Loss of Apm1, the μ 1 Subunit of the Clathrin-Associated Adaptor-Protein-1 Complex, Causes Distinct Phenotypes and Synthetic Lethality with Calcineurin Deletion in Fission Yeast

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Calcineurin is a highly conserved regulator of Ca^{2+} signaling in eukaryotes. In fission yeast, calcineurin is not essential for viability but is required for cytokinesis and Cl^- homeostasis. In a genetic screen for mutations that are synthetically lethal with calcineurin deletion, we isolated a mutant, *cis1-1/apm1-1*, an allele of the *apm1*⁺ gene that encodes a homolog of the mammalian μ 1A subunit of the clathrin-associated adaptor protein-1 (AP-1) complex. The *cis1-1/apm1-1* mutant as well as the *apm1*-deleted (Δ *apm1*) cells showed distinct phenotypes: temperature sensitivity; tacrolimus (FK506) sensitivity; and pleiotropic defects in cytokinesis, cell integrity, and vacuole fusion. Electron micrographs revealed that Δ *apm1* cells showed large vesicular structures associated with Golgi stacks and accumulated post-Golgi secretory vesicles. Δ *apm1* cells also showed the massive accumulation of the exocytic v-SNARE Syb1 in the Golgi/endosomes and a reduced secretion of acid phosphatase. These phenotypes observed in *apm1* mutations were accentuated upon temperature up-shift and FK506 treatment. Notably, Apm1-GFP localized to the Golgi/endosomes, the spindle pole bodies, and the medial region. These findings suggest a role for Apm1 associated with the Golgi/endosome function, thereby affecting various cellular processes, including secretion, cytokinesis, vacuole fusion, and cell integrity and also suggest that calcineurin is involved in these events.

INTRODUCTION

Calcineurin is a Ca^{2+} /calmodulin-dependent serine/threonine protein phosphatase that plays a crucial role in mediating Ca^{2+} -dependent signaling in various organisms (Klee *et al.*, 1979). Two important immunosuppressive drugs, namely, cyclosporin A and tacrolimus (FK506) are potent and specific inhibitors of calcineurin activity (Liu *et al.*, 1991). Calcineurin dephosphorylates and regulates transcription factors, such as nuclear factor of activated T cells (Klee *et al.*, 1979; Crabtree and Olson, 2002) and Elk-1 in mammals (Tian and Karin, 1999), Crz1/Tcn1 in budding yeast (Stathopoulos and Cyert, 1997), and Prz1 in fission yeast (Hirayama *et al.*, 2003), thereby influencing various

cellular functions, including T-cell activation, muscle development, cardiac hypertrophy, and ion homeostasis. Aside from transcriptional factors that are important calcineurin targets, calcineurin also has been reported to regulate various physiological functions, such as apoptosis and intracellular trafficking in higher eukaryotes, and cell wall synthesis and vacuolar morphology in both budding and fission yeasts (Conboy and Cyert, 2000; Cheng *et al.*, 2002; Sugiura *et al.*, 2002). However, the mechanisms underlying these roles remain unclear.

We have been studying calcineurin signal transduction pathway in fission yeast *Schizosaccharomyces pombe* because this system is amenable to genetic analysis and has many advantages in terms of its relevance to higher systems. *S. pombe* has a single gene encoding the catalytic subunit of calcineurin, *ppb1*⁺, that is essential for cytokinesis (Yoshida *et al.*, 1994) and chloride ion homeostasis; and calcineurin acts antagonistically with the Pmk1 mitogen-activated protein kinase pathway (Sugiura *et al.*, 1998, 1999, 2003). The cell-separation and chloride-sensitive phenotypes as well as the functional interaction between calcineurin and Pmk1 are

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very different from those reported for calcineurin-null cells in budding yeast (Nakamura *et al.*, 1993; Mendoza *et al.*, 1994; Pozos *et al.*, 1996; Sugiura *et al.*, 2002), suggesting that the signaling pathways regulated by calcineurin or the molecules functionally interacting with calcineurin may be distinct in these distantly related yeasts.

To identify components that functionally interact with the calcineurin-mediated pathway, we used the immunosuppressant drug FK506, which specifically inhibits the activity of calcineurin, in a genetic screen and searched for the mutations that display synthetic lethality with the calcineurin-null mutation. By this genetic screen, we identified the Rab family small GTPase Ypt3/Its5, and demonstrated the functional connection between calcineurin and Ypt3/Its5 in membrane trafficking and cytokinesis (Cheng *et al.*, 2002).

Here, we report the identification and characterization of *cis1-1*, a new addition to the series of immunosuppressant- and temperature-sensitive mutants. The *cis1*⁺ gene is identical to the *apm1*⁺ gene (SPBP16F5.07), which encodes a protein that is highly similar to the mammalian μ 1A subunit of the clathrin-associated adaptor protein complex 1 (AP-1) and to the budding yeast *Saccharomyces cerevisiae* Apm1. Hence, we renamed the *cis1-1* mutant as *apm1-1* mutant. The AP-1 mediates protein sorting at the *trans*-Golgi network (TGN) (Boehm and Bonifacino, 2001), and the μ subunits have been implicated in cargo selection. Three sorting signals for selection into clathrin-coated vesicles have been identified, and of these, the best characterized is the YXX ϕ signal, which binds to the μ subunits of both AP-1 and AP-2 (Ohno *et al.*, 1995). Recent genetic approach has begun to reveal a role for μ 1 subunit in membrane trafficking in various organisms. Mouse "knock-outs" of μ 1A-adapatin gene cause embryonic lethality, and the role for μ 1A-adapatin for the endosome-to-TGN transport has been reported previously (Zizioli *et al.*, 1999; Meyer *et al.*, 2000). In *S. cerevisiae*, the involvement of AP-1 in transport from endosomes to the TGN of chitin synthase III has been reported (Valdivia *et al.*, 2002). However, the deletion of AP-1 subunit genes yielded no discernible phenotypes (Yeung *et al.*, 1999), except when combined with a temperature-sensitive allele of the clathrin heavy chain gene, and hence the physiological roles of AP-1 complex still remain unclear in yeasts.

Here, we present the evidence that, in contrast with its homolog in budding yeast, a mutation and a deletion of Apm1, the μ 1 subunit of AP-1 complex in fission yeast, caused distinct phenotypes, namely, temperature and FK506 sensitivities, and pleiotropic defects in cytokinesis, cell wall integrity, vacuole fusion, and secretion. Notably, these pleiotropic defects caused by the loss of Apm1 function were exacerbated in the presence of the calcineurin inhibitor FK506, strongly suggesting the involvement of calcineurin in these biological processes. This article provides the first demonstration of a genetic and functional interaction in vivo between calcineurin and the clathrin-associated AP-1 complex.

MATERIALS AND METHODS

Strains, Media, and Genetic and Molecular Biology Methods

S. pombe strains used in this study are listed in Table 1. The complete medium, YPD, YES, and the minimal medium, EMM, have been described previously (Moreno *et al.*, 1991; Toda *et al.*, 1996). Standard genetic and recombinant-DNA methods (Moreno *et al.*, 1991) were used except where noted. FK506 was provided by Fujisawa Pharmaceutical (Osaka, Japan).

Table 1. *S. pombe* strains used in this study

Strain	Genotype	Reference
HM123	<i>h</i> ⁻ <i>leu1-32</i>	Our stock
HM528	<i>h</i> ⁺ <i>his2</i>	Our stock
KP1248	<i>h</i> ⁻ <i>leu1-32 ura4-294</i>	Our stock
KP356	<i>h</i> ⁻ <i>leu1-32 cis1-1/apm1-1</i>	This study
KP630	<i>h</i> ⁻ <i>leu1-32 ura4-D18 apm1::ura4</i> ⁺	This study
KP119	<i>h</i> ⁺ <i>leu1-32 ura4-D18 ppb1::ura4</i> ⁺	Our stock
KP456	<i>h</i> ⁻ <i>leu1-32 ura4-D18</i>	Our stock
KP162	<i>h</i> ⁻ <i>leu1-32 ypt3-i5/its5-1</i>	Our stock
KP1276	<i>h</i> ⁺ <i>his2 leu1-32 ura4-D18 ade6-M216 ypt7::ura4</i> ⁺	This study
KP2032	<i>h</i> ⁻ <i>leu1-32 ura4-D18 apm1::ura4</i> ⁺ <i>apm1-GFP::leu1</i> ⁺	This study
KP2035	<i>h</i> ⁻ <i>leu1-32 ura4-294 GFP-syb1</i> ⁺ <i>::ura4</i> ⁺	This study

Isolation of *cis1-1/apm1-1* Mutant

The *cis1-1/apm1-1* mutant was isolated in a screen of cells that had been mutagenized with nitrosoguanidine. Cells of strain HM123 were mutagenized with 300 μ M nitrosoguanidine (Sigma-Aldrich, St. Louis, MO) for 60 min (~10% survival) as described previously (Moreno *et al.*, 1991). Mutants were spread onto YPD plates to give ~1000 cells/plate and incubated at 27°C for 4 d. The plates were then replica-plated onto plates containing 0.5 μ g/ml FK506 or 0.2 M MgCl₂. Mutants that showed both FK506 sensitivity and MgCl₂ sensitivity were selected and designated as chloride- and immunosuppressant-sensitive (*cis*) mutants. The original mutants isolated were backcrossed three times to wild-type strains HM123 and HM528.

Cloning and Tagging of the *cis1*⁺/*apm1*⁺ Gene

To clone *cis1*⁺ gene, *cis1-1* mutant (KP356) was grown at 27°C and transformed with an *S. pombe* genomic DNA library constructed in the vector pDB248 (Beach *et al.*, 1982). *Leu*⁺ transformants were replica-plated onto YPD plates at 36°C, and the plasmid DNA was recovered from transformants that showed plasmid-dependent rescue. These plasmids complemented both the immunosuppressant sensitivity and temperature sensitivity of the *cis1-1* mutant. By DNA sequencing, the suppressing plasmids fell into two classes, with one class containing the *apm1*⁺ gene (SPBP16F5.07), and another class containing a gene distinct from the *apm1*⁺ gene. Characterization of the gene contained in the second class will be reported elsewhere. To investigate the relationship between the cloned *apm1*⁺ gene and *cis1-1/apm1-1* mutant, linkage analysis was performed as follows. The entire *apm1*⁺ gene was subcloned into the pUC-derived plasmid containing *S. cerevisiae* *LEU2* gene and integrated by homologous recombination into the genome of the wild-type strain HM123. The integrant was mated with the *cis1-1/apm1-1* mutant. The resulting diploid was sporulated, and tetrads were dissected. In total, 30 tetrads were dissected. In all cases, only parental ditype tetrads were found, indicating allelism between the *apm1*⁺ gene and the *cis1-1/apm1-1* mutation (our unpublished data). For ectopic expression of proteins, we used the thiamine-repressible *nmt1* promoter (Maundrell, 1993). Expression was repressed by the addition of 5 μ M thiamine to EMM and was induced by washing then incubating the cells in EMM lacking thiamine.

To assess the subcellular localization, Apm1 was tagged at its C terminus with green fluorescent protein (GFP) carrying the S65T mutation. To express Apm1-GFP in fission yeast, the entire open reading frame and 0.8 kb of upstream sequences from the fission yeast *apm1*⁺ gene were inserted in front of the GFP gene so that the C terminus of Apm1 was joined to the N terminus of GFP. The resultant construct was subcloned into the pJK148 integrating vector containing the *leu1*⁺ gene (Keeney and Boeke, 1994) to give pKB4677. To obtain the chromosome-borne Apm1-GFP fusion protein under the endogenous promoter control, wild-type strain (KP456) or Δ *apm1* cells (KP630) were transformed with pKB4677 and were integrated into the chromosome at the *leu1*⁺ gene locus of KP456 (*h*⁻ *leu1-32 ura4-D18*) or KP630 (*h*⁻ *leu1-32 ura4-D18 apm1::ura4*⁺) (see below for *apm1* deletion). The resultant strain (*h*⁻ *leu1-32 ura4-D18 apm1::ura4*⁺ *apm1-GFP::leu1*⁺) did not show temperature sensitivity and FK506 sensitivity, indicating that Apm1-GFP is fully functional.

The fission yeast *sad1*⁺ gene codes for a spindle pole body (SPB) component (Hagan and Yanagida, 1995), and the Sad1-DsRed plasmid was created as described previously (Nabeshima *et al.*, 1998), except for the use of DsRed (Matz *et al.*, 1999) instead of GFP.

Deletion of the *apm1*⁺ Gene

A one-step gene disruption by homologous recombination was performed as described in Rothstein (1983). The *apm1::ura4*⁺ disruption was constructed as

follows. The *Hind*III fragment containing the *apm1*⁺ gene was subcloned into the *Hind*III site of pGEM-13Zf(+) (Promega, Madison, WI). Then, a *Bam*HI-*Sph*I fragment containing the *ura4*⁺ gene was inserted into the *Bam*HI-*Sph*I site of the previous construct. The fragment containing the disrupted *apm1*⁺ gene was transformed into haploid cells. Stable integrants were selected on medium lacking uracil, and disruption of the gene was checked by genomic Southern hybridization (our unpublished data).

Electron Microscopy

Conventional electron microscopy was performed as described previously (Sato *et al.*, 1996) with some modification. Cells were collected, washed with distilled water, and then fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, at 4°C for 2 h. Cells were washed with the same buffer and then postfixed with 3% potassium permanganate in distilled water at room temperature for 90 min. After washing with distilled water, cells were embedded in 2% agarose, and then block staining was carried out with 2% uranyl acetate in distilled water at 4°C for >30 min. Blocks were dehydrated with alcohols in graduated series and were embedded in Quetol 653 mixture. Ultrathin sections were stained with uranyl acetate and lead citrate and then were observed with a transmission electron microscope (Hitachi H-7000) at 75 kV.

The quantification of the micrographs was carried out as described previously (Cheng *et al.*, 2002). Electron-dense profiles were counted as large vesicles if they were 100–150 nm in diameter. Thirty cells were counted for each strain.

Measurement of the Acid Phosphatase Secretion

Acid phosphatase secretion was assayed with some modifications (Craighead *et al.*, 1993; Tanaka and Okayama, 2000). Cells were grown to log phase in EMM at 27°C, pelleted, washed twice with EMM, and resuspended in fresh EMM at 27 or 36°C. Samples were taken at 0 h (time of resuspension) and at hourly intervals thereafter. For each sample, 1 ml of culture was centrifuged, and 500 μ l of the supernatant was added to 500 μ l of substrate solution (2 mM *p*-nitrophenyl phosphate, 0.1 M sodium acetate, pH 4.0; prewarmed to 30°C) and incubated at 30°C for 5 min. Reactions were stopped by the addition of 500 μ l of 1 M sodium hydroxide. The absorbance at 405 nm was measured, by using the 0-h sample as a blank control. Detection of the GFP-fused *pho1*⁺ acid phosphatase was performed as described previously (Cheng *et al.*, 2002).

Light Microscopic Analysis

Standard techniques were used for microscopy (Hagan and Hyams, 1988). For microscopic observation, cells were grown to exponential phase in YPD medium at 27°C, shifted to various conditions as indicated in the figure legends, and washed with phosphate-buffered saline (pH 7.0). To visualize the DNA and septum, cells were stained with 4,6-diamidino-2-phenylindole and Calcofluor, respectively. Cells were examined by differential interference contrast and fluorescence microscopy by using Axioskop or Axiovert microscopes (Carl Zeiss, Thornwood, NY). Photographs were taken with SPOT2 or AxioCam digital cameras (Diagnostic Instruments, Sterling Heights, MI). Images were processed with the CorelDRAW software (Corel, Ottawa, Ontario, Canada).

Localization of GFP-Syb1

Syb1, the synaptobrevin equivalent in fission yeast (Edamatsu and Toyoshima, 2003), was amplified, ligated to the C terminus of GFP and was subcloned into the pREP vectors. To obtain the chromosome-borne GFP fusion protein, the resultant plasmid containing the *ura4*⁺ marker was integrated into the chromosome at the *ura4*⁺ gene locus of KP1248 (*h*[−] *leu1*-32 *ura4*-294).

Staining of Vacuoles with Lucifer Yellow

The staining with Lucifer yellow is described in Murray and Johnson (2001). Briefly, cells were grown to an exponential phase in YES medium, harvested with centrifugation for 3 min at 4°C, resuspended in fresh YES medium containing 5 mg/ml Lucifer yellow carbonyl hydrazine (Sigma-Aldrich), and incubated at 27°C for various periods in time-course experiments. Aliquots were harvested at times indicated, washed three times with the medium, and fluid-phase endocytosis was microscopically observed under the fluorescence microscope.

N-[3-Triethylammoniumpropyl]-4-[*p*-diethylaminophenylhexatrienyl] Pyridinium Dibromide (FM4-64) Labeling

The staining with FM4-64 is as described previously (Vida and Emr, 1995) with some modifications. Briefly, cells were grown to exponential phase in YPD medium at 27°C, harvested with centrifugation at 4°C, resuspended in ice-cold YPD medium, FM4-64 was added to a final concentration of 80 μ M, and the cells were incubated at 0°C for 30 min. Then, the cells were harvested with centrifugation at 700 $\times$ g for 3 min at 4°C, washed by resuspending in ice-cold fresh YPD to remove free FM4-64, and incubated at 27°C. Then, the cells were harvested at the designated periods (5 min to visualize the Golgi/

endosomes, 60 min to visualize the vacuoles, or various time periods for time-course experiments), washed with ice-cold phosphate-buffered saline), and immediately examined under a fluorescence microscope.

Two-Hybrid Analysis

The vectors used for the two-hybrid assay were pAS2.1, which encodes the DNA binding domain of Gal4, and pGAD GH, which expresses the activation domain (Matchmaker two-hybrid system 2; BD Biosciences Clontech, Palo Alto, CA). The Gal4 DB domain open reading frame in pAS2.1 was fused with the full-length Sad1 coding sequence, and the pGAD GH was fused with the full-length Apm1 coding sequence. The two-hybrid assay was performed according to the instructions of the system manufacturer.

RESULTS

Isolation of the *cis1-1* Mutant

We developed a simple genetic screen for mutants that depend on calcineurin for growth by using FK506, a specific inhibitor of calcineurin. Previous work from our laboratory has identified the *ypt3*⁺/*its5*⁺ gene that encodes a homolog of mammalian Rab11 and has suggested the functional connection between Ypt3/Its5 and calcineurin in the fission yeast membrane trafficking (Cheng *et al.*, 2002). To isolate new molecules that functionally interact with calcineurin in the membrane trafficking, we further searched for mutants that are sensitive to the immunosuppressive drug FK506 and isolated the *cis1-1* mutant. As shown in Figure 1, *cis1-1* mutants grew equally well compared with the wild-type cells at 27°C. However, *cis1-1* mutant cells could not grow at 36°C nor could they grow on YPD plate containing FK506 at the permissive temperature, whereas wild-type cells grew normally (Figure 1). The *cis1-1* mutants also failed to grow in the presence of 0.2M MgCl₂ and 0.4M KCl where the wild-type cells grew well (Figure 1). As predicted, no double mutant was obtained at any temperature by the genetic cross between *cis1-1* and calcineurin deletion (Δ *ppb1*) (our unpublished data), indicating that *cis1-1* and Δ *ppb1* are synthetically lethal.

The *cis1-1* Is an Allele of the *apm1*⁺ Gene that Encodes a Homolog of Mammalian μ 1A Subunit of the Clathrin Adaptor Protein Complex 1

The *cis1*⁺ gene was cloned by complementation of the temperature-sensitive growth defect of the *cis1-1* mutant cells (Figure 1, +*apm1*⁺). The *cis1*⁺ gene also complemented all the phenotypes associated with the *cis1-1* mutant (Figure 1). Nucleotide sequencing of the cloned DNA fragment revealed that *cis1*⁺ gene is identical to the *apm1*⁺ gene (SPBP16F5.07), which encodes Apm1 protein of 426 amino acids that is highly similar to *Homo sapiens* μ 1A and *S. cerevisiae* Apm1 (Figure 2A). Linkage analysis was performed (see MATERIALS AND METHODS) and indicated allelism between the *apm1*⁺ gene and the *cis1-1* mutation. It should be noted that the structural similarity between the human μ 1A and the fission yeast Apm1 is remarkably higher (62%), compared with that between human μ 1A and the budding yeast Apm1 (32%).

To characterize the mutation site in the *apm1-1* mutant, genomic DNA from *apm1-1* mutant was isolated, and the full-length coding region of the *apm1-1* was sequenced. The G-to-A nucleotide substitution caused a tryptophan to be altered to a termination codon at the amino acid position 343, and resulted in a truncated protein product lacking 83 amino acids downstream of the mutation. This suggests the functional importance of its C-terminal amino acids (Figure 2B).

Figure 1. Mutation in the *cis1*⁺/*apm1*⁺ gene causes immunosuppressant-, chloride- and temperature-sensitive phenotypes. The immunosuppressant and temperature sensitivities of the *cis1-1/apm1-1* mutant cells and *cis1/apm1*-deleted (Δ *apm1*) cells are shown. Cells transformed with the multicopy vector pDB248 or the vector containing the *cis1*⁺ gene were streaked onto each plate containing YPD, YPD plus 0.5 μ g/ml FK506, YPD plus 0.2 M MgCl₂, or YPD plus 0.4 M KCl and then incubated for 4 d at 27°C or 3 d at 36°C, respectively.

Disruption of *cis1*⁺/*apm1*⁺ Gene

We constructed a null mutation in the *apm1*⁺ gene (see MATERIALS AND METHODS) and found that *apm1* deletion was viable (Figure 1, Δ *apm1* + vector), indicating that Apm1 is not essential for cell viability. The Δ *apm1* cells also showed temperature sensitivity at 36°C and FK506 sensitivity at 27°C, as well as chloride sensitivity similar to that of *apm1-1* cells (Figure 1, Δ *apm1* + vector). Consistently, no double mutant was obtained at any temperature by the genetic cross between Δ *apm1* and calcineurin deletion (Δ *ppb1*) (our unpublished data).

The Δ *apm1* Cells Accumulate Abnormal Membranous Structures and Presumptive Post-Golgi Secretory Vesicles

Recent studies on embryonal fibroblast cell line deficient in μ 1A adaptin point to its role in protein sorting at the TGN or endosomes (Boehm and Bonifacio, 2001). This prompted us to investigate the trafficking pathway in which the fission yeast Apm1 is involved. In general, electron microscopic analysis of mutants defective in the membrane trafficking have been shown to accumulate an organelle or a vesicular intermediate of the secretory compartments that precede the step in which they first function (Novick *et al.*, 1981; Kaiser and Schekman, 1990; Cheng *et al.*, 2002). To determine whether Δ *apm1* cells accumulate such structures, cells were examined by electron microscopy.

First, we examined the Golgi structures of Δ *apm1* cells and compared them with those of wild-type cells. Golgi structures of Δ *apm1* cells were larger than those in wild-type cells, were frequently observed to form stacks, and were occasionally swollen at their periphery (Figure 3B, a–c). Notably, large vesicular structures associated with Golgi stacks were observed in Δ *apm1* cells, suggesting that Apm1 is involved in vesicle formation at the TGN (Figure 3B, b and c). The accumulation of abnormal electron-dense membranous

structures that were ~500 nm in diameter were also observed in Δ *apm1* cells (Figure 3B, a, c, and e, white arrowheads). These abnormal membranous structures, whose identity is unknown, may likely be “Berkeley bodies” known to represent abnormal Golgi membranes as they were often connected to the Golgi stacks. These structures, although negligible in wild-type cells, were observed in ~10% of Δ *apm1* cells at the permissive temperature and markedly increased upon temperature up-shift and FK506 treatment.

Another striking feature of the Δ *apm1* cells was the accumulation of large vesicles resembling putative post-Golgi secretory vesicles (Armstrong *et al.*, 1993) at 27°C, which became more remarkable when the mutant cells were shifted to 36°C or when treated with FK506. As shown in Figure 3A, a, even at 27°C, Δ *apm1* cells exhibited an accumulation of large vesicles (100–150 nm in diameter), whereas these were negligible in the wild-type strain. The numbers of large vesicles in Δ *apm1* cells were quantified and compared with those in wild-type cells. At 27°C, Δ *apm1* cells exhibited an abundance of large vesicles (Figure 3C), similar to the phenotype of *ypt3-i5* mutant cells (Cheng *et al.*, 2002). When the cells were shifted to the restrictive temperature or when treated with FK506, the numbers of large vesicles in Δ *apm1* cells markedly increased, whereas the wild-type cells treated in a similar manner remained almost unaffected (Figure 3, A–C). Notably, the accumulation of vesicles after FK506 treatment was often detected near the plasma membrane, the active regions of secretion (Figure 3A, f, black arrow). Interestingly, the accumulation of presumptive post-Golgi secretory vesicles and abnormal Golgi-like structures were also characteristic of the *ypt3-i5* mutants that we reported previously (Cheng *et al.*, 2002). Similar electron microscopic observations were obtained with the *apm1-1* mutant cells (our unpublished data).

Figure 2. Alignment of protein sequences of *S. pombe* Cis1/Apm1 with related proteins from human and *S. cerevisiae*. (A) Amino acid sequences are aligned using the Clustal W program. Filled boxes indicate the identical amino acids. Sp, *S. pombe*; Hs, *H. sapiens*; and Sc, *S. cerevisiae*. (B) Schematic representation of the mutation site of the *cis1-1/apm1-1* mutation and the resulting truncated gene products are shown.

Collectively, these results suggest a role for Apm1 associated with the Golgi complex, particularly in traffic between the Golgi and the endocytic pathway, and also suggest that calcineurin is involved in the regulation of these cellular events.

The Δ apm1 Cells Show Defective Secretion of Acid Phosphatase

The massive accumulation of putative post-Golgi vesicles prompted us to examine the role of Apm1 in the secretory pathway by using a different approach. For this, we determined the ability of Δ apm1 cells to secrete acid phosphatase, a protein that follows the classical secretory pathway from the endoplasmic reticulum to the extracellular periplasmic space (see MATERIALS AND METHODS). The Δ apm1 cells secreted much less acid phosphatase than wild-type cells at 27°C (Figure 3D). On temperature up-shift to 36°C, the secretion of acid phosphatase was markedly increased in wild-type cells, whereas that of the Δ apm1 cells remained unchanged (Figure 3D). Interestingly, when the Δ apm1 cells were treated with FK506, the cells displayed a reduction in

the secretion of acid phosphatase, whereas the wild-type cells were unaffected by the treatment (Figure 3D).

To demonstrate the direct effect of Δ apm1 on secretion, we used GFP fused with the *pho1*⁺ leader peptide (SPL-GFP), because this fusion protein is secreted through the same pathway as Pho1 acid phosphatase (Cheng et al., 2002). We tried to determine whether the Δ apm1 cells showed a defect in secreting SPL-GFP to the medium. At the permissive temperature, there was a marked decrease in the amount of secreted SPL-GFP in the growth media in Δ apm1 cells compared with wild-type cells (Figure 3E, 4 h at 27°C, 8 h at 27°C, Extra). On temperature up-shift to 36°C or FK506 treatment, the secretion of SPL-GFP was abolished in the Δ apm1 cells, whereas no remarkable change was seen in wild-type cells (Figure 3E, 4 h at 36°C, 8 h at 27°C + FK506, Extra). No remarkable difference in the amount of SPL-GFP detected in the internal cell extracts of all these samples was observed (Figure 3E, Intra).

These results are consistent with a defect in the secretory pathway associated with Δ apm1, and the inhibition of calcineurin activity exacerbates the defective secretion of the Δ apm1 cells, in agreement with the observations obtained from the electron microscopic analyses.

Localization of GFP-fused Synaptobrevin in Δ apm1 Cells

In addition, we visualized Syb1, the synaptobrevin in fission yeast, as a GFP-fusion protein (Edamatsu and Toyoshima, 2003), because synaptobrevin is a vesicle-associated membrane protein and copurifies with secretory vesicles. As a secretory vesicle SNARE, Syb1 would be expected to cycle between the cell surface and the endocytic pathway.

As shown in Figure 4, the fluorescence of GFP-Syb1 in wild-type cells could be detected as punctate structures in the cytoplasm (Figure 4, wt arrowheads), and enriched in the medial region and cell ends (Figure 4, wt arrows). In contrast, in Δ apm1 cells cultured at 27°C, GFP-Syb1 failed to localize to the cell surface and the medial regions (Figure 4, Δ apm1). Instead, they were observed as large dots with bright fluorescence in the cytoplasm at 27°C (Figure 4, 27°C Δ apm1, arrowheads). On temperature up-shift or FK506 treatment, the accumulation of GFP-Syb1 become more remarkable in Δ apm1 cells, whereas no significant change was observed in wild-type cells (our unpublished data).

Wild-type cells and Δ apm1 cells expressing GFP-Syb1 were loaded with FM4-64 dye for 5 min and examined by fluorescence microscopy. At early times after dye uptake, FM4-64 is endocytosed normally but accumulated at an endosomal compartment rather than at the vacuole (Vida and Emr, 1995). In wild-type cells, most of the GFP-Syb1 dots in the cytoplasm also fluoresced with FM4-64, indicating the colocalization at the Golgi/endosomes (Figure 4, wt arrowheads). Remarkably, the massive accumulation of GFP-Syb1 in Δ apm1 cells also colocalized with FM4-64 (Figure 4, Δ apm1, arrowheads), indicating that Syb1 accumulated in endosomes due to a defect in AP-1-dependent membrane traffic.

The Apm1-GFP Localizes at the Golgi/Endosomes, the Spindle Pole Bodies, and the Medial Regions When Expressed under Its Endogenous Promoter

Next, we examined the localization of Apm1 protein. Experiments were performed with GFP-tagged Apm1 expressed chromosomally under its endogenous promoter in Δ apm1 cells. These cells expressing Apm1-GFP grow normally (our unpublished data), suggesting that GFP-tagged Apm1 is capable of replacing the function of the wild-type protein. Apm1-GFP localized diffusely to the cytoplasm as dot-like

Figure 3. $\Delta apm1$ cells show aberrant accumulation of membranous structures and defects in acid phosphatase secretion. (A) Wild-type cells (wt) and $\Delta apm1$ cells were analyzed by electron microscopy. Electron micrographs of the representative cells are shown: $\Delta apm1$ cells at 27°C (a), wild-type cells (b), and $\Delta apm1$ cells (c) grown at 36°C for 8 h; wild-type cells (d), and $\Delta apm1$ cells treated with FK506 for 8 h grown at 27°C (e and f). Black arrows point to the accumulation of large vesicles (100–150 nm). White arrow points to the abnormal multiple-septum in $\Delta apm1$ cells. The boxed regions in c and e are enlarged in B, d and e, respectively. N, nucleus; V, vacuoles. Bar, 1 μ m. (B) Examples of stacked Golgi-cisternae in $\Delta apm1$ cells were displayed: $\Delta apm1$ cells at 36°C for 8 h (a) and treated with FK506 for 8 h grown at 27°C (b and c). White arrowheads indicate electron-dense membranous structures (a, c, and e). Details of large vesicles and abnormal membranous structures in $\Delta apm1$ cells. Accumulation of large vesicles in $\Delta apm1$ cells at 36°C for 8 h (d), and treated with FK506 for 8 h grown at 27°C (e). Bar, 0.25 μ m. (C) Quantification of large putative post-Golgi vesicles in wild-type and $\Delta apm1$ cells, which were cultured at 27 or 36°C for 8 h, or 27°C with the addition of FK506 for 12 h. Bars represent the mean number of large vesicles in 30-cell sections. Data are normalized to a density per 100 μ m². (D) Defective secretion of acid phosphatase in $\Delta apm1$ cells. Wild-type and $\Delta apm1$ cells were assayed for secreted acid phosphatase activity as indicated. The data presented are representative of three independent experiments. (E) Defective secretion of SPL-GFP to the growth medium in $\Delta apm1$ cells. Wild-type and $\Delta apm1$ cells expressing SPL-GFP were cultured at 27 or 36°C for 4 h or at 27°C with or without the addition of FK506 for 8 h. Proteins were separated by SDS-PAGE and analyzed by Western blotting by using anti-GFP antiserum. Extra, the trichloroacetic acid-precipitate of supernatant of 5×10^6 cells; Intra, cell extract of 1×10^6 cells.

Figure 4. GFP-fused synaptobrevin failed to localize to the cell surface but instead accumulated at the Golgi/endosomes in $\Delta apm1$ cells. Wild-type (wt) and $\Delta apm1$ cells ($\Delta apm1$) expressing chromosome-borne GFP-Syb1 cultured in YPD medium at 27°C were incubated with the dye FM4-64 for 5 min at 27°C to visualize early endosomes. The GFP-Syb1 localization and the FM4-64 fluorescence were examined under the fluorescence microscope. Bar, 10 μ m.

structures (Figure 5A, arrowheads) and the nucleus. Surprisingly, the strong fluorescence was observed at the spindle pole bodies and the medial region (Figure 5A, double arrowheads and arrows, respectively).

We examined whether the dot-like fluorescence of Apm1-GFP colocalized with FM4-64 at an early stage of endocytosis. After 5 min of dye uptake, most of the FM4-64-positive structures showed colocalization with Apm1-GFP structures (Figure 5B, arrowheads). This suggests that the dot-like structures of Apm1-GFP represent Golgi/endosomal compartments, consistent with its role at the TGN.

To confirm whether the focal staining of Apm1-GFP next to the nucleus is a spindle pole body, it was coexpressed with Sad1-DsRed, a well-known component of the spindle pole body, under their endogenous promoters. Notably, the fluorescence of Apm1-GFP in the nucleus clearly colocalized with that of Sad1-DsRed (Figure 5C, double arrowheads). The binding between Apm1 and Sad1 was also confirmed by two-hybrid analysis (Figure 5D). Thus, Apm1 colocalizes and interacts with Sad1 protein at the spindle pole body.

The $\Delta apm1$ Cells Exhibit Defects in Cytokinesis

Electron micrographs of $\Delta apm1$ cells treated with FK506 showed morphological defects, such as highly elongated cells with abnormal multisepta (Figure 3A, f, white arrow), that were clearly more severe than those observed in wild-type cells treated with FK506 (Figure 3A, d), suggesting a role for Apm1 in cytokinesis. These findings, together with

the localization of Apm1-GFP to the spindle pole body and the medial region (Figure 5), prompted us to examine cytokinesis of $\Delta apm1$ cells. Cells grown to mid-log phase at 27°C in liquid YPD medium were subjected to a temperature up-shift or to the medium containing FK506 at 27°C. On shifting to 36°C for 6 h, the frequency of septated cells in $\Delta apm1$ cells significantly increased (38%), whereas the septation index of wild-type cells remained unchanged (Figure 6B, left). Strikingly, the magnitude of the defect in $\Delta apm1$ cells was even more pronounced 6 h after the addition of FK506, because nearly 80% of the $\Delta apm1$ cells were septated, compared with 30% of wild-type cells (Figure 6B, right). At the restrictive temperature and upon FK506 treatment, microscopic analysis revealed that the $\Delta apm1$ cells are highly elongated. A thick septum that was brightly stained with Calcofluor and the unusual multiple septation were frequently observed in the $\Delta apm1$ cells (Figure 6A, arrowheads). Similar abnormal morphological phenotypes also were observed in the *apm1-1* mutants (our unpublished data). Together, these results are consistent with the observations by electron microscopy, indicating that Apm1 is implicated in cytokinesis and that the loss of calcineurin activity accentuates the defective cytokinesis of $\Delta apm1$ cells.

The $\Delta apm1$ Cells Exhibit Defects in Vacuolar Fusion Induced by Osmotic Stress

The localization of Apm1-GFP in an endosomal compartment suggests that Apm1 may be involved in vacuolar function. Because electron micrographs of the $\Delta apm1$ cells also revealed that vacuoles were highly fragmented (Figure 3A, c, e, and f), we wanted to ascertain whether Apm1 is required for the proper vacuolar function. We then examined the vacuolar fusion induced by osmotic stress in wild-type strain and $\Delta apm1$ cells. When the cells were labeled with FM4-64 for 60 min, the $\Delta apm1$ cells were highly fragmented compared with wild-type cells, consistent with the findings obtained by electron microscopy (Figure 7B). Hypotonic stress causes a dramatic fusion of vacuoles in *S. pombe* (Bone *et al.*, 1998). When cells were collected, washed, and resuspended in water for 90 min, the wild-type cells had evidently large vacuoles that resulted from vacuole fusion (Figure 7A, wt). In contrast, vacuoles remained small and numerous in $\Delta apm1$ cells suspended in water, indicating a defect in the vacuole fusion (Figure 7A, $\Delta apm1$). However, in $\Delta apm1$ cells, pulse-chase analysis revealed that the maturation of carboxipeptidase Y was not impaired significantly even at the restrictive temperature or with FK506 treatment (our unpublished data), suggesting that vacuolar protein sorting was unaffected in $\Delta apm1$ cells.

Endocytosis Is Not Impaired in $\Delta apm1$ Cells

As shown in Figure 7A, vacuoles were normally labeled with FM4-64, a vital dye that is internalized in living cells through endocytosis and that accumulates at the vacuole (Vida and Emr, 1995). To convincingly conclude that endocytosis is not affected in $\Delta apm1$ cells, we performed a time-course experiment carried out with FM4-64. Within 10 min of incubation at 27°C, small fluorescent dots occurred in the cytoplasm (Figure 7B, 10 min) that became increasingly brighter over the next 20 min (Figure 7B, 30 min). The staining of these endosomal intermediates subsequently decreased concomitantly with the occurrence of FM4-64 in vacuolar membranes (Figure 7B, 60 min), and after 90 min staining was predominant in the vacuoles (Figure 7B, 90 min). In $\Delta apm1$ cells, FM4-64-labeled endosomal intermediates has kinetics similar to that seen in wild-type cells, and subsequent delivery to the vacuole was not impaired (Figure

Figure 5. Apm1-GFP is concentrated at the Golgi/endosomes, the spindle pole body, and the medial region. (A) Wild-type cells, expressing chromosome-borne Apm1-GFP under its endogenous promoter, were examined by fluorescence microscopy. Arrowheads indicate the dot-like structures, double arrowheads indicate the strong fluorescence of the spindle pole bodies, and arrows point to the concentrated fluorescence at the medial region. (B) Apm1-GFP localizes at the Golgi/endosomes. Wild-type cells, expressing chromosome-borne Apm1-GFP were incubated with the dye FM4-64 for 5 min to visualize early endosomes. Arrowheads indicate the dot-like structures of Apm1-GFP and early endosomes stained with FM4-64, respectively. (C) Apm1-GFP colocalizes with Sad1-DsRed at the spindle pole body. Wild-type cells, coexpressing Apm1-GFP and Sad1-DsRed under their endogenous promoter, were examined by fluorescence microscopy. Double arrowheads indicate the strong fluorescence of the spindle pole bodies. Bar, 10 μ m. (D) Apm1 interacts with Sad1 in the yeast two-hybrid system. AH109 budding yeast cells were transformed with each plasmid and spread onto the indicated SD media. BD indicates binding domain and AD indicates activation domain, respectively.

7B, Δ apm1). However, because the vacuoles in *apm1* mutants are smaller and highly fragmented, it is difficult to convincingly conclude that the dye reached up to the vacuoles or the late endosomes.

Consequently, another time-course experiment was performed using the membrane-impermeable fluorescence dye Lucifer yellow to monitor the fluid phase endocytosis (Riezman, 1985). Vacuoles of wild-type and Δ apm1 cells were seen as fluorescence-positive compartments after 30-min incubation, and no significant delay of dye uptake during the time course was observed in Δ apm1 cells (Figure 7C). In contrast, no uptake of Lucifer yellow was observed in an endocytosis-defective mutant as in the case of *ypt7* disruption strain (Bone *et al.*, 1998) (our unpublished data). Collectively, these results indicate that endocytosis is not impaired in Δ apm1 cells.

Apm1 Is Required for Cell Wall Integrity

The Δ apm1 cells also showed cell wall integrity defects, as judged by the hypersensitivity to β -glucanase, even at the

permissive temperature of 27°C. The Δ apm1 cells lysed faster than the wild-type cells (Figure 8A). The sensitivity of Δ apm1 cells was as severe as that of the *ypt3-i5* mutant (Figure 8A), which is also involved in membrane trafficking and is defective in the cell wall integrity (Cheng *et al.*, 2002). Defects in cell wall integrity can sometimes be compensated for by increases in the osmolarity of the growth media (Levin and Bartlett-Heubusch, 1992; Yada *et al.*, 2001). Consistently, the Δ apm1 cells were capable of forming colonies at 36°C on rich YPD plates containing 1.2 M sorbitol (Figure 8B), and the cytokinesis abnormality caused by the *apm1* mutation also was alleviated by osmotic stabilization (our unpublished data). Therefore, the temperature-sensitive and the aberrant morphological phenotypes caused by the loss of Apm1 function were ascribable, at least in part, to the defects in cell wall integrity. Notably, the osmotic stabilizer failed to suppress the FK506 sensitivity of the *apm1-1* cells and Δ apm1 cells (Figure 8B).

Figure 6. The $\Delta apm1$ cells are defective in cytokinesis. (A) Fluorescence micrographs of wild-type (wt) and $\Delta apm1$ cells stained with Calcofluor. Cells were shifted to the restrictive temperature (36°C) for 6 h, or FK506 was added for 4 h and incubated at 27°C and then stained with Calcofluor to visualize cell wall and septum. Bar, 10 μ m. (B) Percentage of cells forming a division septum at each time point in wild-type and $\Delta apm1$ cells after the shift to 36°C (left) or the addition of FK506 at 27°C (right). Values are the average of three independent experiments with 500 cells counted for each time point. Standard deviations between experiments were <10%.

Genetic Interaction between $apm1^+$ and $ypt3^+$ Genes

As described above, the phenotypes of $ypt3$ -i5 and $apm1$ -1 were very similar. This prompted us to examine the genetic interaction between $apm1^+$ and $ypt3^+$ genes. We constructed a dominant-negative mutant form of the Ypt3 protein, in which the conserved Ser24 among Rab11/Ypt3 proteins was mutated to Asn (Ypt3S24N) as indicated in our previous study (Cheng *et al.*, 2002). In the same study, wild-type cells expressing Ypt3S24N from the attenuated *nmt1* promoter (Maundrell, 1993) exhibited severe FK506-sensitive growth defect, whereas cells expressing wild-type Ypt3 grew well in the presence of FK506, indicating that the inhibition of Ypt3 activity is lethal in combination with inactivation of calcineurin. The same expression vector, pREP41-Ypt3S24N, was used in this study to express the dominant-negative Ypt3, Ypt3S24N, in $\Delta apm1$ cells as well as in wild-type cells. As shown in Figure 8C, expression of Ypt3S24N strongly inhibited the growth of $\Delta apm1$ cells, whereas it had no effect on the growth of wild-type cells. Furthermore, no double

mutant was obtained from the genetic cross between $\Delta apm1$ cells and $ypt3$ -i5 mutants, indicating the synthetic lethal interaction (our unpublished data).

DISCUSSION

The isolation of an immunosuppressant-sensitive mutation of the $apm1^+$ gene has revealed that Apm1, a μ 1 subunit of AP-1 complex, functionally interacts with calcineurin. To our knowledge, the functional and genetic interaction of AP-1 complex and calcineurin has not been documented in budding yeast or in any other system.

In addition, we have demonstrated that the loss of Apm1 function causes defects in cellular processes such as secretion, cytokinesis, vacuole fusion, and cell wall integrity. Remarkably, the endogenous intracellular localization of Apm1 was visualized in the spindle pole body, the medial region, as well as in the Golgi/endosomes. This situation, in clear contrast to the budding yeast, which shows no discernible phenotypes when AP-1 complex is deleted, emphasizes that the study of Apm1 in fission yeast may be very productive to analyze the membrane trafficking events regulated by AP-1 complex and that Apm1 in fission yeast may have very unexpected functions.

In this study, we have revealed a role for Apm1 in membrane traffic associated with the Golgi/endosomes in fission yeast, based on electron microscopic analyses. In particular, the electron micrographs showing enlarged Golgi stacks and large vesicular structures associating with Golgi stacks observed in $\Delta apm1$ cells (Figure 3B) strongly suggest the role for Apm1 in vesicle formation at the TGN, consistent with the conventional models of Apm1 functions in other organisms. The findings that a vesicle-associated protein synaptobrevin, Syb1, failed to localize to the sites of polarized secretion in $\Delta apm1$ cells, and instead accumulated at the Golgi/endosomal compartments, also support the role for Apm1 in membrane trafficking associated with the Golgi/endosomes. Furthermore, the localization of Apm1-GFP in the Golgi/endosomes as evidenced by the colocalization with FM4-64 is consistent with its role in these organelles.

On the other hand, the internalization steps of the endocytic process is not impaired in $\Delta apm1$ cells, as evidenced by the time-course experiments using the FM4-64 fluorescent dye and Lucifer yellow (Figure 7). Together with its high sequence homology to *H. sapiens* μ 1A and *S. cerevisiae* Apm1, these results strongly suggest that the fission yeast Apm1 is involved in AP-1 complexes that are associated with clathrin-coated vesicles derived from the TGN and is not involved in AP-2 complexes that are associated with the endocytic clathrin-coated vesicles for internalization.

Interestingly, we found that $\Delta apm1$ cells also showed phenotypes compatible with a defect in secretory vesicle docking/fusion to the plasma membrane. These include the accumulation of putative post-Golgi secretory vesicles (100–150 nm) and the defective secretion of acid phosphatase. Also, the defects in cytokinesis or cell wall integrity observed in $\Delta apm1$ cells may reflect the defects in secretion of the cell wall biosynthetic enzymes to the plasma membrane.

Notably, the accumulation of putative post-Golgi secretory vesicles and aberrant Golgi-like structures, together with the various phenotypes conferred by the loss of Apm1 function are reminiscent of those of Ypt3/Its5 protein that we have previously identified in the same genetic screen for mutations that are synthetically lethal with calcineurin deletion. Consistently, the synthetic lethal interaction was observed between $apm1^+$ and $ypt3^+$ genes, suggesting that these two genes share an overlapping function in membrane

Figure 7. Vacuole fusion is defective, but internalization step of endocytosis is not impaired in $\Delta apm1$ cells. (A) $\Delta apm1$ cells are defective in vacuole fusion. Wild-type (wt) and $\Delta apm1$ cells were grown in YPD medium at 27°C. Cells were harvested, labeled with FM 4-64 fluorescent dye (see MATERIALS AND METHODS), resuspended in water, and examined by fluorescence microscopy. Photographs were taken after 90 min. Bar, 10 μm . (B) Time-course analysis of FM4-64 internalization. Wild-type (wt) and $\Delta apm1$ cells were incubated with FM4-64 for 30 min at 0°C in YPD media. The washed cells were viewed with a fluorescence microscope (FM4-64) and differential microscopy. (C) Time-course analysis of Lucifer yellow (LY) internalization. Wild-type (wt) and $\Delta apm1$ cells were incubated in YES medium containing Lucifer yellow (5 mg/ml) and were processed as indicated in B. Bar, 10 μm .

trafficking, presumably affecting the same stage of a pathway. We demonstrated that Ypt3 is involved at multiple steps of the fission yeast membrane trafficking events, namely, at the exit from the *trans*-Golgi as well as at the later step of the exocytic pathway (Cheng *et al.*, 2002). Similarly, Apm1 also may be involved in secretory vesicle docking/fusion to the plasma membrane, which might explain the synthetic lethal interaction between *apm1* null and *ypt3* mutation. Alternatively, given the observations that in *apm1* mutants, acid phosphatase clearly failed to occur in the medium, but it did not accumulate inside the cell, the secretory protein might be diverted to the vacuoles and degraded in *apm1* mutants. Rather than indicating two separate functions, this might be consistent with a single underlying function in the Golgi/endosome region explaining all the trafficking phenotypes.

Interestingly, FK506 treatment exacerbated the phenotypes of $\Delta apm1$ cells related to membrane trafficking, in-

cluding secretory vesicle accumulation, defects in acid phosphatase secretion, as well as the accumulation of GFP-Syb1 in endosomes, suggesting that calcineurin also is involved in these cellular processes. Calcineurin plays a key role in maintaining cell wall integrity by regulating the enzymes involved in cell wall synthesis, such as Cps1/Drc1/Its2 at the transcriptional level (our unpublished data). Therefore, impairment of both Apm1 and calcineurin, the two key players in cell wall synthesis, might explain the synthetic lethal interaction. Nevertheless, it is possible that calcineurin plays a role in the membrane trafficking pathway at step(s) where Apm1 or Ypt3 function. In support of this view is the finding that the FK506 sensitivity caused by the loss of Apm1 was not suppressed by the addition of sorbitol to the media (Figure 8B). This strongly suggests the involvement of calcineurin in aspects other than the regulation of cell wall integrity.

Figure 8. *Δapm1* cells show defective cell wall integrity similar to *ypt3-i5* mutants and are hypersensitive to the expression of the dominant-negative mutant of Ypt3. (A) Cell wall digestion of wild-type, *Δapm1*, and *ypt3-i5/its5-1* mutants cells by β -glucanase. Cells exponentially growing in YPD medium were harvested and incubated with β -glucanase (Zymolyase) at 30°C with vigorous shaking. Cell lysis was monitored by the measurement of optical density at 660 nm (the value before adding the enzyme was taken as 100%). (B) High osmolarity suppressed the temperature-sensitive phenotype but not the FK506-sensitive phenotype of the *apm1-1* mutant and *Δapm1* cells. Cells were streaked onto YPD plate or YPD + FK506 with or without 1.2 M sorbitol and incubated at 36 or 27°C for 3 d. (C) *Δapm1* cells were hypersensitive to the expression of the dominant-negative *ypt3* mutant. The dominant-negative mutant of Ypt3, Ypt3S24N (Cheng *et al.*, 2002) was expressed in wild-type (wt) and *Δapm1* cells, and cells were streaked onto EMM plate then incubated for 4 d at 27°C.

In conclusion, our studies have defined a role of Apm1 in membrane trafficking, thereby affecting various cellular processes. Our results also may indicate a very unexpected function for Apm1 protein in cytokinesis, dependent or independent of its role in membrane traffic, as suggested by its localization in the medial region and the spindle pole body colocalizing and interacting with a spindle pole body component Sad1. Also, the genetic screen utilizing distinct phenotypes of *apm1* mutants, or the synthetic lethal screen with calcineurin deletion in fission yeast may contribute to the further identification of the new components involved in the membrane trafficking pathway.

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