Mutational Analysis of Csc1/Vps4p: Involvement of Endosome in Regulation of Autophagy in Yeast

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Key words: autophagy/vacuole/endosome/AAA protein

ABSTRACT. In the yeast *Saccharomyces cerevisiae*, autophagy, a bulk protein degradation in the vacuole, is induced in response to nutrient starvation. In a screen for mutations that result in induction of autophagy even in the presence of nutrients, we have isolated four mutants representing two *csc* complementation groups. These mutants induce autophagy of which activity is represented by activation of truncated alkaline phosphatase that is designed to be expressed in the cytosol. *CSC1* was cloned by complementation of loss of viability phenotype of *csc1-1* mutant and shown to be identical to *END13/VPS4/GRD13*. Though *csc1-1* mutation is recessive, cells of $\Delta csc1$ do not induce autophagy in rich media, suggesting that *csc1-1* allele is not a complete loss-of-function. Csc1p is a member of novel ATPase family named AAA protein including Sec18p/NSF, Cdc48p/p97, and Pas8p. Mutation site in *csc1-1* is found in the SRH region that is highly conserved among AAA proteins. Cells of *csc1-1* show sorting defect of CPY and the appearance of the class E compartment. These mutant phenotypes suggest the role of the protein that is involved in the traffic among the Golgi, endosome, and the vacuole in autophagy.

In the yeast *Saccharomyces cerevisiae*, the vacuole is a major site of intracellular protein degradation. Two processes are responsible for the uptake of proteins into the vacuole, autophagy and endocytosis. Autophagy in yeast is induced under various nutrient starvation conditions (30). The whole process is equivalent to autophagy in mammalian cells and associated with a dynamic membrane flow (2). Starvation induces formation of autophagosomes which consist of a double membrane enclosing a portion of the cytosol non-selectively (2). The outer membrane of autophagosome immediately fuses with the vacuolar membrane and the resulting inner membrane structure, the autophagic body, is delivered to the vacuole (2, 30). The autophagic body is rapidly decomposed by vacuolar enzymes (2, 30). Cells lacking proteinase B (PrB), a serine proteinase in the vacuole, accumulate autophagic bodies in the vacuole, so that degradation of autophagic bodies depend on the activity of PrB. Ultrastructural analyses on the membranes

of autophagosomes and autophagic bodies showed that these membranes are apparently different from those of vacuole, nucleus, rough ER (rER), mitochondrion, and plasma membrane (1). In mammalian cells, some morphological studies demonstrate that the membranes of autophagosomes contain markers of rER (7, 8) or post-Golgi membrane (36, 37). The membrane dynamics of autophagic process are quite unique, hence the origin and formation of autophagosome are important problems that remain to be solved.

In endocytosis, extracellular or cell surface molecules are transported to the vacuole via endocytic pathway by passing through two sequential intermediates (12). The internalized molecules are first transferred to an early endosome that is a peripheral organelle and heterogeneous in appearance, then to the late endosome, and finally to the vacuole where they are degraded (12). The late endosome is a prevacuolar organelle that corresponds to the prevacuolar compartment of the biosynthetic pathway of the vacuole. The endocytic and biosynthetic pathways to the vacuole converge at the late endosome/prevacuolar compartment (24). A subset of vacuolar protein sorting mutants (class E *vps*) showed an exaggerated form of late endosome/prevacuolar compartment, called class E compartment, that accumulates an endocytic marker, FM4-64 (33), the Golgi protein Vps10p (the carboxypeptidase Y sorting receptor), vacuolar H⁺-ATPase and endocytosed Ste3p (the a fac-

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Abbreviations: ADH, alcohol dehydrogenase; ALP, alkaline phosphatase; API, aminopeptidase I; CPY, carboxypeptidase Y; ECL, epichemiluminescence; PMSF, phenylmethylsulfonylfluoride; PrB, proteinase B; SD, synthetic glucose medium; SCD, synthetic glucose medium containing casamino acid; SGal, synthetic galactose medium; SD(-S), synthetic sulfate free medium.

tor receptor) (23, 25). Electron microscopic analysis revealed that the class E compartment has a unique structure consisting of stacks of curved membrane cisternae (25).

Recently, the relationship between the autophagic and endocytic pathways have been clarified light on (17, 18). Both autophagy and endocytosis are pathways of transport to the vacuole/lysosome. In mammalian cells, Liou *et al.* (17) reported that the autophagic and endocytic pathways merge in a multi-stage fashion. Newly formed autophagosomes have the capacity to fuse with endocytic structures in the maturation process (17).

To know how biogenesis of autophagosome is regulated, we isolated mutants that are abnormal in regulation of autophagy. We report here the isolation and characterization of novel yeast mutants that show autophagy even in nutrient conditions (*csc*; Constitutive Sequestration of Cytosol by autophagy). Sequence analysis revealed that *CSC1* is allelic to *END13/VPS4/GRD13* (3, 19, 22) that plays important roles in the endocytic and vacuolar biosynthetic pathways. This finding would demonstrate that the membrane traffic among the Golgi, endosome, and the vacuole is involute, and may provide a genetic and biochemical means to examine this process in detail.

MATERIALS AND METHODS

Materials and reagents. Fast Red TR salt, α 2-macroglobulin, α -naphthyl phosphate, bovine serum albumin (BSA), ethylmethansulfonate (EMS), pepstatin A, and phenylmethylsulfonylfluoride (PMSF) were purchased from Sigma (St. Louis, MO). Other reagents were purchased from Wako Chemical (Tokyo, Japan) or Nakarai (Kyoto, Japan) unless

otherwise stated.

Strains, media, and genetic methods. *S. cerevisiae* strains used in this study are listed in Table I. YEPD medium contained 1% yeast extract (Difco Laboratories, Inc., MI), 2% polypeptone (Daigo-eiyo, Japan), and 2% glucose. The composition of synthetic glucose medium (SD) was 0.67% yeast nitrogen base (Difco), 2% glucose and, if needed, appropriate amino acids. SCD medium was SD medium containing 0.5% casamino acid (Difco). The composition of synthetic galactose medium (SGal) was 0.67% yeast nitrogen base, 5% galactose, 0.2% sucrose, 0.5% casamino acid, and appropriate amino acids. The synthetic sulfate-free medium [SD(-S)] was prepared as described by Rothblatt and Schekman (26). Solid media were prepared by adding 2% agar. Cell number was determined with a hemocytometer.

Standard yeast genetic techniques, such as crossing, sporulation of diploids, and tetrad dissection, were performed as described by Kaiser *et al.* (14). Yeast transformation was performed as previously described (11). DNA manipulations and DNA-mediated transformation of *E. coli* JM109 were performed by routine procedures (27).

Isolation of *csc* mutants. TN124 cells harboring *pho8Δ60* (21) were mutagenized by EMS to 40–50% survival and incubated on YEPD plates for 3 days at 30°C. The colonies were replica-plated onto fresh YEPD, incubated for 6 h at 30°C, and screened for autophagy in a rich medium as follows. Reaction mixture of total 5 ml (50 mM Tris-HCl [pH 9.0], 5 mM MgCl₂, 5 mg/ml Fast Red TR salt, 0.5 mg/ml α -naphthyl phosphate, 1 mM PMSF, 1 μ g/ml pepstatin A, 1% agar), prewarmed at 55°C, was overlaid onto colonies on YEPD plates, and incubated for 15 min at room temperature. Mutant colonies exhibited vacuolar alkaline phosphatase (ALP) activity in a nutrient-rich condition were detected as red colonies in a background of pink colonies. Red colonies were selected and

Table I. YEAST STRAINS USED IN THIS STUDY.

Strain	Genotypes	Source
TN121	<i>MATa trp1 leu2 pho13::URA3 pho8::pho8Δ60</i>	(21)
TN124	<i>MATa trp1 ura3 pho13::LEU2 pho8::pho8Δ60</i>	(21)
TN122	<i>MATa ura3 prb1::TRP1 pho13::LEU2 pho8::pho8Δ60</i>	(21)
TN123	<i>MATa ura3 apg1::TRP1 pho13::LEU2 pho8::pho8Δ60</i>	(21)
YW5-1B	<i>MATa leu2 trp1 ura3</i>	Y. Wada
KS11-10B	<i>MATa csc1-1 trp1 ura3 pho13::LEU2 pho8::pho8Δ60</i>	This study
KS11-1A	<i>MATa csc1-1 trp1 ura3 pho13::LEU2 pho8::pho8Δ60</i>	This study
KS11-2C	<i>MATa csc1-1 trp1 ura3</i>	This study
KS4-4D	<i>MATa csc1-2 trp1 ura3 pho13::LEU2 pho8::pho8Δ60</i>	This study
KS95-10C	<i>MATa csc2-1 trp1 ura3 pho13::LEU2 pho8::pho8Δ60</i>	This study
KS100	<i>MATa/α leu2/leu2 trp1/trp1 ura3/ura3</i>	This study
KS101	<i>MATa csc1-1 prb1::TRP1 ura3 pho13::LEU2 pho8::pho8Δ60</i>	This study
KS103	<i>MATa csc1-1 apg1::TRP1 ura3 pho13::LEU2 pho8::pho8Δ60</i>	This study
KS104	<i>MATa csc2-1 prb1::TRP1 ura3 pho13::LEU2 pho8::pho8Δ60</i>	This study
KS105	<i>MATa csc2-1 apg1::TRP1 ura3 pho13::LEU2 pho8::pho8Δ60</i>	This study
KSL12C	<i>MATa csc1::LEU2 trp1 pho13::URA3 pho8::pho8Δ60</i>	This study

incubated in liquid YEPD medium containing 1 mM PMSF for 3 to 5 h at 30°C, and observed by a light microscope to select those which accumulated autophagic bodies in the vacuole.

Assay of ALP activity. Cells were incubated in appropriate media to a density of $1\sim 2 \times 10^7$ cells/ml at 30°C, harvested by centrifugation, and washed in distilled water. Yeast lysate was prepared by disrupting the cells in ice-cold reaction buffer (50 mM Tris-HCl [pH 9.0], 5 mM MgCl₂, 1 mM PMSF, 1 µg/ml pepstatin A) and removed the cell debris by centrifugation at $10,000 \times g$ for 5 min (21). ALP activity was assayed by the method as described by Torriani (31). Reaction mixture (200 µl) contained 50 mM Tris-HCl (pH 9.0), 5 mM MgCl₂, 0.64 mg/ml *p*-nitrophenyl phosphate, 1 mM PMSF, 1 µg/ml pepstatin A, and the cell lysate as enzyme was incubated at 30°C for 20 to 30 min. The reaction was stopped by addition of 200 µl of 10% trichloroacetic acid. Then 400 µl of saturated sodium bicarbonate was added to the solution and the absorbance at 420 nm was measured by a spectrophotometer. Protein concentration of the lysate was assayed with the BCA Protein Assay Reagent (Pierce, Rockford, IL) using BSA as standard. The reaction time and amount of the cell lysate were within the linear range for these parameters. A unit of activity was defined as an absorbance of 420 nm per mg protein per min.

Cloning and sequencing of the *CSC1* gene. The *CSC1* gene was cloned by complementation of the loss of viability phenotype of *csc1-1* mutant under nitrogen-starvation condition. Cells of KS11-10B (*csc1-1*) were transformed with a yeast genomic library constructed on YCp50, spread onto SCD (-Ura), and incubated for 3 days at 30°C. A total of approximately 10,000 transformants at a density of $2\sim 4 \times 10^2$ colonies per plate were replica-plated onto SD(-N) plates containing 10 µg/ml phloxine B and incubated at 30°C for 3 days. The starvation-resistant cells forming white colonies were selected, and incubated in liquid SCD(-Ura) medium containing 1 mM PMSF for 3 to 5 h. The cells were observed under a light microscope to select those that did not accumulate autophagic bodies in the vacuole. Plasmids were isolated from those cells and reintroduced into the cells of KS11-10B (*csc1-1*) for the ability to confer the starvation-resistance and induction of autophagy in a rich medium.

The cloned insert was mapped by integration of plasmid pKS306-H1.8, constructed by subcloning of a 1.8-kb of *Hind*III fragment of the isolated plasmid into the *Hind*III site of pRS306 (29). pKS306-H1.8 was linearized at the unique *Bgl*II site and transformed into the wild-type (*ura3*) strain. The resultant strain was crossed with KS11-1A (*csc1-1*) and spores from the resultant diploids were dissected. Sixteen tetrads were analyzed and showed parental ditypes for the Ura⁺ and Csc⁺ phenotypes.

The DNA sequence of *CSC1* was obtained by generating exonuclease III — Mung bean nuclease deletion constructs from both directions of plasmid pKS11-H1.8, constructed by subcloning of the 1.8-kb minimal complementing fragment into

the *Hind*III site of pBluescript II KS⁺ (Stratagene, CA). Double-stranded plasmid DNA templates used for sequencing were isolated by a standard alkaline lysis method and denatured before sequencing. Overlapping clones were sequenced with KS (5'-cgaggtcgacggtatcg-3') and SK (5'-tctagaactagtggtatc-3') — specific primers according to the dideoxynucleotide-chain termination method using the Sequence Pro kit (Toyobo, Tokyo, Japan).

Cloning and sequencing of the mutant alleles. Mutant alleles were isolated from the genomic DNA of the *csc1-1* and *csc1-2* strains. First, about 2.8-kb *Eco*RI/*Apa*I fragments of *csc1-1* and *csc1-2* genomic DNA were subcloned into *Eco*RI/*Apa*I site of pBluescript II KS⁺. Generated plasmids were then amplified in *E. coli* JM109, and digested with *Hind*III. Approximately 1.8-kb *Hind*III fragments were then subcloned into *Hind*III site of pBluescript II KS⁺. Sixteen transformants of each were analyzed whether they contained *csc1-1* or *csc1-2* by polymerase chain reaction with specific oligodeoxynucleotide primers of *CSC1*. *csc1-1* and *csc1-2* were sequenced by ABI PRISM Dye Terminator Cycle Sequencing kit (Perkin Elmer, Foster City, CA) with specific oligodeoxynucleotide primers.

Gene disruptions. Single step disruptions of the chromosomal genes were performed with linear DNA fragments. To disrupt the *PRB1* gene, pST100 (21) was digested with *Sal*I and introduced into KS11-10B and KS95-10C, respectively. Disruption of the *APG1* gene was performed by introducing *Sca*I digested pTN4 (21) into KS11-10B and KS95-10C, respectively. To disrupt the *CSC1* gene, blunt-ended *Acc*I-*Bgl*II fragment of pKS11-H1.7, constructed by cloning the 1.7-kb *Hind*III-*Hinc*II fragment of pKS11-1.8 into *Hind*III/*Hinc*II site of pBluescript II KS⁺, was replaced to blunt-ended *Hind*III-*Bam*HI fragment of pJJ252 (13) that contains *LEU2* to produce pKS11Δ*leu*. Resultant plasmid was digested with *Hind*III/*Hinc*II and introduced into KS100 and TN121. In all cases, correct integration was verified by Southern blot analysis using the epichemiluminescence (ECL) system (Amersham, UK).

Construction of plasmids. The 1.8-kb *Hind*III fragment containing *CSC1* or *csc1-1* was cloned into the *Hind*III site of pRS424 (29) to obtain pKS401 and pKS402, respectively. pMB24 (3) containing *CSC1/VPS4*^{K179A} that have a mutation in the ATPase domain was kindly provided by S.D. Emr (U.C.S.D.). pMB24 was digested with *Hind*III and the 1.8-kb fragment was cloned into the *Hind*III site pRS424 to create pKS403. To construct pKS404 that contains *csc1*^{K179A E291K}, the 360-bp *Bst*EII/*Nco*I fragment of pKS402 was replaced by the 360-bp *Bst*EII/*Nco*I fragment of pMB24.

*Bam*HI site was generated by a polymerase chain reaction at base position -4 of the *CSC1* or *csc1-1* open reading frame. *Bam*HI/*Hind*III fragment containing *CSC1* or *csc1-1* was subcloned into pYES2, which is a 2 µ yeast expression vector containing the *GAL1* promoter (Invitrogen, San Diego, CA).

Preparation of antibody against Csc1p. A GST-*CSC1* fu-

sion gene was constructed by cloning a blunt-ended 390-bp *StyI-RsaI* fragment, encoding the carboxy-terminal 117 amino acids of Csc1p, into blunt-ended *Bam*HI site of pGEX-2T (Pharmacia Biotech, Uppsala, Sweden), and was used to transform *E. coli* strain BL21 (DE3). The fusion protein was purified by Glutathione Sepharose 4B (Pharmacia Biotech) according to manufacturer's instruction. The purified fusion protein was used to immunize a rabbit to generate anti-Csc1p antiserum by Shibayagi (Gunma, Japan).

SDS-PAGE and immunoblotting. SDS-PAGE was performed according to Laemmli (16). Electrophoresed proteins were transferred electrophoretically to a nitrocellulose membrane in a semi-dry transfer apparatus at 100 mA for 1 h. The membrane was blocked and then incubated for one hour with primary antibody in TBST (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, and 0.05% Tween 20), 10% nonfat dry milk at the following dilutions: anti-ALP (21), and anti-Kex2p (21) sera were used at 1:500 dilutions, anti-alcohol dehydrogenase (2) and anti-Csc1p sera were used at 1:10,000 dilutions. The membrane was then washed three times for 10 min each in TBST, incubated with a 1:10,000 dilution of either goat anti-rabbit IgG conjugated to horseradish peroxidase or goat anti-rabbit IgG conjugated to ALP (both from Jackson ImmunoResearch, West Grove, PA) in TBST containing 10% nonfat dry milk for 1 h, and washed three times for 10 min each in TBST. Membrane-bound antibodies were then detected by the ECL system (Amersham) or the bromochloroindolyl phosphate/nitro blue tetrazolium substrate.

Subcellular fractionation. Yeast strain YW5-1B (wild-type) and KS11-2C (*csc1-1*) were grown at 30°C to $1\sim 2 \times 10^7$ cells/ml in YEPD medium and harvested by centrifugation. Spheroplasts were generated and collected as described by Nishikawa *et al.* (20). The spheroplasts were suspended in lysis buffer (50 mM Tris-HCl [pH 7.5], 0.2 M sorbitol, 1 mM EDTA, 1 mM PMSF) and dounced for 20 times in an ice-cold Dounce homogenizer. The resultant lysate was centrifuged at $500 \times g$ for 5 min at 4°C to remove unbroken spheroplasts and cells. The supernatant was centrifuged at $100,000 \times g$ for 60 min at 4°C in a TLA100.3 rotor (Beckman Instruction, Palo Alto, CA) to separate into soluble and particulate fractions. Each fraction was subjected to SDS-PAGE and immunoblotting analysis.

Radiolabeling and Immunoprecipitation. Radiolabeling and immunoprecipitation were performed as described by Wada *et al.* (34) with modifications. Yeast cells were grown at 30°C to $1\sim 2 \times 10^7$ cells/ml in SD medium and converted to spheroplasts. The spheroplasts were resuspended in SD(-S) containing 0.7 M sorbitol, 1 mg/ml of BSA and 100 μ g/ml of α 2-macroglobulin. The cells were incubated at 30°C for 30 min and then labeled for 10 min at 30°C with 25 μ Ci/ 10^7 cells of Tran³⁵S-label (ICN, Irvine, CA). Chase was initiated by the addition of 5 $\times$ chase solution (25 mM methionine, 5 mM cysteine, 5% glucose, 2.5% yeast extract). Following a 0-, 15- or 45-min chase period, the cultures were centrifuged at $13,000 \times g$ for 1 min to generate an intracellular (pellet) fraction and

an extracellular (supernatant) fraction. The amount of CPY in each fraction was determined by immunoprecipitation, SDS-PAGE, and fluorography.

Vital staining. Every cell culture examined was grown at 30°C to $1\sim 2 \times 10^7$ cells/ml in YEPD or SCD(-Trp) medium. FM4-64 (Molecular Probes Inc., Eugene, OR) staining was performed as described by Vida and Emr (33).

RESULTS

Isolation of mutants which induce autophagy in a nutrient condition. Autophagy is induced under the starvation conditions, but not under the nutrient-rich condition (2, 21, 30, 32). Therefore, there some mechanism must exist for regulation of autophagy. To elucidate that mechanism, we searched for mutants that showed autophagy even in rich medium.

We have previously developed a biochemical method to monitor autophagy in yeast (21). Pho8 Δ 60p, a vacuolar alkaline phosphatase which lacks N-terminus 60 amino acids containing transmembrane region, is designed to be expressed in the cytosol. This protein is transported to the vacuole in an autophagy-dependent manner and acquires phosphatase activity as the result of processing by vacuolar proteinase (21). Therefore, mutant cells which show autophagy in a nutrient-rich condition are predicted to exhibit elevated ALP activity.

Yeast cells expressing Pho8 Δ 60p were mutagenized by EMS, and approximately 30,000 colonies were screened for ALP activity in a rich medium, YEPD. Mutant cells exhibiting high ALP activity under the nutrient condition were then observed with a light microscope, and four mutants accumulated autophagic bodies in the vacuole when incubated in YEPD medium containing 1 mM PMSF (Fig. 1). Cross with the wild-type strain revealed that these four mutations were recessive. Tetrad analyses strongly suggested that the phenotypes of mutants were derived from single nuclear gene mutations. Cross among these mutants revealed that they could be assigned to two complementation groups, 1)

Fig. 1. Accumulation of autophagic bodies in *csc* mutants. Cells of TN124 (wild-type), KS11-10B (*csc1-1*), and KS95-10C (*csc2-1*) were incubated in YEPD medium to $0.5\sim 1 \times 10^7$ cells/ml and 1 mM PMSF was added to the medium. Cells were further incubated for 3~5 h and visualized by phase-contrast microscopy. The bar indicates 5 μ m.

Table II. ACTIVATION OF PHO8Δ60P IN THE *csc* MUTANTS.

	ALP activity (A420/min/mg protein)		
	—	<i>Δprb1</i>	<i>Δapg1</i>
Wild-type	0.135	0.122	0.133
<i>csc1-1</i>	0.212	0.124	0.140
<i>csc2-1</i>	0.491	0.117	0.147

csc1-1, *csc1-2* and *csc1-3*, and 2) *csc2-1* (*csc*; Constitutive Sequestration of Cytosol by autophagy).

Degradation of autophagic bodies depends on the activity of proteinase B (PrB) (30). To determine whether the activation of Pho8Δ60p occurred within the vacuole, we disrupted the *PRB1* gene of the *csc* mutants. ALP activities of the mutant cells growing in YEPD medium were markedly affected by *PRB1* disruption, though that of wild-type was not (Table II). This indicates that the activation of Pho8Δ60p in these *csc* mutants occurred within the vacuole.

To determine whether the activation of Pho8Δ60p in the mutants reflects autophagy, we constructed double mutants for *csc* and *Δapg1*. The Apg1 protein (Apg1p) is a serine/threonine protein kinase and its kinase activity is essential for autophagy (Matsuura *et al.*, in press). ALP activities of the *csc1-1* and *csc2-1* strains were restrained by *APG1* deletion to the basal level (Table II). This implies that the activation of Pho8Δ60p in the *csc* mutants reflects autophagy. From these results, we concluded that the *csc* mutants actually induce autophagy even in a rich medium.

CSC1 is allelic to *END13/VPS4/GRD13*. In the course of characterization of mutants, we found that *csc1-1* mutant cells lose viability under the nitrogen-starvation condition. Tetrad analysis revealed that the loss of viability under the nitrogen-starvation condition co-segregated with the phenotype of autophagy in a rich medium (data not shown). By complementation of this loss of viability phenotype of KS11-10B (*csc1-1*), the *CSC1* gene was cloned from a yeast genomic library. Three plasmids that conferred starvation-resistance were recovered. The minimal complementing region was mapped by subcloning and retransformation into the *csc1-1* strain, and encompassed by a 1.8-kb *HindIII* fragment. To confirm that this genomic clone contained *CSC1*, the 1.8-kb *HindIII* fragment was subcloned into an integrating vector containing the *URA3* gene, and transformed into the wild-type (*Ura*[−]) strain after linearizing at the unique *Bgl*III site. An *Ura*⁺ transformant was then crossed to the KS11-1A (*csc1-1*) strain. Tetrad analysis of the resulting diploid showed that all spores from 16 tetrads were parental ditypes (*Ura*⁺ *Csc*⁺, *Ura*[−] *Csc*[−]), strongly suggesting that this clone contains *CSC1*.

Sequencing analysis revealed that this fragment contains an open reading frame predicted to encode a pro-

Fig. 2. Amino acid sequence alignment of AAA module of Csc1p, Sec18p, Yta10p, and S4. The solid lines mark the ATP binding motif and the SRH region. Amino acid change in the Csc1^{E291K}p is indicated by asterisk.

tein with a molecular weight of 48.2 kDa. A BLAST search with the *CSC1* gene revealed that *CSC1* is identical to *VPS4/END13/GRD13* (GenBank accession number U25842) (3, 19, 22). Csc1 protein (Csc1p) belongs to the AAA (ATPases Associated to a variety of cellular Activities) protein family (5). Despite extensive sequence conservation, the member of the family fulfill a large diversity of cellular functions (5).

To determine the mutation site within the *csc1-1* allele, we cloned the 1.8-kb *HindIII* fragment from genomic DNA of KS11-10B. Sequence analysis of the fragment revealed single transition of G to A at base position +871 within the *CSC1* open reading frame (Fig. 2B). This change results in an amino acid substitution of Glu to Lys in the SRH (Second Region of Homology) region that is highly conserved among the AAA protein family, but whose function is still unknown (5). We isolated another independent allele *csc1-2*, but sequence analysis revealed the same amino acid substitution in it.

csc1-1 is a gain-of-function allele. We generated null mutant of the *CSC1* gene whereby a significant part of the protein-coding region of the gene was deleted. A disruptant of *CSC1* (*Δcsc1*) was viable and had no significant growth defect. We then analyzed the effect of the *CSC1* deletion on autophagy in the nutrient-rich condition by activation of Pho8Δ60p (Fig. 3). Unexpectedly, *Δcsc1* cells did not show ALP activity which reflects autophagy in the growing condition (SCD medium). To test whether *csc1*^{E291K} (*csc1-1*) behaves as a gain-of-function mutation, *csc1*^{E291K} was overexpressed in *Δcsc1* cells

Fig. 3. ALP activity of Pho8Δ60p under nutrient condition. Cultures of $\Delta csc1$ (KSL12C *pho8Δ60*) carrying pRS424 (vector) or pKS401 (2 μ *CSC1*) or pKS402 (2 μ *csc1^{E291K}*) or pKS403 (2 μ *csc1^{K179A}*) or pKS404 (2 μ *csc1^{K179A}E291K*) and TN124 (*pho8Δ60*) carrying pYES2 (vector), pYES2/*CSC1*, or pYES2/*csc1^{E291K}* were grown in YEPD or SGal medium and ALP activities of these cultures were measured as described in Materials and Methods. The values were indicated as differences from the activity of wild-type.

and the phenotype was examined by ALP activity. $\Delta csc1$ cells harboring *csc1^{E291K}* allele on a high-copy number (2 μ) plasmid showed elevated ALP activity. Furthermore, wild-type cells showed elevated ALP activity when *csc1^{E291K}* was overexpressed under the *GAL1* promoter. These results demonstrate that the *csc1^{E291K}* is a gain-of-function allele.

Since Csc1p is a member of the AAA protein family, we determined the requirement of ATPase activity. *csc1^{K179A}* and *csc1^{K179A}E291K*, that have both K179A in the ATPase domain and E291K in the SRH region, were introduced into $\Delta csc1$ cells and the ALP activities were measured. *csc1^{K179A}* did not act as a gain-of-function allele, because $\Delta csc1$ cells expressing *csc1^{K179A}* did not show significant ALP activity in a rich medium. $\Delta csc1$ cells harboring double mutant allele on high-copy plasmids did not show significant ALP activity in a rich medium, which was comparable to the ALP activity of the cells harboring a mutation only in the ATPase domain (Fig. 3). This result implies that the ATPase activity is necessary for the induction of autophagy by

Fig. 4. Immunoblot analysis of yeast extracts using anti Csc1p antibodies. (A) Yeast extracts were prepared from TN124 (WT), KS11-10B (*csc1-1*), KSL12C ($\Delta csc1$), and KSL12C carrying pKS401 (2 μ *CSC1*). (B) Spheroplasts of YW5-1B (wild-type) and KS11-2C (*csc1-1*) were osmotically lysed with dounce homogenization. The homogenates (T) were then spun in an ultracentrifuge at 100,000 $\times$ g for 1 h. The pellet (P) and supernatant (S) fractions were then subjected to immunoblot analysis with antiserum specific to Csc1p.

*csc1^{E291K}*p.

Both wild-type and mutant Csc1p are soluble proteins. We generated Csc1p-specific antiserum against GST-Csc1 fusion protein. Immunoblotting analysis of wild-type cells identified a single protein of 50 kDa which was not detected in $\Delta csc1$ cells (Fig. 4A lane 1 and 3). Csc1^{E291K}p was also detected with the antiserum as a 50-kDa polypeptide (Fig. 4A lane 2).

Subcellular fractionation was performed to assess the intracellular location of Csc1p. Spheroplasts of the wild-type (YW5-1B) and *csc1-1* (KS11-2C) strains were lysed and cleared by centrifugation at 500 $\times$ g. The cleared cell lysates were subjected to centrifugation at 100,000 $\times$ g to generate pellet (P100) and supernatant (S100) fractions. The resulting fractions were subjected to immunoblotting analyses with antisera that recognize either Csc1p or various marker proteins. Both wild-type and mutant proteins were present mainly in the supernatant fraction indicating that it is soluble under these conditions (Fig. 4B lane 1–3, 4–6), although small amounts were detected in the pellet fractions, indicating the association of Csc1p and Csc1^{E291K}p to membranes or large protein complex.

***csc1-1* mutant has a defect in the endosomal function.** The *CSC1* was isolated by three independent screenings for mutants defective in vacuolar protein sorting (3), endocytosis (19), and Golgi protein retention (22), which suggests the role of the Csc1p in the endosomal function. We analyzed the effect of the *csc1-1*

mutation on the endosomal functions.

Transport of CPY through the secretory pathway could be monitored by the appearance of three different forms, a 67-kDa ER form (p1CPY), a 69-kDa Golgi-modified form (p2CPY), and a 61-kDa mature form (mCPY). In the late-Golgi, CPY is sorted out from the secreting proteins by the sorting receptor Vps10p (4, 6). In the class E *vps* mutants, the defect in the retrieval pathway of Vps10p leads to a partial missorting of CPY to the cell surface (23). To determine the effect of *csc1-1* mutation on the endosomal function, we analyzed the transport of CPY by pulse-chase experiment. After the termination of the chase period, the intracellular (I) and extracellular (E) fractions were separated and the immunoprecipitated CPY species were analyzed by SDS-PAGE. In wild-type (TN124) cells, CPY was completely converted to the mature form and detected only in the intracellular fractions, demonstrating proper delivery (Fig. 5 lane 1–3). On the other hand, in cells of *csc1-1* (Fig. 5 lane 4–6) as well as $\Delta csc1$ (Fig. 5 lane 7–9), a portion of CPY was detected as p2-Golgi form in the extracellular fractions.

CSC1/VPS4 is categorized into the class E *vps* mutants (24). One characteristic phenotype of the class E *vps* mutants is appearance of the class E compartment which represents an exaggerated version of the prevacuolar/late endosomal compartment (25). The class E compartment could be stained transiently with the fluorescent lipophilic dye, FM4-64, that is transported to the vacuole by endocytosis (33). Logarithmic growing cells were incubated in YEPD medium containing FM4-64 for 15 min, and chased for 45 min in fresh YEPD medium. In wild-type cells, only the vacuolar membranes were stained with the dye (Fig. 6A). Cells of $\Delta csc1$ showed brightly stained spots adjacent to the vacuoles (Fig. 6B). This is a typical staining pattern of the class E

Fig. 6. Endocytosis determined with FM4-64. Wild-type (TN124), *csc1-1* (KS11-10B), and $\Delta csc1$ (KSL12C) cells were treated with the vital endocytic marker FM4-64 for 15 min followed by a 45-min chase period. The stained cells were visualized by Nomarski (left panels) and fluorescence (right panels) microscopy. The bar indicates 5 μ m. The arrowheads indicate the class E compartment.

compartment. Cells of *csc1-1* exhibited small spots adjacent to the vacuoles of which fluorescence was weaker than that of $\Delta csc1$ (Fig. 6C). These results suggest that

Fig. 5. Pulse-chase experiment of CPY sorting. Spheroplasts of TN124 (wild-type), KS11-10B (*csc1-1*), and KSL12C ($\Delta csc1$) were pulse-labeled with Tran³⁵S for 10 min and chased for 45 min. The cultures were then separated to intracellular (I) and extracellular (E) fractions. CPY was immunoprecipitated and analyzed by SDS-PAGE.

the mutant strain has some defect in the endosomal functions.

DISCUSSION

We have succeeded in isolating yeast mutants which showed autophagy in a nutrient-rich condition by means of a biochemical method for autophagy (21). The *csc* mutants isolated in this screening fell into two complementation groups, one of which, *CSC1*, encodes an ATPase that is a member of the AAA protein family. The AAA proteins are involved in diverse cellular activities, such as membrane fusion (10, 35) and organelle biogenesis (9). The key feature of the family is a highly conserved module of 230 amino acids present in one or two copies in each protein (5). The contrast between functional diversity and structural conservation of the module suggests that they play essential roles at key points of the cellular machinery (5).

The *csc1-1* (*csc1*^{E291K}) allele contains a mutation which results in an amino acid substitution in the SRH region. This region is highly conserved among the AAA protein family, so some pivotal roles should be addressed to this region (5). Surprisingly, though the *csc1-1* mutation is recessive, a disruptant of the *CSC1* gene does not cause autophagy in the nutrient-rich condition. In wild-type cells, overexpression of the *csc1-1* allele under the *GAL1* promoter leads the *csc* phenotype, indicating that *csc1-1* is a gain-of-function allele. Double mutation analysis of the SRH region (E291K) and the ATPase domain (K179A) of Csc1p revealed that the ATPase activity is required for the *csc* phenotype autophagy under the nutrient-rich condition. These findings suggest that *csc1-1* confers novel function during induction of autophagy. One possible explanation is that the SRH region of Csc1p interacts with a molecule essential for autophagy.

In our laboratory, 14 mutants which have the defect in autophagy (*apg*) were isolated (32). None of them could form autophagosomes, but were normal for general starvation response pathways such as glycogen accumulation and induction of vacuolar proteins. The phenotypic similarities among the 14 *apg* mutants suggest that the Apg proteins act together at close in the autophagic pathway. Recent studies revealed that the *APG* genes are also required for transport of aminopeptidase I (API) (28). API is synthesized as a precursor in the cytoplasm and targets directly to the vacuole (15). Autophagy is induced only under the starvation condition, whereas transport of API occurs even in a nutrient condition. These raise the possibility that the Apg proteins play roles in the formation of autophagosome, rather than the starvation signaling for induction of autophagy. Analyses of double mutants of *csc1/apg1* and *csc2/apg1* indicated that the *APG1* gene is required for

the *csc* phenotype. This suggests that Apg1p and other Apg proteins function around the late-endosome.

CSC1 is identical to *VPS4/END13/GRD13* that is supposed to play a role in the endosomal function (3, 19, 22). Indeed, we found that the endosomal function is impaired in the *csc1-1* and disruptant strains, including the sorting defect of CPY and the appearance of class E compartment. Emr's group proposed that the class E Vps proteins might be involved either directly in the formation of the transport intermediates from the endosome or in the maintenance of a dynamic endosomal structure required for transport out of the compartment. One interesting point is the morphological characteristic of the class E *vps* mutants, the accumulation of class E compartment which consists of stacks of curved membrane cisternae (25). During the formation of autophagosome, it appears as a cupped-shape structure which has different membrane characteristics from the rER, Golgi, and the vacuole (1, 2). These imply that the late endosome has a critical role in the formation of autophagosome. Δ *csc1* cells showed a severe defect in autophagy under starvation conditions (our unpublished observation), indicating that Csc1p is involved in the autophagic process. A recent study on mammalian cells has revealed that the autophagic and endocytic pathways merge in a multi-stage fashion (17). Further analysis is required to assess the relationship between autophagy and endosomes.

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(Received for publication, June 4, 1997

and in revised form, June 27, 1997)