The penta-EF-hand protein ALG-2 interacts directly with the ESCRT-I component TSG101, and Ca^{2+} -dependently co-localizes to aberrant endosomes with dominant-negative AAA ATPase SKD1/Vps4B

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ALG-2 (apoptosis-linked gene 2) is a Ca^{2+} -binding protein that belongs to the PEF (penta-EF-hand) protein family. Alix (ALG-2-interacting protein X)/AIP1 (ALG-2-interacting protein 1), one of its binding partners, interacts with TSG101 and CHMP4 (charged multivesicular body protein 4), which are components of ESCRT-I (endosomal sorting complex required for transport I) and ESCRT-III respectively. In the present study, we investigated the association between ALG-2 and ESCRT-I. By a GST (glutathione S-transferase) pull-down assay using HEK-293T (human embryonic kidney 293T) cell lysates, endogenous TSG101 and two other exogenously expressed ESCRT-I components [hVps28 (human vacuolar protein sorting 28) and hVps37A] were shown to associate with GST–ALG-2 in the presence of Ca^{2+} . By the yeast two-hybrid assay, however, a positive interaction was observed with only TSG101 among the three ESCRT-I components, suggesting that ALG-2 associates with hVps28 and hVps37A indirectly through TSG101. Using various deletion mutants of TSG101, the central PRR (proline-rich region) was found to be sufficient for interaction with ALG-2 by the GST-pull-down assay.

Direct binding of ALG-2 to the TSG101 PRR was demonstrated by an overlay assay using biotin-labelled ALG-2 as a probe. In immunofluorescence microscopic analysis of HeLa cells that overexpressed a GFP (green fluorescent protein)-fused ATPase-defective dominant-negative form of SKD1/Vps4B (GFP–SKD1^{E235Q}), ALG-2 exhibited a punctate distribution at the perinuclear area and co-localized with GFP–SKD1^{E235Q} to aberrant endosomes. This punctate distribution of ALG-2 was markedly diminished by treatment of HeLa cells with a membrane-permeant Ca^{2+} chelator. Moreover, a Ca^{2+} -binding-defective mutant of ALG-2 did not co-localize with GFP–SKD1^{E235Q}. Our findings suggest that ALG-2 may function as a Ca^{2+} -dependent accessory protein of the endosomal sorting machinery by interacting directly with TSG101 as well as with Alix.

Key words: apoptosis-linked gene 2 (ALG-2), calcium-binding protein, EF-hand, endosomal sorting complex required for transport (ESCRT), multivesicular endosome, TSG101.

INTRODUCTION

ALG-2 (apoptosis-linked gene 2) is a 22 kDa calcium-binding protein that was originally identified as a proapoptotic protein by a genetic death trap screen [1]. Expression of ALG-2 antisense RNA protects mouse T-cell hybridoma 3D0 from apoptosis induced by various stimuli such as T-cell receptor cross-linking. Although ALG-2-deficient mice showed no abnormality in development and immune functions [2], it was recently suggested that ALG-2 mediates endoplasmic-reticulum-stress-induced cell death [3]. ALG-2 belongs to a PEF (penta-EF-hand) protein family that consists of sorcin, grancalcin, peflin and the large and small subunits of conventional calpains and ALG-2 is the most conserved PEF protein in eukaryotes [4–7]. ALG-2 forms a homodimer or heterodimer with peflin [8] and has been shown to interact in a Ca^{2+} -dependent manner with some proteins, such as Alix (ALG-2-interacting protein X)/AIP1 (ALG-2-interacting protein 1) [9,10], annexin VII and annexin XI [11,12], by directly binding to their PRRs (proline-rich regions) [11–13].

Alix has recently been recognized as a regulator that functions in the endosomal pathway [14,15]. In addition to antagonizing EGFR (epidermal growth factor receptor) down-regulation by the Cbl-SETA/CIN85 complex [16], Alix is involved in endosomal sorting of EGFR and budding of retroviruses from plasma membranes by interacting with TSG101, CHMP4 (charged multivesicular body protein 4) and Gag proteins [17–21]. TSG101 and CHMP4 are components of ESCRT-I (endosomal sorting complex required for transport I) and ESCRT-III respectively. CHMP6, a CHMP4-binding partner, is a myristoylated component of ESCRT-III and interacts with an ESCRT-II component, EAP20 [22]. ESCRTs and their associating proteins are conserved from yeast to humans and function in the sorting of ubiquitinated cargoes into intraluminal vesicles that are generated by inward budding of the endosomal membrane of the so-called MVB (multivesicular body) or MVE (multivesicular endosome) (see [23–25], for reviews). Enveloped RNA viruses usurp the endosomal sorting system for budding of virus particles from plasma membranes (see [26] for a review).

Abbreviations used: AD, GAL4 transcription-activation domain; ALG-2, apoptosis-linked gene 2; AIP1, ALG-2-interacting protein 1; Alix, ALG-2-interacting protein X; BAPTA/AM, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester); BD, GAL4 DNA-binding domain; bio-ALG-2, biotin-labelled ALG-2; CC, coiled-coil; CHMP, charged multivesicular body protein; EEA1, early endosome antigen 1; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; EGF, epidermal growth factor; EGFR, EGF receptor; ESCRT, endosomal sorting complex required for transport; GFP, green fluorescent protein; GST, glutathione S-transferase; HEK-293T cells, human embryonic kidney 293T cells; HSP90, heat-shock protein 90; Vps, vacuolar protein sorting; hVps, human Vps; Lamp-1, lysosome-associated membrane protein-1; LBPA, lysobisphosphatidic acid; mAb, monoclonal antibody; MVB, multivesicular body; pAb, polyclonal antibody; PEF, penta-EF-hand; PRR, proline-rich region; SB, steadiness box; UEV, ubiquitin E2 variant.

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TSG101 has a UEV (ubiquitin E2 variant) domain, PRR, CC (coiled-coil) region and SB (steadiness box) domain. The UEV domain contains a sequence similar to that of ubiquitin E2 conjugating enzyme. Although the UEV domain is unable to catalyse ubiquitin transfer due to lack of a catalytic cysteine residue required for thioester bond formation with ubiquitin, it functions as an ubiquitin-binding domain [27]. The membrane-associated structural proteins of enveloped RNA viruses such as retrovirus Gag and Ebola virus VP40 are ubiquitinated, and TSG101 plays a role in budding of virus particles [28–30]. Moreover, the UEV domain recognizes PTAP/PSAP motifs found not only in these viral proteins [28] but also in the N-terminal part of human Alix/AIP1 PRR [21].

Previously, we reported that a region containing PxY repeats in the central part of Alix/AIP1 PRR was sufficient for binding to ALG-2 [13]. Since the binding sites for TSG101 and ALG-2 are separated in Alix/AIP1, at least on the primary structural level, we investigated in the present study whether ALG-2 associates with TSG101 through interaction with Alix/AIP1. Unexpectedly, however, we found that ALG-2 directly binds to TSG101 in a Ca^{2+} -dependent manner, and the results obtained suggest that ALG-2 participates in a Ca^{2+} -regulated modulator in membrane trafficking by interaction with both TSG101 and Alix/AIP1.

MATERIALS AND METHODS

Antibodies

The following mouse mAbs (monoclonal antibodies) were used: anti-TSG101 mAb (4A10) (GeneTex, San Antonio, TX, U.S.A.), anti-GFP (green fluorescent protein) mAb (B-2) (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-HSP90 (heat-shock protein 90) mAb (Transduction Laboratories, San Diego, CA, U.S.A.), anti-FLAG mAb (M2) and anti-Myc mAb (9E10) (Sigma). Rabbit pAbs (polyclonal antibodies) raised against Myc-epitope oligopeptide, FLAG tag and GFP were purchased from Santa Cruz Biotechnology, Sigma and Invitrogen respectively. Preparation of anti-ALG-2 rabbit pAb has been described previously [31]. Peroxidase-conjugated goat anti-rabbit and anti-mouse IgG antibodies were obtained from Jackson Immuno-research Laboratories (West Grove, PA, U.S.A.). Cy3-labelled anti-rabbit antibodies used for indirect immunofluorescence analyses were purchased from Amersham Biosciences. See Supplementary Material and methods at <http://www.BiochemJ.org/bj/391/bj3910677add.htm> for details of other antibodies used.

Mammalian expression plasmids

pBj-Myc-TSG101 and pBj-Myc-TSG101 Δ C were constructed previously [30]. pBj-Myc-TSG 101UEV was obtained by creating a stop codon in pBj-Myc-TSG101 Δ C with a PCR-based QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) using the following primers: 5'-GTCTTCTCTCG-TCCTTAAGCGGCATCCTATCCGC-3' and 5'-GCGGATAGG-ATGCCGCTTAAGGACGAGAGAAGAC-3'. pGFP-TSG101 was constructed by ligating a BamHI fragment derived from pBj-Myc-TSG101 into pEGFP-C1 (ClonTech, Palo Alto, CA, U.S.A.). To construct pGFP-TSG101 PRR, a cDNA fragment encoding a PRR of TSG101 (residues 146–218) was first amplified by PCR using forward and reverse primers containing additional overhanging BglII and EcoRI sites respectively, and a BglII–EcoRI fragment was subcloned into the BglII–EcoRI sites of pEGFP-C1. A cDNA encoding hVps28 (human vacuolar protein sorting 28) was amplified by PCR from the human spleen Marathon-Ready cDNA library (ClonTech). After KpnI sites had

been introduced by the standard PCR method into the hVps28 cDNA fragment at both the 5'- and 3'-ends, a KpnI fragment was subcloned into the KpnI site of pcDNFL that had an insertion of FLAG sequence between the XbaI and KpnI sites of pcDNA3.1/Hygro (Invitrogen), and the obtained plasmid was designated pFLAG-hVps28. The insertion of the hVps28 cDNA at the KpnI site of pEGFP-C3 (ClonTech) resulted in construction of pGFP-hVps28. A cDNA encoding hVps37A was obtained by PCR amplification from a HeLa cDNA library that had been cloned in the vector pGAD GH (ClonTech). EcoRI sites were introduced by the standard PCR method into the hVps37A cDNA fragment at both 5'- and 3'-ends, and the EcoRI fragment was subcloned into pEGFP-C2 (ClonTech) to construct pGFP-hVps28.

Construction of mammalian expression plasmids pFLAG-Alix, pFLAG-ALG-2 and pFLAG-ALG-2^{E47A/E114A} was described previously [8, 13]. pGFP-ALG-2 was constructed by ligating an EcoRI–SalI fragment derived from pFLAG-ALG-2 into the EcoRI–SalI sites of pEGFP-C1. Plasmids for expression of short Alix fragments fused with GFP (pGFP-Alix 735–751 and pGFP-Alix 795–827) were constructed by inserting double-stranded oligonucleotide blocks encoding amino acid residues 735–751 and 795–827 of Alix into pEGFP-C2 and pEGFP-C1 respectively, essentially as described previously [13]. pGFP-SKD1^{E235Q} [32] was kindly provided by Dr T. Yoshimori (National Institute of Genetics, Mishima, Japan).

Expression and purification of GST (glutathione S-transferase) fusion proteins

The following plasmids were constructed for expression of GST-fused ALG-2 proteins in *Escherichia coli*. pFLAG-ALG-2^{ΔGF122} lacking Gly¹²¹ and Phe¹²² was constructed by PCR-based site-directed mutagenesis using pFLAG-ALG-2 as a template. pFLAG-ALG-2, pFLAG-ALG-2^{ΔGF122} and pFLAG-ALG-2^{E47A/E114A} [13] were digested with EcoRI and XhoI, and each EcoRI–XhoI DNA fragment encoding the respective wild-type and mutant ALG-2 proteins lacking a FLAG epitope was subcloned into the EcoRI–XhoI sites of pGEX4T-3 (Amersham Biosciences). The constructed plasmids were designated pGST-ALG-2, pGST-ALG-2^{ΔGF122} and pGST-ALG-2^{E47A/E114A} respectively and used for transformation of *E. coli* BL21 cells. GST fusion proteins were purified by binding to glutathione–Sepharose 4B beads (Amersham Biosciences) according to the manufacturer's instructions. GST fusion proteins were eluted from Sepharose beads with 10 mM GSH in 50 mM Tris/HCl (pH 8.5) and dialysed against 50 mM Tris/HCl (pH 7.5) and then stored at 4 °C until use.

Cell culture, transfection and treatment with BAPTA/AM [bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester)]

HEK-293T (human embryonic kidney 293T) and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) at 37 °C under humidified air containing 5% CO₂. One day after HEK-293T or HeLa cells had been seeded, the cells were transfected with the expression plasmid DNAs by the conventional calcium phosphate precipitation method or by using FuGENE6 (Roche, Indianapolis, IN, U.S.A.) and cultured for 24 h. In the case of treating cells with intracellular Ca²⁺ chelator or Ca²⁺ ionophore, the cells were incubated with a medium containing either 10 µM BAPTA/AM (dissolved in DMSO) for 1 h or 10 µM A23187 for 10 min before analysis.

Subcellular fractionation

Subcellular fractionation was performed essentially as described previously [8]. The post-nuclear P2/3 fraction (100 000 g pellet) was subjected to membrane floatation analysis essentially as described previously [33]. Briefly, the P2/3 fraction was resuspended in 0.17 ml of 20 mM Hepes/NaOH (pH 7.4), 0.1 mM dithiothreitol and 8 % (w/w) sucrose, and then mixed with 0.33 ml of 61 % sucrose. The sucrose cushion was overlaid with three additional layers of 35 % (0.65 ml), 30 % (0.45 ml) and 8 % (0.4 ml) sucrose. The gradient was subjected to centrifugation at 100 000 g (40 000 rev./min by a Beckman TLS-55 rotor) for 6 h at 4 °C, and fractions were collected from top to bottom of the 2 ml centrifuge tube by an automatic density gradient fraction collector (Avantec model CHD255AA).

GST pull-down assay

HEK-293T cells transfected with various expression vectors were harvested and washed with PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄ and 1.5 mM KH₂PO₄, pH 7.3) and then lysed in lysis buffer A {10 mM Hepes/NaOH, pH 7.4, 142.5 mM KCl, 0.2 % Nonidet P40, 0.1 mM Pefabloc, 25 µg/ml leupeptin, 1 µM E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane] and 1 µM pepstatin}. Either 1 mM CaCl₂ or 100 mM EGTA was added to supernatants obtained by centrifugation at 10 000 g (cleared lysates) to a final concentration of 10 µM or 1 mM respectively and then the mixtures were incubated with 10 µg of GST or GST fusion proteins that had been immobilized on glutathione–Sepharese beads for 3 h at 4 °C. After the beads had been recovered by a low-speed centrifugation and washed three times with the lysis buffer A used for binding, the bead-bound proteins (pull-down products) were subjected to SDS/PAGE followed by Western blotting using PVDF membranes (Immobilon-P; Millipore, Bedford, MA, U.S.A.). Signals were detected by the chemiluminescence method using Super Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL, U.S.A.).

Immunofluorescence microscopic analysis

HeLa cells grown on coverslips were fixed in 4 % (w/v) paraformaldehyde/PBS and permeabilized in 0.1 % Triton X-100/PBS. After blocking with 0.1 % gelatin/PBS, the cells were incubated first with primary antibodies either at 4 °C overnight or at room temperature (22–25 °C) for 1 h and then with secondary antibodies at room temperature for 1 h. Finally, they were mounted with anti-fading solution [25 mM Tris/HCl, pH 8.7, 10 % polyvinyl alcohol, 5 %, v/v, glycerol and 2.5 % 1,4-diazobicyclo (2,2,2)-octane]. The fluorescence signals of GFP- and Cy3-labelled secondary antibody (anti-rabbit or anti-mouse IgG) were analysed with a confocal laser-scanning microscope, LSM5 PASCAL (Carl Zeiss, Thornwood, NY, U.S.A.).

Yeast two-hybrid assay

The MATCHMAKER Two-Hybrid System, including yeast strain (AH109) and vectors, was obtained from ClonTech. To construct the expression vectors for AD (GAL4 transcription-activation domain) fusion proteins (prey), each cDNA encoding one among ALG-2, TSG101, hVps28 and hVps37A was subcloned into pGAD424 by the conventional gene manipulation method. Construction of pGBT9-ALG-2 and construction of pGAD424-Alix 681–868 were described previously [11,13]. To construct expression vectors of the BD (GAL4 DNA-binding domain) fusion proteins (bait), cDNAs encoding full-length TSG101 and

an Alix fragment (amino acid residues 681–868) were subcloned into pGBT9 and pGBKT7 respectively. Interaction was evaluated by growth on nutrient dropout medium plates as described previously [11].

Miscellaneous

An overlay assay using bio-ALG-2 (biotin-labelled ALG-2) was performed as described previously [11] except that the signals were detected by the same chemiluminescence method as that used for Western blotting in the present study. Mutations by site-directed mutagenesis, insertion of oligonucleotide blocks and recombinant plasmid constructs were essentially confirmed by nucleotide sequencing with an automated fluorescent sequencer, ABI PRISM 310 (PE Applied Biosystems, Foster City, CA, U.S.A.), using a BigDyeTM terminator cycle sequencing ready reaction kit (PE Applied Biosystems).

RESULTS

Ca²⁺-dependent association between ALG-2 and TSG101

To determine whether ALG-2 interacts with endogenous TSG101 in a Ca²⁺-dependent manner, we first performed a pull-down assay using recombinant GST–ALG-2 immobilized on glutathione–Sepharese beads and lysates of HEK-293T cells in the presence or absence of Ca²⁺. Endogenous TSG101 was detected by Western blotting using an anti-TSG101 antibody in the pellets co-precipitated with GST–ALG-2 in the presence of 10 µM Ca²⁺, whereas the signal for TSG101 was barely detectable in the presence of EGTA (Figure 1A). TSG101 was not detected under either condition in the pull-down products of GST that were used as a negative control (Ctrl). Next, to clarify whether the requirement of Ca²⁺ for ALG-2–TSG101 interaction was due to the action of Ca²⁺ on the ALG-2 molecule itself or due to an effect on ESCRT-I and/or associating proteins, we employed Ca²⁺-binding-defective ALG-2 mutants for the binding assay. ALG-2 binds to three Ca²⁺ molecules at two high-affinity sites, EF1 and EF3, and one low-affinity site, EF5 [34,35]. It was previously shown that double mutation of glutamic residues to alanines in both EF1 [E47A (Glu⁴⁷ → Ala)] and EF3 (E114A) abolishes the ability of ALG-2 to bind Ca²⁺ and Alix/AIP1 [13,34]. Tarabykina et al. [36] reported that an alternatively spliced isoform of mouse ALG-2, designated ALG-2.1, which lacks the two amino acid residues Gly¹²¹ and Phe¹²², has a lower sensitivity to Ca²⁺ than does full-length ALG-2, and that ALG-2.1 lacks the ability to interact with mouse Alix. We found a similar two-residue-shorter ALG-2 sequence in the human EST (expressed sequence tag) database and designated the isoform ALG-2^{ΔGF122} in the present study. As shown in Figure 1(B), TSG101 was not detected in the pull-down products by either GST–ALG-2^{E47A/E114A} or GST–ALG-2^{ΔGF122} in the presence of Ca²⁺.

Binding of ALG-2 to ESCRT-I

When transiently overexpressed in HEK-293T cells, Myc-epitope-tagged or GFP-tagged TSG101 was insoluble in the lysis buffer containing non-ionic detergents. To overcome this problem, we co-expressed epitope-tagged hVps28 (FLAG–hVps28 or GFP–hVps28), another component of ESCRT-I, according to the report by Martin-Serrano et al. [37]. Indeed, we could recover most of the exogenously expressed Myc-TSG101 from the soluble fraction of co-transfected HEK-293T cells (see Supplementary Figure S1 at <http://www.BiochemJ.org/bj/391/bj3910677add.htm>). Both epitope-tagged TSG101 and hVps28 were pulled

Figure 1 Ca²⁺-dependent pull-down of ESCRT-I components with GST-ALG-2

HEK-293T cells were lysed with lysis buffer A (10 mM Hepes/NaOH, pH 7.4, 142.5 mM KCl, 0.2% Nonidet P40, 0.1 mM Pefabloc, 25 µg/ml leupeptin, 1 µM E-64 and 1 µM pepstatin), and the cleared lysate was incubated with glutathione-Sepharose beads carrying GST (Ctrl) or GST-ALG-2 (ALG-2) in the presence of 1 mM EGTA or 10 µM CaCl₂, followed by low-speed centrifugation. After washing with lysis buffer A used for binding, the pellets (pull-down products) were analysed by Western blotting (WB) using anti-TSG101 antibody. **(B)** The cleared lysate of HEK-293T cells was incubated with glutathione-Sepharose beads carrying GST (Ctrl), GST-ALG-2 (WT), GST-ALG-2^{E47A/E114A} (E47A/E114A) or GST-ALG-2^{ΔGF122} (ΔGF122) in the presence of 10 µM CaCl₂, and the pull-down products were analysed by Western blotting. **(C)** The cleared lysates of HEK-293T cells expressing Myc-TSG101, GFP-hVps28 and GFP-hVps37A were subjected to GST-ALG-2-pull-down assays in the presence of 10 µM CaCl₂, and the pull-down products were analysed by Western blotting using either anti-Myc pAb (left panel) or anti-GFP mAb (right panel).

down with GST-ALG-2 (see Supplementary Figure S2 at <http://www.BiochemJ.org/bj/391/bj3910677add.htm>). A third component of mammalian ESCRT-I, Vps37, has been recently identified and four genetically different isoforms (Vps37A–Vps37D) have been reported [38–40]. We further examined whether ALG-2 interacts with ESCRT-I containing Vps37. As shown in Figure 1(C), GFP-hVps37A was also pulled down with GST-ALG-2 from the lysate of HEK-293T cells co-expressing Myc-TSG101, GFP-hVps28 and GFP-hVps37A. To examine the effects of Alix on association of ALG-2 with TSG101, we performed a GST-ALG-2-pull-down assay using lysates of the HEK-293T cells that had been co-transfected with pGFP-TSG101, pGFP-hVps28 and either pFLAG-Alix or an empty vector. Although we expected an increase in the amount of co-precipitated GFP-TSG101 in the case of co-expression with FLAG-Alix, intensities of the detected

Figure 2 Yeast two-hybrid assay

Yeast AH109 cells were co-transformed with the expression plasmids for fusion proteins of both BD (bait) and AD (prey) and selected on a double dropout (SD/-Trp/-Leu) medium plate. Each colony was streaked on selection medium plates of double dropout and quadruple dropout (SD/-Trp/-Leu/-His/-Ade) and incubated for 6 days at 30 °C.

signals in the presence and absence of co-expressed FLAG-Alix were not significantly different (see Supplementary Figure S2 at <http://www.BiochemJ.org/bj/391/bj3910677add.htm>). These results raised two possibilities: (i) the endogenous level of Alix is sufficient to mediate the indirect association between ALG-2 and TSG101 and (ii) ALG-2 directly associates with component(s) of ESCRT-I.

To explore the possibility of direct binding of ALG-2 to each component of ESCRT-I, we performed a yeast two-hybrid assay using proteins fused with the BD and proteins fused with the AD as bait and prey respectively. Yeast transformants expressing BD-ALG-2 grew well on a quadruple dropout medium (SD/-Trp/-Leu/-His/-Ade) plate when co-expressed with AD-TSG101, AD-Alix 681–868 (containing PRR, positive control) or AD-ALG-2 (dimer formation, positive control) but did not grow when co-expressed with one among AD-hVps28, AD-hVps37A and AD (negative control) (Figure 2), indicating specific interaction between ALG-2 and TSG101 in this assay. The yeast transformant expressing BD-Alix 681–868 and AD-Alix 681–868 also grew well. Positive self-interactions of both full-length proteins of TSG101 and Alix revealed by yeast two-hybrid assays were previously reported by other groups [19,21]. The C-terminal one-fifth of Alix was found to be sufficient for self-interaction in the present study.

ALG-2-binding domain in TSG101

Previously, the UEV domain of TSG101 was shown to interact with the PSAP motif in Alix [21]. To delineate the essential domain in TSG101 for interaction with ALG-2, we prepared

Figure 3 Determination of ALG-2-binding domain in TSG101

(A) TSG101 is schematically represented with four defined domains: UEV, PRR, CC and SB. Myc- or GFP-tagged TSG101 full-length and truncated mutants contain fragments of TSG101 indicated by residue numbers. HEK-293T cells were co-transfected with pGFP-hVps28 and expression vectors for full-length or deletion mutants of Myc-tagged TSG101 (B) or transfected with pGFP-TSG101 PRR (C). After 48 h, cells were lysed in the presence of 10 μ M CaCl₂ and the cleared lysates were subjected to pull-down assays with glutathione–Sepharose beads carrying GST (Ctrl) or GST–ALG-2 (ALG-2), and then the co-precipitated proteins were analysed by Western blotting using anti-Myc pAb (B) or anti-GFP mAb (C).

two C-terminally truncated mutants that were tagged with Myc epitope and one deletion mutant encoding only PRR (residues 146–218) that was fused with GFP (Figure 3A). Positive signals were detected in the GST–ALG-2-pull-down products for Myc-TSG101 ΔC and Myc-TSG101 (Full) as expected (Figure 3B). However, the pull-down products did not contain Myc-TSG101 UEV, suggesting that the interaction between TSG101 and ALG-2 requires PRR of TSG101. On the other hand, GFP-TSG101 PRR could be pulled down (Figure 3C), indicating that PRR is sufficient for the interaction with ALG-2.

Direct binding of ALG-2 to TSG101 PRR

Although the results of the yeast two-hybrid assay implied a direct interaction between ALG-2 and TSG101, the possibility of indirect interaction mediated by their associating proteins in yeast cells could not be excluded. To obtain further evidence of direct interaction between ALG-2 and TSG101, we first attempted to prepare recombinant GST–TSG101 PRR in *E. coli* but could not express the protein, probably due to an extremely high sensitivity to proteolysis in bacteria. We therefore employed the overlay method using bio-ALG-2, which had been successfully used to

Figure 4 ALG-2 overlay assay

HEK-293T cells were transfected with the expression vectors of GFP fused with TSG101 PRR or fused with Alix C-terminal fragments. The cleared cell lysates were immunoprecipitated with anti-GFP pAb. The pellets were subjected to SDS/PAGE and transferred on to a PVDF membrane that was then probed either with biotin-labelled ALG-2 (bio-ALG-2) for overlay assay (upper panel) or with anti-GFP mAb for Western blotting (lower panel).

detect direct Ca²⁺-dependent interactions between ALG-2 and its target proteins blotted on to a PVDF membrane after SDS/PAGE [11–13]. In HEK-293T cells, we expressed GFP-TSG101 PRR and two Alix truncated mutants, GFP–Alix 735–751 (containing a CIN85-binding motif; see [41] and H. Shibata, H. Takahashi and M. Maki, unpublished work) and GFP–Alix 795–827 (containing ALG-2-binding PxY repeats [13]), as a negative and a positive control respectively. As shown in Figure 4, signals of bio-ALG-2 were detected for GFP–TSG101 PRR and GFP–Alix 795–827 but not for negative controls (GFP and GFP–Alix 735–751).

Ca²⁺-dependent localization of ALG-2 to SKD1^{E235Q}-induced aberrant endosomal compartments and membranous fractions

Protein molecules involved in endosomal sorting are known to accumulate on endosomal membranes in cells expressing dominant-negative AAA ATPase Vps4/SKD1 in yeast and mammals [17,32,42–45]. In HeLa cells transiently expressing GFP-tagged SKD1^{E235Q}, which is identical with Vps4B and lacks the ability of ATP hydrolysis [32], fluorescent signals of FLAG–ALG-2 detected by anti-FLAG mAb (M2) and Cy3-labelled anti-mouse IgG antibody (red) exhibited a punctate pattern in the perinuclear area and merged with the fluorescent signals of GFP–SKD1^{E235Q} (green) (Figure 5A, a–c). In contrast, the Ca²⁺-binding-defective mutant FLAG–ALG-2^{E47A/E114A} showed a diffuse distribution pattern in the cytoplasm and nucleus even when GFP–SKD1^{E235Q} was co-expressed (Figure 5A, d–f).

When endogenous ALG-2 was detected with anti-ALG-2 pAb, a similar heavily punctated subcellular distribution of ALG-2 and its partial co-localization with GFP–SKD1^{E235Q} were observed in HeLa cells expressing GFP–SKD1^{E235Q} (Figure 5B, a–c) but only fine dotted distribution of ALG-2 was observed in untransfected cells in the same microscopic field (right bottom corner). To clarify further whether the co-localization of ALG-2 with GFP–SKD1^{E235Q} depends on Ca²⁺, we examined the effect of BAPTA/AM, a membrane-permeant Ca²⁺ chelator, on the ALG-2 distribution. Addition of BAPTA/AM to the culture medium of HeLa cells for 1 h before immunostaining caused a diffuse distribution pattern of ALG-2 in the cytoplasm but did not change the distribution pattern of GFP–SKD1^{E235Q} as representatively shown in Figure 5(B, d–f). We quantified the effect of BAPTA/AM on the ALG-2 distribution by counting the number

Figure 5 Ca^{2+} -dependent co-localization of ALG-2 with GFP-SKD1^{E235Q}

(A) HeLa cells were co-transfected with pGFP-SKD1^{E235Q} and either with pFLAG-ALG-2 (a–c) or with pFLAG-ALG-2^{E47A/E114A} (d–f). After 24 h, cells were subjected to immunofluorescence confocal microscopic analysis by direct visualization of GFP-SKD1^{E235Q} (a and d, green) and by staining with anti-FLAG mAb and Cy3-labelled goat anti-mouse IgG antibody (b and e, red). Merged images of a and b and of d and e are shown in c and f respectively. Scale bar, 10 μm . (B) HeLa cells transiently expressing GFP-SKD1^{E235Q} were incubated in the medium containing a vehicle (0.1% DMSO) (a–c) or 10 μM BAPTA/AM (d–f) for 1 h before subjecting to immunofluorescence confocal microscopic analysis using anti-ALG-2 pAb and Cy3-labelled goat anti-rabbit IgG antibody; a and d, GFP-SKD1^{E235Q}; b and e, ALG-2; c and f, merged images of a and b and of d and e respectively. Scale bar, 10 μm . (C) Efficiency of co-localization of ALG-2 with transiently overexpressed GFP-SKD1^{E235Q} in the presence (closed bar) or absence (open bar) of BAPTA/AM was quantified by counting the numbers of GFP-SKD1^{E235Q}-expressing cells that exhibited co-localization and non-co-localization between endogenous ALG-2 and GFP-SKD1^{E235Q}. Results are expressed in terms of percentage of co-localized cells and are means $\pm$ S.D. for triplicate transfection experiments.

of cells showing co-localization of ALG-2 with GFP-SKD1^{E235Q}. BAPTA/AM decreased the number of co-localized cells from 76 to 25% (Figure 5C).

Figure 6 Subcellular fractionation

(A) HEK-293T cells treated with 0.1% DMSO (mock, Ctrl), 10 μM BAPTA/AM or 10 μM A23187 were homogenized and fractionated into total cell lysate (T), crude nuclear/cytoskeletal (P1), membrane (P2/3) and cytosolic (S) fractions by the differential centrifugation as described previously [8]. ALG-2 and a cytosolic marker protein HSP90 were detected by Western blotting using anti-ALG-2 pAb and anti-HSP90 mAb respectively. (B) The post-nuclear membrane fractions (P2/3) were separated from HEK-293T cells that had been treated with BAPTA/AM for 1 h or A23187 for 10 min. After resuspending in 46% sucrose solutions, the membrane fractions were overlaid with three additional layers of sucrose concentrations of 35, 30 and 8% in a stepwise manner, and then the membranes were separated by centrifugation at 100 000 g for 6 h. The gradients were fractionated from top to bottom into 24 fractions. The P2/3 fractions (left panel) and the collected odd number fractions from 3 to 11 (right panel), which correspond to light membrane fractions, were subjected to Western blotting using anti-ALG-2 pAb and anti-Lamp-1 mAb.

These data suggested that ALG-2 might change the subcellular localization in a Ca^{2+} -dependent manner. To confirm this idea biochemically, we performed the subcellular fractionation of the cells treated with BAPTA/AM or A23187, an ionophore for calcium ion. In the differential centrifugation, endogenous ALG-2 was recovered from all fractions: crude nuclear (P1), membrane (P2 and P3; designated P2/3) and cytosolic (S) fractions (Figure 6A). Detection of ALG-2 in P1 fraction is consistent with the result of immunofluorescence microscopic observations (Figures 5A and 5B) and this agrees with our previous report that endogenous ALG-2 was recovered from purified nuclear fraction of Jurkat cells [8]. Functions of ALG-2 in the nucleus remain to be elucidated. By treatment of cells with BAPTA/AM, the amount of ALG-2 slightly increased in the cytosolic fraction (S) and decreased in the membrane fractions (P2/3). On the other hand, ALG-2 was barely recovered from the cytosolic fraction from the lysate of A23187-treated cells. In contrast, HSP90, which largely localizes to cytosol, did not change its distribution with or without Ca^{2+} stimulation. The membrane fractions (P2/3) were further subjected to floatation analysis by sucrose-density-gradient centrifugation. A large part of ALG-2 was recovered from Lamp-1 (lysosome-associated membrane protein-1)-rich fractions, which is inferred to be present in the upper part of the 30% sucrose cushion (fractions 3–11) (Figure 6B). BAPTA/AM decreased the amount of ALG-2 in the light membrane fractions as well as in the P2/3 fractions.

DISCUSSION

TSG101 was originally discovered as a tumour-susceptible gene by an assay for cellular transformation on random gene disruption

[46]. TSG101 is essential for embryonic development and closely linked to cancer by playing important roles in transcriptional regulation, cell growth and cell cycle ([47] and references therein). Recently, however, more attention has been paid to the function of TSG101 in the endosomal protein sorting system [24,48,49] and the budding of enveloped RNA viruses from the plasma membranes [26,28]. On the other hand, Alix was originally found as an interacting partner of the PEF protein ALG-2 [6,9,10], but it is now recognized as a multifunctional adaptor protein involved in protein sorting from the plasma membrane to multivesicular endosomes [15].

We explored the possibility that ALG-2 plays a role as a Ca²⁺-dependent switching factor in the MVB sorting system. Binding to the PRR of Alix may induce conformational change of Alix to expose its multiple protein-interacting sites, for example, the PSAP motif, which is recognized by TSG101. In order to test this idea, we performed a GST–ALG-2 pull-down assay using cleared lysate of HEK-293T cells. As expected, endogenous TSG101 could be pulled down with GST–ALG-2 in the presence of Ca²⁺ (Figure 1). Association between ALG-2 and TSG101 was observed by a co-immunoprecipitation assay using transiently overexpressed epitope-tagged proteins (see Supplementary Figure S3 at <http://www.BiochemJ.org/bj/391/bj3910677add.htm>). Moreover, ALG-2 was found to associate not only with TSG101 but also with two other human ESCRT-I components, hVps28 and hVps37A (Figure 1C). In contrast with our expectation, however, co-expression of Alix did not increase the amount of pulled down GFP–TSG101 (see Supplementary Figure S2 at <http://www.BiochemJ.org/bj/391/bj3910677add.htm>). This can be explained by the surprising finding that ALG-2 interacts with PRR present in the central part of TSG101 (Figure 3) and that this interaction is due to direct binding as shown by a bio-ALG-2 overlay assay (Figure 4). Although the amino acid sequence of this region does not have an obvious similarity to the ALG-2-binding sequence containing the defined PxY repeats in Alix [13], TSG101 has YPP repeats (residues 149–151, 178–180 and 189–191) and PYP repeats (173–175, 188–190 and 195–197). The recognition motif of ALG-2 seems to be flexible on the primary sequence level, but the polyproline II helix containing tyrosine residues might be essential for the interaction [13]. This information will be helpful to search for novel candidates of ALG-2-interacting partners in the future.

The finding of direct interaction between ALG-2 and TSG101 has raised a new question of whether an ALG-2 homodimer functions as a bridging factor that binds both Alix and TSG101 and helps Alix–TSG101 interaction or, alternatively, whether Alix competes with TSG101 for binding to ALG-2. We investigated these possibilities by examining the effects of co-expression of Alix on the amount of ALG-2 co-immunoprecipitated with TSG101, but no clear differences were found (results not shown). Moreover, the amount of TSG101 co-immunoprecipitated with epitope-tagged Alix was not significantly influenced by the presence or absence of Ca²⁺ (results not shown). Endogenous levels of Alix and ALG-2 might be excessively high compared with the level of TSG101, and exogenous expression of Alix may not sufficiently increase the molar ratio of Alix to ALG-2 to exhibit the effects. We also attempted *in vitro* interaction analysis using recombinant proteins. Unfortunately, production of recombinant GST-fused PRRs of either TSG101 or Alix in *E. coli* was not successful, and we could not determine whether the ALG-2 homodimer is capable of binding two targets simultaneously. The potential self-dimerization (or oligomerization) of both TSG101 and a C-terminal fragment of Alix (residues 681–868) containing the ALG-2-binding site, as observed in the yeast two-hybrid assay (Figure 2), may also hamper stoichiometric analysis.

In the endosomal sorting pathway, TSG101/Vps23, Vps28 and Vps37 form an approx. 350 kDa protein complex called ESCRT-I [37–39,49], which plays a role in recognition and transfer of ubiquitinated cargo proteins from the Hrs–STAM complex to ESCRT-II [23–25] and also functions in the budding of enveloped RNA viruses (see [26] for a review). We have demonstrated that ALG-2 interacts with endogenous TSG101 by the GST–ALG-2 pull-down assay in a Ca²⁺-dependent manner (Figures 1A and 1B). Although all three exogenously co-expressed ESCRT-I components (Myc–TSG101, GFP–hVps28 and GFP–hVps37A) were pulled down with GST–ALG-2 (Figure 1C), neither hVps28 nor hVps37A showed interaction with ALG-2 but TSG101 did show interaction with ALG-2 by the yeast two-hybrid assay (Figure 2). Thus it is likely that ALG-2 can bind not only TSG101 in the free form but also TSG101 in the complex (ESCRT-I).

The interaction between ALG-2 and TSG101 was found to be Ca²⁺-dependent and a Ca²⁺ concentration of 10 μ M was sufficient (Figure 1), as already shown, for interaction with annexins and Alix [11–13]. Extracellular calcium ions are concomitantly taken up by endocytosis and are released from endosomes during acidification caused by vacuolar ATPase [50]. It is possible that the local concentrations of Ca²⁺ in the immediate vicinity of the endosomes are high enough for ALG-2 to interact with TSG101 and Alix. We investigated whether ALG-2 localizes to endosomes and associates with the MVB sorting machinery. By immunofluorescence microscopic analysis using specific antibodies to ALG-2 and organelle markers, we observed a fine dotted distribution of ALG-2 throughout the cytoplasm and nucleus in HeLa cells. However, endogenous ALG-2 did not co-localize with any known endosomal markers such as EEA1 (early endosome antigen 1; early endosome marker), Lamp-1 (late endosome and lysosome marker), LBPA (lysobisphosphatidic acid; MVB marker), transferrin receptor and clathrin in HeLa cells (see Supplementary Figure S4 at <http://www.BiochemJ.org/bj/391/bj3910677add.htm>). ALG-2 did not co-localize with either internalized rhodamine-labelled EGF or dextran (results not shown). Meanwhile, by biochemical cellular fractionation, ALG-2 was recovered from the light membrane fractions and its recovery was decreased by treatment with a membrane-permeant Ca²⁺ chelator BAPTA/AM (Figure 6). These findings suggest that ALG-2 Ca²⁺-dependently localizes to unknown membrane structures, whose densities approximate to those of the Lamp-1-rich fractions.

There is the possibility that ALG-2 associates with endosomal membranes transiently and dissociates too fast to detect the co-localization with endosomal markers under the conditions used. Alternatively, since both TSG101 in ESCRT-I and Alix are essentially cytosolic proteins and only small amounts of these proteins are transiently recruited to endosomal membranes during cargo sorting, their membrane-localized concentrations may not be high enough to detect the ALG-2 interaction under physiological conditions. To settle this issue, we induced aberrant endosomes by overexpression of GFP–TSG101 without co-expression of FLAG–hVps28 in mammalian cells as reported in [51]. When FLAG–ALG-2 was expressed alone in HeLa cells, FLAG–ALG-2 exhibited a fine-dotted like distribution throughout the cytoplasm as well as in the nucleus and slightly accumulated in the perinuclear region [13]. By co-expression with GFP–TSG101, however, FLAG–ALG-2 changed its distribution to a punctate pattern and co-localized well with GFP–TSG101 (see Supplementary Figure S5 at <http://www.BiochemJ.org/bj/391/bj3910677add.htm>). A similar punctate distribution pattern of overexpressed GFP–TSG101 was also observed without FLAG–ALG-2 co-expression, indicating that, unlike Vps28, ALG-2 has no solubilizing effect (results not shown). Although co-localization of GFP–TSG101 with FLAG–ALG-2 implies their interaction on aberrant

endosomes, the possibility of mere aggregation of the two overexpressed proteins into the 'aggresome' cannot be excluded under the experimental conditions used [52].

On the other hand, aberrant endosomes induced by overexpression of dominant-negative AAA ATPase Vps4/SKD1, GFP-SKD1^{E235Q}, have been well characterized and known to accumulate endosomal sorting cargoes and machinery [17,32,43–45,49]. We also observed that overexpression of GFP-SKD1^{E235Q} in HeLa cells caused perinuclear accumulation of EEA1, Lamp-1 and LBPA, among which the latter two late endosomal markers well co-localized with GFP-SKD1^{E235Q} (Supplementary Figure S6 at <http://www.BiochemJ.org/bj/391/bj3910677add.htm>). In the present study, localization of ALG-2 to this so-called E235Q compartment was demonstrated to be Ca²⁺-dependent by two experimental results: (i) loss of co-localization with the Ca²⁺-binding-defective ALG-2 mutant FLAG-ALG-2^{E47A/E114A} (Figure 5A) and (ii) decrease of ALG-2 co-localization by treatment of HeLa cells with a membrane-permeant Ca²⁺ chelator, BAPTA/AM (Figures 5B and 5C). We previously showed that Alix as well as CHMP4b, an ESCRT-III component and interacting partner of Alix, co-localized with GFP-SKD1^{E235Q} [17]. Thus recruitment of ALG-2 to the E235Q compartment appears to be triggered by interaction with both TSG101 and Alix in the presence of Ca²⁺ released from the aberrant endosomes. Although a biological function of ALG-2 has been suggested in apoptosis, knowledge of its physiological functions is still limited. The present finding of ALG-2 interaction with TSG101, in addition to the previous findings of binding to Alix and annexins shed new light on the understanding of biological functions of ALG-2 in membrane trafficking.

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