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# EH Domain Proteins Pan1p and End3p Are Components of a Complex That Plays a Dual Role in Organization of the Cortical Actin Cytoskeleton and Endocytosis in *Saccharomyces cerevisiae*

HSIN-YAO TANG,[1] ALAN MUNN,[2]† AND MINGJIE CAI[1]*

*Institute of Molecular and Cell Biology, National University of Singapore, Singapore 119260, Singapore,[1] and Department of Biochemistry, Biozentrum, University of Basel, CH-4056 Basel, Switzerland[2]*

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**Several proteins from diverse organisms have been shown to share a region of sequence homology with the mammalian epidermal growth factor receptor tyrosine kinase substrate Eps15. Included in this new protein family, termed EH domain proteins, are two yeast proteins, Pan1p and End3p. We have shown previously that Pan1p is required for normal organization of the actin cytoskeleton and that it associates with the actin patches on the cell cortex. End3p has been shown by others to be an important factor in the process of endocytosis. End3p is also known to be required for the organization of the actin cytoskeleton. Here we report that Pan1p and End3p act as a complex in vivo. Using the *pan1-4* mutant which we isolated and characterized previously, the *END3* gene was identified as a suppressor of *pan1-4* when overexpressed. Suppression of the *pan1-4* mutation by multicopy *END3* required the presence of the mutant Pan1p protein. Coimmunoprecipitation and two-hybrid protein interaction experiments indicated that Pan1p and End3p associate with each other. The localization of Pan1p to the cortical actin cytoskeleton became weakened in the *end3* mutant at the permissive temperature and undetectable at the restrictive temperature, suggesting that End3p may be important for proper localization of Pan1p to the cortical actin cytoskeleton. The finding that the *pan1-4* mutant was defective in endocytosis as severely as the *end3* mutant under nonpermissive conditions supports the notion that the association between Pan1p and End3p is of physiological relevance. Together with results of earlier reports, these results provide strong evidence suggesting that Pan1p and End3p are the components of a complex that has essential functions in both the organization of cell membrane-associated actin cytoskeleton and the process of endocytosis.**

Endocytic pathways play a variety of important roles in eucaryotic cells. They are involved, for example, in the efficient uptake of essential nutrients into the cell interior, the remodeling of plasma membrane protein composition, and the regulation of mitogenic signal transduction across the plasma membrane (33, 34, 39, 46). Upon ligand binding, cell surface receptors are first mobilized into invaginated membrane structures, such as clathrin-coated pits, which pinch off to form vesicles carrying receptors and their ligands (30, 32, 46). These endocytic vesicles fuse with an endosomal compartment, where some of the receptors are dissociated from their ligands and recycled to the cell surface, whereas other receptors, together with the bound ligands, are transported to the lysosome for degradation (30, 39, 42, 46). In the case of growth factor receptors, endocytosis and lysosomal degradation of the receptor-ligand complex leads to depletion of receptors from the cell surface and attenuation of mitogenic signalling triggered by extracellular growth factors. This process is known as ligand-induced receptor down-regulation (39, 42).

One of the most extensively studied examples of ligand-induced down-regulation of cell surface receptors via the endocytic pathway is that of the epidermal growth factor receptor (EGFR). Binding of ligand to EGFR increases the tyrosine kinase activity of the receptor, resulting in its autophosphorylation and phosphorylation of many intracellular substrates (16). Evidence has accumulated to suggest that the activation of the EGFR kinase by ligand binding serves two functions. On one hand, it triggers a cascade of phosphorylation reactions leading to activation of the cell cycle machinery and promotion of DNA synthesis and cell division. On the other hand, it is also a prelude to down-regulation of the mitogenic signal transduction pathway. Studies using mutant EGFR demonstrate that the tyrosine kinase activity of EGFR, and its autophosphorylation, is necessary for efficient ligand-stimulated receptor endocytosis (22, 29). It has been further shown that the intracellular phosphotyrosine-containing domain of EGFR interacts with the clathrin-associated adaptor complex AP-2 (28, 43), suggesting a role for tyrosine phosphorylation in the recruitment of the receptor into clathrin-coated pits. Autophosphorylation of the receptor, however, may not be sufficient for its endocytosis, because in vitro experiments have suggested the requirement of other cellular factors for recruiting the receptor into coated pits (21, 22). Eps15, a protein originally identified on the basis of its rapid phosphorylation by EGFR upon ligand binding (9), may be one such factor. This speculation arises from the recent finding that Eps15 constitutively associates with AP-2 complexes on the plasma membrane (3) and, indeed, colocalizes with AP-2 and clathrin (45). Eps15 belongs to a newly identified protein family sharing a conserved sequence of approximately 70 amino acids, termed the EH

* Corresponding author. Mailing address: Institute of Molecular and Cell Biology, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore. Phone: (65)7723382. Fax: (65)7791117. E-mail: mcbcaimj@leonis.nus.sg.

† Present address: Institute of Molecular Agrobiology, Singapore 118240, Singapore.

TABLE 1. Yeast strains used

| Name | Relevant genotype |
|---|---|
| CRY1 | *MAT***a** *ade2 his3 leu2 trp1 ura3* |
| CRY2 | *MAT*α *ade2 his3 leu2 trp1 ura3* |
| YHT99 | *MAT***a** *ade2 his3 leu2 trp1 ura3 pan1-4* |
| YHT139 | *MAT*α *ade2 his3 leu2 trp1 ura3 pan1*Δ::*HIS3* pRS316-PAN1 |
| YHT151 | *MAT*α *ade2 his3 leu2 trp1 ura3 end3*Δ::*LEU2* |
| YHT153 | *MAT***a**/α *ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 PAN1/pan1-4 END3/end3*Δ::*LEU2* |
| YHT167 | *MAT***a** *ade2 his3 leu2 trp1 ura3 end3*Δ::*LEU2* |
| YHT199 | *MAT*α *ade2 his3 leu2 trp1 ura3 pan1*Δ::*HIS3* pRS316-HA-PAN1 |
| YHT215 | *MAT***a** *ade2 his3 leu2 trp1 pan1-4 ura3*::*URA3-PAN1* |
| YHT237 | *MAT***a** *ade2 his3 leu2 trp1 ura3* pGAL-HA-PAN1 |
| YHT238 | *MAT***a** *ade2 his3 leu2 trp1 ura3* pGAL-HA-PAN1 pGAL-*myc*-END3 |

(Eps15 homology) domain (50). The physiological function of the EH domain is unknown.

Two EH domain-containing proteins from the yeast *Saccharomyces cerevisiae*, Pan1p and End3p, participate in cortical actin cytoskeleton-related processes (2, 44). Pan1p is required for normal organization of the cortical actin cytoskeleton (44). Loss of activity or overproduction of Pan1p results in an abnormal distribution of the actin cytoskeleton on the cell cortex (44). Pan1p also colocalizes with cortical actin patches, suggesting that it may be a component of these structures (44). Recent reports also identified Pan1p as a protein required for the fluid-phase endocytosis (51) and the normal level of endocytosis of the yeast mating pheromone α-factor (49). End3p is similarly involved in both the organization of the cortical actin cytoskeleton and endocytosis (2). The *end3* mutation was identified in a genetic screen for mutants defective in endocytosis of α-factor and shown to specifically affect the internalization step of this process (2, 31). *END3* has subsequently been found to be required for internalization of a variety of other yeast plasma membrane proteins, such as the ABC transporter Ste6p, and the uracil and inositol permeases (17, 20, 47).

In this report, we describe the isolation of a multicopy suppressor of a *pan1* temperature-sensitive mutant (*pan1-4*). Interestingly, the suppressor is found to be *END3*. The relevance of this genetic interaction is supported by the demonstrations that Pan1p and End3p associate with each other in vivo and that the *pan1-4* mutant is defective in both receptor-mediated and fluid-phase endocytosis. These findings suggest a dual role for the Pan1p-End3p complex in the organization of the cortical actin cytoskeleton and the process of endocytosis.

## MATERIALS AND METHODS

**Strains, media, and general methods.** The yeast strains used are listed in Table 1. Rich (YPD), synthetic complete (SC), and dropout media were prepared as described previously (35). Wild-type cells were grown at 30°C. Temperature-sensitive mutants were propagated at the permissive temperature of 24°C and analyzed at the restrictive temperature of 37°C. Genetic manipulations were performed according to standard methods described by Rose et al. (35). Recombinant DNA methodology was performed as described by Sambrook et al. (38). Lucifer yellow carbohydrazide (LY) was obtained from Sigma. PCRs were performed with Vent polymerase (New England Biolabs) as recommended by the manufacturer. DNA was sequenced by using Sequenase (U.S. Biochemical), and sequences were compared to entries in the GenBank database, using the BLAST server at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, Md.).

**Primers.** The primers used in this study were HTP1 (5′ ACCTGCCTCTAT GCAAC 3′), HTP2 (5′ TAGGACATTGGGGATTG 3′), HTP3 (5′ CGAGAA TTCATTACAGCGGGCGTA 3′), HTP4 (5′ CGGGGATCCCTATGCATTGG TAATCTCATT 3′), HTP5 (5′ GCCGAATTCAAGGTTTACTCTGCG 3′), HTP6 (5′ CACGTCGACTTCAGAAATTAGTATACATA 3′), HTP7 (5′ CGA GAATTCATGGCACCAACCACTTTCAA 3′), HTP8 (5′ CGAGAATTCATG CCCCAGCAAAGC 3′), HTP9 (5′ GCCGAATTCATTTATACCAAATCC 3′), HTP10 (5′ CGAGAATTCATGTATAACCCGTACC 3′), HTP11 (5′ CGGCT CGAGCTATTTATAAAACAAAGATTT 3′), HTE1 (5′ CGGAATTCCCCAT GGCCAAGTTGGAACAA 3′), HTE2 (5′ GGAACTCGAGTTCTGCCCATT GTC 3′), HTE3 (5′ GGCGAATTCATATTTGATATGGTT 3′), HTE4 (5′ CGGGATCCATGAACACGTGTTTAAATTAT 3′), HTE5 (5′ CCGGAATTC CCAGCAAGTTTACCA 3′), HTE6 (5′ CAGGAATTCTCGAGTTCCGAT AGC 3′), HTE7 (5′ CCGGAATTCAAAAAGAGTGAAAAAACT 3′), and HTE8 (5′ GGGAATTCTTATCGGTGTCAATTGAT 3′).

**Isolation of multicopy suppressor of *pan1-4*.** Strain YHT99 (*pan1-4*) was transformed with a yeast genomic library constructed in the 2μm plasmid pRS425. About 50,000 transformants were tested for growth at 37°C. DNA of temperature-resistant transformants was extracted and transformed into *Escherichia coli* XL-1 Blue. The primers HTP1 and HTP2, which amplify a region within the essential domain of *PAN1* (44), were used in PCR to identify plasmids containing the *PAN1* gene. Plasmids that did not contain the *PAN1* gene were then analyzed further by restriction digestions and sequencing.

**Plasmid constructions.** The plasmids used are listed in Table 2. To generate pRS316-HA-PAN1, a unique *Asp*718 site was first inserted by PCR immediately after the initiation codon of *PAN1*. An *Asp*718 cassette containing three tandem repeats of the hemagglutinin (HA) epitope (YPYDVPDYAG) was then ligated in frame to generate pRS316-HA-PAN1. To construct pGAL-*myc*-END3, PCR was first used to amplify the coding sequence of *END3* with an *Asp*718 site inserted after the ATG codon. This was then cloned into pRS315 containing the *GAL1* promoter. Subsequently a cassette containing the Myc tag (EQKLI SEEDL) was inserted in frame into the *Asp*718 site to generate pGAL-*myc*-END3.

**Construction of yeast strains.** The gene disruption of *PAN1* (*pan1*Δ::*HIS3*) has been described previously (44). To disrupt *END3*, the *END3* gene was first digested with *Ava*I and *Stu*I. The *Ava*I site was then blunted, and a fragment containing the *LEU2* gene was cloned into it. This resulted in the deletion of *END3*, leaving behind only 47 and 180 nucleotides from the 5′ and 3′ ends, respectively, of the coding sequence. The *LEU2* gene together with *END3* flanking sequences (*end3*Δ::*LEU2*) was subsequently transformed into CRY2 to generate YHT151. The deletion was confirmed by PCR. YHT153 was generated by mating YHT99 with YHT151. YHT167 was one of the spores containing the *end3*Δ::*LEU2* obtained by sporulating YHT153. YHT139 and YHT199 were generated by sporulating the *pan1*Δ::*HIS3* heterozygous diploid (44) transformed with pRS316-PAN1 and pRS316-HA-PAN1, respectively, and selecting for spores that were $His^+$ and $Ura^+$. YHT215 was obtained by transforming YHT99 with the *Stu*I-linearized pRS306-PAN1. The *Stu*I site is located within the *URA3* gene of pRS306 (41). Transformants that were $Ura^+$ and temperature resistant were chosen. YHT237 was generated by transforming CRY1 with pGAL-HA-PAN1. YHT238 was CRY1 transformed with pGAL-HA-PAN1 and pGAL-*myc*-END3.

Derivatives of CRY1, YHT99, YHT167, and YHT215 that are $Bar1^-$ were created by first selecting *lys2* mutations on α-aminoadipate (40) and then transforming these $Lys2^-$ strains to lysine prototrophy with the *Eco*RI fragment of pEK3. This fragment carries a disruption of the *BAR1* gene marked with *LYS2* (18).

**Immunoprecipitation.** Cells were grown at 30°C to mid-log phase ($A_{600}$ = 0.9 to 1.2) in 150 ml of SC medium lacking the appropriate amino acids, washed once in solution A (0.9% NaCl, 1 mM $NaN_3$, 10 mM EDTA, 50 mM NaF), and resuspended in 900 μl of solution B (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris-HCl [pH 7.2], 100 mM sodium orthovanadate, 15 mM *p*-nitrophenylphosphate, 1 mM phenylmethylsulfonyl fluoride, protease inhibitors). Cells were then lysed at 4°C by vortexing with 500-μm-diameter acid-washed glass beads. After centrifugation at 15,000 × *g* for 20 min, the supernatant was transferred to a new tube and the protein concentration was measured. For immunoprecipitation, cell lysates (about 3 mg) were incubated for 1 h at 4°C with mouse anti-HA antibody 12CA5 (Boehringer Mannheim) to precipitate HA-tagged proteins or with mouse anti-Myc antibody 9E10 (Calbiochem) to precipitate Myc-tagged proteins. This was followed by an incubation with protein A-Sepharose beads (Pharmacia) for 1 h at 4°C. The beads were then washed five times with solution C (1% Nonidet P-40, 150 mM NaCl,

TABLE 2. Plasmids used

| Name | Construct |
|---|---|
| pRS314-END3 | *END3* (1.9-kb *Xba*I/*Cla*I) in pRS314 |
| pRS424-END3 | *END3* (1.9-kb *Xba*I/*Cla*I) in pRS424 |
| pRS426-END3 | *END3* (1.9-kb *Xba*I/*Cla*I) in pRS426 |
| pRS314-PAN1 | *PAN1* (5.8-kb *Bam*HI/*Eco*RI) in pRS314 |
| pRS424-PAN1 | *PAN1* (5.8-kb *Bam*HI/*Eco*RI) in pRS424 |
| pRS306-PAN1 | *PAN1* (5.8-kb *Bam*HI/*Eco*RI) in pRS306 |
| pRS316-HA-PAN1 | HA-tagged *PAN1* under its own promoter in pRS316 |
| pGAL-*myc*-END3 | Myc-tagged *END3* under *GAL1* promoter control in pRS315 |
| pGAL-HA-PAN1 | HA-tagged *PAN1* under *GAL1* promoter control in pRS316 |
| pPAN1.1 | *PAN1* (1–385) in pGAD424 |
| pPAN1.2 | *PAN1* (384–846) in pGAD424; made with primers HTP3 and HTP4 |
| pPAN1.3 | *PAN1* (734–1480) in pGAD424; made with primers HTP5 and HTP6 |
| pPAN1.4 | *PAN1* (431–846) in pGAD424; made with primers HTP7 and HTP4 |
| pPAN1.5 | *PAN1* (520–846) in pGAD424; made with primers HTP8 and HTP4 |
| pPAN1.6 | *PAN1* (577–846) in pGAD424; made with primers HTP9 and HTP4 |
| pPAN1.7 | *PAN1* (384–606) in pGAD424; made with primers HTP3 and HTP11 |
| pPAN1.8 | *PAN1* (1–606) in pGAD424; made with primers HTP10 and HTP11 |
| pPAN1.9 | *PAN1* (1–846) in pGAD424; made with primers HTP10 and HTP4 |
| pEND3.0 | *END3* (1–349) in pGBT9; made with primers HTE1 and HTE4 |
| pEND3.1 | *END3* (1–254) in pGBT9; made with primers HTE1 and HTE2 |
| pEND3.2 | *END3* (72–349) in pGBT9; made with primers HTE3 and HTE8 |
| pEND3.3 | *END3* (208–349) in pGBT9; made with primers HTE5 and HTE4 |
| pEND3.4 | *END3* (254–349) in pGBT9; made with primers HTE6 and HTE4 |
| pEND3.5 | *END3* (296–349) in pGBT9; made with primers HTE7 and HTE4 |

0.5% sodium deoxycholate, 50 mM Tris-HCl [pH 7.2], 5 mM EDTA, 1 mM sodium orthovanadate), and the immune complexes were released by boiling in sample buffer for 5 min. After centrifugation, samples were loaded on SDS–7.5 or 10% polyacrylamide gels for detecting either Pan1p or End3p. After electrophoresis, the separated proteins were electroblotted onto Immobilon-P membranes (Millipore). The HA-tagged Pan1p and End3p and the Myc-tagged End3p were detected by using mouse anti-HA 12CA5, affinity-purified rabbit anti-End3p (2), and mouse anti-Myc 9E10, respectively, followed by either horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies (Jackson ImmunoResearch Laboratories). Antibody-antigen complexes were visualized with the Amersham ECL system.

**Two-hybrid assays.** The Clontech MATCHMAKER system was used in two-hybrid analysis. All *PAN1* and *END3* constructs were fused to the *GAL4* activation domain of pGAD424 and the DNA binding domain of pGBT9, respectively. pPAN1.1 was generated by ligating the 1.2-kb *Eco*RI fragment from pMC213 (44) to the *Eco*RI site of pGAD424. PCR performed in three separate reactions was used to generate the other constructs, using Vent polymerase and the primers described in Table 2. Plasmids were cotransformed into the yeast strain SFY526, and β-galactosidase activities were measured in at least three different isolates of each transformation. Activities were expressed as Miller units.

**Endocytosis assays.** Internalization of $^{35}$S-labeled α-factor was measured as described by Munn and Riezman (26). The assays were carried out at 24°C and at 37°C with a 15-min preshift before α-factor addition. Internalization was calculated by dividing the internalized counts (pH 1 resistant) by the total cell-associated counts (pH 6 resistant) for each time point. The accumulation of LY was performed as described by Dulic et al. (8), with slight modifications. Overnight cultures, grown in YPD at 24°C, were diluted to fresh YPD and allowed to grow until early log phase. LY was added to a final concentration of 5 mg/ml and incubated for another hour at 24°C. Cells were then washed five times in phosphate-buffered saline containing 10 mM sodium azide and observed with a Zeiss Axioplan microscope. In control experiments, cells without LY incubation displayed no staining, indicating that the fluorescence observed is due to the LY accumulation and not to the *ade2* fluorophore, which is seen only in stationary-phase cells grown in adenine-limiting conditions.

**Double immunofluorescence staining of Pan1p and actin.** YHT151 (*end3*Δ::*LEU2*) was transformed with pRS314-END3 and pGAL-HA-PAN1 individually or in combination. Transformants were grown at 24°C in SC medium containing 2% galactose but lacking the appropriate amino acids. When required, the cells were shifted to 37°C for 2 h. Cells were then fixed with formaldehyde and processed for double immunofluorescence staining as described by Tang and Cai (44). The mouse anti-HA 12CA5 and guinea pig antiactin antibodies were used as the primary antibodies followed by rhodamine-conjugated goat anti-mouse and fluorescein-conjugated donkey anti-guinea pig antibodies (Jackson ImmunoResearch Laboratories).

## RESULTS

***END3* is a multicopy suppressor of the *pan1-4* mutation.** Originally, Pan1p was thought to be a poly(A) nuclease (36), but this finding was later retracted (37). We have recently isolated and characterized a mutation in *PAN1* (*pan1-4*) that causes severe disorganization of the actin cytoskeleton and defects in cell cycle progression (44). To identify other factors that may interact with Pan1p, we isolated multicopy suppressors of *pan1-4*. Strain YHT99 (Table 1) containing the *pan1-4* mutation was transformed with a yeast genomic DNA library constructed in the multicopy vector pRS425 (5). From an estimated 50,000 transformants, we isolated 35 that were viable at 37°C. The plasmid DNA was extracted and analyzed by PCR using *PAN1* gene-specific primers. Of these 35 transformants, 23 were found to contain the *PAN1* gene. The remaining 12 plasmids all contained a 3.6-kb *Eco*RI fragment. After subcloning and retransformation, this 3.6-kb *Eco*RI fragment was sufficient to support the growth of the *pan1-4* mutant at 37°C when a multicopy vector was used. Further digestion of this fragment with the restriction enzyme *Xho*I resulted in 0.9- and 2.7-kb fragments, neither of which was able to suppress the temperature sensitivity of *pan1-4* (data not shown). The DNA region embracing the *Xho*I site was then sequenced and found to be from the coding region of a previously identified gene, *END3*. To confirm that the suppression of *pan1-4* by this piece of DNA was solely due to the *END3* gene, the 1.9-kb *Xba*I/*Cla*I fragment containing only the *END3* gene (2) was subcloned into pRS424 to generate plasmid pRS424-END3. As shown in Fig. 1A, this construct was sufficient to allow the *pan1-4* cells to grow at the restrictive temperature nearly as well as the mutant cells carrying the *PAN1* gene on a single-copy vector (pRS314 [41]). However, when the same *END3* fragment was inserted into the single-copy vector pRS314 (pRS314-END3), its ability to support growth of the mutant at 37°C was lost (Fig. 1A). This result indicates that the *END3* gene is a multicopy suppressor of *pan1-4*.

As we reported previously, *pan1-4* mutant cells exhibit de-

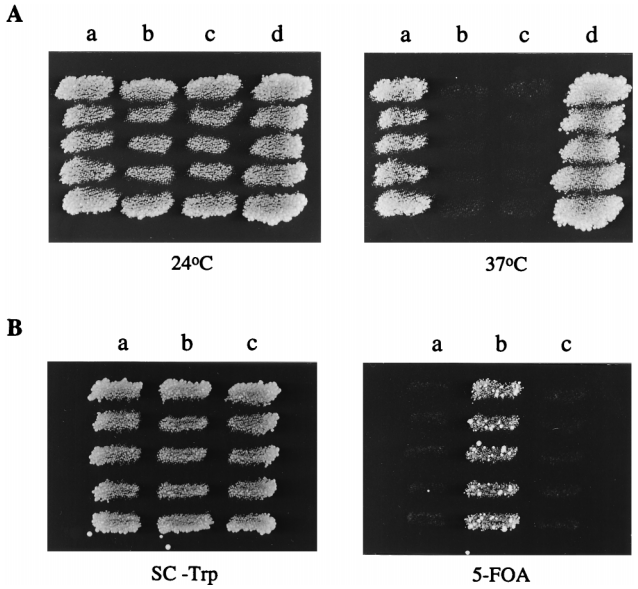


FIG. 1. *END3* is a multicopy suppressor of the *pan1-4* mutant. (A) YHT99 (*pan1-4*) cells transformed with pRS424-END3 (a), pRS314-END3 (b), pRS424 (c), and pRS314-PAN1 (d) were patched onto Trp-deficient SC plates at 24°C (left) and replica plated at 37°C (right). Only pRS424-END3 and pRS314-PAN1 were able to complement the $Ts^-$ phenotype of *pan1-4*. (B) YHT139 (*pan1*Δ pRS316-PAN1) cells transformed with pRS424 (a), pRS424-PAN1 (b), and pRS424-END3 (c) were patched onto Trp-deficient SC plates (left) and replica plated onto 5-FOA plates (right) at 24°C. pRS424-END3 could not replace pRS316-PAN1 in YHT139.

fects in organization of the cortical actin cytoskeleton similar to those found for the *end3* mutant (2, 44). In addition, both End3p and Pan1p belong to the EH domain family of proteins (44, 50). It is therefore conceivable that overexpressed End3p could suppress the *pan1-4* mutation by substituting for the cellular functions of Pan1p. To test this possibility, we examined whether *END3* on a multicopy plasmid was able to complement the lethality of a *pan1* null mutant. *PAN1* is an essential gene at all temperatures (36, 44). A *PAN1* deletion strain, YHT139, complemented by the *PAN1* gene on a *URA3*-containing single-copy plasmid (pRS316-PAN1), was transformed separately with pRS424-END3, pRS424-PAN1, and the multicopy vector pRS424 as a control. Transformants were then tested for growth in SC medium containing 5-fluoroorotic acid (5-FOA), a drug that is toxic to cells harboring a functional *URA3* gene (4a). As shown in Fig. 1B, none of the pRS424-END3 transformants could survive without the *PAN1* gene on the *URA3*-containing plasmid, indicating that *END3* was unable to replace *PAN1* (Fig. 1B). These results suggest that the suppression of *pan1-4* by *END3* requires the presence of the mutant Pan1p protein, and therefore the mechanism of this suppression may be attributable to interactions between these two proteins.

**Synthetic lethality between *pan1-4* and *end3* deletion mutants.** To study further the genetic interaction between the *PAN1* and *END3* genes, we investigated whether combination of the two mutants, *pan1-4* and *end3*Δ, would confer a lethal phenotype. *pan1-4* and *end3*Δ are both temperature-sensitive mutants. They are able to grow at 24°C but not at 37°C. To test for synthetic lethality, we first transformed a diploid yeast strain (YHT153) heterozygous for *pan1-4* and *end3*Δ::*LEU2* alleles with pRS426-END3, a multicopy plasmid containing the *END3* gene and the *URA3* marker. The transformants were selected, and sporulation was induced. Asci capable of forming four viable spores on rich medium were then analyzed for the ability to lose the pRS426-END3 plasmid. Spore colonies grown on YPD plates were replica plated onto SC medium containing 5-FOA and incubated at 24°C. Of 10 tetrads analyzed, 7 contained either one or two segregants whose viability depended on the presence of the pRS426-END3 plasmid, as they failed to grow on 5-FOA-containing medium at 24°C. A representative of each type is shown in Fig. 2. By backcrossing these 5-FOA-sensitive segregants to a wild-type strain, we confirmed them to be the *pan1-4 end3*Δ double mutants (data not shown). All of the segregants capable of growing on 5-FOA-containing medium were found to be either wild type or single-mutant cells (data not shown). This experiment shows that the *pan1-4* and *end3*Δ mutations are synthetically lethal.

**Pan1p forms a complex with End3p in vivo.** To investigate whether Pan1p and End3p physically interact with each other, we performed immunoprecipitation experiments in an attempt to detect complex formation between the two proteins. Pan1p was tagged with the HA epitope at its amino terminus. When expressed from the *PAN1* promoter, the tagged protein was able to complement the *pan1* deletion mutation (data not shown). Protein extract from YHT199, a *pan1* deletion strain kept alive by the HA-tagged *PAN1* on a centromere plasmid (pRS316-HA-PAN1), was incubated with the anti-HA monoclonal antibody 12CA5, and the precipitate was collected. Upon analysis by Western blotting, three major bands with apparent molecular masses of about 100, 140, and 200 kDa were observed (Fig. 3A). The largest (200-kDa) protein has been previously shown to be intact Pan1p (44). The other two proteins with higher mobility were also specific to the HA-PAN1 construct, because they were not observed in immunoprecipitates from the isogenic wild-type strain without a tagged *PAN1* gene. They are likely to be the proteolytic fragments of Pan1p. Similar fragments have been detected by others using antibody against Pan1p (4), and a 140-kDa protein was shown by microsequencing to be a part of Pan1p by Sachs and Deardorff (36). When probed with the affinity-purified anti-End3p antibody (2), End3p was found to be present in the anti-HA immunoprecipitate of HA-Pan1p but not in the control anti-HA immunoprecipitate from cell extract containing no HA-tagged Pan1p (Fig. 3A, right panel). Pan1p and End3p, therefore, associate with each other in vivo.

To reinforce these data, we performed a reciprocal experi-

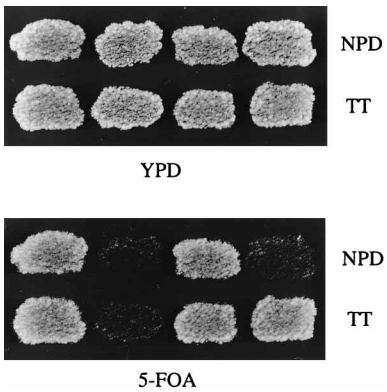


FIG. 2. The *pan1-4* and *end3*Δ::*LEU2* double mutants are synthetically lethal at 24°C. The diploid strain YHT153 (YHT99 × YHT151) was transformed with pRS426-END3. After sporulation, the four segregants resulting from a nonparental ditype (NPD) and a tetratype (TT) were patched onto a YPD plate (top) and then replica plated onto 5-FOA medium (bottom) at 24°C. The cells that could not grow on 5-FOA medium contained both the *pan1-4* and *end3*Δ::*LEU2* mutations.

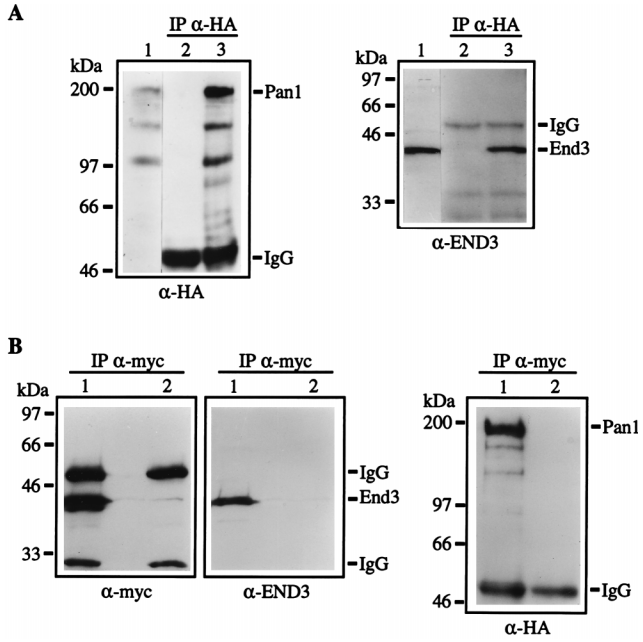


FIG. 3. Pan1p and End3p form a complex in vivo. (A) Total extract of YHT199 (*pan1*Δ pHA-PAN1; lane 1) and proteins immunoprecipitated (IP) with the anti-HA (α-HA) antibody from CRY1 (wild-type; lane 2) and YHT199 (lane 3) were electrophoresed on SDS–7.5% (left) and 10% (right) polyacrylamide gels and blotted to membranes. The blots were then probed with anti-HA (left) and anti-End3p (right). End3p was found to coprecipitate with HA-Pan1p (lane 3). (B) Anti-Myc immunoprecipitates of YHT238 (pGAL-HA-PAN1, pGAL-*myc*-END3; lane 1) and YHT237 (pGAL-HA-PAN1; lane 2) were electrophoresed on SDS–10% (left) and 7.5% (right) polyacrylamide gels and blotted to membranes. One was probed with anti-Myc (left) to detect Myc-End3p. This blot was stripped and reprobed with anti-End3p (middle). The other was probed with anti-HA (right) to detect coprecipitated HA-Pan1p. The lane between lanes 1 and 2 in the left and middle panels is empty.

ment using End3p-specific antibody for immunoprecipitation and then examined the precipitates for the presence of Pan1p. Since the anti-End3p antibody that we obtained was not suitable for immunoprecipitation (32a), we constructed a Myc epitope-tagged *END3* gene under control of the *GAL1* promoter (pGAL-*myc*-END3). In this experiment, we also used the pGAL-HA-PAN1 construct, which is functional in vivo as well (44). The wild-type strain CRY1 containing these two plasmids was grown in galactose-containing medium for preparation of protein extracts. Myc-End3p was precipitated with the anti-Myc monoclonal antibody 9E10, separated by SDS-polyacrylamide gel electrophoresis and transferred to a membrane (Fig. 3B, left panel). The filter was first probed with the monoclonal anti-Myc antibody and then stripped and reprobed with the anti-End3p antibody to confirm that both antibodies recognize the same band on the gel (Fig. 3B, left and middle panels, respectively). Using the anti-HA antibody, the presence of HA-Pan1p was detected in the anti-Myc immunocomplex (Fig. 3B, right panel, lane 1), confirming that Pan1p and End3p form a complex in vivo. As a control for the specificity of the interaction, protein extracts from wild-type cells transformed only with pGAL-HA-PAN1 were subjected to immunoprecipitation using the anti-Myc antibody under the same conditions as described above. As expected, neither End3p nor Pan1p could be detected in this experiment (Fig. 3B).

**The second long repeat of Pan1p binds to the C-terminal repeats of End3p.** Recently, it has been reported that the EH domain may act as a protein-protein interaction motif (50). Since both Pan1p and End3p are members of the EH domain protein family, it is possible that the interaction of the two proteins is through their respective EH domains. To identify the regions in Pan1p and End3p that are involved in their interaction, we used the yeast two-hybrid system (1, 10). Pan1p possesses a number of distinctive structural features (Fig. 4A). At the amino terminus there are two long repeated regions, each containing the sequence LXXQXTG (where X is any amino acid) repeated several times. In addition, each of the long repeats contains an EH domain. The second long repeat also contains a Sla1 homology domain (44), located between the fourth and sixth LXXQXTG repeats. Near the carboxyl terminus, a short motif, QPTQPV, situated about 200 amino acids away from a proline-rich domain at the very terminus, is found to be repeated seven times (36, 44, 48).

The two-hybrid assay enabled us to show a strong interaction between the N-terminal half of Pan1p and End3p. Using various constructs, we localized the region of Pan1p capable of interacting strongly with End3p to the second long repeat (pPAN1.2 [Fig. 4A]). Deletion of three LXXQXTG repeats before the Sla1 domain did not affect the strength of the interaction (pPAN1.4 [Fig. 4A]). However, deletion of a small region containing the Sla1 domain almost completely elimi-

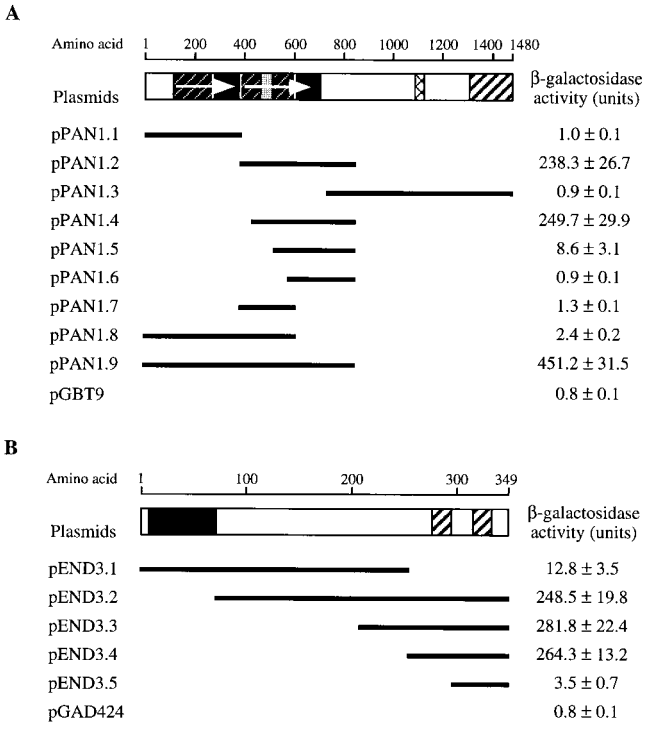


FIG. 4. Domains required for the interaction between Pan1p and End3p. (A) Each deletion mutant of Pan1p (pPAN1.1 to pPAN1.9) was assayed for its ability to interact with the full-length End3p (pEND3.0 [Table 2]). The lines below the diagram of Pan1p represent the regions of Pan1p fused to the *GAL4* activation domain of pGAD424. The white arrows in the diagram indicate the two long repeats. Each long repeat contains the repeating LXXQXTG (white hatched boxes) and an EH domain (solid boxes). The second long repeat also contains a Sla1 homology domain (stippled box). The QPTQPV repeats (cross-hatched box) and the proline-rich domain (black hatched box) at the C terminus are also indicated. (B) Various deletion mutants of End3p (pEND3.1 to pEND3.5) were assayed for interaction with pPAN1.9. The regions of End3p fused to the *GAL4* DNA binding domain of pGBT9 are represented by lines below the End3p diagram. The solid box and the black hatched boxes in the diagram represent the EH domain and the two C-terminal repeats of End3p, respectively. The β-galactosidase values are reported as the averages ± standard deviations for at least three transformants.

nated the interaction, as the β-galactosidase activity dropped from around 240 U to about 9 U (pPAN1.5 [Fig. 4A]). Deletion of the remaining LXXQXTG repeats from this construct while leaving the EH domain intact further reduced the resdual interaction to the control level (pPAN1.6 [Fig. 4A]), demonstrating that the EH domain alone was not sufficient for protein-protein interaction. Nevertheless, this EH domain was absolutely required for the interaction because two other constructs containing the LXXQXTG repeats and the Sla1 domain but without the EH domain (pPAN1.7 and pPAN1.8) failed to show any interaction with End3p (Fig. 4A). Thus, both the Sla1 and EH domains in the second long repeat of Pan1p are required for the interaction with End3p. The first long repeat (pPAN1.1), which also contains an EH domain but lacks the Sla1 domain, did not interact with End3p. However, a construct containing both long repeats (pPAN1.9) interacted with End3p more strongly than the second long repeat alone, elevating the β-galactosidase activity from around 240 U to about 450 U (Fig. 4A). This result suggests that even though the first long repeat displays no interacting activity on its own, it may aid the interaction of the second long repeat with End3p. Inclusion of more sequence from the C-terminal half of the protein did not significantly enhance the interaction with End3p (data not shown).

The End3p protein contains only one EH domain, which is located near the N terminus. In addition, End3p has a region at the C terminus consisting of two short repeats (2). Since the pPAN1.9 construct displayed the highest degree of interaction with End3p, it was used in combination with various *END3* deletion constructs to define the region in End3p that is involved in the interaction (Fig. 4B). A construct comprising the N-terminal two-thirds of the *END3* coding region including the EH domain (pEND3.1) displayed only a weak interaction with Pan1p, and the ability to interact with Pan1p was mostly localized to the C-terminal region of End3p. In fact, a short construct containing the C-terminal repeats (pEND3.4) was sufficient to show a strong interaction (Fig. 4B). Both of the C-terminal repeats might be required for the interaction, since removing one of them abolished the interacting activity of End3p (pEND3.5 [Fig. 4B]). We conclude that the complex formation between Pan1p and End3p is not through EH-EH interaction but is accomplished through the C-terminal repeats of End3p binding to the Sla1 domain- and EH domain-containing second long repeat of Pan1p.

**Association of Pan1p with the cortical actin cytoskeleton is severely affected by the *end3* mutation.** Using an HA-tagged *PAN1* construct, we have shown that Pan1p is localized to cortical actin patches (44). Although fractionation experiments have suggested that End3p may also associate with actin cytoskeleton (2), the attempts to determine the cellular localization of End3p by immunofluorescence have so far produced no conclusive results (data not shown and reference 2). We therefore chose to investigate whether End3p is required for cellular localization of Pan1p by performing double immunofluorescence staining of Pan1p and actin in the *end3* deletion mutant. The YHT151 (*end3*Δ::*LEU2*) strain was transformed with pGAL-HA-PAN1 and pRS314-END3, alone or together, and the transformants were grown in the presence of galactose at 24°C and shifted to 37°C for 2 h before being processed for immunofluorescence staining (Fig. 5). In the cells that contained both plasmids, it is clear that Pan1p was stained as punctate structures that colocalized with cortical actin patches on the plasma membrane at both 24 and 37°C (Fig. 5B and reference 44). However, in the YHT151 cells that contained only pGAL-HA-PAN1 (*end3*Δ), the Pan1p-dependent punctate structures were seen much less frequently and most of the cells displayed a diffuse staining in the cytoplasm at 24°C (Fig. 5C). About 30% of the *end3*Δ cells showed punctate staining that colocalized with cortical actin patches at 24°C, compared to about 60% in cells carrying the *END3* gene under the same condition. When these *end3*Δ cells were shifted to 37°C for 2 h, the Pan1p-dependent punctate staining that colocalized with actin was completely undetectable, and only cytoplasmic staining was observed (Fig. 5D). These data indicate that End3p may be required for the stable association of Pan1p with the cortical actin patches.

**The *pan1-4* mutant is defective in receptor-mediated and fluid-phase endocytosis.** It has been well established that endocytosis (both receptor-mediated and fluid phase) in yeast requires the function of *END3* (2, 31). If the genetic and molecular interactions of Pan1p and End3p were of physiological significance, it would be expected that Pan1p might also be required for the process of endocytosis. Receptor-mediated endocytosis in yeast can be assayed by measuring the rate of α-factor uptake (6). We examined the uptake of α-factor in CRY1 (wild-type), YHT99 (*pan1-4*), YHT167 (*end3*Δ), and YHT215 (*pan1-4*::*PAN1*) strains. To minimize the degradation of α-factor by the Bar1p protease (24), the *bar1* mutation was first introduced into each of the test strains (see Materials and Methods). The internalization of $^{35}$S-labeled α-factor was assayed at both 24 and 37°C. In agreement with the previous report (2), the *end3*Δ mutant displayed a defect in α-factor uptake at both temperatures (Fig. 6). The *pan1-4* mutant showed a similar defect in α-factor uptake (Fig. 6A). At 24°C, the level of α-factor uptake in *pan1-4* was approximately 50% of that exhibited by the wild type, whereas at 37°C, the defect virtually equaled that of the *end3* null mutant (Fig. 6B). Integration of a wild-type copy of the *PAN1* gene at the *URA3* locus in the *pan1-4* mutant restored α-factor internalization at both temperatures (Fig. 6), confirming that the endocytosis defect in the mutant is due to the *pan1-4* mutation.

We also investigated the effect of the *pan1-4* mutation on fluid-phase endocytosis, by testing the ability of the *pan1-4* mutant to accumulate LY in the vacuole. LY is a small fluorescent organic anion that is often used as a marker for fluid-phase endocytosis (8). Its internalization into the vacuole requires energy as well as the functions of some End proteins, including End3p (26, 27, 31). Wild-type, *pan1-4*, and *end3*Δ cells were incubated for 1 h with LY at 24°C, washed, mounted, and viewed under phase-contrast and fluorescence optics. Under phase-contrast optics, both *pan1-4* and *end3*Δ mutants displayed a normal vacuolar morphology (Fig. 7). However, when examined under fluorescence optics, both mutants showed a strong defect in accumulation of LY compared to wild-type cells (Fig. 7). Typically, greater than 90% of wild-type cells exhibited an unambiguous vacuolar staining, whereas only 6% of *end3*Δ cells and 14% of the *pan1-4* cells had clearly stained vacuoles. Therefore, it is evident that even at the permissive temperature, both mutants were severely defective in fluid-phase endocytosis. Strong impairment of fluid-phase endocytosis was similarly observed in *pan1-4* and *end3*Δ mutants at 37°C. In addition, a population of cells were also brightly and uniformly stained by LY, resulting from cell lysis (data not shown). These results demonstrate that *PAN1*, like *END3*, has an essential function in both receptor-mediated and fluid-phase endocytosis in yeast.

While this report was being prepared, two reports describing the endocytic defects of the *pan1* mutants were published (49, 51). Using the LY assay, Zoladek et al. showed that the *mdp* mutants including an allele of *pan1* (*mdp3*), isolated as mutants with abnormal distribution of the mitochondrial protein Mod5p-I, were defective in fluid-phase endocytosis (51). Wendland et al. reported the isolation and characterization of another allele of *pan1*, *pan1-20*, which exhibited a moderate

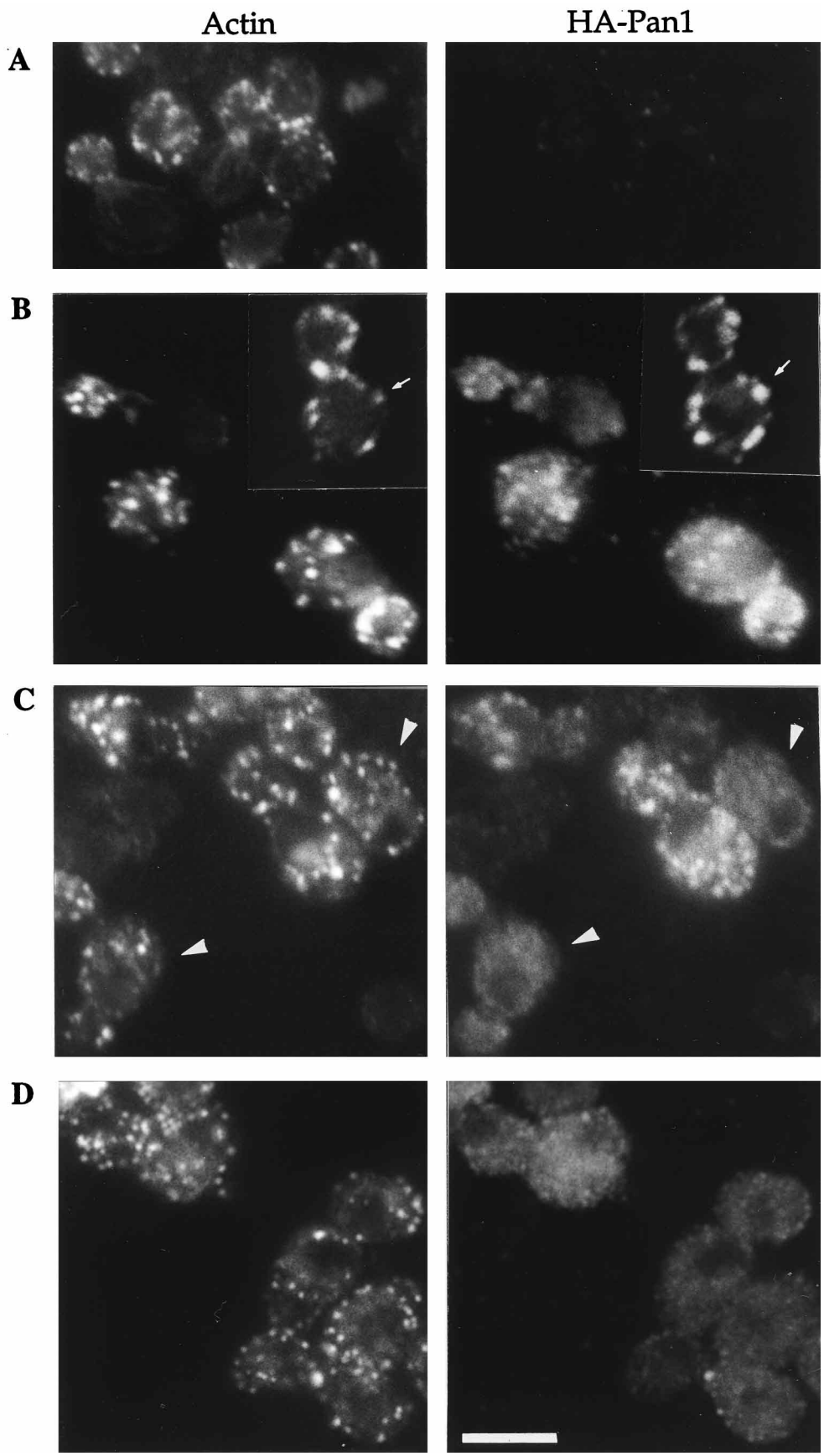
Actin
HA-Pan1
A
B
C
D

FIG. 5. Localization of Pan1p in the *end3* deletion mutant. Yeast cells grown at 24°C in galactose-containing medium were either maintained at 24°C or shifted to 37°C for 2 h before fixation. Cells were fixed with formaldehyde and double labeled with the antiactin (left) and anti-HA 12CA5 (right) antibodies to visualize filamentous actin and HA-Pan1p, respectively. (A) YHT151 (*end3*Δ::*LEU2*) cells containing pRS314-END3 grown at 24°C. (B) YHT151 cells containing pRS314-END3 and pGAL-HA-PAN1 were shifted to 37°C for 2 h. In each insert, the arrow indicates a cell focused to display the localization of actin and Pan1p on the plasma membrane. (C) YHT151 cells containing only pGAL-HA-PAN1 grown at 24°C. The arrowheads indicate examples of cells showing diffuse cytoplasmic staining of HA-Pan1p. The area with no staining contained the nucleus. (D) YHT151 cells containing pGAL-HA-PAN1 shifted to 37°C for 2 h. In these *end3*Δ cells, anti-HA does not display clear punctate staining that colocalizes with actin. Bar, 5 μm.

defect in α-factor internalization at the nonpermissive temperature (49). Our data showing that the *pan1-4* mutant became severely defective in α-factor internalization immediately after the temperature shift to 37°C, as shown in Fig. 6, further strengthened the conclusion of these authors that Pan1p is required for endocytosis in yeast.

## DISCUSSION

In this report, we have described the identification of *END3* as a multicopy suppressor of the *pan1-4* mutant. *END3* is well known for its involvement in the process of receptor-mediated endocytosis. The essential role of *PAN1* in endocytosis is now also confirmed. It is of interest that Pan1p and End3p are both found to be important factors in endocytosis, because the two belong to a new protein family termed EH domain proteins. Proteins in this family have sequence similarity with the mammalian protein Eps15, a substrate of EGFR kinase. Eps15 has emerged recently as a strong candidate for being involved in recruitment of the EGFR and other growth factor receptors into clathrin-coated pits, largely because of its newly discovered ability to associate with the AP-2 adaptor complex on the plasma membrane (3) and with clathrin-coated pits and vesicles (45). These findings raise a very interesting possibility that the EH domain family may designate a new class of factors that are involved in endocytosis and related processes. Pan1p, therefore, may provide valuable solutions to some unanswered questions on receptor-mediated endocytosis, especially with regard to early steps of the process.

**Pan1p and End3p are components of the same functional complex.** The main conclusion of this report is that the two EH domain proteins Pan1p and End3p are components of the same functional complex. This conclusion is strongly supported by the data that we have presented. Association of Pan1p with End3p was first suggested by the interaction between their respective genes. The suppression of the *pan1-4* mutation by multicopy *END3* was not due to functional substitution of *PAN1* by *END3*, as it required the presence of the mutant Pan1p. Furthermore, the *pan1-4* and *end3*Δ mutations were synthetically lethal. A possible explanation for this synthetic lethality is that in the absence of its partner (End3p), the mutant Pan1p (*pan1-4*) becomes unstable or inactive. The *end3* mutant has been tested previously for synthetic lethality with several other mutations affecting proteins involved in actin cytoskeleton organization, such as Sac6 (fimbrin), Abp1 (actin binding protein 1), and Cap1 and Cap2 (actin capping proteins) (2). No synthetic lethality could be detected in these crosses (2), suggesting that the synthetic lethality with the *pan1-4* mutant is specific.

With the use of epitope-tagged constructs, direct evidence for the association between Pan1p and End3p was obtained. Immunoprecipitation experiments demonstrated that End3p could be indirectly precipitated by the antibody specific for tagged Pan1p, and vice versa. Therefore, it is clear that Pan1p and End3p form a complex in vivo. Further investigation using the two-hybrid protein-protein interaction assay confirmed that Pan1p and End3p bind to each other. The two-hybrid assay also provided important information on which regions from the two proteins participate in the interaction. The N-terminal half of Pan1p is composed of two long repeats, each of which contains an EH domain (44). The second long repeat also contains a short region which we call the Sla1 domain, for its high homology with a part of Sla1p (44), yet another protein required for normal organization of the cortical actin cytoskeleton (14). The second long repeat has been shown to be the essential region of *PAN1* (36) and is required for complementation of the *pan1-4* mutation (44). We found that this region is principally responsible for binding to End3p and that both

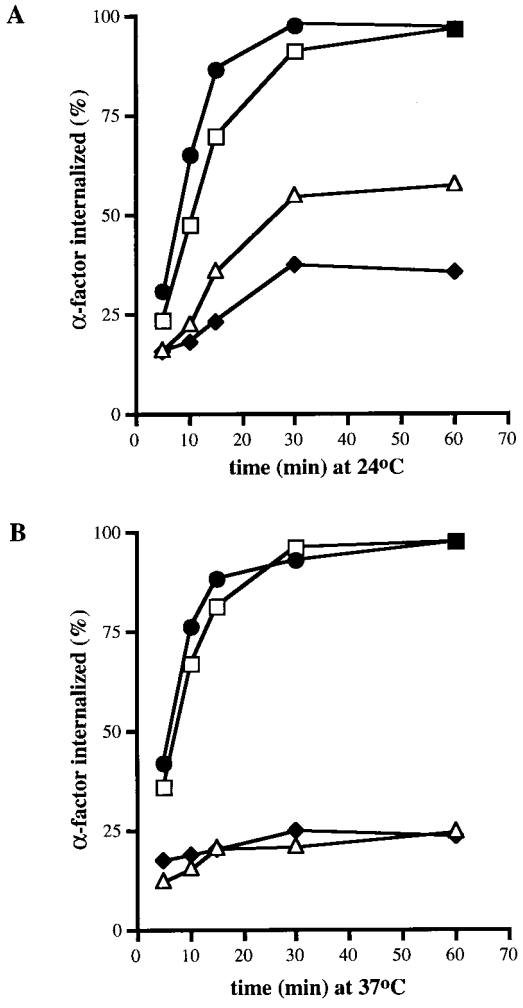


FIG. 6. The *pan1-4* and *end3*Δ mutants are defective in internalization of α-factor. α-Factor uptake assays were performed at 24°C (A) and at 37°C (B) on the *bar1* derivatives of CRY1 (wild-type; ●), YHT99 (*pan1-4*; Δ), YHT167 (*end3*Δ; ◆), and YHT215 (*pan1-4*::*PAN1*; □). In each assay, the cells were preshifted to 24 or 37°C for 15 min before α-factor addition. At the indicated times, samples were taken and processed as described in Materials and Methods. The results shown are the averages of two independent assays.

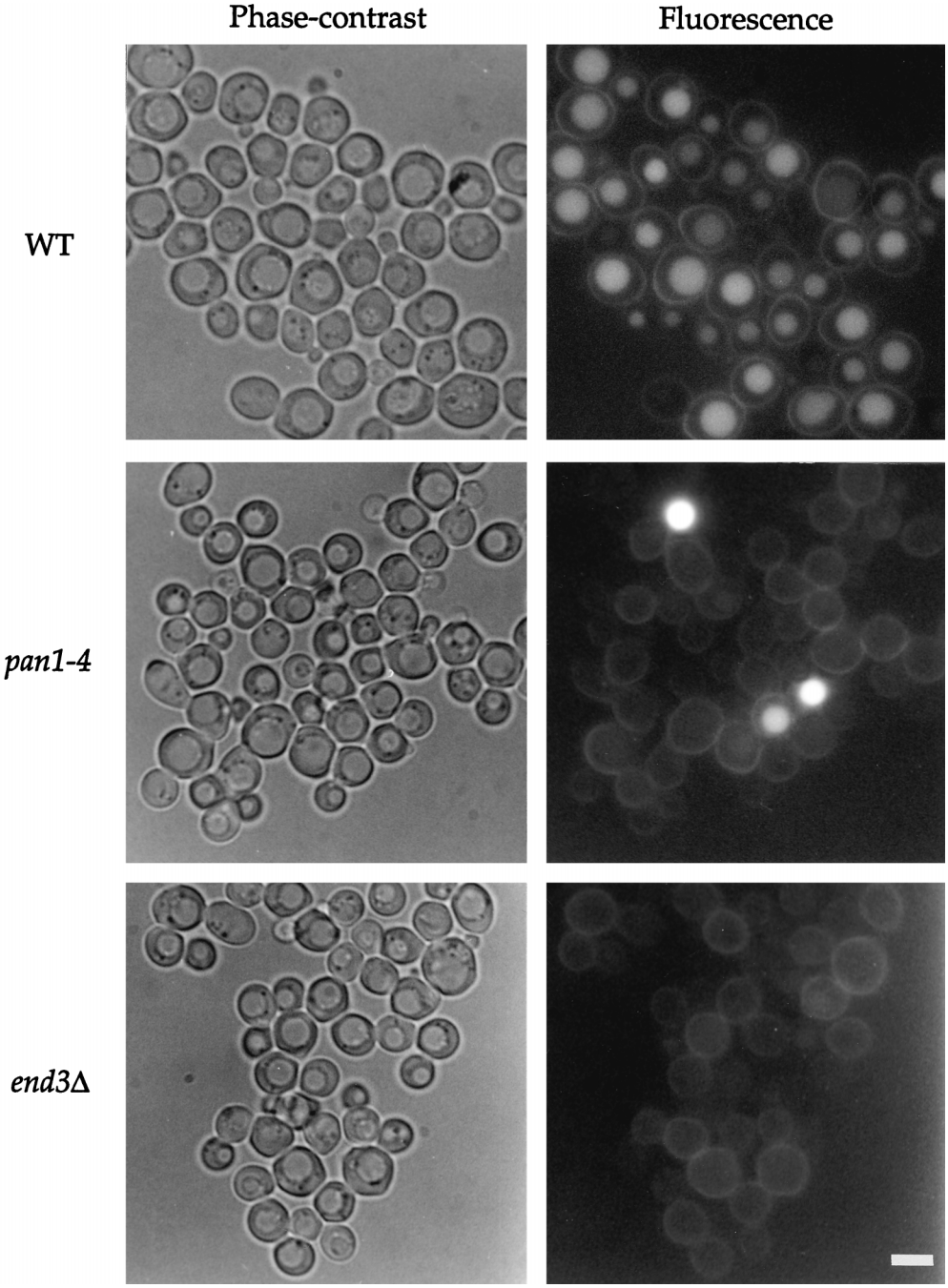


FIG. 7. The *pan1-4* and *end3*Δ mutants are defective in accumulation of LY in the vacuole. The LY assays were performed on the wild-type CRY1 (WT), YHT99 (*pan1-4*), and YHT167 (*end3*Δ) as described in Materials and Methods. Cells grown at 24°C were stained with LY for 1 h at 24°C and then visualized by using phase-contrast (left) and fluorescence (right) microscopy. Vacuoles (large, circular indentation) in all strains are clearly visible under phase-contrast microscopy. However, only the wild-type cells have fluorescent staining in vacuoles under fluorescence optics. Bar, 5 μm.

the EH and the Sla1 domains are required for the binding activity. Neither the EH domain nor the Sla1 domain alone was sufficient to interact with End3p. The first long repeat of Pan1p, which also contains a well-conserved EH domain but lacks the Sla1 domain, was not able to interact with End3p. It was, however, able to enhance the interaction of the second long repeat with End3p, suggesting that it may also participate in complex formation in vivo. The region of End3p responsible for interaction with Pan1p was localized to the C terminus, where two short repeats are found. This region is also essential for End3p function and shares some sequence homology with α-actinins, the actin binding proteins that cross-link actin filaments (2). The observation that this C-terminal region alone was sufficient for binding to Pan1p in the two-hybrid assay does not necessarily suggest that it is the only region of sequence important for the function of End3p, as the N-terminal region comprising the EH domain is also required for α-factor internalization (2). It is therefore possible that the EH domain in

the N terminus of End3p also plays a part in the interaction with Pan1p in vivo or, alternatively, that there are other proteins required for endocytosis that interact with End3p through its EH domain.

The finding that Pan1p and End3p associate with each other through regions that have been identified previously as important for their functions suggests that the interaction between the two proteins contributes significantly to their functions. This suggestion is consistent with the similarity in phenotypes found for the two mutants. Additional support comes from the result that the association of Pan1p with the cortical actin cytoskeleton was greatly diminished in the *end3*Δ mutant. At the permissive temperature, small portions of actin patches in the *end3*Δ mutant still coincided with the Pan1p staining. At 37°C, however, no discernible colocalization of Pan1p and actin was found in the mutant. The failure of Pan1p to associate with the cortical actin patches in the *end3* mutant could be due to the possibility that the complex formation between Pan1p and End3p is required for the localization of Pan1p to actin patches or, alternatively, that the abnormal cortical actin structure in the *end3* mutant is not suitable for Pan1p association. However, we consider the latter possibility less likely because the cortical actin structures in the *end3* null mutant did not undergo dramatic dissolution upon the temperature shift from 24°C, where a significant amount of cells (30%) still exhibited colocalization of Pan1p and the actin patches, to 37°C, where the colocalization was completely undetectable (Fig. 5). It is plausible, therefore, that at 24°C, Pan1p retains a weak ability to associate with the actin cytoskeleton when the End3 protein is absent, and this ability is completely lost at 37°C. This may explain why the *pan1* null mutant is lethal whereas the *end3* null mutant is temperature sensitive (2, 44).

**The Pan1p-End3p complex may link the cytoskeleton to endocytosis.** The actin cytoskeleton has been demonstrated to play an essential role in endocytosis in yeast as well as in certain mammalian cells such as polarized epithelial cells (11, 32). Mutations in *ACT1* and *SAC6*, encoding actin and fimbrin of yeast, respectively, cause defects in the internalization step of endocytosis (18). Similarly, disassembly of actin filaments leads to inhibition of clathrin-dependent endocytosis in MDCK cells (11). The requirement for the actin cytoskeleton in endocytosis in yeast and the apical surface of epithelial cells may be related to contact with the external environment (32). The actin cytoskeleton beneath the cell membrane is likely necessary for endocytosis under turgor pressure (see below), which however, may not be required for endocytosis in those animal cells that are not exposed to the external environment (32). For proteins like Pan1p and End3p, whose functions are implicated in both the actin cytoskeleton and endocytosis, it is perhaps inevitable to ask whether they are required for endocytosis simply because they are required for function of the actin cytoskeleton. Based on the following arguments, we suggest that the Pan1p-End3p complex is involved in modulation of the membrane dynamics and plays a dual and direct role in both endocytosis and actin cytoskeleton organization. First, actin cytoskeleton disorders do not necessarily lead to an endocytic defect. For example, the *myo2-66* (myosin type V) mutant performs endocytosis as well as the wild type despite having extensively delocalized cortical actin (15, 19). Similar observations have also been made with the actin binding proteins profilin and tropomyosin (12, 23, 27). Second, proteins found on the cortical actin cytoskeleton do not necessarily play a role in the process of endocytosis. For example, cells that lack Abp1p, an actin-binding protein exclusively found on the cortical actin patches, can internalize α-factor normally (7, 18). Third, the endocytic defects in both *end3* and *pan1* temperature-sensitive mutants manifest immediately after temperature shift to 37°C (reference 2 and Fig. 6), favoring the possibility that both proteins are directly required for endocytosis. Fourth, the immunostaining data for Pan1p suggest that the plasma membrane structure to which Pan1p is localized may not simply be the cortical actin patches. While most, if not all, cortical actin staining coincided with Pan1p staining, the converse was not true. In many cells, Pan1p staining revealed more punctate membrane structures than actin patches (44). This finding suggests that association of Pan1p with punctate cell membrane structures may not depend on the cortical actin cytoskeleton. What then is the nature of the Pan1p-associated membrane structure? One clue comes from the ultrastructure of the cortical actin cytoskeleton visualized by immunoelectron microscopy (25). The cortical actin patches were shown to be associated with the cell surface via invaginations of plasma membrane around which actin filaments and actin binding proteins are organized (25). Importantly, it was revealed that a large number of prominent invaginations of plasma membrane were not visibly labeled by the antiactin antibody, suggesting that not all surface invaginations correspond to cortical actin patches (25). Our immunolocalization data on Pan1p and actin can be best explained by proposing that the Pan1p-associated membrane structures correspond to sites of invaginated plasma membrane, some of which are not cortical actin patches. If this is the case, Pan1p may be required for function of the invaginated membrane pits. This suggestion is supported by Wendland et al. (49), who found that the membrane invaginations were abnormal in the *pan1* mutant. Finally, that the Pan1p-End3p complex is directly involved in endocytosis is also favored by the observation that the mammalian protein Eps15, which shares a homologous domain with both Pan1p and End3p, associates with AP-2 proteins on the plasma membrane (3) and is indeed localized to invaginated membrane pits (45).

Membrane invaginations are vital to yeast cells. Yeast cells are under enormous internal pressure generated by an osmotic gradient across the plasma membrane (13). Invaginated cell membrane pits are likely the areas where the turgor pressure can be negated (13). Invaginated structures may also be the only areas on the cell membrane capable of budding inward under the turgor pressure. Therefore, the invaginated membrane pits are the natural choice for sites of endocytosis as well as exocytosis. Since the cortical actin cytoskeleton uses the invaginated membrane for its organization, protein factors that function in invaginated membrane structures will be expected to be important for normal organization of cortical actin cytoskeleton as well. Pan1p and End3p, being directly involved in organization of cortical actin cytoskeleton and endocytosis, may be such proteins. In addition to the arguments presented above, the observations that *pan1* and *end3* mutants easily undergo cell lysis at the restrictive temperature (data not shown) and that the mutations can be partially suppressed by high salt- or sorbitol-containing medium (data not shown) further support our suggestion. Therefore, the Pan1p-End3p complex, by functioning in invaginated membrane structures, may play important roles in actin cytoskeleton and membrane dynamics on the cell cortex.

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