. 1C). The LC3-I band is almost undetectable probably because it is less sensitive to detection with the antibody used or because it is more labile than LC3-II in BHK cells.

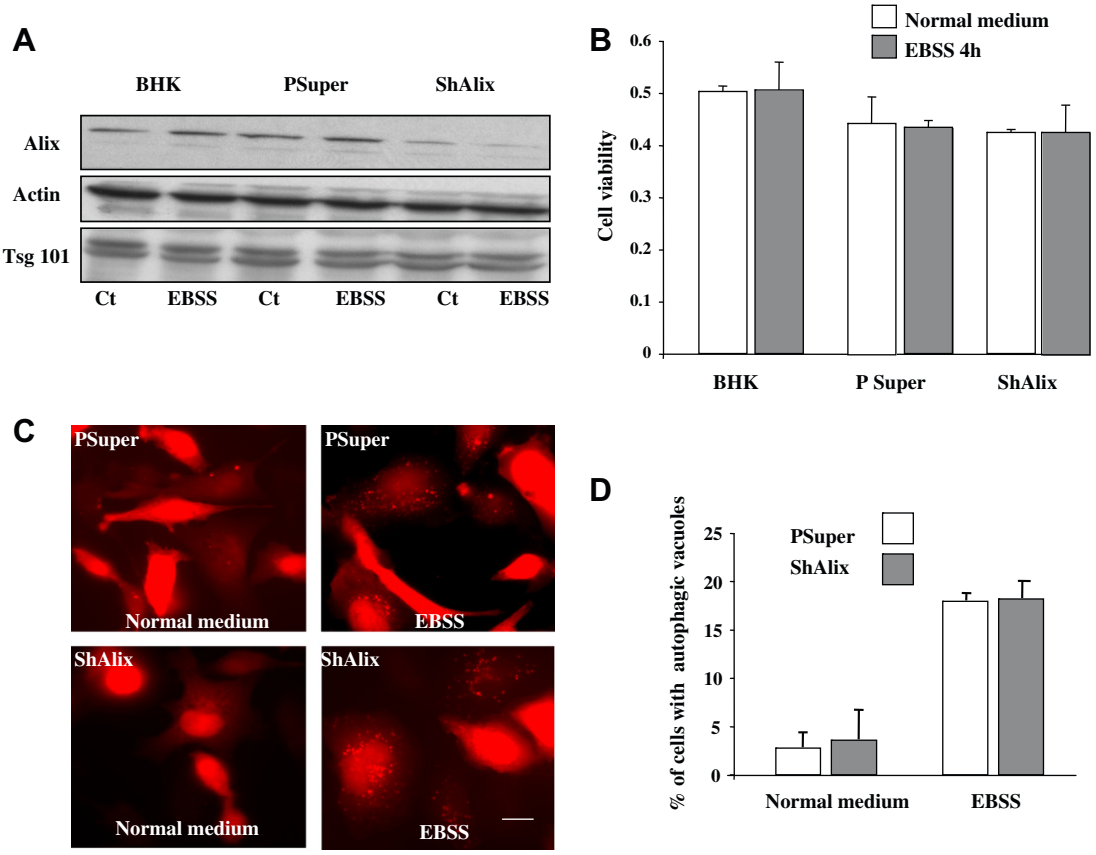

Fig. 2. Autophagy is not disturbed in Alix-depleted BHK cells. (A) Cells from BHK-21 cell lines expressing PSuper or a PSuper coding for an ShRNA against Alix (Sh Alix) were analysed by Western blotting using anti-Alix, anti-Tsg101 and anti-actin antibodies. (B) MTT test on PSuper and shAlix cells incubated in normal or EBSS conditions. (C) PSuper and shAlix cells were tranfected with LC3-RFP and incubated for 4 h in normal medium or medium depleted of amino acids (EBSS), Bars: 5 μm. (D) Number of cells containing LC3-RFP labelled vacuoles estimated in PSuper and shAlix transfected cells. The values reported are those of 200–300 transfected cells counted in three independent experiments.

We next tested whether downregulation of Alix expression could affect autophagy. For this, we used a BHK-21 cell line transfected with a pSuper plasmid expressing shRNA targeted to Alix. Alix expression was reduced by about 70%, whereas expression of actin or Tsg101 remained unchanged (Fig. 2A). As estimated by MTT, downregulation of Alix expression did not affect cell survival in any culture condition (Fig. 2B). The number of cells with vacuolar LC3-RFP staining was equivalent in cells with reduced Alix expression and in controls. This was true in both normal or in amino acid-depleted medium (Fig. 2C and D). Moreover, there was no significant difference in the levels of LC3-II, detected by Western blot analysis of PSuper and shAlix cells (data not shown). Taken together, these observations strongly suggest that unlike ESCRT proteins, Alix is not necessary for the formation of autophagic vacuoles.

Alix does not colocalise with autophagic vacuoles

Because endocytic proteins can be found on autophagosomal membranes [24–26], the localisation of overexpressed FLAG-Alix protein was studied in LC3-GFP transfected cells in normal or in amino acid-depleted medium. As illustrated in Fig. 3A, Alix did not colocalise with LC3-GFP, suggesting that Alix is not present in the autophagosome compartment. Alix-CT both formed and delimited, spherical structures surrounding large ubiquitinated protein aggregates resembling those seen in ESCRT depleted cells ([10,22] and Chatellard-Causse, unpublished data). In contrast with this latter situation where it colocalized around the aggregates, LC3-GFP was never localised on Alix-CT induced spherical structures (Fig. 3B). ESCRT depletion and Alix-CT expression therefore induce the formation of morphologically similar, but distinct compartments.

Alix is not involved in autophagic protein degradation

We went on testing the effect of Alix on protein degradation. For this we quantified the percentage of acid-soluble radioactivity recovered from BHK-21 cells metabolically labelled with [^{14}C] valine; it was almost doubled after cells were switched from DMEM to EBSS (Fig. 4A and B). This increase partly reflects autophagic degradation induced by amino acid-deprivation since the percentage of soluble [^{14}C] was reduced by about 30% in cells cultured in EBSS containing LY294002, a phosphatidyl inositol 3-kinase inhibitor known to block autophagic degradation [29]. Transient overexpression of SKD1(E235Q) significantly reduced autophagic degradation, whereas Alix or Alix-CT expression had no significant effect. Furthermore, the use of shAlix BHK cells, did not reveal any detectable effect of Alix downregulation on the level of autophagic

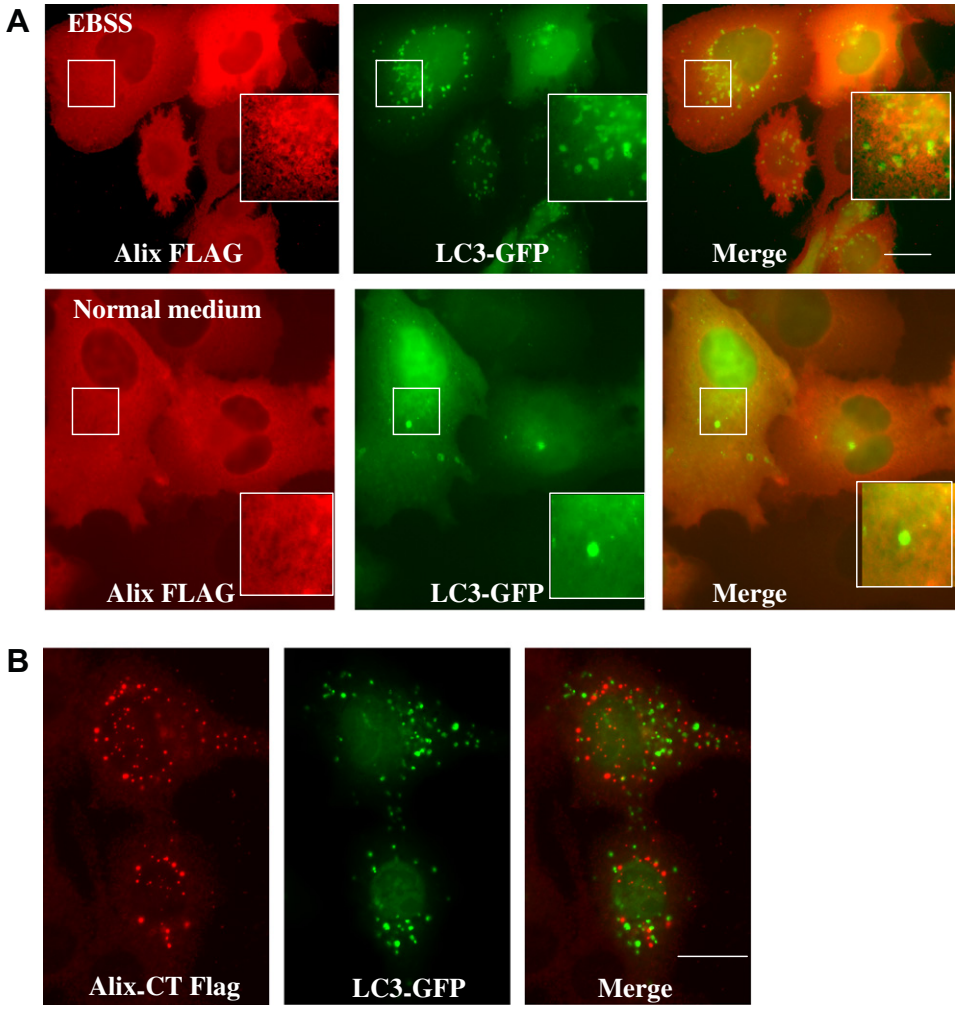

Fig. 3. Alix is not localised on autophagic vacuoles. (A) BHK cells were co-transfected with LC3-GFP and FLAG Alix, before 4 h incubation in normal medium or depleted amino acid medium (EBSS). Cells were then fixed and labelled with a monoclonal anti-FLAG antibody. (B) BHK cells were co-transfected with FLAG Alix-CT, before 4 h incubation in depleted amino acid medium (EBSS). Cells were then fixed and labelled with a monoclonal anti-FLAG antibody. Bars: (A) 5 μm; (B) 2.5 μm.

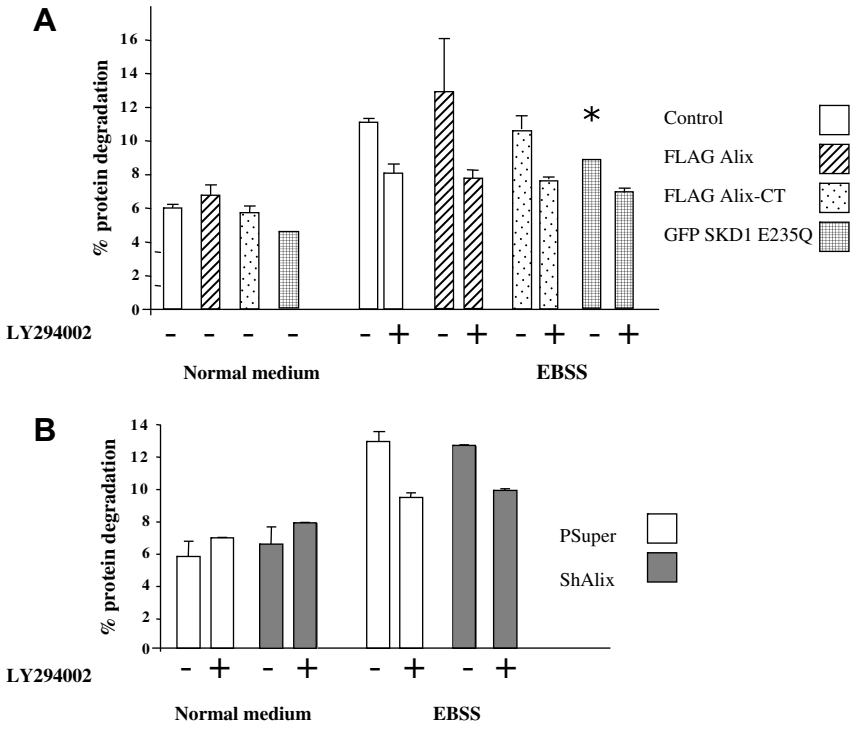

Fig. 4. Alix is not involved in autophagic protein degradation. The rate of [^{14}C] valine-labelled long-lived protein degradation, in normal or in a medium depleted on amino acids (EBSS), was measured in BHK cells expressing Flag tagged Alix proteins (A) or in PSuper shAlix cells (B). LY 294002 was used 4 h at 10 μM. Data are expressed as the percentage of cellular protein degradation during the 4 h chase period. The values reported are the means of three determinations ± SD. * test, $p < 0.05$.

protein degradation (Fig. 4B). Taken together, these results suggest that Alix is not involved in autophagic degradation due to amino acid depletion in BHK-21 cells.

Discussion

Here, we show that Alix downregulation or expression of Alix-CT does not impair autophagy as monitored by LC3 relocalisation or protein degradation, thus challenging the hypothesis that Alix acts as an ESCRT protein in the making of MVBs. ESCRT complexes (0-III) bind sequentially on the cytosolic surface of endosomes where they control the sorting of cargoes and budding of intralumenal vesicles [30]. Activity of the ATPase Vps4/SKD1 allows the dissociation of ESCRT-III complexes from the endosome limiting membrane. Expression of a dominant negative form of the ATPase, SKD1(E235Q), leads to abnormal MVBs [23] and produces accumulation of LC3 in *Drosophila* [22] and in starved HeLa cells [23]. In this latter case, Nara et al. also observed an increase LC3-II, in parallel to partial inhibition of protein degradation [23]; our present study reports similar results using BHK-21 cells. In higher eukaryotes, autophagosomes fuse with endosomal compartments to make amphisomes, which in turn fuse with lysosomes to form autolysosomes [31]. Autophagosomes and amphisomes, but not autolysosomes, form in ESCRT depleted HeLa cells, explaining the increase in the number of LC3 decorated vacuoles [32]. Such an increase was not seen after downregulation of Alix or expression of Alix-CT, which is thought to disorganize the ESCRT pathway by binding to ESCRT-I but not to ESCRT-III. Cells depleted of Tsg101 of ESCRT-I and Vps24 of ESCRT-III, accumulate large ubiquitin positive aggregates which concentrate LC3, a phenomenon probably reflecting their incapacity to eliminate polyubiquitinated proteins [32]. Alix-CT induces the formation of vacuoles containing ubiquitinated aggregates (C. Chatellard-Causse, unpublished) which differ from those detected in ESCRT depleted cells since they did not recruit LC3-GFP.

Our results demonstrating that Alix has no central role in the maturation of autophagosomes seem puzzling in view of its proposed role in the making of MVBs. Alix provides a link between different effectors of intralumenal vesicle budding through binding to proteins of both ESCRT-I [6] and ESCRT-III [7], and to lysobisphosphatidic acid (LBPA), a cone shaped lipid implicated in inward vesiculation [9]. The hypothesis of Alix playing a role in MVB genesis stems from its homology with a yeast protein, Bro-1p, which is required for the sorting of mono-ubiquitinated transmembrane proteins into intralumenal vesicles of MVBs [33]. In *Caenorhabditis elegans* ALX-1 is needed for proper MVB and late endosome function and thereby required for the degradation of membrane proteins [34]. Using electron microscopy, we have reported that Alix downregulation in HeLa cells impairs the budding of intralumenal vesicles inside endosomes [9]. However, contradicting this observation several reports showed that Alix seems to play no role in EGF receptor degradation, a process known to require ESCRT proteins [15–18]. Instead, Doyotte et al. found that sorting of EGF-R and genesis of MVBs require the Alix-related tyrosine phosphatase HD-PTP/PTN23 suggesting that some of the functions attributed to Alix, might in fact be due to HD-PTP [16]. Thus our study demonstrating that neither Alix knock-down nor expression of Alix-CT affect maturation of autophagosomes suggests once more that Alix plays a role clearly distinct from that of ESCRT proteins. Furthermore, our observations demonstrate that the potency of Alix mutants to block cell death *in vivo* and *in vitro* cannot simply be explained by a role of the protein in controlling autophagy, known to be involved in several aspects of cell death.

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