Mammalian class E Vps proteins, SBP1 and mVps2/CHMP2A, interact with and regulate the function of an AAA-ATPase SKD1/Vps4B

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Summary

SKD1 belongs to the AAA-ATPase family and is one of the mammalian class E Vps (vacuolar protein sorting) proteins. Previously we have reported that the overexpression of an ATPase activity-deficient form of SKD1 (suppressor of potassium transport growth defect), SKD1(E235Q), leads the perturbation of membrane transport through endosomes and lysosomes, however, the molecular mechanism behind the action of SKD1 is poorly understood. We have identified two SKD1-binding proteins, SBP1 and mVps2, by yeast two-hybrid screening and we assign them as mammalian class E Vps proteins. The primary sequence of SBP1 indicates 22.5% identity with that of Vta1p from *Saccharomyces cerevisiae*, which was recently identified as a novel class E Vps protein binding to Vps4p. In fact, SBP1 binds directly to SKD1 through its C-terminal region (198-309). Endogenous SBP1 is exclusively localized to cytosol, however it is redirected to an aberrant endosomal structure, the E235Q compartment, in the cells expressing SKD1(E235Q). The ATPase activity of SKD1 regulates both the membrane association of, and assembly of, a large hetero-oligomer protein complex, containing SBP1, which is potentially involved in membrane transport through endosomes and lysosomes. The N-terminal half (1-157) of human SBP1 is identical to lyst-interacting protein 5 and intriguingly, SKD1 ATPase activity significantly influences the membrane association of lyst protein. The SKD1-SBP1 complex, together with lyst protein, may function in endosomal membrane transport. A primary sequence of mVps2, a mouse homologue of human CHMP2A/BC-2, indicates 44.4% identity with Vps2p/Did4p/Chm2p from *Saccharomyces cerevisiae*. mVps2 also interacts with SKD1 and is localized to the E235Q compartment. Intriguingly, the N-terminal coiled-coil region of mVps2 is required for the formation of the E235Q compartment but not for binding to SKD1. We propose that both SBP1 and mVps2 regulate SKD1 function in mammalian cells.

Key words: AAA-ATPase, Vacuolar protein sorting, Endosome, Lysosome, LYST/Beige

Introduction

In eukaryotic cells, there are two major routes to the lysosome/vacuole after exiting the trans-Golgi network (TGN) (Hunziker and Geuze, 1996; Kornfeld and Mellman, 1989). One is the biosynthetic pathway by which the cell delivers newly synthesized lysosomal enzymes and membrane proteins for the biogenesis and maintenance of lysosomes/vacuoles. The transport vesicles carrying the cargo molecules are budded from the TGN and mostly target endosomes prior to arriving at lysosomes/vacuoles. The other route is the so-called endocytic pathway by which the cell delivers various internalized ligands and the receptors from the plasma membrane (PM) to lysosomes/vacuoles for degradation. The ligand-receptor complexes, for instance the EGF/EGF-receptor, are segregated into the internal vesicles of endosomes (Futter et al., 1996). Subsets of receptors that recycle back to the PM, such as transferrin receptor and low-density lipoprotein receptor, are sequestered into clathrin-coated vesicles at recycling endosomes and are removed from the degradative pathways. In both pathways, endosomes play central roles in the sorting of cargo molecules and indeed, endosomes are meeting places for membrane traffic from both routes.

By an extensive genetic analysis in the yeast *Saccharomyces cerevisiae*, more than 50 of the Vps (vacuolar protein sorting) genes involved in membrane transport to vacuoles were isolated (Raymond et al., 1992). The class E Vps family, one of the sub-groups of Vps mutants, exhibits a modest degree of secretion of newly synthesized carboxypeptidase Y (CPY), a soluble vacuolar enzyme, compared with other sub-classes of Vps mutants. In class E Vps mutants, both the 60 kDa V-

ATPase subunit and FM4-64 dye, a tracer of endocytic membranes, appear to be accumulated in a novel compartments adjacent to vacuoles, so-called 'class E compartments'. One of the class E Vps mutants, *vps4*, accumulates vacuolar, endocytic and late-Golgi markers in an aberrant multilamellar pre-vacuolar compartment. Electron microscopy revealed that upon a shift in temperature, exaggerated stacks of curved cisternal membranes are accumulated adjacent to vacuoles in the *vps4ts* mutant (Babst et al., 1997). These aberrant membrane structures found in *vps4* correspond to a typical class E compartment. Based on these and other observations, it is believed that Vps4p function is required for efficient transport out of the pre-vacuolar endosome. The VPS4 gene has been cloned and found to encode a 48 kDa protein that belongs to the protein family of AAA-type ATPases (ATPase associated with cellular activities) (Babst et al., 1998). Biochemical analyses of Vps4p and its mutant defective in ATP hydrolysis revealed that the nucleotide-free or ADP-bound form of Vps4p exist as a dimer, whereas in the ATP-bound state, Vps4p dimers assembled into a decameric complex. This suggests that ATP hydrolysis drives a cycle of assembly and disassembly of Vps4p dimers/decamers, as well as the association of Vps4p with an endosomal compartment in vivo. Moreover, membrane associations of two other class E Vps proteins, Vps24p and Vps32p/Snf7p, are also affected by mutations in VPS4.

It has been shown that two mammalian Vps4ps, SKD1/Vps4B and Vps4A, are involved in membrane transport through endosomes (Bishop and Woodman, 2000; Yoshimori et al., 2000). We have previously shown that overexpression of SKD1(E235Q), the ATPase-deficient form of SKD1 (suppressor of potassium transport growth defect 1), led to perturbation of various membrane transports via endosomes (Fujita et al., 2003). For instance, recycling receptors are accumulated in the E235Q compartments, the aberrant endosomes induced by SKD1(E235Q). The recycling of endolyn and TGN38, from the PM to lysosomes and TGN, respectively, is also severely abrogated by the expression of SKD1(E235Q). As a result of the accumulation of mannose-6-phosphate receptor (MPR) in the E235Q compartments, a newly synthesized lysosomal enzyme, cathepsin D, was hyper-secreted to the outside of the cells. These phenotypes led by the expression of SKD1(E235Q) resembled those found in class E Vps mutants in yeast. SKD1(E235Q) also caused the accumulation of hybrid organelles, the intermediate compartments in the membrane transport between late endosomes and lysosomes. Although the ATPase activity of SKD1 is considered to be of vital importance to membrane transport through endosomes, its molecular function is still poorly understood. In this study, we have cloned and characterized two mammalian class E Vps proteins. We propose that they are required for the function of SKD1 in membrane transport through endosomes.

Materials and Methods

Reagents

Rabbit polyclonal antibodies against SKD1 has been described previously (Fujita et al., 2003). Alexa488- and Alexa594-labeled secondary antibodies were purchased from Molecular Probes (Eugen, OR, USA). Mammalian expression vectors, pcDNA3.1(–) and pcDNA3.1/*Myc*-His(–) were obtained from Invitrogen (Carlsbad, CA, USA). pEGFP-C1 was obtained from Clontech (Palo Alto, CA, USA).

Two-hybrid screening

The PROQUEST two hybrid system kit was obtained from Gibco-BRL (Gaithersburg, MD, USA) and used according to the instructions supplied. Briefly, the pDBLeu-SKD1 construct was used to screen a mouse brain cDNA library. The transformants were plated on synthetic complete (SC) medium lacking histidine, leucine and tryptophan. After 4-5 days of growth at 30°C, the surviving colonies were picked and assayed for β-galactosidase activity. A total of 65 transformants were $\beta\text{-gal}^{+}\text{His}^{+}$. Further screenings, a positive selection on SC lacking uracil and a negative selection on SC containing 5FOA, identified a total of 46 transformants. Plasmid DNAs recovered from the positive clones were sequenced with an ABI PRISM dye terminator cycle sequencing kit and an ABI automated sequencer (model 373A gel electrophoresis detection system or 377 capillary system DNA sequencer, Perkin-Elmer Corp., Applied Biosystems Division, Foster City, CA, USA).

Plasmid construction

SKD1

Plasmids encoding GST-SKD1, GFP-SKD1 and GFP-SKD1(E235Q) were described previously (Fujita et al., 2003). cDNAs encoding the wild-type SKD1 and mutant E235Q were excited from GFP fusion constructs and subcloned into a pcDNA3.1(–) vector.

SBP1

Full-length and deletion mutants of SBP1 were engineered and amplified by the polymerase chain reaction (PCR) method using the following primers: for full-length SBP1(1-309); #1: 5′-ACACGA-ATTCAGATGGCCGCGCTGGCCCCTC-3′ and #2: 5′-GAGAGTC-GACTCAACAGCTGGAGCAGCTGG-3′, ΔC-SBP1(1-129); #1 and #3: 5′-GAGAGCGGCCGCTGTGATGACATCTATTAAAAG-3′, and ΔN-SBP1(198-309); #4: 5′-ACACCTCGAGCAGCATACGACCCA-AGCAAC-3′ and #2, and then subcloned into the vector pGEX-KG (Guan and Dixon, 1991). After verification of their DNA sequence, some were transferred to the appropriate vectors, pThioHis (Invitrogen) and pEGFP-C1 (Clontech) by endonuclease digestion.

mVps2

Plasmids encoding GST-mVps2 fusion protein were created by PCR using a set of primers (#5: 5′-ACCTCGAGCCATGGACCTGTTG-TTTGGGC-3′ and #6: 5′-ACAAGCTTTTCAGTCCCTGCGAAG-GTTC-3′) and then subcloned into pGEX-KG. Plasmids encoding tagged full-length, ΔC- and ΔN- mVps2 were engineered and amplified by PCR using primers for full-length mVps2 (#5 and #7: 5′-ACAGGATCCCAGTCCCTGCGAAGGTTCTTG-3′), ΔC-mVps2 (#5 and #8: 5′-ACCGGATCCGTGGAGGGAAGGTTTGACAG-3′), and ΔN-mVps2 (#9: 5′-ACCTCGAGATGGCAAAGCAAGGCCA-GATG-3′ and #6) and then subcloned into the vector, pcDNA3.1/*Myc*-His(–) or pEGFP-N1.

Bacterial expression of recombinant proteins and purification of antibody

GST-tagged and thioredoxin-His^{6}-tagged fusion proteins were expressed in *E. coli* DH5α. Both fusion proteins were subjected to affinity purification with either a glutathione-Sepharose 4B column (Pharmacia Biotech, Uppsala, Sweden) or nickel column (QIAGEN, Chatsworth, CA, USA). His-Patch thioredoxin-tagged SBP1 was used to immunize Japanese White rabbits. Serum was harvested and a specific polyclonal antibody (pAb) was prepared by affinity purification, using purified GST-SBP1-coupled Sepharose 4B (Pharmacia Biotech).

Northern blotting

Mouse MTN blot (multiple tissues northern blot), containing 2 μg of

purified poly(A)+ RNA from mouse tissues was obtained from Clontech. To obtain a radio-labeled probe, full length-SBP1 and mVps2 cDNA were excised from agarose gel and labeled with ^{32}P using a random primed DNA labeling kit (Takara, Kyoto, Japan). Hybridization was carried out in the ExpressHyb hybridization buffer (Clontech) at 68°C for 1 hour and then the blot was washed according to the manufacturer's instructions.

Western blotting

Mouse tissue lysates (50 μg of each) and cultured cell lysates (10 μg of each) were subjected to SDS-PAGE according to Laemmli's method (Laemmli, 1970) using 10% acrylamide under reducing conditions, after which the gel was processed for western blotting with affinity purified antibody against SBP1 (1 μg/ml) according to standard procedures. In order to detect the 400 kDa lysosomal trafficking regulator (lyst) protein, we used 5% SDS-PAGE gels that did not have a 'stacker' (Pagel, Atto Corporation, Tokyo, Japan). The SDS-PAGE gels were transferred to PVDF membrane (Millipore, Bedford, MA, USA) for 2 hour at 2 mA/cm^2 using a transfer buffer (25 mM Tris and 200 mM glycine) without methanol. The membranes were blocked by incubation in TBST (20 mM Tris-HCl buffer pH 8.0, 0.15 M NaCl and 0.1% Tween 20) with 10% skim milk. They were then incubated with affinity purified antibody against lyst (1:500 dilution) (Perou et al., 1997) and the immunoreacting bands were detected using an enhance chemiluminescence (ECL) detection kit (Pharmacia Biotech) or chemiluminescent peroxidase substrate (Sigma, St Louis, MO, USA) (for lyst blotting).

Surface plasmon resonance spectroscopy

Surface plasmon resonance measurements were carried out on a BIAcoreX following the manufacturer's instructions. The surface of the sensor chip, a carboxymethyldextran matrix, was covalently coupled with either GST-SKD1 or GST (as a reference) via NHS until the desired level of immobilization (~1,500 response unit) was achieved. The indicated concentration of His-patch thioredoxin-tagged SBP1 was injected into the sensor chips at a flow rate of 5 μl/minutes. The response from the reference, which was immobilized GST as described, was subtracted from that of the sample-injected flow cell to correct for changes in the refractive index. The sensor chip was regenerated using 50 mM NaOH at a flow rate of 10 μl/minute for 5 minutes and reused. Data transformation and overlay sensorgrams were prepared using BIAevaluation version 3.1 software (BIACORE K.K., Tokyo, Japan).

GST pull-down assay

SBP1

Purified recombinant proteins, GST-SKD1 and ThioHis-SBP1 (full length, ΔN, ΔC), were used for the GST pull-down assay. GST-SKD1 was coupled to glutathione-Sepharose 4B in the binding buffer (0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 100 mM KCl, 50 mM dithiothreitol, 10% glycerol, 0.1% Triton X-100 and 50 mM Hepes-KOH, pH 7.3) for 1 hour at 4°C. After several washes, His-patch thioredoxin-tagged-SBP1 (full length, ΔN, ΔC), were incubated in the same buffer for 1 hour at 4°C. After five extensive washes, the bound proteins were eluted with Laemli's SDS sample buffer and subjected to SDS-PAGE followed by western blotting with anti-SBP1 antibody.

mVps2

Purified recombinant proteins, GST and GST-SKD1, were coupled to glutathione-Sepharose 4B in binding buffer for 1 hour at 4°C. After several washes, total cell lysates prepared from HeLa cells transfected with mVps2-GFP chimeras (full length, ΔN, ΔC), were incubated in the same buffer for 4 hours at 4°C. After five extensive washes, the bound proteins were eluted with Laemli's SDS sample buffer and subjected to SDS-PAGE followed by western blotting with anti-GFP antibody.

Cell culture and plasmid transfection

HeLa, U251, NRK and 3Y1 rat fibroblast cells were cultured in Dulbecco's modified Eagle's medium (Sigma) with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD, USA). Human fibroblasts, GM05565 and GM02075, were obtained from Coriell Institute for Medical Research (Camden, NJ, USA) and cultured in Dulbecco's modified Eagle's medium (Sigma) with 20% fetal bovine serum (Life Technologies). Mouse NIH3T3 fibroblast cells were cultured in Dulbecco's modified Eagle's medium (Sigma) with 10% calf serum (Life Technologies). Transient transfection with FUGENE6 (Roche Molecular Biochemicals, Indianapolis, IN) was carried out according to the manufacturer's instructions.

Total cell lysate preparation and subcellular fractionation

Mouse tissues (brain, liver, kidney, spleen, lung and heart) were minced and homogenized with a Dounce homogenizer in 1% TritonX-100/PBS. Total protein was extracted on ice for 30 minutes and the insoluble materials were sedimented by centrifugation (10,000 ***g*** for 30 minutes). The protein concentration was determined using a Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and bovine serum albumin as a standard. For the subcellular fractionation of cultured cells, cells were washed with PBS and harvested with 0.25 M sucrose containing 50 mM Hepes-KOH buffer (pH 7.3) and 1 mM EDTA. The collected cells were homogenized by passage through a 23G needle 10-20 times. After low speed centrifugation (650 ***g*** for 5 minutes), the post nuclear supernatant was centrifuged at 105,000 ***g*** for 1 hour at 4°C and the resultant pellet and supernatant were saved as total membrane and cytosol fractions, respectively.

Gel filtration

The infected and uninfected HeLa cells were harvested with a binding buffer (see GST pull-down assay) containing 1 mM PMSF, 1 μg/ml of leupeptin and pepstatin, and solubilized by freezing and thawing. The resultant cell lysates were centrifuged for 5 minutes at low speed (700 ***g***) and the supernatant was passed through a 0.45 μm filter prior to being applied to a Superdex 200 gel filtration column in a SMART system (Pharmacia Biotec). Molecular mass was estimated using a High Molecular Weight Calibration kit (Pharmacia Biotec) containing standard markers, thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa) and bovine serum albumin (66 kDa).

Cell fixation and immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature and permeabilized with 0.05% saponin for 5 minutes. Fixed cells were washed with 50 mM NH_4Cl for quenching and then in PBS for 15 minutes. After blocking treatment with 1% bovine serum albumin (BSA) in PBS, the cells were incubated with primary antibodies in 1% BSA/PBS for 1 hour at the following dilutions; anti-SBP1 (rabbit pAb, affinity purified; 5 μg/ml) and anti-SKD1 (rabbit pAb, affinity purified; 5 μg/ml). Secondary goat anti-rabbit that had been conjugated with Alexa-594 or Cy3 was used at 5-10 μg/ml.

Results

Cloning of SKD1 binding proteins

A yeast two-hybrid screening was used to isolate proteins that interact with SKD1. A GAL4 DNA binding domain/SKD1

fusion construct was made in the vector pDBLeu and expression of the fusion protein was confirmed by western blot analysis of total yeast extract with anti-SKD1 antiserum (data not shown). By screening a mouse brain cDNA library with mouse SKD1 as bait, we obtained 46 positive colonies from 1.1×10^6 primary transformants (see Materials and Methods). From the positive transformants, we recovered plasmids encoding possible SKD1-binding proteins, and sequenced and determined their open reading frames (ORFs). We named one of them SBP1 (SKD1 binding protein 1; GeneBank/DDBJ accession number AK002611). Interestingly, one of the class E Vps proteins from *Saccharomyces cerevisiae*, Vta1p (Yeo et al., 2003), showed the highest homology to SBP1 in the yeast genomic database, with 22.5% of amino acid identity through the entire sequence (Fig. 1A). Interestingly, the very C terminus of SBP1 (275-309) and Vta1p (298-330) had 54.3% amino acid identity. In addition to a sequence similarity, the binding ability of SBP1 to SKD1/Vps4B compelled us to assume that SBP1 is the functional homologue of Vta1p, since Vta1p has been shown to bind to yeast Vps4p (Yeo et al., 2003). A protein-protein BLAST search (blstp) of SBP1 produced a match with five possible human SBP1 homologues, DRG-1 (AK000051; 90.3% identity), CAB66619 (AL136684; 90.0%), HSPC228 (AF151062; 86.7%), My012 (AF060225; 78.3%) and Lip5 (AF141341; 53.2%). However, based on the following evidences, we have concluded that human SBP1 has only one gene (i.e. dose not have isoforms) and DRG-1 is the most reliable cDNA sequence of it. First, the DRG-1 sequence perfectly matches with the human genome sequence located in chromosome 6 (data not shown). Second, there is only one gemomic sequence corresponding to human SBP1 in the human genome database. Third, the other four cDNA sequences are highly related, but have a few bases of nucleotide deletions, insertions and substitutions. It is hard to assume that these small alterations are derived from RNA splicing. Therefore, we concluded that human SBP1 (DRG-1) and Lip5 are in fact the same protein encoded by the same cDNA, although the C-terminal half of DRG-1 (158-307) and Lip5 (158-228) are distinct.

Another ORF (GeneBank/DDBJ accession number AK005267) was highly homologous to human BC-2/CHMP2A (BT008100; 99.5% identity) (Howard et al., 2001) and yeast Vps2p/Did4p (44.4% identity), both class E Vps proteins (Babst et al., 2002a) (Fig. 1B). Vps2p homologues have many synonyms (Did4p/Ren1p/Grd7p/Chm2p in yeast and CHMP2A/BC-2 in human), and we have called this ORF protein mVps2 (mouse homologue of Vps2p).

Structural features of SBP1 and mVps2

SBP1 and mVps2 consisted of 309 and 222 amino acids with a deduced molecular mass of 33.9 kDa and 25.1 kDa, respectively (Fig. 1C). SBP1, Vta1p, mVps2 and Vps2p share several common structural features. They are all small coiled-coil-forming proteins. They have an N-terminal enriched in basic amino acids, whereas the C terminus is acidic. SBP1 contained two possible coiled-coil domains in the N-terminal (55-86) and very C-terminal (279-306) regions. Vta1p contains two coiled-coil regions, however the relative location of the second coiled-coil domain of Vta1p to SBP1 is slightly shifted to the N terminus. mVps2 has two coiled-coil regions at both N terminus (12-59) and very C terminus (195-222) of molecule, while Vps2p lacks the second coiled-coil region. The isoelectric points of the full-length, N half (1-155) and C half (156-309) of SBP1 are 5.85, 9.25 and 4.28, respectively. As well, that of the full-length, N half (1-111) and C half (112-222) of mVps2 are 5.79, 11.27 and 4.03, respectively. They also indicated slower mobility in SDS-PAGE than that expected from the calculated molecular mass. Despite its predicted molecular mass, 33.9 kDa, SBP1 is identified as a 45 kDa single band by western blotting (Fig. 1D). Since a bacterially expressed SBP1 was also 45 kDa (data not shown), the slower mobility of SBP1 in the SDS-PAGE is unlikely to be due to any post-translational modifications, such as a glycosylation or lipid modification. Indeed, TOFF-mass spectrometric analysis revealed a purified recombinant SBP1 of 33,515 Da, which is almost the value predicted, 33,891 Da (data not shown). The molecular mass of a bacterially expressed mVps2 determined by SDS-PAGE, 35 kDa, was approximately 10 kDa larger than that calculated based on the amino acid composition (25.1 kDa) (Fig. 1E). Since other class E Vps proteins, yeast Snf7p and Vps2p also exhibited slower mobility on SDS-PAGE (Babst et al., 2002a), this may be an intrinsic property of highly charged small coiled-coil-forming proteins.

Tissue distribution of SBP1 and mVps2

Northern blotting analyses revealed that the mRNAs of SBP1 and mVps2 are transcribed as a single message (1.45 kb and 1.15 kb, respectively) and expressed in most tissues and at relatively high levels in the heart, brain, liver and kidney (Fig. 1F,G). Western blotting analysis also confirmed the ubiquitous expression of SBP1 in the isolated mouse tissues, brain, liver, kidney, spleen, lung and heart (Fig. 1D). Our antibody raised against recombinant mouse SBP1 cross-reacted with human and rat SBP1 and the immunoblotting of lysates from HeLa, NRK and NIH3T3 cells showed single bands (data not shown).

Interaction between SBP1 and SKD1 in vitro

Five independent yeast two-hybrid clones encoded SBP1 with different sized cDNAs (Fig. 2A). All five cDNAs were then reintroduced into Mav203 cells transformed with pDBLeu-SKD1 to examine their 1:1 interaction with SKD1. Three independent two-hybrid analyses, an X-gal assay (Fig. 2A; 2), His- (Fig. 2A; 3) and Ura- (Fig. 2A; 4) indicated that all isolated cDNA clones specifically interact with full-length SKD1. We next tested the protein interaction of SBP1 with SKD1 using surface plasmon resonance (SPR) spectroscopy and a GST pull-down assay. SPR spectroscopy revealed that bacterially expressed recombinant SBP1 bound to SKD1 in a concentration-dependent manner (Fig. 2B). The signal at a concentration of 150 μg/ml reached a plateau about 5 minutes after injection at ~1000 response units. After switching the injection solution from the protein-containing buffer to the dissociation buffer, there was little decrease of signal intensity, indicating that more than 90% of the SBP1 injected was still associated with SKD1 on the sensor chip. An apparent equilibrium dissociation constant of 53 nM was obtained though an analysis performed with BIAevaluation 3.1 software using 1:1 Languir with mass transfer model. Although this

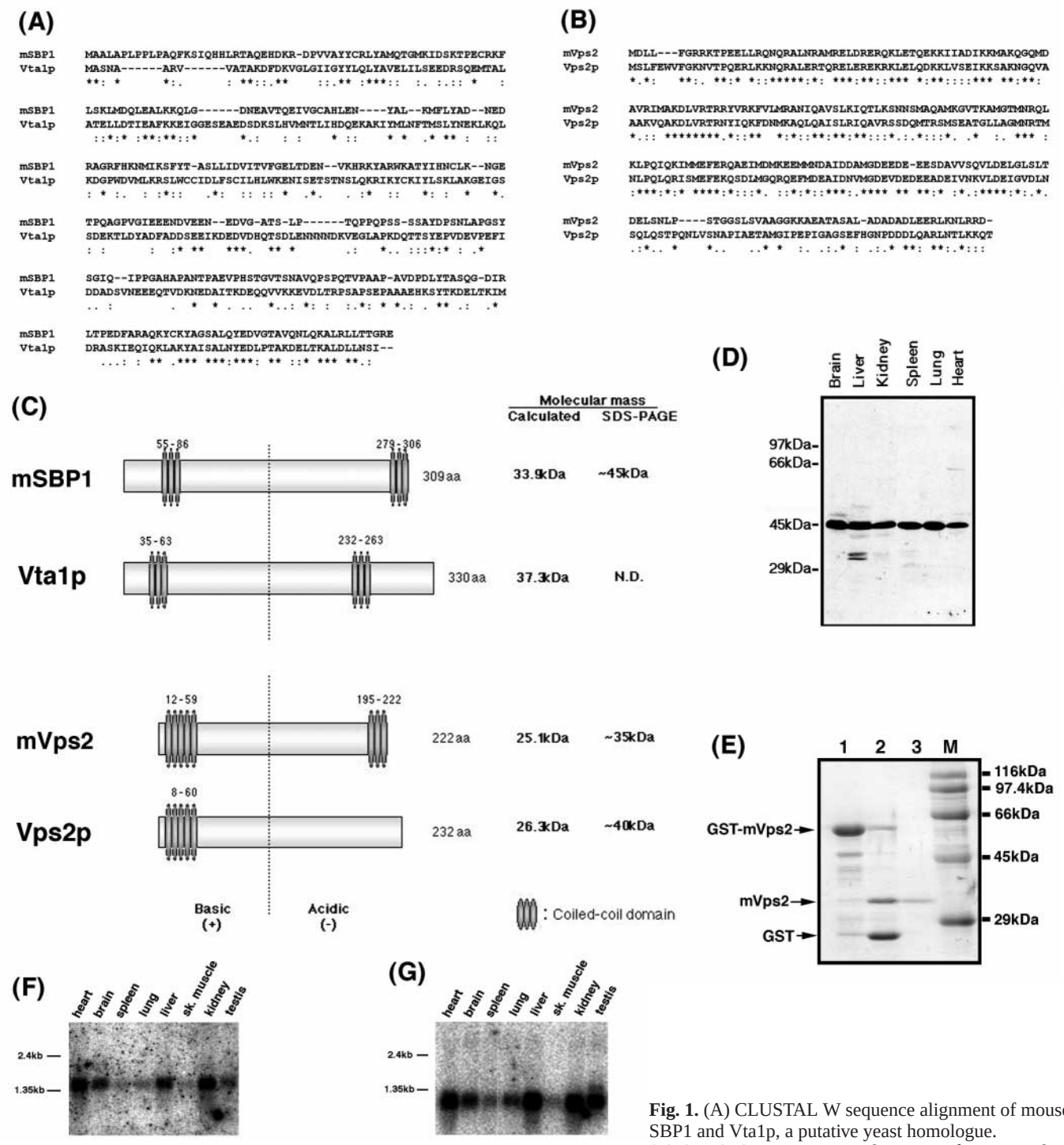

Fig. 1. (A) CLUSTAL W sequence alignment of mouse SBP1 and Vta1p, a putative yeast homologue. (B) CLUSTAL W sequence alignment of mouse and yeast Vps2p. (C) Domain structures of SBP1, Vta1p, mVps2 and Vps2p. Predicted coiled-coil domains are marked with coils. The dotted line through the center of molecules divides the proteins into their positively charged N-terminal and negatively charged C-terminal domains. Molecular masses are given on the right, as calculated from their amino acid composition and determined by SDS-PAGE. Data of Vps2p was described previously (Babst et al., 2002a). N.D. is not determined. (D) Western blotting analysis of SBP1 in mouse tissues. Total extracts from various mouse tissues (50 μg of protein) were run on 10% SDS-PAGE and analyzed by western blotting with affinity purified anti-SBP1 polyclonal antibody. (E) SDS-PAGE analyses of bacterially expressed mVps2. GST-mVps2 was purified with glutathione-beads (lane 1) and subjected to thrombin cleavage on the beads (lane 2). Subsequently, the mVps2 portion was recovered by sedimentation of the beads with centrifugation (lane 3). Coomassie Blue staining revealed that both GST-mVps2 (~60 kDa) and thrombin-cleaved mVps2 (~35 kDa) migrated slower than expected from their molecular masses (51.1 and 25.1 kDa, respectively). (F) Northern blotting analysis of SBP1. Full-length SBP1 cDNA was labeled with ^{32}P by random priming and hybridized to multiple tissue northern blots (Clontech) under high stringency conditions. 2 μg of poly(A) + RNA from various mouse tissues was loaded in each lane. (G) Northern blotting analysis of mVps2. As for SBP1, mVps2 mRNA was detected on Multiple Tissue northern blots (Clontech) using full-length mVps2 cDNA as probe.

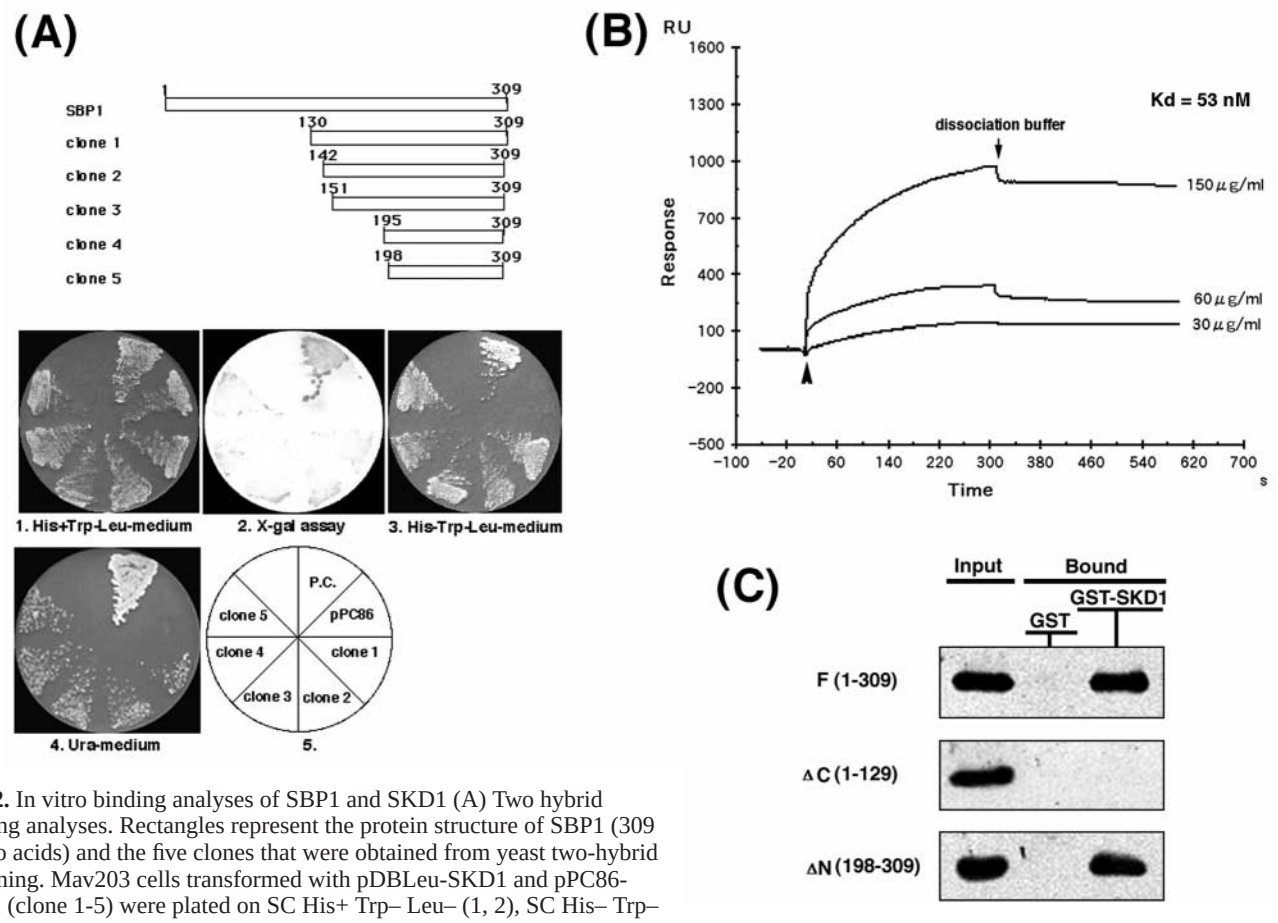

Fig. 2. In vitro binding analyses of SBP1 and SKD1 (A) Two hybrid binding analyses. Rectangles represent the protein structure of SBP1 (309 amino acids) and the five clones that were obtained from yeast two-hybrid screening. Mav203 cells transformed with pDBLeu-SKD1 and pPC86-SBP1 (clone 1-5) were plated on SC His+ Trp– Leu– (1, 2), SC His– Trp– Leu– (3) and SC His– Trp– Leu– Ura– (4) plates. An X-gal assay was performed on the nitrocellulose filter (2). Mav203 cells, transformed with the two plasmids supplied with the kit (pPC97-Fos and pPC86-Jun) as a positive control (P.C.), or with the empty vector pPC86 and pDBLeu-SKD1 as a negative control (pPC86) are indicated. (B) SPR spectroscopy. Indicated concentrations (30, 60, and 150 μg/ml) of His-thioredoxin-SBP1 (full length) in binding buffer were injected over the immobilized GST-SKD1 on a CM5 sensor chip. An arrowhead and arrow indicate the sample and dissociation buffer injection points, respectively. (C) GST pull-down assay. GST or GST-SKD1 was immobilized on glutathione-sepharose prior to incubation with purified His-thioredoxin-SBP1s [full length (1-309), ΔN (198-309), and ΔC (1-129)]. Bound proteins were eluted with SDS sample buffer and processed for immunoblotting with anti-SBP1 antibody.

value should be considered only an estimate, it is nonetheless clear that the interaction is of high affinity. Since the shortest clone (clone 5) obtained from the two-hybrid screening consisted of one third of the full-length SBP1 (111 amino acids, 198-309, Fig. 2A) and obviously lacked the first coiled-coil domain, the protein interaction of SBP1 with SKD1 should occur through the C-terminal region. The GST pull-down assay (Fig. 2C) indicated that full-length SBP1 and ΔN-SBP1 could interact with SKD1, while ΔC-SBP1 could not. These results suggest that the C-terminal region of SBP1 (198-309) is responsible for the protein interaction with SKD1.

SBP1 redistributes to the membrane and forms a large hetero-oligomer protein complex with SKD1 in the presence of SKD1(E235Q)

SBP1 does not have any possible membrane-anchoring domain, such as a hydrophobic stretch or lipid modification sequence. Conventional subcellular fractionation revealed that endogenous SBP1 and SKD1 were exclusively localized to cytosol in rat 3Y1B fibroblast cells (Fig. 3A, control). Interestingly, when the cells were infected with an adenovirus encoding GFP-SKD1(E235Q) and expressed GFP-SKD1(E235Q), significant amounts of endogenous SBP1 and SKD1 were redistributed to the membrane fraction (Fig. 3A, GFP-SKD1(E235Q)). Some AAA-ATPases form a large hetero-oligomer protein complex with accessory proteins in an ATP binding-dependent manner (Kondo et al., 1997; Sollner et al., 1993). The ATP-bound form of *N*-ethylmaleimide-sensitive fusion protein (NSF) forms a 20S complex with α/β-SNAPs and SNAREs and VCP/p97 exists as a 740 kDa hetero-oligomer protein complex with p47. It is also known that the 20S complex is disassembled by the ATPase activity of NSF. We have tested the possibility that SKD1 and SBP1 form a large hetero-oligomer protein complex by gel filtration analysis. In control cells, SKD1 and SBP1 were separately fractionated as approximately ~50 kDa and ~160 kDa globular molecules, respectively (Fig. 3B). Despite the fact that SKD1 interacted with SBP1 in vitro, unexpectedly, SKD1 may exist as a monomer and/or form an unstable complex with SBP1 in vivo in control cells. In contrast, SBP1 formed an oligomer protein complex in control cells. Consistently, several high molecular mass bands (~90, ~135 and ~180 kDa), were generated by un-cleavable cross-linker, disuccinimidyl

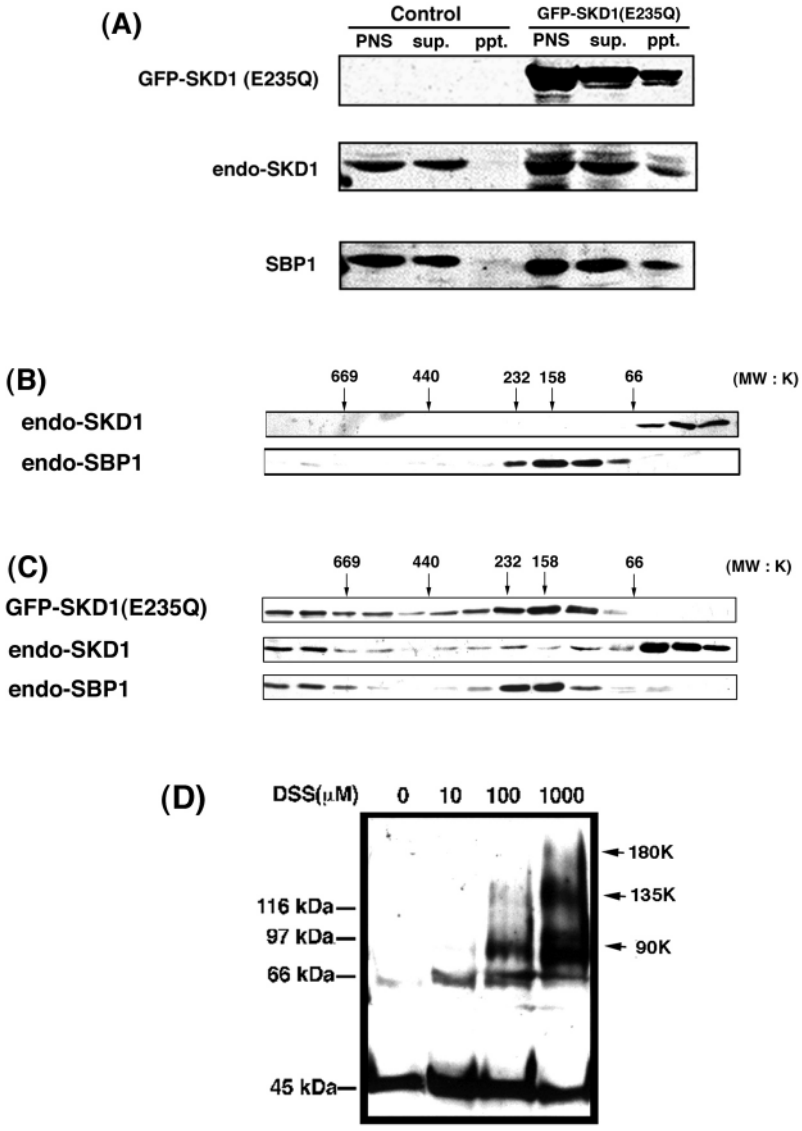

Fig. 3. Effects of SKD1(E235Q) on the subcellular localization and oligomer formation of SBP1. (A) Post nuclear supernatant prepared from control and GFP-SKD1(E235Q) adenovirus-infected rat 3Y1 fibroblast cells were fractionated into cytosol (sup.) and membrane (ppt.) by centrifugation at 105,000 ***g*** for 60 minutes. Comparable amounts of each fraction were processed for immunoblotting with anti-SKD1 and anti-SBP1 antibodies. (B) A cell lysate prepared from HeLa cells with binding buffer was fractionated by size exclusion chromatography on a Superdex 200 column. The collected fractions were processed for immunoblotting with anti-SKD1 and anti-SBP1 antibodies. Positions of molecular mass standards are indicated by arrows. (C) A cell lysate prepared from GFP-SKD1(E235Q) adenovirus-infected HeLa cells was fractionated and analyzed as described in B. (D) Chemical cross-linking of SBP1. NIH3T3 cell lysates were incubated with non-cleavable cross-linker DSS (0, 10, 100 and 1000 μM) for 60 minutes and then processed for immunoblotting with anti-SBP1 antibody.

suberate (DSS) in a concentration-dependent manner (Fig. 3D). Interestingly, when cells were infected with an adenovirus encoding GFP-SKD1(E235Q), significant amounts of endogenous SKD1 and SBP1 and the ectopically expressed GFP-SKD1(E235Q) were co-fractionated above 669 kDa (Fig. 3C). Therefore, we assume that SKD1(E235Q) facilitates the formation of a large hetero-oligomer protein complex by incorporation of both endogenous SBP1 and SKD1. This large complex may be associated with membranous structures, most probably corresponding to the E235Q compartments observed by immunofluorescence analysis (see below).

Endogenous SBP1 specifically localizes to E235Q compartments but not to vacuoles induced by the expression of rab5QL

Consistent with the subcellular fractionation results, the immunofluorescence analyses revealed that endogenous SBP1 did not localize to any membranous structures and was exclusively found in cytosol (Fig. 4, red in A and B, non-transfected cells). Interestingly, when the cells expressed SKD1(E235Q), the endogenous SBP1 redistributed into the E235Q compartments and co-localized well with SKD1 (Fig. 4A, transfected cells). In contrast, wild-type SKD1 did not affect the distribution of the endogenous SBP1 (Fig. 4B, transfected cells). Expression of rab5QL, which indicates a defect in endosomal membrane transport and leads to the formation of enlarged endosomes (Simonsen et al., 1998), did not alter the localization of either SBP1(Fig. 4C) or SKD1 (data not shown). These results suggest that SBP1 is redistributed to the endosomal membrane through specific protein interaction with SKD1(E235Q). We next examined which domain of SBP1 is required for the localization to E235Q compartments by expressing GFP-SBP1 chimeric proteins [full length, ΔC (1-129) and ΔN (198-309)] with SKD1(E235Q). On single transfection, all the chimeric proteins localized to the cytosol (Fig. 5, left columns). Consistent with the results obtained from in vitro binding experiments, both full-length and N-terminal-deleted GFP-SBP1 redistributed to E235Q compartments, as did endogenous SBP1, while the C-terminal-deleted one was retained in the cytosol (Fig. 5, right columns, green).

Endogenous lyst protein is redistributed to the membrane in the presence of SKD1(E235Q)

A Blast search revealed that the N-half of human SBP1 is identical to a partial sequence of human lyst interacting protein 5, Lip5 (Tchernev et al., 2002). Lip5 was isolated by yeast two-hybrid screening, using an internal sequence of the lyst protein (corresponding to 6586-7449 bp, Q2133-G2420: 287 amino acids) as bait. Thus, we wondered if SBP1 accompanies a lyst protein and recruits it to the E235Q compartments. First, we tested, using immunoblotting, whether the antibody against Beige protein, a mouse homologue of lyst, can react with human lyst. The antibody was raised with the carboxyl-terminal 89 kDa of the murine Beige protein, which contains the highly conserved BEACH domain and seven WD40 repeats

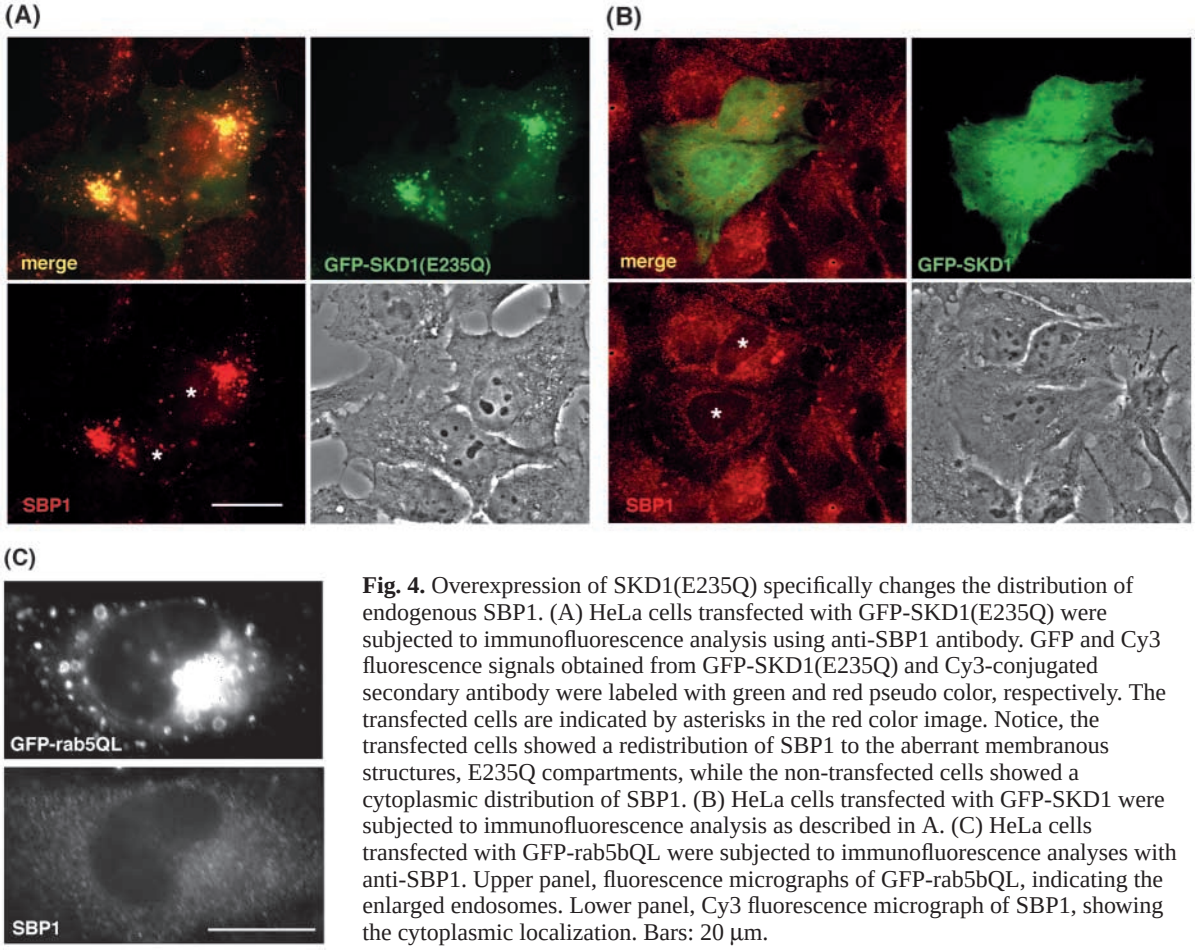

Fig. 4. Overexpression of SKD1(E235Q) specifically changes the distribution of endogenous SBP1. (A) HeLa cells transfected with GFP-SKD1(E235Q) were subjected to immunofluorescence analysis using anti-SBP1 antibody. GFP and Cy3 fluorescence signals obtained from GFP-SKD1(E235Q) and Cy3-conjugated secondary antibody were labeled with green and red pseudo color, respectively. The transfected cells are indicated by asterisks in the red color image. Notice, the transfected cells showed a redistribution of SBP1 to the aberrant membranous structures, E235Q compartments, while the non-transfected cells showed a cytoplasmic distribution of SBP1. (B) HeLa cells transfected with GFP-SKD1 were subjected to immunofluorescence analysis as described in A. (C) HeLa cells transfected with GFP-rab5bQL were subjected to immunofluorescence analyses with anti-SBP1. Upper panel, fluorescence micrographs of GFP-rab5bQL, indicating the enlarged endosomes. Lower panel, Cy3 fluorescence micrograph of SBP1, showing the cytoplasmic localization. Bars: 20 μm.

(Perou et al., 1997). This region has 95% identity with human lyst in amino acid sequence (Wang et al., 2000). As expected, an approximately 400 kDa band, which corresponded to human lyst, was detected in the GM05655 lane (Fig. 6A). The 400 kDa band was missing in the GM02075 lane, since GM02075 cells have a single base duplication in codon 40, GCA to GGCA, which results in a very short truncated form (Barbosa et al., 1996). Thus, we concluded that the antibody to mouse Beige protein could cross-react with human lyst protein. Next, we examined the subcellular distribution of lyst by conventional subcellular fractionation following immunoblotting. In control cells, lyst was exclusively localized to cytosol. Interestingly, in cells expressing GFP-SKD1(E235Q), a small but significant amount of lyst protein was redistributed to the membrane fraction (Fig. 6B). These results strongly suggested that SBP1 bound to lyst and recruited it to E235Q compartments.

Next, we have tested if overexpression of either wild-type SKD1 or SBP1 can restore the phenotype seen in the fibroblast cells derived from a patient with Chediak-Higashi syndrome (CHS), i.e. decrease the size of lysosomes in CHS fibroblast. In control fibroblast cells, the enlarged LAMP1-positive late endosomes/lysosomes were seen by the overexpression of SKD1(E235Q) as described previously (Fig. 6C, a,b). They were quite similar to the giant lysosomes seen in the CHS fibroblast cells (Fig. 6C; arrows). However, expression of neither wild-type SKD1 (Fig. 6C, c,d) nor SBP1 (Fig. 6C, e,f) altered the size of giant lysosomes in CHS fibroblasts. Interestingly, endogenous SBP1 redistributed to E235Q compartments, even in the CHS fibroblasts (Fig. 6D). These results imply that SKD1 and SBP1 may regulate the membrane association of lyst, however the function of lyst cannot be replaced by them, i.e. lyst may function downstream of SKD1 and SBP1.

mVps2 regulates membrane association of SKD1

Consistent with the data from yeast Vps2p (Babst et al., 2002a), we observed, by immunofluorescence microscopy, that mVps2-myc was mainly localized to the cytoplasm (Fig. 7A, a). In yeast, the membrane association of Vps4p requires the presence of both Vps2p and Vps24p, and the deletion of either resulted in the cytoplasmic dispersion of Vps4(E233Q) (Babst et al., 2002a). To test the role of mVps2 in the membrane association of SKD1, we made two deletion mutants of mVps2-myc that lacked one of the two coiled-coil domains (ΔN- and ΔC-mVps2) and attempted the co-transfection of each with either the wild-type or E235Q form of SKD1. Single transfection of full-length mVps2-myc and ΔC-mVps2-myc revealed that they exclusively localized to the cytosol (Fig. 7A, a,g), while ΔN-mVps2-myc mainly localized to the nucleus with some cytosolic distribution (Fig. 7A, d). Double transfection of full-length mVps2-myc with SKD1(E235Q), but not with wild-type SKD1, caused the redistribution

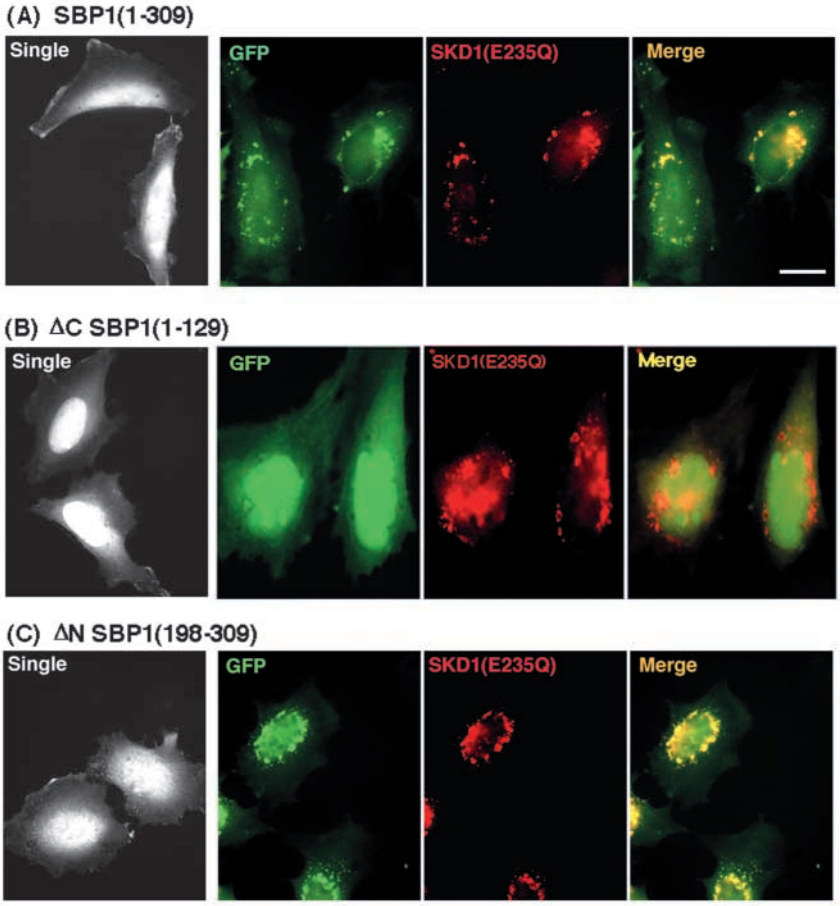

Fig. 5. Localization of GFP-SBP1 to the E235Q compartments requires C terminus coiled-coil domain. HeLa cells transfected with plasmids encoding GFP-SBP1 deletion mutants and SKD1(E235Q) were subjected to immunofluorescence analysis using anti-SKD1 antibody. Left column (black and white), GFP fluorescence micrographs of the cells transfected with GFP-SBP1 (1-309) (A), ΔC (1-129) (B) and ΔN (198-309) (C). Right three columns, GFP (green), Alexa594 fluorescence (red) and the merged images were obtained from the cells double transfected with SKD1(E235Q) and full-length GFP-SBP1 (1-309) (A), ΔC (1-129) (B) and ΔN (198-309) (C), respectively. Notice, full-length and ΔN SBP1 were redistributed to the E235Q compartments, while ΔC mutant was not. Bar, 20 μm.

of mVps2-myc to E235Q compartments (Fig. 7A, b,c). Interestingly, co-transfection of ΔN-mVps2-myc led to the complete redistribution of SKD1(E235Q) to cytosol and inhibited the formation of E235Q compartments (Fig. 7A, f). We also tested the GFP tag at the C terminus of mVps2 and analyzed the effects on the formation of E235Q compartments. Quantitative analyses revealed that in more than 60% of the cells double-transfected with SKD1(E235Q) and ΔN-mVps2, the formation of E235Q compartments were abrogated (Fig. 7B). These observations suggested that the N-terminal coiled-coil portion of mVps2 is responsible for the membrane association of SKD1(E235Q). To assess mVps2 interaction with SKD1, we performed GST pull-down analyses. Bacterially expressed ΔN- and ΔC-mutants of GST-mVps2 chimeras were quite unstable, thus we used HeLa cell lysates transiently expressing mVps2-GFP chimeras for the GST pull-down analyses. In these experiments, equal amounts of GST-SKD1 fusion protein (and GST alone) adsorbed to glutathione-Sepharose beads were incubated with the cell lysates transfected with mVps2-GFP chimeras. Immunoblot analysis revealed that the beads coupled with GST-SKD1, but not GST, specifically pulled down full-length mVps2-GFP from the lysates (Fig. 7C; lane 1 in middle panel). Unexpectedly, both ΔN- and ΔC- mutants of mVps2-GFP chimeras were also associated with SKD1 (Fig. 7C; lanes 2 and 3 in middle panel). Thus, neither of the coiled-coil domains of mVps2 are necessary for the binding to SKD1 and probably the central region of mVps2 is sufficient to interact with SKD1.

Discussion

We have cloned two SKD1 binding proteins, SBP1 and mVps2. The primary structures of SBP1 and mVps2 are homologous to yeast class E Vps proteins, Vta1p and Vps2p, respectively. Moreover, the following evidence supports that both SBP1 and mVps2 are functionally relevant to mammalian class E Vps proteins. First, they are small coiled-coil proteins, have clusters of charged amino acids, and exhibit slow mobility in SDS-PAGE. These features are common to some class E Vps proteins, such as ESCRT-III (endosomal sorting protein complex required for transport) members (Vps2p, Vps20p, Vps24p and Snf7p) (Babst et al., 2002a). Second, they redistributed to E235Q compartments when the cells expressed SKD1(E235Q). It has been reported that mVps23 and mVps28 are localized to the Vps4-A(E233Q)-induced aberrant endosomal compartments (Bishop and Woodman, 2001). Third, subsets of class E Vps proteins tend to form hetero-oligomer protein complexes, such as ESCRT-I (Vps23p, Vps28p and Vps37) (Katzmann et al., 2001), ESCRT-II (Vps22p, Vps25p and Vps36p) (Babst et al., 2002b) and ESCRT-III (Babst et al., 2002a). SBP1 also forms a large hetero-oligomer complex in the presence of the ATP-bound form of SKD1. This large complex may be the novel protein complex that regulates endosomal membrane sorting.

Our in vitro protein interaction analyses and the expression of deletion mutants of SBP1 revealed the importance of the C-terminal domain of SBP1 for the binding to SKD1. We also found that the very C-terminal regions of mouse and yeast SBP1 are well conserved. Consistent with our results, the very C-terminal domain of Vta1p (265-330/end) is sufficient for binding to Vps4p (Yeo et al., 2003). The fact that five independent clones identified by yeast two-hybrid screening encode SBP1 strongly supports SBP1 as one of the best binding partners of SKD1 in mammalian cells. Indeed, SPR analysis revealed a stable complex of SBP1-SKD1 in vitro. Previously, we reported that E235Q compartments are derived from multiple endogenous membranes, early and late endosomes, and lysosomes (Fujita et al., 2003). GFP-SKD1(E235Q) co-localizes with several endosomal/lysosomal

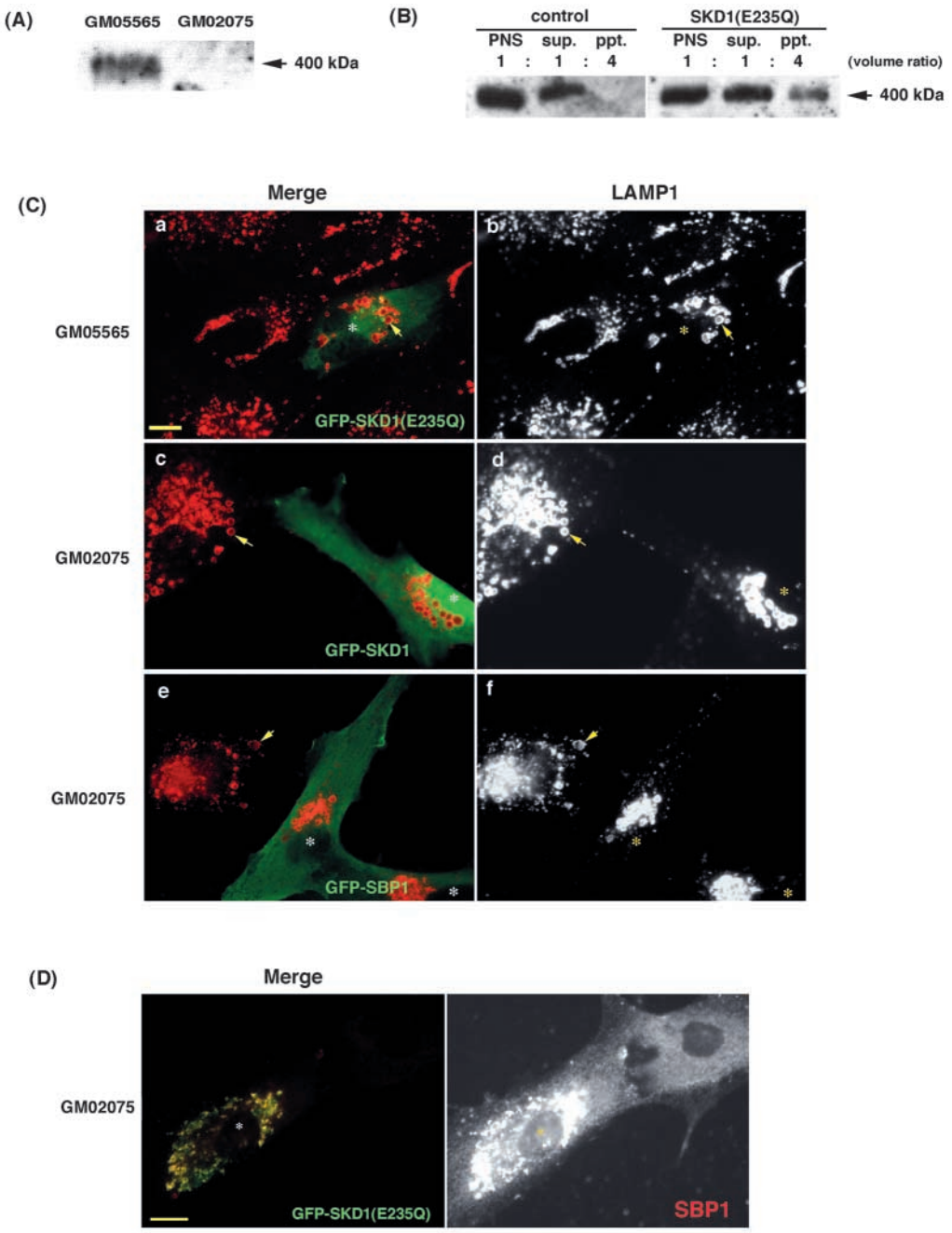

Fig. 6. SKD1(E235Q) increases the membrane association of lyst and enlarges lysosomes, but neither SKD1 nor SBP1 can decrease the size of lysosome in CHS fibroblast. (A) The pAb against murine Beige protein can detect human lyst protein on immunoblotting. Total cell lysates prepared from control human fibroblast (GM05655) and fibroblast cells obtained from a CHS patient (GM02075) were subjected to 5% SDS-PAGE and processed for immunoblotting. (B) PNS, cytosol (sup.) and membrane (ppt.) fractions prepared from control and GFP-SKD1(E235Q) adenovirus-infected U251 cells were subjected to immunoblotting analysis with anti-lyst antibody. The numbers above each lane represent the volume ratio of all fractions loaded on the SDS gel. Notice, a significant amount of lyst was associated with the membrane in the cells expressing GFP-SKD1(E235Q). (C) Overexpression of GFP-SKD1(E235Q) in control fibroblast cells (asterisks in a and b) formed enlarged lysosomes (arrows in a and b), which resembled the phenotype seen in CHS fibroblasts (arrows in c-f). However, neither wild-type GFP-SKD1 (asterisks in c and d) nor GFP-SBP1 (asterisks in e and f) can decrease the size of giant-lysosomes in CHS fibroblasts. Bar, 20 μm. (D) Transfection of GFP-SKD1(E235Q) in CHS fibroblasts did not inhibit the formation of or redistribution of SBP1 to E235Q compartments (asterisk indicated transfected cell). Scale bar: 20 μm.

markers but the extent of the co-localization varies. For instance, the transferrin receptor accumulates at aberrant endosomes and co-localized with some but not all GFP-SKD1(E235Q). These observations reflect the complexity of the origin of the endosomal/lysosomal membrane and multiple functions of SKD1 in membrane transport. Nevertheless, SBP1 is perfectly co-localized with SKD1(E235Q) at E235Q compartments. Thus, SBP1 may have an essential role for the function of SKD1.

Like other AAA-ATPases (Kondo et al., 1997; Sollner et al., 1993), SKD1 and SBP1 form a large hetero-oligomer protein complex, dependent on the ATP-bound state of SKD1. In contrast to yeast Vps4, which exists as a homo-dimer in the presence of the ADP-bound form and is assembled into a homo-decamer in the presence of the ATP-bound form (Babst et al., 1998), SKD1 exists as a monomer and may form the hetero-oligomer with SBP1 in the presence of the ATP-bound form, SKD1(E235Q). In control cells, SBP1 exists as an oligomer protein complex (estimated as ~160 kDa by size exclusion chromatography and 180 kDa by cross-link experiment; Fig. 3B,D), while in SKD1(E235Q)-expressing cells, it forms a higher order hetero-oligomer complex with SKD1 (over 669 kDa; Fig. 3C). The complex is specifically formed by the expression of SKD1(E235Q), thus it is probable that the ATPase activity of SKD1, as in the case of NSF, is required for the disassembly of the complex (May et al., 2001). The complex is probably localized to the aberrant endosomal structures derived from early and late endosomes and

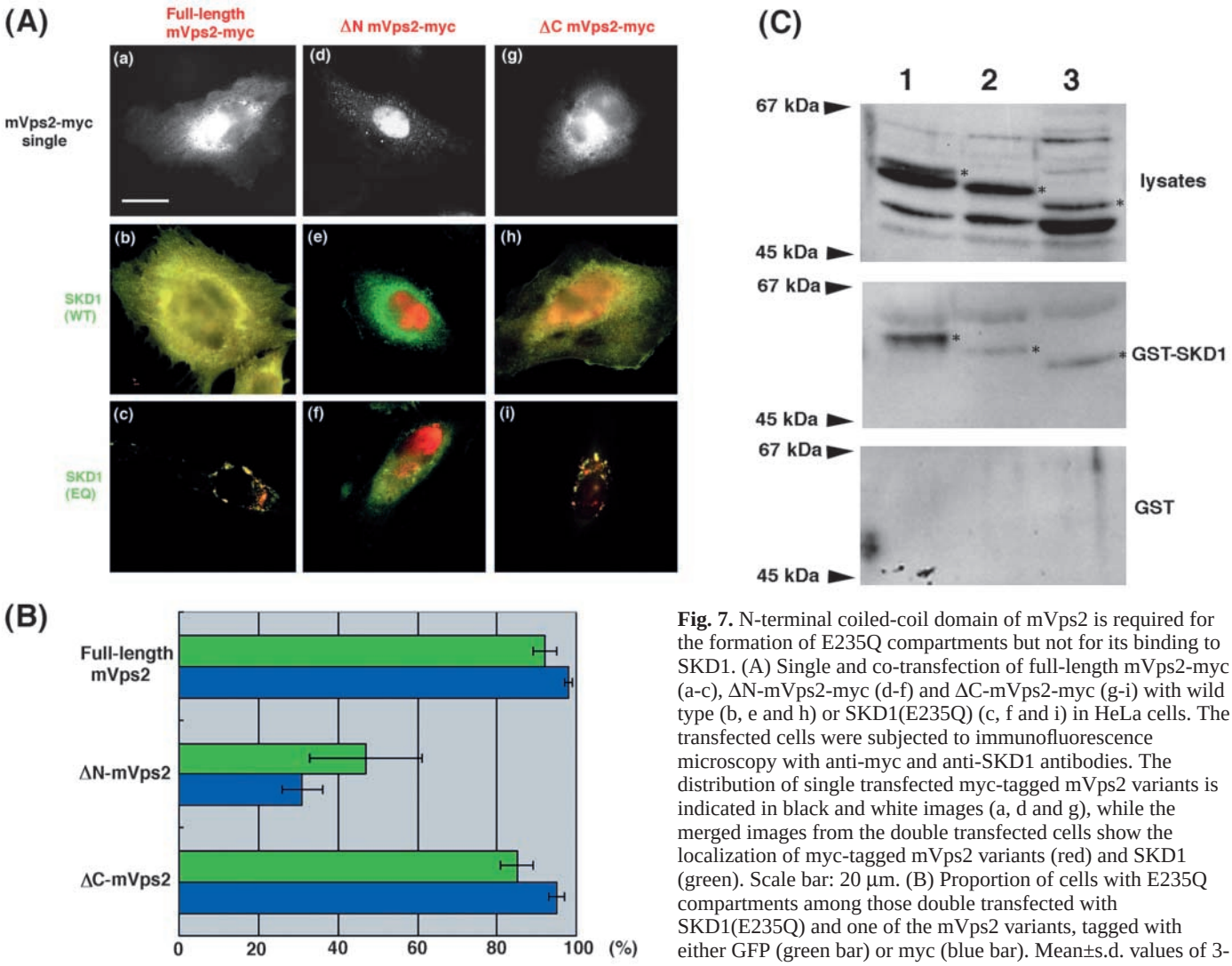

Fig. 7. N-terminal coiled-coil domain of mVps2 is required for the formation of E235Q compartments but not for its binding to SKD1. (A) Single and co-transfection of full-length mVps2-myc (a-c), ΔN-mVps2-myc (d-f) and ΔC-mVps2-myc (g-i) with wild type (b, e and h) or SKD1(E235Q) (c, f and i) in HeLa cells. The transfected cells were subjected to immunofluorescence microscopy with anti-myc and anti-SKD1 antibodies. The distribution of single transfected myc-tagged mVps2 variants is indicated in black and white images (a, d and g), while the merged images from the double transfected cells show the localization of myc-tagged mVps2 variants (red) and SKD1 (green). Scale bar: 20 μm. (B) Proportion of cells with E235Q compartments among those double transfected with SKD1(E235Q) and one of the mVps2 variants, tagged with either GFP (green bar) or myc (blue bar). Mean±s.d. values of 3-5 independent experiments are shown. (C) GST pull-down analyses of mVps2-GFP chimeras. Top panel shows immunoblots of cell lysates prepared from HeLa cells transiently expressed full-length mVps2-GFP (lane 1), ΔC-mVps2-GFP (lane 2) and ΔN-mVps2-GFP (lane 3). The cell lysates were incubated with GST-SKD1 (middle panel) or GST alone (bottom panel) adsorbed to glutathione-Sepharose beads. The bound proteins were eluted and immunoblotted with anti-GFP antibody. Asterisks indicated the GFP fusion protein bands of the predicted sizes, ~61, 57 and 55 kDa, respectively.

lysosomes. The proper recycling of the complex between the cytosol and membrane and/or the cycle of assembly and disassembly may be required for the regulation of membrane transport through endosomal/lysosomal compartments. While we cannot rule out the possibility that other proteins are associated with the complex, our experiments failed to identify them. Furthermore, because of the size discrepancy of the SBP1 monomer, predicted by SDS-PAGE (45 kDa) and amino acid composition (33.9 kDa), it is hard to accurately determined the stoichiometry of the oligomer complex. Future experiments will be aimed at identifying other components of the complex and determining their precise stoichiometry.

Both SKD1(E235Q) and rab5QL, a GTPase-deficient form of rab5 increase the size of early endosomes and perturb the membrane transport through them. However, their predicted molecular mechanisms are distinct. SKD1(E235Q) most probably acts on the vesicle budding from endosomes, while rab5QL probably accelerates the homotypic fusion of early endosomes (Simonsen et al., 1998). Indeed, we found that SKD1(E235Q) induced the membrane association of SBP1 while rab5QL did not (Fig. 4C). This result clearly demonstrates that SKD1 and rab5 regulate endosomal membrane transport independently. Interestingly, despite the distinct molecular mechanisms of SKD1(E235Q) and rab5QL, the resultant morphological alterations on late endosomes and lysosomes are quite similar; both induce the enlargement (Fujita et al., 2003; Rosenfeld et al., 2001). Based on our results and those of others, we assume that the biogenesis of lysosomes is highly ascribed to the membrane traffic at early endosomes. Maturation and/or the ‘kiss and run’ model, rather than vesicle transport, may be feasible to explain why early endosomal dysfunction severely affects the morphology of lysosomes (Mullins and Bonifacino, 2001; Storrie and Desjardins, 1996).

The homology search of SBP1 reveals that human SBP1 is identical to lyst interacting protein 5, Lip5p (Tchernev et al.,

2002). Lyst is a large cytosolic protein containing WD domains at its C terminus (Barbosa et al., 1996). A genetic defect in both lyst and beige, a mouse homologue of lyst, leads to either enlargement and/or dysfunction of lysosome-related organelles, such as melanosomes, dense granules and cytolytic granules (Burkhardt et al., 1993; Shiflett et al., 2002; Ward et al., 2000). Several lines of evidence suggest that the lyst/beige protein is responsible for fission, rather than fusion. *beige*/CHS giant lysosomes are capable of fusing with other lysosomes and overexpression of the Beige protein resulted in smaller lysosomes (Perou and Kaplan, 1993a; Perou and Kaplan, 1993b). Intriguingly, we have shown here that cytosolic lyst protein is redistributed to the membrane in the cells expressing SKD1(E235Q) (Fig. 6B). These findings strongly suggest that the membrane association of lyst is regulated by SKD1-SBP1/lip5 complex and is required for proper endosomal/lysosomal transport, most probably for the fission steps. However, overexpression of either SKD1 or SBP1 did not restore the formation of giant lysosomes in fibroblasts from CHS patients (Fig. 6C). These results imply that lyst functions downstream of the SKD1-SBP1/Lip5 complex. Indeed, the fibroblast cells of CHS patient are still capable of forming and redistributing SBP1 to E235Q compartments (Fig. 6D). Such results imply that the formation of E235Q compartments and the membrane association of SBP1 did not require the function of the lyst protein. Despite the many studies of lyst and its homologues, the precise molecular function of the lyst protein is still not well understood. The study of the SKD1-SBP1/Lip5p complex may provide a breakthrough in the study of the function of the lyst protein and the molecular mechanism behind the formation of giant lysosomes in CHS patients.

In mammalian cells, Vps4 and ESCRT-I (Vps23, Vps28) homologues have been identified and analyses have suggested that the function of these proteins is well conserved (Bishop and Woodman, 2001). Indeed, a BLAST search revealed that all yeast ESCRT-III subunits have mammalian homologues with an amino acid identity of 23-44% (data not shown). Most of our data and that of others suggest that in mammalian cells, the ESCRT model serves a similar function in the delivery of membrane proteins to lysosomes. In yeast, it has been postulated that Vps2p forms a sub-complex with Vps24p and creates an ESCRT-III with another sub-complex, Vps20p-Snf7p to recruit Vps4p to the endosomal membrane. Moreover, Vps2 mutants in yeast are unable to recruit Vps4p to the membrane. We revealed that two coiled-coil domains of mVps2 are not required for the binding to SKD1 (Fig. 7C), nevertheless, ΔN-mVps2 inhibited the formation of E235Q compartments (Fig. 7A, B). Thus, we assume that ΔN-mVps2 may form the incomplete ESCRT-III complex that is unable to recruit SKD1 to the endosomal membrane and the complex function in a dominant-negative manner. Since mVps2 exclusively localizes to cytoplasm and has no association with the membrane, mVps2 may only function for the association of SKD1 with the ESCRT-III complex. Possibly mVps20, which contains a conserved myristoylation acceptor site at the N terminus, may be responsible for the association of the SKD1-ESCRT-III complex with the endosomal membrane.

There are many other possible SKD1-interacting proteins, including Rnd2 and CHMP family (Howard et al., 2001; Tanaka et al., 2002; von Schwedler et al., 2003), however, it is not clear yet if they all have significant roles in the function of SKD1/Vps4 in membrane transport through endosomes. Intriguingly some mammalian class E Vps proteins, including ESCRT complexes, are also required for HIV budding at the PM (Garrus et al., 2001). A coherent network of their protein-protein interactions has been reported (von Schwedler et al., 2003). Functional studies of SKD1 and its binding partners may provide us novel understanding of the molecular basis of not only the cargo sorting at multi-vesicular bodies but also retrovirus budding at the PM in mammalian cells.

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