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Penta-EF-hand protein ALG-2 functions as a Ca^{2+}-dependent adaptor that bridges Alix and TSG101

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ABSTRACT

Alix and TSG101, known to physically interact with each other, have Pro-rich regions that are bound by ALG-2 Ca^{2+}-dependently. We investigated the role of ALG-2 in the Alix-TSG101 association by pulldown assays using Strep-tagged Alix and its various mutants. The ALG-2-binding site was required for the Ca^{2+}-dependent pulldown of TSG101 using HEK293T cells, whereas the PSAP sequence, a binding motif for the UEV domain of TSG101, was dispensable. Alix-TSG101 association was not observed using ALG-2-knockdown cells but became detectable by addition of the purified recombinant ALG-2 protein in the assay mixtures. Exogenous expression of mGFP-fused ALG-2 also restored the pulldown capability of Strep-Alix, but an alternatively spliced shorter ALG-2 isoform and a dimerization-defective mutant were incompetent. Based on the X-ray crystal structure model showing the presence of one ligand-binding site in each molecule of an ALG-2 dimer, we propose that Ca^{2+}-loaded ALG-2 bridges Alix and TSG101 as an adaptor protein.

Introduction

Alix (ALG-2-interacting protein X), a homologue of yeast Bro1p, is a cytoplasmic 95-kDa protein that functions in regulation of endocytosis, intraluminal vesicle formation of multivesicular bodies, budding of enveloped RNA viruses, cell death, and cell adhesion in mammals (see Refs. [1–3] for reviews). These multiple functions depend on interactions with various cellular and viral proteins at different regions. The N-terminal Bro1 domain associates with CHMP4 proteins, which are components of endosomal sorting complex required for transport (ESCRT)-III. The middle V domain associates with the p6 domain of HIV-1 Gag [3]. The C-terminal Pro-rich region is less conserved among organisms, but mammalian Alix proteins commonly contain binding motifs for TSG101, CIN85, Src, endophilin A1, and ALG-2 [1–4]. Association of Alix with TSG101 is particularly interesting because TSG101 is a component of ESCRT-I and Alix has a potential ability to bridge ESCRT-I and ESCRT-III [3,5,6]. The UEV domain of TSG101 recognizes a PTAP motif [7] and binds to the PSAP sequence present in the Pro-rich region of Alix [5].

ALG-2, a 22-kDa Ca^{2+}-binding protein, possesses five serially repetitive EF-hand motifs (EF1 to EF5) and belongs to the penta-EF-hand (PEF) family [8]. Previously, we reported that ALG-2 bound Ca^{2+}-dependently to TSG101 at its Pro-rich region [9]. Using lysates of cells exogenously expressing Strep-tagged Alix, endogenous TSG101 has been shown to be recovered in the pulldown products of Strep-Alix in the presence of Ca^{2+}, but not in the absence of Ca^{2+} [10]. Recently, we have determined the X-ray crystal structure of the complex between the PEF domain of ALG-2 and a peptide of the ALG-2-binding site in Alix [11]. ALG-2 forms a dimer and each monomer molecule in the dimer accepts one Alix peptide molecule, indicating the possibility that an ALG-2 dimer bridges two binding molecules and functions as a Ca^{2+}-dependent adaptor protein. In this study, we further investigated the role of ALG-2 in Alix-TSG101 interaction and found that the ALG-2-binding site, but not the PSAP sequence, of Alix is important for the Alix-TSG101 complex formation and that depletion of ALG-2 by RNA interference (RNAi) abolished the interaction. Based on these results together with the results of pulldown assays using ALG-2 proteins, we propose that the ALG-2 dimer functions as a Ca^{2+}-dependent adaptor protein that bridges Alix and TSG101.

Materials and methods

Antibodies and recombinant ALG-2 protein. The following mouse monoclonal antibodies (mAbs) were used for Western blotting: anti-Strep-tag II (IBA GmbH), anti-TSG101 (4A10, GeneTex), and anti-GFP (B2, Santa Cruz). Preparations of rabbit polyclonal antibodies (pAbs) against ALG-2 and Alix were described previously

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[12]. Recombinant human ALG-2 protein was purified by affinity chromatography using a column immobilizing an ALG-2-binding peptide as described previously [13].

Plasmid constructions. Construction of a Strep-tagged mammalian expression vector for wild-type (WT) human Alix was described previously [10]. Expression vectors of Alix mutants in the Pro-rich region were obtained by PCR-based site-directed mutagenesis using specific primers (Supplementary Table), a QuikChange Site-Directed Mutagenesis Kit (Stratagene) and pCMV-3xFLAG-Alix as a template. After confirming the mutations by nucleotide sequencing, the respective EcoRI fragments were subcloned into the EcoRI site of pEXPR-IBA105-B [10]. A full-length isoform of ALG-2 was designated ALG-2^{WT} to discriminate it from the alternatively spliced shorter isoform (lacking $Gly^{121}Phe^{122}$) [14], ALG-$2^{\Delta GF122}$, and a mutant. Silent mutations that render ALG-2 constructs resistant to RNAi were obtained by substituting nucleotides (without changing encoded amino acids) within the RNAi-responsible sequence. Expression vectors for ALG-$2^{\Delta GF122}$ and an amino acid substitution mutant of Tyr-180 to Ala (ALG-2^{Y180A}) were constructed from an RNAi resistant form of ALG-2 cDNA. For more information on plasmid constructions, see Supplementary data.

Cell culture, DNA transfection and Strep-pulldown assays. An ALG-2-knockdown HEK293 cell line (ALG-2_{KD} cells) was established by expression of the short hairpin RNA specific for the ALG-2 mRNA as described previously [12]. For more information, see Supplementary data. One day after cells had been seeded, they were transfected with the expression plasmid DNAs by the conventional calcium phosphate precipitation method and cultured for 24 h. Strep-pulldown assays were performed essentially as described previously [10]. Chemiluminescence signals of Western blotting were detected by LAS-3000mini (Fujifilm) using Super Signal West Pico Chemiluminescent Substrate (PIERCE).

Results

Strep-pulldown assays for Ca^{2+}-dependent Alix-TSG101 association

To investigate which region in Alix is required for the Ca^{2+}-dependent association between Alix and TSG101, we performed pulldown assays using various Alix mutants that lack binding sites for either ALG-2 [15] or TSG101 [5] (Fig. 1A). Cleared cell lysates of HEK293T cells expressing Strep-tagged Alix of either wild-type (WT) or respective mutants were incubated with Strep-Tactin Sepharose beads in the presence of 5 mM EGTA, a Ca^{2+}-chelating reagent, or 100 μM $CaCl_2$. After washing the beads, bound proteins (pulldown products) were subjected to Western blotting using antibodies against TSG101, ALG-2, and Strep-tag. As shown in Fig. 1B, in the presence of EGTA, immunoreactive signals of TSG101 and ALG-2 were not detected in any pulldown products. In the presence of Ca^{2+}, however, these signals were detected not only in the pulldown products of the Strep-tagged wild-type Alix (WT) but also in those of the two 717-PSAP-720 sequence mutants: substituted with alanines (ASAA) and deletion (ΔPSAP). On the other hand, neither TSG101 nor ALG-2 was pulled down with Alix

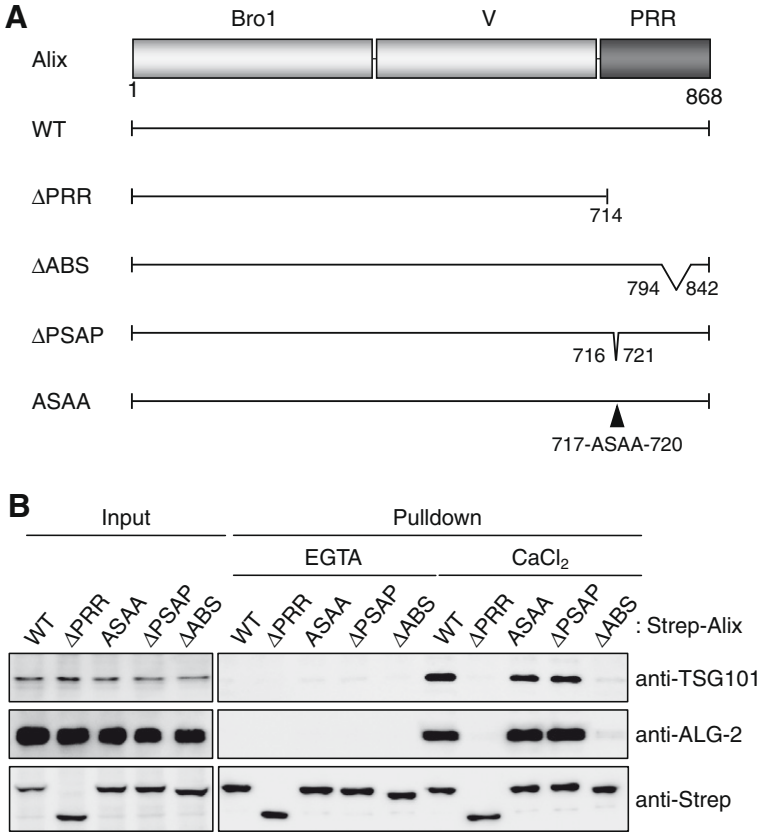

Fig. 1. Essential region in Alix for Ca^{2+}-dependent association with TSG101. (A) Schematic representations of wild-type (WT) and mutants of human Alix used in this study. Bro1, Bro1 domain; V, V domain; PRR, Pro-rich region; ABS, ALG-2-binding site. The numbers indicate amino acid residue numbers (aa). Wedge shapes and closed triangle in mutant proteins indicate deletions (795–841 aa, 717–720 aa) and substitution (717-PSAP-720 to ASAA), respectively. (B) Pulldown assays using Strep-tagged Alix mutants. HEK293T cells were transfected with expression vectors of Strep-tagged $Alix^{WT}$, $Alix^{\Delta PRR}$, $Alix^{ASAA}$, $Alix^{\Delta PSAP}$ or $Alix^{\Delta ABS}$. The cleared cell lysates (Input) were incubated with Strep-Tactin Sepharose beads in 0.5 ml of the binding mixture in the presence of 5 mM EGTA or 100 μM $CaCl_2$ at 4 °C overnight. After washing, proteins bound to the beads (Pulldown) were subjected to SDS–PAGE and Western blotting using respective antibodies indicated.

deletion mutants of the entire Pro-rich region (ΔPRR) and ALG-2-binding site (ΔABS).

Requirement of ALG-2 for the formation of Alix-TSG101 complex

Results of mutant analyses indicate the importance of the ALG-2-binding site in Alix for efficient Alix-TSG101 interaction *in vitro*. To obtain direct evidence that ALG-2 is involved in this interaction, we established an ALG-2-knockdown HEK293 cell line (ALG-2_{KD} cells) by RNAi using a short hairpin RNA expression vector and then performed Strep-Alix pulldown assays. As shown in Fig. 2A, immunoreactive bands corresponding to ALG-2 were barely detectable in the cleared lysates (Input) of ALG-2_{KD} cells, in contrast to the intense signals in those of the parental HEK293 cells. Both TSG101 and ALG-2 were pulled down with Strep-Alix^{WT} in the parental cells but not in the ALG-2_{KD} cells. Neither proteins were detected in the pulldown products of Strep-Alix$^{\Delta ABS}$. Next, we investigated whether exogenously added recombinant ALG-2 compensates Alix-TSG101 association. As shown in Fig. 2B, the amount of TSG101 that was pulled down by Strep-Alix^{WT}, but not by Strep-Alix$^{\Delta ABS}$, increased in proportion to the amount of added recombinant ALG-2 protein.

Analyses of ALG-2 isoform and mutant for Alix-TSG101 association

We previously showed that an alternatively spliced ALG-2 isoform, designated ALG-2$^{\Delta GF122}$, and an amino acid substitution mutant in which Tyr-180 was replaced with alanine (ALG-2^{Y180A}) lost the ability to bind Alix and TSG101 by glutathione-*S*-transferase (GST) pulldown assays [9,11,13]. When the isoform and the mutant in the RNAi-resistant forms were fused with either mGFP or Strep-tag and coexpressed in the ALG-2_{KD} cells, neither endogenous Alix nor TSG101 was pulled down with Strep-ALG-2$^{\Delta GF122}$ or -ALG-2^{Y180A}, but these proteins were pulled down with Strep-ALG-2^{WT} in the presence of Ca^{2+} (Fig. 3A). In the X-ray crystal structure of the complex between ALG-2 and Alix-ABS peptide, the side chains of Phe-122 and Tyr-180 interact with the Alix peptide [11]. Tyr-180 that forms a bottom of one hydrophobic pocket to accept a part of the Alix peptide is derived from the counter molecule of the ALG-2 dimer. While both Strep-ALG-2^{WT} and Strep-ALG-2$^{\Delta GF122}$ pulled down mGFP-ALG-2^{WT} and mGFP-ALG-2$^{\Delta GF122}$, respectively, in the presence and absence of Ca^{2+}, Strep-ALG-2^{Y180A} did not pull down mGFP-ALG-2^{Y180A} under the same conditions, indicating the importance of Tyr-180 for dimerization of ALG-2.

Next, we investigated whether there is a correlation between the ALG-2-dependent Alix-TSG101 interaction and capacities of ALG-2 to bind to Alix and TSG101 by Strep-Alix pulldown assays in which ALG-2 depletion was complemented by exogenous expression of RNAi-resistant mGFP-ALG-2. As shown in Fig. 3B, exogenously expressed mGFP-ALG-2^{WT} was pulled down with coexpressed Strep-Alix as efficiently as endogenous ALG-2 in the parental cells, but mGFP-ALG-2^{Y180A} and mGFP-ALG-2$^{\Delta GF122}$ were barely pulled down. Coexpression with mGFP-ALG-2^{WT} increased the amount of pulled-down TSG101, whereas coexpression with mGFP-ALG-2$^{\Delta GF122}$ or mGFP-ALG-2^{Y180A} showed no significant effects on TSG101 as well as endogenous ALG-2. An immunoreactive band of TSG101 was detected in the pulldown product from ALG-2_{KD} cells when mGFP-ALG-2^{WT} was expressed but not in the case of mGFP (Control), mGFP-fused ALG-2$^{\Delta GF122}$ or ALG-2^{Y180A}.

Discussion

The interaction between TSG101, a component of ESCRT-I, and Alix was first shown in studies on roles of ESCRTs and their associated proteins in HIV-1 budding [5,6]. The recombinant UEV domain of TSG101 was shown to bind specifically to a GST-fused Alix peptide (714–723) containing the PSAP sequence in binding assays *in vitro* [5]. However, the estimated dissociation constant was significantly higher (K_D = 142 μM) than those estimated for TSG101 UEV interaction with HIV-1 peptide of unubiquitinated p6 (K_D = 27 μM) and ubiquitinated p6 (K_D = 2.3 μM) that contained a PTAP sequence [16]. Thus, further investigations of the full-length

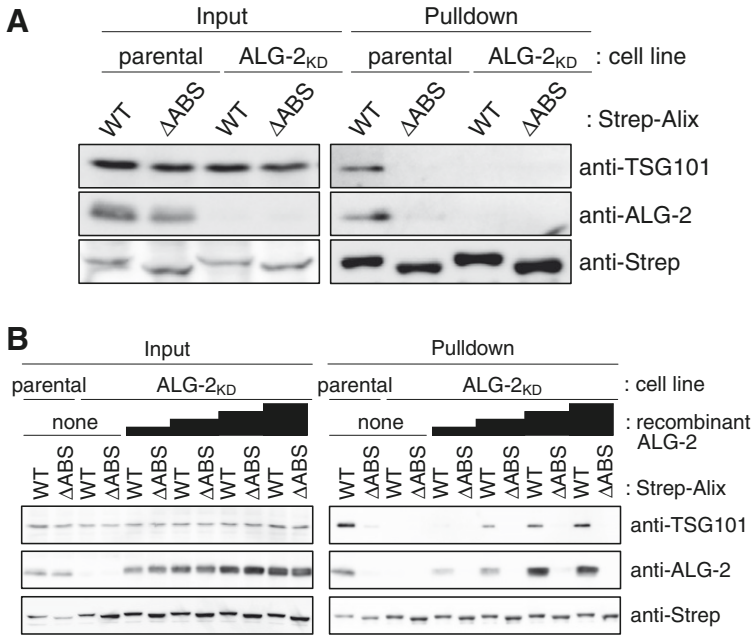

Fig. 2. Strep-Alix pulldown assays using ALG-2-knockdown cells and complementation by recombinant ALG-2. (A) Loss of interaction between Alix and TSG101 in the ALG-2-knockdown (ALG-2_{KD}) HEK293 cells. Parental and ALG-2_{KD} cells were transfected with either pStrep-Alix^{WT} or pStrep-Alix$^{\Delta ABS}$. The cleared cell lysates (Input) were subjected to Strep-pulldown assays in the presence of 100 μM $CaCl_2$. (B) Restoration of Ca^{2+}-dependent Alix-TSG101 association by recombinant ALG-2. The cleared cell lysates were subjected to Strep-pulldown assays in the presence of increasing amounts (0, 0.1, 0.2, 0.3, 0.4 μg) of purified recombinant human ALG-2 protein in 0.5 ml of the binding mixture containing 100 μM $CaCl_2$.

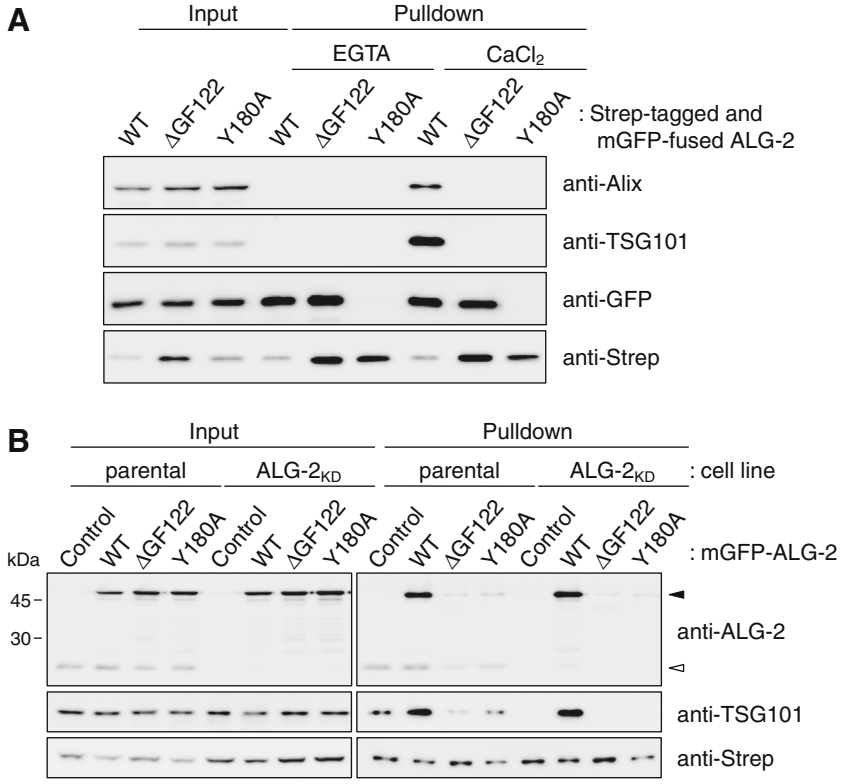

Fig. 3. Analyses of ALG-2 isoform and mutant for Alix-TSG101 association. (A) Strep-ALG-2 pulldown assays for characterization of ALG-2 isoform and mutant. ALG-2_{KD} cells were cotransfected with each pair of RNAi-resistant forms of vectors expressing Strep-tagged and mGFP-fused ALG-2 (ALG-2^{WT}; ALG-$2^{\Delta GF122}$; ALG-2^{Y180A}). The cleared cell lysates (Input) were subjected to pulldown assays as described in the legend to Fig. 1B. Western blotting was performed using respective antibodies indicated. Relative amounts of analyzed respective proteins in the samples of Input compared to Pulldown: anti-Alix, 1.5%; anti-TSG101, 1.5%; anti-GFP, 3%; anti-Strep, 15%. (B) Strep-Alix pulldown assays for complemented ALG-2 proteins in ALG-2_{KD} cells. Parental and ALG-2_{KD} cells were cotransfected with pStrep-$Alix^{WT}$ and with either pmGFP-C1 (control), pmGFP-ALG-2^{WT}, pmGFP-ALG-$2^{\Delta GF122}$ or pmGFP-ALG-2^{Y180A}. The cleared cell lysates (Input) were subjected to Strep-Alix pulldown assays. Western blotting was performed using respective antibodies indicated. Endogenous ALG-2 (open arrowhead) and mGFP-fused ALG-2 proteins (closed arrowhead) were detected using anti-ALG-2 antibody.

proteins of Alix and TSG101 and cellular environments such as presence of other cellular factors were needed to understand the mechanism of Alix-TSG101 interaction.

In this study we performed Strep-pulldown assays using Strep-tagged Alix and its mutants in mammalian cells. In these assays, endogenous TSG101 was pulled down with Alix in the presence of Ca^{2+} but not in the absence of Ca^{2+} (Fig. 1B), confirming our previous results [10]. We found that the PSAP sequence in Alix is dispensable but that the ALG-2-binding site (ABS) is essential for the Ca^{2+}-dependent Alix-TSG101 association (Figs. 1B and 2). Recently, CEP55 (centrosomal protein 55) was reported to associate with both TSG101 and Alix [17,18]. Although the CEP55-binding site in Alix overlaps with the ALG-2-binding site [11,19], the observed Alix-TSG101 interaction in our pulldown assays depends on ALG-2, but it may not depend on CEP55 on the basis of the following observations: (i) the interaction is Ca^{2+}-dependent (Fig. 1B) and (ii) depletion of ALG-2 by RNAi abolished the Alix-TSG101 interaction (Fig. 2A) and the interaction was recovered and augmented by exogenously added recombinant ALG-2 protein in a dose-dependent manner (Fig. 2B) as well as by exogenous expression of mGFP-ALG-2^{WT} in ALG-2-knockdown (ALG-2_{KD}) cells (Fig. 3B). This complementation was not achieved by mGFP-ALG-$2^{\Delta GF122}$ and mGFP-ALG-2^{Y180A} (Fig. 3B) that lack abilities to bind to Alix and TSG101 (Fig. 3A).

The X-ray crystal structure of the complex between ALG-2 and the Alix-ABS peptide indicates that ALG-2 forms a dimer through the paired fifth EF-hands (EF5) and that each ALG-2 molecule in the dimer has one Alix-binding site, *i.e.*, two binding sites in the dimer [11]. A bottom of one of the hydrophobic pockets accepting the Alix peptide is formed by Tyr-180 that is positioned at EF5 of the counter ALG-2 molecule in the dimer. Substitution of Tyr-180 to Ala caused disruption of dimer formation and the mutant was unable to bind to Alix and TSG101, indicating that monomeric ALG-2 does not have the capacity to bind to these proteins. Therefore, we propose that ALG-2 in a dimeric form functions as a Ca^{2+}-dependent adaptor that bridges Alix and TSG101 (Fig. 4). In this model, Alix directly associates with TSG101 through a weak interaction between the PSAP sequence in Alix and the UEV domain of TSG101, and this complex is stabilized by the Ca^{2+}-bound ALG-2 dimer, which binds to the Pro-rich regions of the respective target

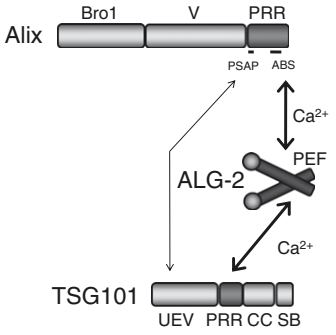

Fig. 4. A model of Ca^{2+}-dependent adaptor function of ALG-2. Each molecule of ALG-2 dimer binds to the Pro-rich regions (PRRs) of Alix and TSG101, respectively, in a Ca^{2+}-dependent fashion. The UEV domain of TSG101 and PEF domain of ALG-2 recognize the PSAP sequence and ALG-2-binding site (ABS) in Alix, respectively. Abbreviations of domains or regions: CC, coiled coil; PEF, penta-EF-hand; SB, steadiness box; UEV, ubiquitin E2 variant; V, V domain.

proteins. The alternatively spliced isoform, ALG-2$^{\Delta GF122}$, is inactive for this function for Alix-TSG101 bridging (Fig. 3B). Since ALG-2 also forms a heterodimer with a closely related PEF protein named peflin [20], the bridging activity of ALG-2 may depend not only on the level of the ALG-2 protein itself but also on ratios of the short isoform and peflin in the cell. Since ALG-2 binds to a variety of cytoplasmic, nuclear and membrane proteins in addition to Alix and TSG101 [4,12,13,21,22], specificities of Ca^{2+}-dependent adaptor activities of ALG-2 need to be clarified in future studies.

Note added in proof

Knock down of ALG-2 with 90% reduction of the protein did not completely abolish colocalization of exogenously expressed Alix and TSG101 in HeLa cells by fluorescence microscopic analyses (data not shown) suggesting that the role of ALG-2 is to enhance the affinity for Alix for TSG101 as suggested by these over expression experiments but the involvement of other proteins in this interaction cannot be ruled out and is presently under consideration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.06.015.

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