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Fission Yeast Epsin, Ent1p is Required for Endocytosis and Involved in Actin Organization

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In this study, we have characterized an essential gene *ent1*$^+$ encoding fission yeast epsin, which is similar to mammalian and budding yeast endocytic protein epsins. The *S. pombe* Ent1p contains ENTH (epsin amino-terminal homology) domain at its amino terminus, two copies of a ubiquitin-interacting motif (UIM) immediately carboxyl-terminal to the ENTH domain, three NPF motifs in the carboxyl-terminal half, and the clathrin-binding motif at the carboxyl terminal. When repressed the expression of *ent1*$^+$ gene, the conditional *ent1* gene knockout cells showed a marked defect in internalization of fluorescent dyes, suggesting that Ent1p is essential for endocytosis. Changes in conserved amino acid residues within ENTH domain in *ent1* mutant cells revealed temperature-sensitive defect in actin organization and cell morphology. The Ent1p bound PI(4,5)P_2 and PI(3,5)P_2 immobilized onto nitrocellulose *in vitro* and also weakly bound PI(3,4)P_2, PI(3,4,5)P_3, PI4P and PI5P. Surprisingly, the localization of Ent1p-GFP was not affected even in the *its3-1* cells, in which the level of PI(4,5)P_2 was severely reduced, suggesting that PI(4,5)P_2 may not be essential for proper localization of Ent1p at endocytic sites. Our findings indicate that *S. pombe* Ent1p is an essential component in endocytic process, and involved in actin organization and cell morphogenesis.

Endocytosis is an essential process in which extracellular fluid and portions of the plasma membrane are internalized towards the cytoplasm to form small vesicles. Cells take up nutrients, down-regulate the signaling activities of receptor/ligand complexes and allow viruses to gain entry through endocytosis. Recently, a great deal of attention has been focused on studying cytosolic proteins that cooperate with each other to select the content of endocytic vesicles. The clathrin-dependent endocytic pathway is the best-characterized of the multiple endocytic pathways. Many cytosolic proteins are required for clathrin- dependent endocytosis, including the coat protein clathrin, the adaptor protein AP2, and a class of accessory factors (9). Accessory factors have been suggested to play a regulatory rather than a structural role in clathrin-coated vesicle formation; however, the precise functions of most accessory factors have not yet been demonstrated.

One accessory protein is epsin, which is evolutionarily conserved from yeast to humans. Several lines of evidence have indicated an important roles for epsin in the internalization step of endocytosis (7,15,19,21). Epsins from all species share several features, including an epsin amino-terminal homology (ENTH) domain that binds phosphatidylinositol-4,5-bisphosphate (PI(4,5)P_2), two or three copies of the ubiquitin interacting motif (UIM) that binds ubiquitin, and a carboxyl terminal region containing several short peptide sequences that serve as ligands for binding to endocytic machinery components (7,15,20,21).

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We have taken the approach of using the fission yeast *Schizosaccharomyces pombe* as a model for endocytosis, because this system is amenable to genetic analysis and has many advantages in terms of its relevance to higher eukaryotes. The fission yeast *S. pombe* genome sequence encodes only a single epsin homologue. The budding yeast *S. cerevisiae* genome sequence encodes four homologues, Ent1p through Ent4p. The comparatively smaller number of epsin in fission yeast makes it a simple model organism in the study on functions of epsin.

Here, we describe the characterization of *S. pombe* epsin Ent1p and show that Ent1p is required for endocytosis and is involved in actin organization using temperature-sensitive *ent1* allele. We also provide evidence that $PI(4,5)P_2$ may not be essential for proper localization of Ent1p at endocytic sites, despite Ent1p binding to $PI(4,5)P_2$ *in vitro*.

MATERIALS AND METHODS

Strains, Media, and Genetic Techniques

S. pombe strains used in this study are listed in Table I. The complete medium, YPD, and the minimal medium, EMM, have been described previously (11,17). Standard genetic and recombinant DNA method (11) were used except where noted.

Gene disruptions are denoted by lowercase letters representing the disrupted gene followed by two colons and the wild-type gene marker used for disruption (for example, ent*1*::*ura4*$^+$) and also denoted by an abbreviation of the gene preceded by Δ (for example, Δ*ent1*). Proteins are denoted by Roman letters, only the first letter is capitalized, and added letter "p" at the end (for example, Ent1p).

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

Strains	Genotype	Source
HM123	*h*$^-$ *leu1-32*	Our stock
HM528	*h*$^+$ *his2*	Our stock
Kp456	*h*$^-$ *leu1-32 ura4-D18*	Our stock
5A/1D	*h*$^-$/*h*$^+$ *leu1-32/leu1-32 ura4-D18/ura4-D18 his2/+ ade6-M210/ade6-M216*	Our stock
KP405	*h*$^-$ *leu1-32 cdc3-6*	Balasubramanian *et al*. (1994)
KP409	*h*$^-$ *leu1-32 cdc8-134*	Balasubramanian *et al*. (1992)
KP637	*h*$^-$ *leu1-32 cps8-188*	Ishiguro *et al*.
KP167	*h*$^-$ *leu1-32 its3-1*	Zhang *et al*.
KP1410	*h*$^-$/*h*$^+$ *leu1-32/leu1-32 ura4-D18/ura4-D18 his2/+ ade6-M210/ade6-M216 ent1*$^+$/*ent1*::*ura4*$^+$	This study

Cloning and Tagging of the E*nt1*$^+$ Gene

The *ent1*$^+$ gene was amplified by PCR with the genomic DNA of *S. pombe* as a template. The sense primer used for PCR was 5'-GA AGA TCT CAT ATG GCA TTT TCA GCG TTA GCT TAT AAT TTG T-3' (*Bgl*II site and start codon underlined) and the antisense primer was 5'-GA AGA TCT TTA TAA ATC AAT TAG AGA GCC AAC TTGGG-3' (*Bgl*II site and stop codon underlined). The amplified product was digested with *Bgl*II, and the resulting fragment was subcloned into Bluescript SK(+).

For ectopic expression of proteins, we used the thiamine-repressible *nmt1* promoter at various levels of expression (10). Expression was repressed by the addition of 4 μg/ml thiamine to EMM, and was induced by washing and incubating the cells in EMM lacking

thiamine. To express Ent1p-GFP, the open reading frame of *ent1*$^+$ without stop codon was ligated to the N-terminus of the GFP carrying the S65T mutation (4). The GFP-fused gene was subcloned into pREP1 vector.

Site-Directed Mutagenesis

Site-directed mutagenesis was performed using the Quick Change mutagenesis kit (Stratagene, La Jolla, CA). Single nucleotide substitutions were introduced into two conserved residues within ENTH domains, resulting in substitutions of serine for Glycine-90 (G90S) and alanine for threonine-107 (T107A). Mutated PCR fragment was subcloned into the integrating vector containing the *ura4*$^+$ gene (pKB3765). Wild-type strain (Kp456) was transformed with pKB3765 and a portion of mutated DNA was integrated into the chromosome at the *ent1*$^+$ gene locus of Kp456.

Disruption of E*nt1*$^+$ Gene

A one-step gene disruption by homologous recombination was performed as describe in Rothstein (1983) (14). The *ent1*::*ura4*$^+$ disruption was constructed as follows. 5'-untranslated region and partial open reading frame of the *ent1*$^+$ gene was amplified by PCR with the genomic DNA of *S. pombe* as a template. The sense primer used for PCR was 5'-GA AGA TCT TCA CGT AGA ACT GCG GTA AGT TTC CTC GCA ATC TA-3' (*Bgl*II site underlined) and the antisense primer was 5'-ATA AGA ATG CGG CCG CAC GTT GAG GCT GCA TAA ATC CAG T-3' (*Not*I site underlined). The amplified product was digested with *Bgl*II and *Not*I, and the resulting fragment was subcloned into Bluescript SK(+). Cloned fragment of the ent*1*$^+$ gene in the Bluescript vector was digested with *Eco*RI and *Xho*I. Then, a *Eco*RI/*Xho*I fragment containing the *ura4*$^+$ gene was inserted into the *Eco*RI and *Xho*I site of the previous construct, causing the interruption of the *ent1*$^+$ gene. Diploid cells were transformed by the fragment containing disrupted *ent1*$^+$ gene. Stable integrants were selected on medium lacking uracil, and disruption of the gene was verified by genomic Southern hybridization.

Binding of Recombinant Proteins to Phosphoinositides Immobilized onto Nitrocellulose

Nitrocellulose membranes containing immobilized phospholipids (PIP-Strips membranes, Echelon, Salt Lake City, UT), blocked with TBS-T buffer with 3% fatty acid-free bovine serum albumin, were incubated with cell extract containing GFP-tagged recombinant Ent1 protein for 1 h at room temperature and washed. The bound proteins were detected by immunoblotting using an anti-GFP rabbit polyclonal antibody.

Microscopic Analysis

For actin staining, cells were fixed in 3 % formaldehyde in phosphate buffered saline (PBS) for 30 min and to 50 µl of fixed cell suspension 1 µl of 100 µg/ml rhodamine-labeled phalloidin (Molecular Probe) was added. After 30 min at room temperature the excess phalloidin was washed away with PBS. Cells were dried on coverslips treated with poly-L-lysine.

Vacuolar staining was conducted using 5- (and 6-)carboxy-2',7'-dichlorofluorescein diacetate (CDCFDA) or FM4-64 (Molecular Probes Inc., Eugene, OR) as described (13,18). Cells were grown to early-log phase in YPD at 27 ^{o}C. One ml cells were pelleted at 300 x g for 30 s and resuspended in 0.5 ml of medium containing 12 µM CDCFDA or 10 µM FM4-64. After 30 min labeling, the cells were centrifuged at 13,000 × g for 1 min, washed by resuspending in YPD to remove free CDCFDA or FM4-64, collected by centrifugation at 13,000 × g for 1 min, and then resuspended in YPD. Cells were examined and photographed by differential interference contrast (DIC) and fluorescent microscopy using an Axioskop microscope (Carl Zeiss Inc., Thornwood, NY) equipped with SPOT2 digital camera

(Diagnostic Instruments Inc., Sterling Heights, MI). Images were processed with the CorelDRAW software (Corel Corporation Inc., Ottawa, ON).

RESULTS

Identification of the *S. Pombe Ent1*$^+$ Gene

A BLAST program search using the peptide sequence of ENTH domain of *S. cerevisiae*

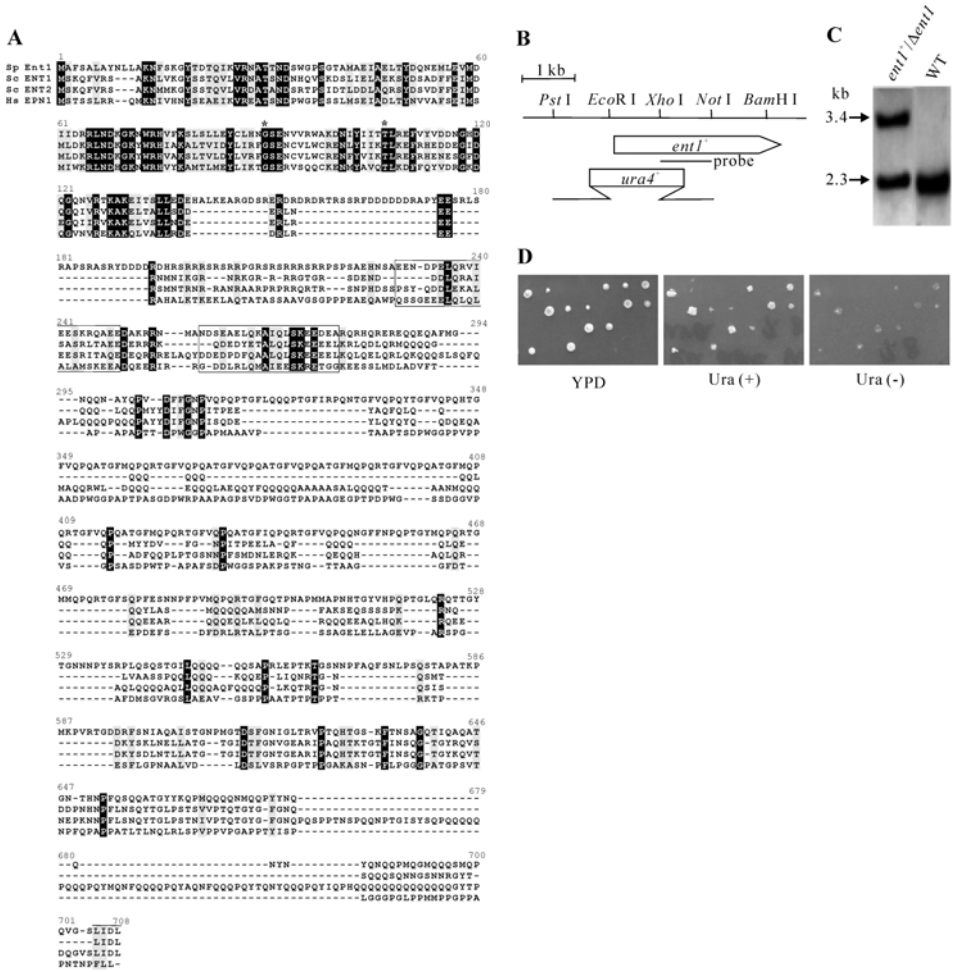

Fig. 1. (A) Alignment of protein sequences of *S. pombe* Ent1p with related proteins from *S. cerevisiae* and human. Amino acid sequences are aligned performed using the Clustal W program (16). Identical amino acids are shaded in black, and conservative substitutions are shaded in gray. Putative tandem UIMs are boxed (Residues 230-249 and 258 - 277). The clathrin-binding motif (LIDL) is indicated at the carboxyl terminus. Gly-90 and Thr-107, highly conserved residues in the ENTH domain are indicated by asterisks. Sp, *S. pombe*; Sc, *S. cerevisiae*; Hs, *Homo sapiens*. **(B)** A restriction map of the genomic *ent1*$^+$ locus, and the plasmid construct used for the *ent1*$^+$ gene disruption. **(C)** Southern blot analysis showing proper disruption of the chromosomal gene. Genomic DNAs from a wild-type *ent1*$^+$/*ent1*$^+$ diploid (WT) and the heterozygous *ent1*$^+$/*ent1*::*ura4*$^+$ diploid (*ent1*$^+$/Δ*ent1*) were digested with *Eco*RI/*Bam*HI, and resolved on a 0.8% agarose gel. DNAs were blotted onto Hybond-N$^+$ (Amersham Pharmacia Biotech) and hybridized with the labeled probe as indicated in Panel B. The deletion results in the production of a new band of 3.4 kb in addition to the 2.3-kb parental band. **(D)** Tetrad analysis of a heterozygous diploid. Diploid which consisted of one copy of wild-type *ent1*$^+$ and its complete deletion allele was manipulated and incubated at 27ºC on YPD plate (YPD) supplemented with adenine sulfate for 6 days. Then, growing colonies on YPD plate were replica plated onto EMM plate containing uracil (Ura(+)) or no uracil (Ura(-)).

Ent1p (21) against the *S. pombe* protein data base at the Sanger center (www.sanger.ac.uk) revealed an open reading frame, SPCC162.07, exhibiting significant similarity to Ent1p (score = 389, p = $9.1e^{-36}$, identities = 71/134 (52%), positives = 99/134 (73%)). We named the gene *ent1*$^+$. As shown in Fig. 1A, the *ent1*$^+$ gene encodes a protein of 706 amino acids that contains ENTH (epsin amino-terminal homology) domain at its amino terminus highly homologous to that of budding yeast Ent1p. Like all epsin homologues (20), *S. pombe* Ent1p comprises two copies of a ubiquitin-interacting motif (UIM) immediately carboxyl-terminal to the ENTH domain, three NPF motifs that may function as ligands for EH-domain-containing proteins in the carboxyl-terminal half, and the clathrin-binding consensus amino-acid motif at the carboxyl terminal. The presence of these various motifs in the carboxyl-terminal endocytic-machinery-binding domain is a common feature of epsins, suggesting that *S. pombe* Ent1p may also function as an important component of an endocytic complex.

Isolation and Disruption of *Ent1*$^+$ Gene

The *ent1*$^+$ gene was amplified by PCR with the genomic DNA of *S. pombe* as a template and was subcloned into Bluescript SK(+) (see Materials and Methods). As a first step to analyze Ent1p function, the *ent1*$^+$ gene was knocked out in a diploid by homologous recombination using the *ura4*$^+$ marker gene (Fig. 1B). Southern blotting of the genomic DNA of one such transformant confirmed that the wild-type gene had been replaced by the derivative containing the *ura4*$^+$ insertion (Fig. 1C). Tetrad analysis of the heterozygous diploid showed two viable (Ura-) and two inviable spores (Fig. 1D), indicating that *ent1*$^+$ gene is essential for cell growth.

Endocytic Defect in *Ent1* Knockout Cells

Ent1$^+$ gene knockout cells were inviable. To generate conditional *ent1*$^+$ gene knockout cells, the heterozygous diploid *ent1* knockout cells *(*Δ*ent1/ent1*$^+$) were transformed by pREP1-*ent1*$^+$-GFP plasmid, in which the Ent1p-GFP fusion protein is expressed under the control of the *nmt1* promoter. Tetrad analysis of these transformants indicated that viable *ent1* gene deleted haploid cells (Ura+) bearing pREP1-*ent1*$^+$-GFP plasmid can be recovered only when the plasmid protein expression was induced. This result suggests that Ent1p-GFP fusion protein is functional.

The endocytic function on *ent1* knockout cells bearing pREP1-*ent1*$^+$-GFP plasmid was assessed by examining the internalization of the fluorescent dye FM4-64 and CDCFDA. A lipophilic styryl dye, FM4-64 inserts into the plasma membrane of cells, is internalized to punctate cytosolic compartments, and is ultimately delivered to the vacuoles of yeast (18). CDCFDA is internalized from the plasma membrane and hydrolysed in the vacuoles to a fluorescent, membrane-impermeant species (13). Numerous small vacuoles were visualized in the wild-type cells using these vital dyes (Fig. 2). *ent1* knockout cells with pREP1-*ent1*$^+$-GFP plasmid were able to internalize both FM4-64 and CDCFDA similar to the wild-type cells when the plasmid protein expression was induced in the absence of thiamine (Fig. 2, promoter ON). In contrast, when the expression of Ent1p-GFP fusion protein was repressed in the presence of thiamine (Fig. 2, promoter OFF), *ent1* knockout cells with pREP1-*ent1*$^+$-GFP plasmid exhibited a marked defect in internalization of FM4-64 and CDCFDA. In this case, FM4-64 staining was not observed both at the vacuolar membrane and in the cytoplasm. These results suggest that Ent1p is required for endocytosis, mainly internalization step.

Ent1p is Involved in Actin Organization and Cell Morphogenesis

To better understand the function of Ent1p, we constructed conditional *ent1* mutant,

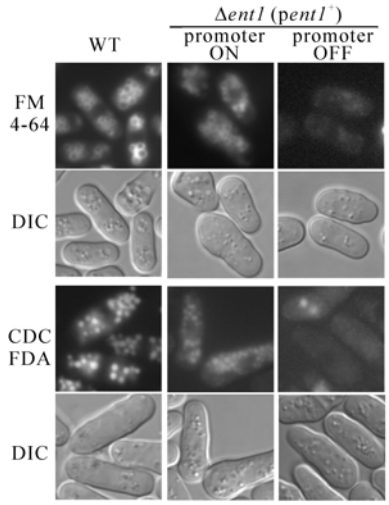

Fig. 2. *ent1* knockout cells are defective in endocytosis. Wild-type (WT) cells and *ent1* knockout cells with pREP1-*ent1*$^{+}$-GFP plasmid (Δ*ent1*(p*ent1*$^{+}$)) were labeled with 12 μM CDCFDA or 10 μM FM4-64 for 30 min. Cells were washed in YPD medium, resuspended in fresh YPD, and observed under a fluorescent microscope. Expression of Ent1p-GFP protein in the Δ*ent1* (p*ent1*$^{+}$) was induced by removal of thiamine from medium (promoter ON).

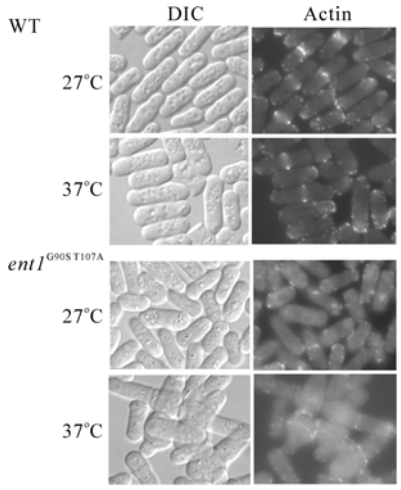

Fig. 3. Disorganization of actin patches and aberrant cell morphology in *ent1*(G90ST107A) mutant cells. Wild-type (WT) and in *ent1*(G90ST107A) were grown in YPD medium at 27°C and then 37°C for 3h. Cells were fixed and stained with rhodamine-labeled phalloidin (Molecular Probe).

which is endowed with amino acid substitutions in conserved residues within ENTH domains. Both Glycine-90 and Threonine-107 within ENTH domains are highly conserved residues. It is reported that mutations in corresponding amino acid residues caused temperature-sensitive alleles of *ENT1* in budding yeast (21). Therefore, we generated and analyzed *ent1*(G90S), *ent1*(T107A), and *ent1*(G90ST107A) mutant strains. In contrast to temperature-sensitivity of budding yeast *ent1*(G87S) and *ent1*(T104A) mutants (corresponding to *ent1*(G90S) and *ent1*(T107A) mutants in fission yeast, respectively), neither fission yeast *ent1*(G90S) mutant nor *ent1*(T104A) mutant showed no

temperature-sensitivity. Furthermore, *ent1*(G90ST107A) double mutant exhibited no temperature-sensitive growth defect (data not shown).

Next, we examined the morphological change in wild-type and *ent1*(G90ST107A) mutant. Microscopic observation revealed that *ent1*(G90ST107A) exhibited cell morphology and F-actin localization similar to those of wild-type at 27°C (Fig. 3). Interestingly, on temperature up-shift to 37°C for 3 h, *ent1*(G90ST107A) showed marked aberrant cell morphology and depolarization of F-actin. This result suggests that Ent1p is involved in actin organization and cell morphogenesis.

Intracellular Localization of GFP-fused Ent1p

We examined the localization of Ent1p in the wild-type cells by expressing the Ent1p-GFP fusion protein under the control of the *nmt1* promoter. When *nmt1-ent1*$^{+}$-GFP was expressed in wild-type cells, Ent1p-GFP was localized to punctate structures at the cell periphery and the medial region, which resemble those of F-actin (Fig. 4A, 5B).

The fact that F-actin and Ent1p localized to the similar region of the cell prompted us to determine whether the actin cytoskeleton was required for the localization of Ent1p-GFP. To disrupt the actin cytoskeleton, the *ts* profilin mutant *cdc3*, the *ts* tropomyosin mutant *cdc8*, and the *ts* actin mutant *cps8* (2,3,6) were transformed with the Ent1p-GFP fusion plasmid and incubated at restrictive temperature. Under these conditions, the F-actin patches associated with the cell periphery and the actomyosin ring structure failed to form, resulting in mitosis without cytokinesis. Ent1p localization to patches both at the cell periphery and the medial region was not affected (Fig. 4B). Therefore, the localization of Ent1p-GFP is not dependent on the integrity of the F-actin cytoskeleton.

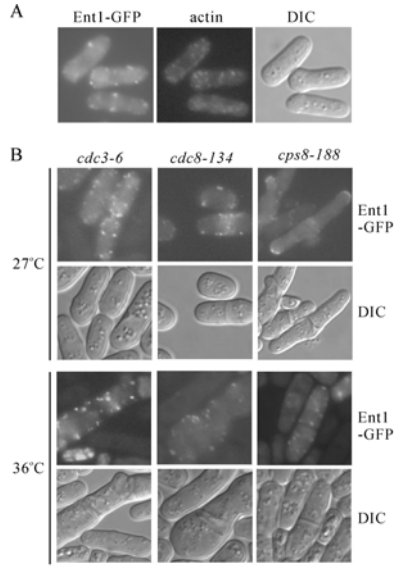

Fig. 4. Localization of Ent1p-GFP is not dependent on the integrity of the F-actin cytoskeleton. **(A)** Wild-type cells expressing plasmid-borne Ent1p-GFP were fixed and stained with rhodamine-phalloidin. **(B)** *cdc3-6* and *cdc8-134*, *ts* mutants with defects in actin-binding proteins and *cps8-188* actin *ts* mutant cells expressing plasmid-borne Ent1p-GFP were grown at 27°C and then 36°C for 6h. Cells were processed for fluorescent microscopy.

Ent1p Binds to Phosphoinositides *in Vitro*

The ENTH domains of mammalian epsins and budding yeast epsins bind

phosphoinositides (1,7). Therefore, we tested whether the Ent1p ENTH domain also interacts with lipids. GFP-fused Ent1p (Ent1p-GFP) protein was expressed from thiamine-repressible *nmt1* promoter on pREP1 vector in wild-type fission yeast cells. Nitrocellulose strips containing a variety of immobilized phospholipids were incubated with cell extract containing Ent1p-GFP protein. The Ent1p-GFP significantly bound $PI(4,5)P_2$ and $PI(3,5)P_2$ immobilized onto nitrocellulose *in vitro* and also weakly bound other phosphoinositides including $PI(3,4)P_2$, $PI(3,4,5)P_3$, PI4P and PI5P (Fig. 5A). The PLCδ PH domain fused to

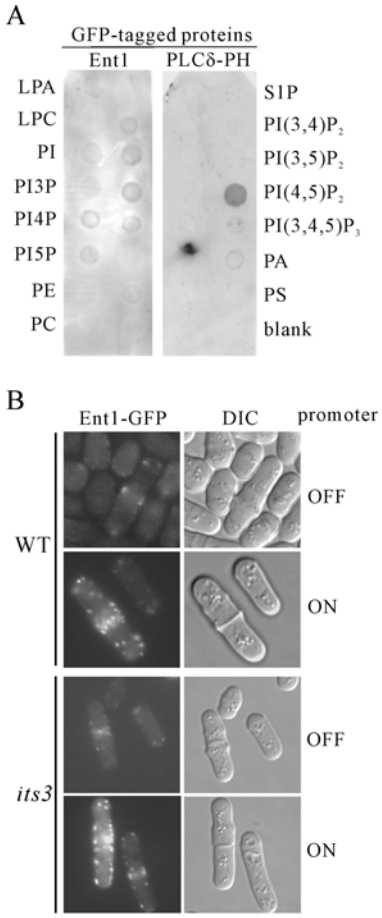

Fig. 5. Ent1p binds to phosphoinositides. **(A)** Nitrocellulose membranes bearing differential immobilized phosphoinositides were incubated with fission yeast cell extracts containing Ent1p-GFP protein (Ent1) or PLCδ PH domain fused to GFP (PLCδ-PH) for 1 h at room temperature and washed, the bound proteins were detected by immunoblotting using an anti-GFP rabbit polyclonal antibody. **(B)** Intracellular localization of Ent1p-GFP in wild-type (WT) and in *its3-1* mutant (*its3*) cells. Wild-type and *its3-1* cells harboring pREP1-*ent1*$^+$-GFP plasmid were grown in EMM medium in the presence (promoter OFF) and the absence (promoter ON) of 4 μg/ml thiamine. Cells were processed for fluorescent microscopy.

GFP (GFP-PH) was expressed in wild-type fission yeast and cell extract was prepared. PH domain from PLCδ has been shown to have a high binding affinity for plasma membrane $PI(4,5)P_2$ (5,12,22). Consistently, GFP-PH specifically bound $PI(4,5)P_2$ immobilized onto

nitrocellulose (Fig. 5A).

Given that Ent1p binds plasma membrane PI(4,5)P_2, we predicted that Ent1p localization to the plasma membrane would be perturbed in *its3-1* mutant cells, in which the level of PI(4,5)P_2 was severely reduced (22). Unexpectedly, Ent1p-GFP was localized to punctate structures at the cell periphery and the medial region even in *its3-1* mutant cells, similar to that in the wild-type (Fig. 5B). This finding suggests that Ent1p may physiologically bind phosphoinositides other than PI(4,5)P_2.

DISCUSSION

In this study, we have demonstrated that Ent1p is essential for cell viability and indispensable for endocytosis in fission yeast through the construction and functional analysis of *ent1* gene knockout mutant. *S. pombe* Ent1p is 706 amino acids in length and shows structural similarity with *S. cerevisiae* Ent1p and Ent2p. A high degree of sequence similarity between the proteins occurs in the amino-terminal region, which is known as the ENTH domain (8). Moreover, the protein exhibits various motifs (UIM, NPF and the clathrin-binding motif) of endocytic-machinery-binding domain in the carboxyl-terminal half as well as epsins of other organisms. This structural similarity suggests that *S. pombe* Ent1p functions as an important component of an endocytic complex.

The budding yeast has *ENT1* and *ENT2* genes, which encode two epsin homologues with redundant functions (21). Cells lacking either gene alone are viable, however, cells in which both *ENT1* and *ENT2* are deleted are inviable. On the other hand, the fission yeast genome sequence encodes only a single epsin homologue. We clearly demonstrated that fission yeast *ent1* knockout cells were inviable and that viable *ent1* knockout cells could be recovered only if a plasmid bearing functional *ent1*$^+$ gene was present and the expression of *ent1*$^+$ gene was induced. These data indicate that *ent1* is essential for cell viability.

The *ent*1$^+$ gene can be conditionally expressed from thiamine-repressible *nmt1* promoter on pREP1 vector in *ent1* knockout cells bearing pREP1-*ent1*$^+$-GFP plasmid. This allowed us to analyze physiological function of Ent1p. On repression of Ent1p production, *ent1* knockout cells with pREP1-*ent1*$^+$-GFP plasmid showed a severe defect in endocytosis, especially in the internalization step. Therefore, fission yeast epsin Ent1p is also required for endocytosis similar to mammalian and budding yeast epsins.

The ENTH domain is highly conserved from yeast to humans (8). By using internal deletions within ENTH domain and generating temperature-sensitive alleles of *ENT1* in budding yeast, it is demonstrated that this domain is required for the essential functions of epsins (21). To better understand the functional importance of ENTH domain, we constructed conditional *ent1* mutant (*ent1*(G90ST107A)), which has amino acid substitutions in conserved residues within ENTH domains. Unlike *S. cerevisiae ent1*^{ts} mutants, *S. pombe ent1*(G90ST107A) mutant showed no temperature-sensitive growth defect. However, *ent1*(G90ST107A) showed disorganization of F-actin and marked aberrant cell morphology on temperature up-shift to 37^{o}C, suggesting that Ent1p is involved in actin organization and cell morphogenesis. This also indicates that ENTH domain is required for essential functions of Ent1p in fission yeast as well as all epsins of other organisms.

Previous studies have been shown that EHTN domains bind phosphoinositides in mammalian cells (7) and budding yeast (1), and we have confirmed this for the fission yeast Ent1p. To test whether PI(4,5)P_2 is required for Ent1p recruitment to the plasma membrane, we observed the localization of Ent1p-GFP in *its3-1* mutant cells, in which the level of PI(4,5)P_2 was severely reduced (22). It was surprising to find that the localization of Ent1p-GFP was not affected in *its3-1* mutant cells. Moreover, *its3-1* mutant cells are not

defective in endocytosis (data not shown). Taken these together, PI(4,5)P_2 may not be essential for proper localization of Ent1p at endocytic sites via ENTH domain interaction. It is reported that a major contribution to membrane binding is provided by the recognition of ubiquitin by Ent1p UIM (ubiquitin-interacting motif) s at 4^{o}C (1). Therefore, it is suggested that Ent1p recruitment to membrane may be primarily UIM-dependent under possible alteration of membrane biophysical properties due to reduced level of PI(4,5)P_2 in the *its3-1* mutant cells. Alternatively, it is possible that EH domain-containing proteins like *S. cerevisiae* Ede1p may recruit Ent1p to membranes via EH/NPF interactions (1), cooperatively with or independently of Ent1p binding to lipids.

In conclusion, we characterized *S. pombe* epsin *ent1*$^{+}$ and provide evidence that Ent1p is required for endocytosis, and is involved in actin organization and cell morphogenesis. We also found that Ent1p may be recruited to the plasma membrane via other interactions (i.e. ubiquitin-UIM) other than ENTH-PI(4,5)P_2 interaction, despite Ent1p binding to PI(4,5)P_2 *in vitro*. The order of events for ENTH domain- and UIM-mediated recruitment of Ent1p to sites of endocytosis remains to be determined.

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