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The *END3* Gene Encodes a Protein that Is Required for the Internalization Step of Endocytosis and for Actin Cytoskeleton Organization in Yeast

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Two *Saccharomyces cerevisiae* mutants, *end3* and *end4*, defective in the internalization step of endocytosis, have previously been isolated. The *END3* gene was cloned by complementation of the temperature-sensitive growth defect caused by the *end3* mutation and the *END3* nucleotide sequence was determined. The *END3* gene product is a 40-kDa protein that has a putative EF-hand Ca^{2+}-binding site, a consensus sequence for the binding of phosphotidylinositol 4,5-bisphosphate (PIP_2), and a C-terminal domain containing two homologous regions of 17–19 aa. The EF-hand consensus and the putative PIP_2-binding sites are seemingly not required for End3 protein function. In contrast, different portions of the End3p N-terminal domain, and at least one of the two repeated regions in its C-terminus, are required for End3p activity. Disruption of the *END3* gene yielded cells with the same phenotype as the original *end3* mutant. An $end3^{ts}$ allele was obtained and this allowed us to demonstrate that End3p is specifically involved in the internalization step of endocytosis. In addition, End3p was shown to be required for proper organization of the actin cytoskeleton and for the correct distribution of chitin at the cell surface.

INTRODUCTION

During endocytosis, cells internalize portions of their plasma membrane and substances from the external medium. The internalized material can be nutrients such as iron (Hemmaplardh and Morgan, 1976; Karin and Mintz, 1981), vitamin B12 (Newmark *et al.*, 1970; Sennett *et al.*, 1981), cholesterol (Anderson *et al.*, 1977), toxins, and even large particles such as viruses (Helenius *et al.*, 1980; Sandvig and Olsnes, 1981). Growth factors and hormones are also internalized after binding to their receptors. This internalization leads to a down-regulation of the induced response by clearance of the responsive elements from the membrane. Endocytosis also plays a role in the transcellular transport of immunoglobulins (Kühn and Kraehenbuhl, 1982) and in transcytosis between the basolateral and the apical surfaces of polarized cells (Bartles *et al.*, 1987; Matter *et al.*, 1990). Two different types of endocytosis can be measured: one is fluid-phase mediated and nonspecific and the other is receptor mediated and involves the binding of ligands to specific receptors. In the case of receptor-mediated endocytosis, receptor-ligand complexes are clustered in coated pits that subsequently bud from the plasma membrane and become coated vesicles. The internalized molecules next appear in early endosomes, then late endosomes, and eventually in lysosomes where they are degraded (Helenius *et al.*, 1983; Gruenberg and Howell, 1989).

Most of the studies on endocytosis to date have been performed on mammalian cells using morphological or biochemical approaches. The morphological approaches have allowed the identification of a variety of distinct organelles involved in the pathway (Goldstein *et al.*, 1979; Helenius *et al.*, 1983). The identification of some of the components involved in the process, such as clathrin, adaptins, and, recently, rab proteins (Pearse and Robinson, 1990; Bucci *et al.*, 1992), and the study of their roles in vitro have involved a biochemical approach. The genetics of the yeast *Saccharomyces cerevisiae* provides a powerful approach to identify novel components of the endocytic machinery and to directly analyze the function of the proteins in vivo.

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Table 1. Strains of *S. cerevisiae*

Strains	Genotype	Source
RH144-3D	*MATa his4 ura3 leu2 bar1-1*	Laboratory collection
RH444	*MATa his4 ura3 leu2 bar1-1* / *MATα his4 ura3 leu2 bar1-1*	Laboratory collection
RH266-1D	*MATa end3-1 his4 ura3 leu2 bar1-1*	Laboratory collection
RH1995	*MATa his4 ura3 leu2 bar1-1 end3Δ1::URA3*	This study
RH2386	*MATa his4 ura3 leu2 bar1-1 end3Δ1::URA3* / *MATα his4 ura3 leu2 bar1-1 end3Δ1::URA3*	This study

Two externally added markers have been used to follow endocytosis in *S. cerevisiae:* lucifer yellow CH (LY) for fluid-phase endocytosis and α-factor for receptor-mediated endocytosis. LY is a small fluorescent organic anion and α-factor is a peptide mating factor that binds to a specific receptor on the surface of haploid *MATa* cells. The pheromone receptors are polytopic membrane proteins that, upon binding of the pheromone, transmit a signal via tripartite G-proteins. This signal triggers a program of physiological changes necessary for conjugation (Cross *et al.*, 1988; Marsh *et al.*, 1991). Chvatchko *et al.* (1986) showed that upon binding to its receptor, α-factor is internalized in a time-, temperature-, and energy-dependent way. Pheromone internalization is accompanied by a down-regulation of its receptor from the plasma membrane (Jenness and Spatrick, 1986), which suggests, but does not directly demonstrate, that α-factor and its receptor are taken up together as a complex. The pheromone is then delivered, via two intermediate compartments, to the vacuole (Singer and Riezman, 1990; Singer-Krüger *et al.*, 1993) where it is degraded, thereby enhancing the cellular recovery from the effects of α-factor (Reneke *et al.*, 1988; Rohrer *et al.*, 1993). Therefore, two distinct steps can be distinguished in the α-factor endocytic pathway: its internalization upon binding to its receptor and its transport to the vacuole.

Ypt7p, a yeast homologue of the mammalian GTP-binding protein Rab7, has been found to play a role in the transport of α-factor from the late endosome to the vacuole (Wichmann *et al.*, 1992; Schimmöller and Riezman, 1993). Recently, actin and Sac6p (the yeast homologue of mammalian fimbrin) have been shown to play an essential role in the internalization step of endocytosis, suggesting that a functional actin cytoskeleton is required for this step. Analysis of endocytosis of pheromone receptors in a clathrin heavy chain (*chc1*) conditional mutant suggests that clathrin plays a role at this step, implying endocytosis through coated pits (Tan *et al.*, 1993). Furthermore, two other mutants, *end3* and *end4*, strongly affected in the internalization step, have been isolated (Raths *et al.*, 1993). Neither mutant grows at 37°C. The original *end4* allele is thermosensitive (ts) for endocytosis, which allowed us to demonstrate that End4p is required for the internalization step of endocytosis of α-factor but not in its subsequent transport to the vacuole. This demonstration was not possible with the *end3* mutant because it was defective in pheromone uptake at all temperatures, implying, surprisingly, that yeast cells do not need the endocytic pathway to survive at 24°C.

In the present work, we have cloned and sequenced the *END3* gene and characterized some primary structure-function relationships of the End3 protein. The gene was disrupted, and a thermosensitive allele was obtained, allowing the demonstration that End3p is specifically required at the internalization step of endocytosis. Furthermore, we present evidence that End3p plays an important role in actin cytoskeleton organization, in bipolar budding of diploid cells, and in proper chitin distribution at the cell surface.

MATERIALS AND METHODS

Strains and Media

The strains of *S. cerevisiae* used in these experiments are described in Table 1. *Escherichia coli* media and yeast media have been described elsewhere (Maniatis *et al.*, 1989; Dulic *et al.*, 1991). YPUAD medium contains 1% yeast extract, 2% peptone, 2% glucose, and 30 mg/l each of uracil and adenine sulphate.

Material, Genetic, and Nucleic Acids Techniques

^{35}S-labeled α-factor was prepared from biosynthetically labeled yeast cells overproducing the pheromone (Dulic *et al.*, 1991). Vent polymerase (New England Biolabs, Beverly, MA) was purchased from Flow Laboratories (Basel, Switzerland). Endonucleases, T4-DNA ligase, and Klenow enzyme were from Biofinex (Praromen, Switzerland) or Boehringer-Mannheim (Rotkreuz, Switzerland). The yeast genomic library contained partially *Sau*3A-digested genomic DNA from the yeast strain MH145-4B (Δ(*mat*) *130-141::CAN1-14 mfa2-lacZ, rme, ura3, leu2, his4, met, ade6, HLMa*) inserted into *Bam*HI-digested pSEY8 (Emr *et al.*, 1986) and was obtained from Dr. M. Hall (Biocenter, Basel University, Switzerland).

Yeast mating, sporulation, and tetrad analysis were done by standard methods as described by Sherman *et al.* (1986). DNA manipulations in bacteria were done as described in Maniatis *et al.* (1989). Yeast chromosomal DNA was prepared as described previously (Riezman *et al.*, 1983). Southern blot hybridization was carried out as described by Southern (1975) with DNA fragments labeled by the random primer labeling technique developed by Feinberg and Vogelstein (1983). The random primer labeling kit was from Boehringer-Mannheim. Yeast transformation was done by the method of Ito *et al.* (1983).

DNA Cloning, Sequencing, and Analysis

To clone the *END3* gene, the RH266-1D strain was transformed with the yeast genomic library described above. Selection was applied for Ura prototrophy and then for colony formation at 37°C. Overlapping clones that rescued the *end3* phenotype were isolated; all of them had in common a 1.9-kilobase (kb) *Xba* I/*Cla* I fragment. The 1.9-kb *Xba* I/*Cla* I complementing fragment was digested into smaller fragments that were cloned into the polylinker cloning sites of the pBSK$^-$ vector. DNA sequencing of these fragments was performed according to the method of Sanger *et al.* (1977) using the Sequenase 2.0 kit from United States Biochemical (LucernaChem, Luzern, Switzerland). The DNA sequence was determined from both strands. DNA primers were either the universal M13 sequencing primers or synthetic primers complementary to parts of the *END3* DNA sequence. The *END3* DNA and protein sequences were analysed using the University of Wisconsin Genetics Computer Group programs (1991).

Disruption of the END3 Gene

The *Xba* I/*Cla* I complementing fragment was inserted in pBSK$^-$ opened with *Xba* I and *Cla* I yielding the pSR1 plasmid. To perform the disruption, a *Bam*HI site was created 13 base pair (bp) upstream of the *END3* ATG codon. For this purpose, nucleotide T369 was mutated into C, giving the pSR10 plasmid. The pHB1 plasmid was then constructed to perform the disruption. It was derived from the pSR10 plasmid and the pSR16 plasmid (obtained by insertion of the *Hind*III fragment of pUC1318-URA3 containing the *URA3* gene into pBSK$^-$ opened with *Hind*III). pUC1318-URA3 was constructed by M. Egerton by insertion of the 1.1-kb *Hind*III fragment from pFL1 containing the URA3 gene into pUC1318. pHB1 was obtained by inserting the *Bam*HI/*Sal* I fragment containing the *URA3* gene from pSR16 into pSR10 opened with *Bam*HI/*Xho* I. RH444 was then transformed with a *Ssp* I fragment from pHB1 carrying the *end3Δ1::URA3* allele and disruption was allowed to occur by homologous recombination. The disruption was verified by southern blotting of *Xba* I/*Cla* I digested genomic DNA using the radiolabeled *Xba* I/*Cla* I complementing fragment as probe. The enzymes were chosen for the digestion based on the fact that *Xba* I and *Cla* I are flanking *END3* and that *Cla* I is present in the *URA3* fragment.

Construction of END3 Mutants

Plasmid pHB3 was constructed by inserting the *Xba* I/*Kpn* I fragment containing *END3* from pSR10 into Ycplac111 (Giez and Sugino, 1988) opened with *Xba* I and *Kpn* I. Mutations in *END3* were generated using standard polymerase chain reaction (PCR) protocols (Ho *et al.*, 1989). To avoid undesired changes in the sequence, Vent Polymerase was used (New England Biolabs, Beverly, MA). A unique *Mlu* I site was introduced at codon 126 in pHB3 (yielding pHB3M plasmid) to facilitate the recloning of the PCR-amplified mutagenized fragments into pHB3. This mutation replaced a methionine with an alanine and it did not affect the End3p function. The entire PCR fragments that were subcloned into *END3* were sequenced.

α-Factor Internalization and Degradation Assays

Internalization and degradation of α-factor was assayed as described by Dulic *et al.* (1991) using biosynthetically labeled ^{35}S α-factor. The cells were grown overnight in YPUAD to 10^7 cells/ml, collected by centrifugation, washed in YPUAD, and resuspended to 10^9 cells/ml in ice-cold YPUAD. ^{35}S α-factor was then added and allowed to bind for 1 h on ice.

For the uptake experiments, cells were centrifuged and resuspended in prewarmed 24 or 37°C (for the *END3* thermosensitive allele) YPUAD medium. After various incubation times, 100-μl aliquots were withdrawn in duplicates and diluted into either pH 6 or pH 1.1 buffer at 0°C. Cells were collected on GF/C filters (Whatman, Basel, Switzerland) that were dried and counted in a liquid scintillation counter (1900TR, Packard, Groningen, Netherlands) upon addition of 5 ml of Emusifier Safe (Packard).

For the degradation experiments, cells were centrifuged and resuspended to the same density (10^9 cells/ml) in YPUAD prewarmed at 15°C. After 20-min incubation, the cells were centrifuged at 4°C and resuspended in YPUAD medium prewarmed at 37°C. At the indicated times, 100-μl aliquots were taken and diluted in pH 6 or pH 1.1 buffer. Intact and degraded α-factor were extracted and resolved using preparative 2.2-mm-thick silica gel 60 plates (Merck, Darmstadt, Germany) and the n-butanol:propionic acid:water, 50:25:35 (vol/vol/vol) solvent system (Dulic *et al.*, 1991). The plates were then fluorographed using XAR-5 films (Kodak, Rochester, NY) after coating with EN^{3}HANCE spray (Dupont de Nemours International, S.A., Regensdorf, Switzerland).

For the uptake experiments made after a preincubation at 37°C (see Figure 5, A and B), the cells were resuspended in YPUAD prewarmed at 37°C and incubated 10 min at this temperature before the addition of ^{35}S α-factor. The incubation was continued at 37°C, and at various times 100-μl aliquots were withdrawn in duplicate and treated as described above for the other uptake experiments.

Rhodamine-Phalloidin Staining of Actin

Labeling of actin with rhodamine-phalloidin was performed essentially as described previously (Adams and Pringle, 1984; Kilmartin and Adams, 1984). Briefly, cells were grown to early log phase and fixed by the direct addition of formaldehyde (3.7% final) to the medium buffered at pH 6.5. After 2 h fixation, the fixative was removed by washing two times with water and two times with PBS (53 mM Na_2HPO_4, 13 mM NaH_2PO_4, 75 mM NaCl). The cell walls were not removed and the cells were then permeabilized 10 min using 0.2% Triton X-100 in PBS. After two washes in PBS, the cells were incubated 1–2 h with rhodamine-phalloidin (1.5 μM final concentration) at 24°C. After several washes in PBS, the cell pellet was resuspended in a drop of mounting medium (p-phenylenediamine/90% glycerol) and was then applied to a slide. Cells were photographed using TMAX400 films (Kodak) and a Axiophot microscope (Zeiss, Thornwood, NY) equipped for fluorescence. Rhodamine-phalloidin was purchased from Sigma Chemical (St. Louis, MO).

Calcofluor Staining of Chitin

For labeling of chitin, fixed (as described above) and washed cells were incubated with Calcofluor White M2R (Sigma Chemical) at 1 mg/ml in water for 5 min. They were then washed and resuspended in water and mounted on glass slides as described by Adams and Pringle (1984).

Generation and Affinity Purification of Polyclonal Antibodies Against End3p

Anti-End3p antibodies were raised in rabbits against the synthetic peptide KRHELQALQAEIN (aa 336–349) using the immunization protocol described by Singer-Krüger *et al.* (1993). For affinity purification of antibodies, 3 mg of the immunizing peptide were coupled to 1.5 ml of the Affi10 gel resin (Bio-Rad, Richmond, CA) according to the protocol provided by the manufacturer. Two milliliters of anti-End3p antiserum were added to the resin and incubated for 2 h at room temperature. The unbound proteins were removed by several washes with 0.1 M Hepes pH 7.5 supplemented or not with 0.1% Triton X-100 or 500 mM NaCl. The bound IgG were eluted using 0.2 M glycine pH 2.3. After immediate neutralization with 1 M Tris-base, protein-containing fractions were pooled and concentrated using centricon 30 devices (Amicon, Danvers, MA).

Yeast Cell Extracts and Fractionation Experiments

To analyze the expression of the mutated End3 proteins, whole cell extracts of the producing cells were prepared. Two OD_{600} units of

A

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1    ctctagagtaacttgaccatttcccttaacatcaagttccttgaacaaacacgcataccg
61   tatatctcgctgcttgtccgtctcgcaatttttcaacagcatttagaccgtcattcgatt
121  gaattaaacgttatttgccccttcaaatattcaatatttcccatcgctaataaaaaagct
181  gcatttttatttattacttttttaaatatcatatttgacgatcgtgtaggaccctacgat
241  ttaacatatcaagaatgaatgaaaagaacgtcagaaacacaaggttaattgcctgttata
301  atcagacgattgtaatataaccatagagttagtgggtattggaaaggccggtaaagataa
361  cagGGATCTctgaaaaatgcccaagttggaacaatttgaaataaaaaaatactggcaaat
                  M  P  K  L  E  Q  F  E  I  K  K  Y  W  Q  I   15
421  cttctcgggtttgaaaccaatagagaataaggtaaaccatgaccaggttttaccaatttct
     F  S  G  L  K  P  I  E  N  K  V  N  H  D  Q  V  L  P  I  L   35
481  ttataattccaaattagattcatcggttctaaacaagatttggtttttagctgatattga
     Y  N  S  K  L  D  S  S  V  L  N  K  I  W  F  L  A  D  I  D   55
541  tgacgacgacaatttagactttgaggaatttgtaatttgcatgagattaatatttgatat
     D  D  D  N  L  D  F  E  E  F  V  I  C  M  R  L  I  F  D  M   75
601  ggttaataaaaacattagctctgttccagatgaattgcctgattggttgattccagggag
     V  N  K  N  I  S  S  V  P  D  E  L  P  D  W  L  I  P  G  S   95
661  taaggtgaatctaatcaaagaaagaaagaagcgcaagcaaatagaaaacgcagacttgcc
     K  V  N  L  I  K  E  R  K  K  R  K  Q  I  E  N  A  D  L  P  115
721  tccaaagaaggaaatcaaagtagattggtacatgtctccagatgatttgaatcaatatga
     P  K  K  E  I  K  V  D  W  Y  M  S  P  D  D  L  N  Q  Y  E  135
781  aaaaatttataatagctgtgcaaaactaaccgatggtactattacattcaacgaactgtc
     K  I  Y  N  S  C  A  K  L  T  D  G  T  I  T  F  N  E  L  S  155
841  aacaaaacttttccacaaaatttttttaacatcagtaagacagatttaaataaggtttggag
     T  K  L  S  T  K  F  F  N  I  S  K  T  D  L  N  K  V  W  S  175
901  tttaattaacccacaaaatttgccatcaattgatagagatcctacttttttattttattca
     L  I  N  P  Q  N  L  P  S  I  D  R  D  P  T  F  Y  F  I  H  195
961  ctgtttaagacaaagaaatgatttgggtgctgaaattccagcaagtttaccaaattctct
     C  L  R  Q  R  N  D  L  G  A  E  I  P  A  S  L  P  N  S  L  215
1021 ggcggaggtgtgcaatataaaaacaactgagctatgatttacggtcctctcaacctcctac
     A  E  V  C  N  K  K  Q  L  S  Y  D  L  R  S  S  Q  P  P  T  235
1081 aaagagaaaagaagaagctaacgaagtggacaatctgcgtgacaatgggcagaaCTCGAG
     K  R  K  E  E  A  N  E  V  D  N  L  R  D  N  G  Q  N  S  S  255
1141 ttccgatagcagtggcagtaacgtgctttcaaatgaagacagtataaagcaaaaatatgc
     S  D  S  S  G  S  N  V  L  S  N  E  D  S  I  K  Q  K  Y  A  275
1201 ttctctgacagatgatcaagttgcgaacatgagagaacaattagaaggccttttgaacta
     S  L  T  D  D  Q  V  A  N  M  R  E  Q  L  E  G  L  L  N  Y  295
1261 caaaaagagtgaaaaaactcaaggaggctcaaaactttctaagcggattaacatcaggtc
     K  K  S  E  K  T  Q  G  G  S  K  L  S  K  R  I  N  I  R  S  315
1321 gataactgatgacttggataacattgaacagcaggttgaagtattggagaattacttgaa
     I  T  D  D  L  D  N  I  E  Q  Q  V  E  V  L  E  N  Y  L  N  335
1381 caataagagacacgaattgcaagcattacaagcagaaatcaattgacaccgataattaat
     N  K  R  H  E  L  Q  A  L  Q  A  E  I  N  *                 349
1441 tttatgtacatgaatgtgtaatatttactgtttgttatttcacttgtaaacttttttgat
1501 ttgaacttcaatctatccagtaacggtgtggtgtatacttatttttttcctttttcttctct
1561 gccggaatttcataaaatttttactgttcagcgttctttacatatttatatataatagaata
1621 tgtcatataaagaaacagtgacttagtttctgttttacttttttttctcctatttctaccg
1681 tgattttgattagttttttctttaattgttgctattcactgtgcttgtgttaaatcttga
1741 tgatggtacttgaaccctctgaataatgataatatacctctttacagtaactatctttct
1801 ttattgggtgctttttaaatagttccactatttccatcatactcattctcacgcttcagc
1861 tagatagagctgttgtagtaaactcaactgctttagtgaataatttaaacacgtgttcat
1921 cgat
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B

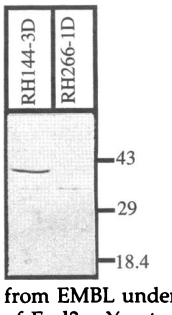


Figure 1. Sequence of the *END3* gene and immunodetection of its product. (A) Sequence of *END3*. The nucleotide sequence of the 1.9-kb *Xba* I/*Cla* I fragment that contains the *END3* gene is shown. The deduced amino acid sequence is shown below in one letter amino acid code. Nucleotide 369 was mutated to C to create the *Bam*HI site that, together with the *Xho* I site (in capitals), was used to construct the disruption. The putative EF-hand Ca^{2+}-binding loop is underlined and the consensus sequence for PIP_2-binding is in italics. The two regions that are homologous are double underlined. The *END3* sequence data are available from EMBL under accession number X79473. (B) Immunodetection of End3p. Yeast cell extracts from RH144-3D and RH266-1D were prepared as indicated in MATERIALS AND METHODS and analyzed by immunoblot with End3p antibody. The same amount of cell extract was loaded in each case. Molecular weight markers are indicated on the side.

cells in mid-log phase were harvested by centrifugation. They were lysed by the addition of 1 ml TE buffer (10 mM Tris pH 7, 1 mM EDTA) containing 0.2 M NaOH and 0.5% β-mercaptoethanol and incubated 10 min on ice. Trichloroacetic acid (TCA) (10% final concentration) was then added and the samples were further incubated 10 min on ice. Precipitates were collected after 10-min centrifugation at 10 000 × *g*. After removal of TCA by an acetone wash, they were solubilized in 100 μl of 2× Laemmli sample buffer, heated 3 min at 95°C, and 50 μl was loaded on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting.

The preparation of the cell extracts used for the fractionation experiments was performed as described by Singer and Riezman (1990). After the addition or not of Na_2CO_3 (100 mM final concentration, pH 11) or Triton X-100 (1% final concentration), these extracts were centrifuged according to Singer and Riezman (1990) to obtain the three pellets, P1, P2, and P3, and the three supernatants, S1, S2, and S3. Correction for cell lysis was performed as already described (Singer and Riezman, 1990) using hexokinase as a cytosolic marker.

Electrophoresis and Immunoblotting

SDS-PAGE was performed according to Laemmli (1970). For Western blotting, gels were transfered to nitrocellulose (Towbin *et al.*, 1979) and filters were stained with Ponceau S to assess relative amounts of proteins transfered. After transfer, the membrane was blocked in PBS/3% nonfat dried milk for 15 min at room temperature. Antibody incubations were 1 h and were followed by three 5-min washes in PBS/milk. Antiserum against End3p was used at a dilution of 1:1000. For detection of the antigen, the ECL Western blotting system (Amersham) was used. Chemicals for SDS-PAGE were obtained from Bio-Rad or BDH (Poole, Great Britain) and the nitrocellulose was from Schleicher and Schüll (Dossel, Germany). The amount of End3p in the different cell fractions was quantified by densitometric scanning on a computing densitometer (Molecular Dynamics, Sunnyvale, CA) of fluorographs on Kodak XAR-5 films. Hexokinase antibody was kindly provided by G. Schatz (Biocenter, Basel, Switzerland).

RESULTS

Cloning, Sequencing, and Disruption of the END3 Gene

The *END3* gene was cloned by complementation of the temperature-sensitive growth defect of the *end3* mutant. For this purpose, the *end3* mutant strain (RH266-1D) was transformed with a yeast genomic DNA library on a multicopy plasmid (see MATERIALS AND METHODS). Several plasmids with overlapping inserts were obtained. When subcloned in YCplac33 (Giez and Sugino, 1988), a 1.9-kb *Xba* I/*Cla* I fragment was shown to complement the mutant phenotype. This fragment was sequenced (Figure 1A). A single open reading frame (ORF) (1047 bp) that encodes a putative protein of 349 amino acids with a calculated molecular mass of 40 297 Da could be identified in the complementing fragment.

Antibodies directed against a peptide corresponding to the last 13 aa of the putative protein were generated. Using these antibodies, a protein of 40-kDa molecular weight can be identified on Western blots of wild-type cell extracts (Figure 1B). In the original *end3* mutant cell extracts, no protein was detected by these antibodies (Figure 1B). These data confirm that the ORF identified corresponds to the End3 protein.

To confirm that the cloned DNA was the *END3* locus, strain RH2143 (*Mata end3Δ1::URA3, his4, leu2, bar1-1,* carrying plasmid pHB3) was crossed with RH1829 (*Matα end3-1, his4, ura3, leu2, bar1-1, suc2Δ9*) and the resulting diploid cells were sporulated. The plasmid pHB3 was necessary for this experiment because homozygous *end3* diploids are defective for sporulation (Raths *et al.*, 1993). Twenty-seven tetrads were dissected

and all uracil auxotrophs (strains not carrying the *end3Δ1::URA3* disruption allele) were temperature-sensitive for growth when they had lost the plasmid carrying the wild-type *END3* gene (pHB3). This demonstrates a very tight linkage between the cloned locus and the original *end3-1* mutation, proving that we have indeed cloned the authentic END3 gene and not a suppressor.

A hydropathy plot of End3p did not identify any potential transmembrane domain nor signal peptide. Inspection of the amino acid sequence revealed a number of regions with potential structural or functional significance. A potential EF-hand helix-loop-helix calcium binding site is present at amino acids 44–72. The EF-hand Ca^{2+}-binding motif consists of two α-helices joined by a Ca^{2+}-binding loop. In this structure, the Ca^{2+} ion is coordinated by seven oxygens in a 12-residue sequence that spans the loop and the beginning of the second α-helix. Residues 1, 3, 5, 7, and 9 of the loop, which are generally highly conserved, constitute five of the ligands; the two others are provided by a glutamate residue at position 12 (Babu *et al.*, 1988). In the putative EF-hand Ca^{2+}-binding motif of End3p, residues at positions 1, 3, 5, 7, and 9 in the loop could coordinate Ca^{2+}, and residue 12 at the beginning of the second α-helix is a glutamate (Figure 2). Residues 96–105 constitute a potential polyphosphoinositide (PIP_2)-binding motif that fits the consensus sequence found by Yu *et al.* (1992) for gelsolin and a variety of PIP_2-binding proteins. Furthermore, two regions of the protein (residues 130–180 and 262–314) present a repeating seven-residue pattern of nonpolar and polar or charged residues. This motif is characteristic of coiled-coil α-helical proteins (Smillie, 1979). Finally, two regions of the protein comprising residues 276–295 and 315–333 are homologous to each other (50% identity) (see below).

To determine the effect of End3p function on the cell viability, the *END3* gene was disrupted. This disruption (*end3Δ1::URA3*) removed 254 amino acids at the N-terminus of the protein. The disruption was transplaced to the chromosome by homologous recombination in a diploid strain (RH444) and transformants showing a Ura^+phenotype were recovered. Sporulation and tetrad dissection of several Ura^+ isolates yielded four viable spores per tetrad at 24°C. At 37°C, only two of the spores were viable and they never carried the Ura^+ marker. Southern blot analysis of the haploid isolates demonstrated that in the two Ura^+ spores, the wild-type *END3* gene had been replaced by the disrupted sequence. α-Factor could not be internalized in the *END3*-disrupted cells of the **a** mating type (RH1995). No protein reacting with the anti-End3p peptide antibody could be detected after immunoblot analysis of the Ura^+ haploid cell extracts. This result suggests that the disruption of 254 amino acids in the N-terminal part of the protein does not confer lethality but confers the same phenotype as the original *end3* mutant: thermosensitivity for growth and inability to internalize α-factor at any temperature.

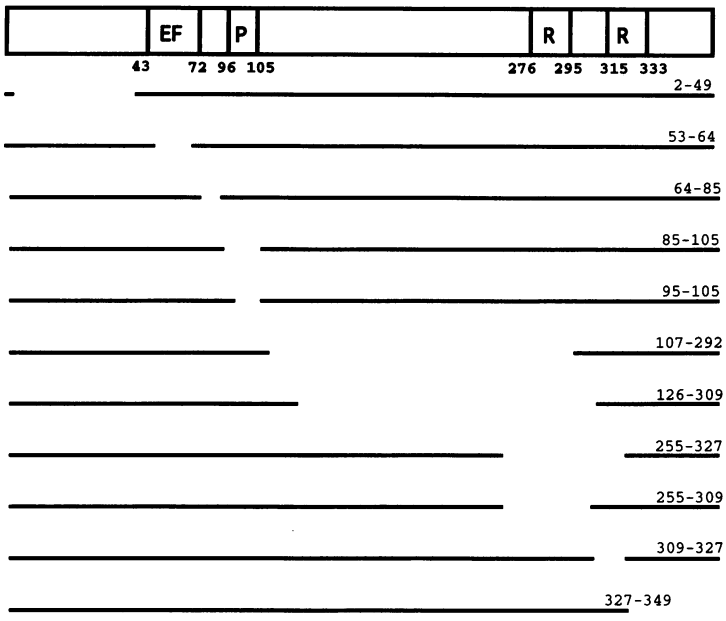
	Helix	Loop (1 3 5 7 9)	Helix (12)	
44	VLNKIWFLA	DIDDDDNLD	FEEFVICMRLI	End3p
10	ELREAFNKI	DIDNSGYVS	DYELQDLFKEA	Fimbrin (1)
50	IIEKIFAVT	DSNKDGKIN	FEEFVSLIQEL	Fimbrin (2)
11	EFKEAFALF	DKDNNGSIS	SSELATVMRSL	Calmodulin (1)
47	EVNDLMNEI	DVDGNHQIE	FSEFLALMSRQ	Calmodulin (2)
84	ELLEAFKVF	DKNGDGLIS	AAELKHVLTSI	Calmodulin (3)

Figure 2. The putative End3p Ca^{2+}-binding site. The potential EF-hand of End3p was compared with EF-hands of chicken fimbrin (Adams *et al.*, 1991) and yeast calmodulin (Geiser *et al.*, 1991). The residues responsible for the coordination of the Ca^{2+} in known EF-hands are numbered. The numbers on the left indicate the positions where the sequences are found in the proteins indicated on the right.

Regions of End3p Important for Its Function

To analyze the structure-function relationships of End3p, a series of deletions were made in the gene as indicated in Figure 3. First of all, the *END3* gene was cloned in Ycplac111 (Giez and Sugino, 1988) containing the *LEU2* marker (pHB3 plasmid). Strain RH1995 transformed with pHB3 internalizes α-factor as efficiently as wild-type cells (RH144-3D) and grows normally at 37°C. Deletions in the *END3* gene were made by PCR as described in MATERIALS AND METHODS. They were cloned back in pHB3M, which corresponds to the pHB3 plasmid where a *Mlu* I site has been introduced without modifying the End3p function. The resulting plasmid was then introduced into the RH1995 strain. Leu^+ transformants were then tested for their ability to grow at 37°C, and the kinetics of α-factor uptake at 24°C were determined to compare the efficiency of the process with cells expressing the entire End3 protein (Figure 4, A and B). S^{35} α-factor was bound to cells on ice, and after removal of the unbound α-factor, internalization was allowed to occur by shifting the temperature to 24°C for different times. The percentage of internalized α-factor at each time was deduced from the percentage of bound α-factor that was not washed off from the cells with a pH 1.1 buffer. Figure 3 summarizes the phenotypes confered on the RH1995 strain by the different *END3* deletions. These results show that diverse regions of the protein are important for its function. The stability of the different truncated proteins was also examined by subjecting the total cell extracts of each mutant strain to Western blot analysis.

Cells expressing an End3 protein deleted from residue 2 to 49 (Δ2–49) or 64 to 85 (Δ64–85) were unable to grow at 37°C and did not internalize α-factor at 24°C. This was also the case for cells expressing Δ85–105; however, deletion of the potential PIP_2-binding site



	Growth at 37°C	Uptake at 24°C
2-49	−	−
53-64	−	−
64-85	−	−
85-105	−	−
95-105	+	+
107-292	+	+
126-309	+	+
255-327	−	−
255-309	+	+
309-327	−	+
327-349	+	+

Figure 3. The *end3* deletion mutants. The numbers in bold delimit the putative EF-hand helix-loop-helix Ca^{2+}-binding site (indicated by **EF**), the putative PIP_2-binding site (indicated by **P**), and the two homologous regions (indicated by **R**). Numbers on the side indicate the position of the deletions. The mutants were created by site directed mutagenesis using PCR. The ability of RH1995 strains expressing these different proteins to grow at 37°C and to internalize α-factor is given on the right side. +, behavior is the same as wild-type cells. −, behavior is the same as the *end3* mutant cells.

(Δ95–105) had no effect in our assay. Because the three nonfunctional proteins were stable (data not shown), we concluded that the regions located between aa 2–49, 64–85, and 85–95 are important for End3p function. When the loop corresponding to the putative EF-hand Ca^{2+} binding site was deleted (Δ53–64), the cells expressing the resulting protein exhibited an *end3* mutant phenotype. This mutant protein was almost undetectable by Western blot analysis (Figure 6B), indicating that this deletion renders the protein unstable. Therefore, such a mutation did not allow us to make any conclusions about the function of the putative Ca^{2+}-binding site.

Two large deletions of End3p (Δ107–292 and Δ126–309) did not affect the function of the protein in our assay. However, the Δ255–327 deletion, although not affecting the protein stability (data not shown), did not restore a End^+ phenotype in the RH1995 strain. It is therefore possible that additional important information for End3p function is present between residues 309 and 327. Cells expressing End3p deleted in this region internalized α-factor as efficiently as wild-type cells at 24°C (Figure 4A) but did not grow at 37°C. Because the inability to grow at 37°C for *end3* mutants was always associated with a defect in endocytosis at 24°C, we suspected that this mutant might encode an *end3* allele that is thermosensitive for α-factor uptake (see below). Taken together, the data obtained from the mutants Δ126–309, Δ255–327, and Δ309–325 suggest that the regions comprising residues 255–309 and 309–325 cannot both be deleted without a loss of function, even though deletion of either one of the regions does not greatly affect End3p function at 24°C. Finally, a deletion in the last 22 amino acids of the protein (Δ327–349) had no effect on End3p function.

The Deletion Mutant Δ309–327 Is an end3 Thermosensitive Allele

Cells expressing the Δ309–327 End3p mutant were tested for α-factor uptake at 37°C. For this study, ^{35}S α-factor was mixed with cells grown at 24°C but preequilibrated for 10 min in YPUAD medium at 37°C. At various times after ^{35}S α-factor addition, aliquots were withdrawn and treated with pH 6 or pH 1.1 buffer to measure the total amount of cell associated radioactivity and the amount of internalized α-factor, respectively. This experiment was also done with wild-type cells, as a positive control, and the results of both experiments are shown in Figure 5A. At the restrictive temperature for growth, cells expressing End3p Δ309–327 internalized very little α-factor. The same result was observed when cells that had been preincubated with α-factor at 0°C to allow pheromone binding were shifted directly to the restrictive temperature (data not shown). Therefore, α-factor uptake is immediately blocked in *end3* Δ*309–327* cells at 37°C.

To characterize this mutant further, we investigated whether the endocytic defect induced at the restrictive temperature could be reversed upon return of the cells to the permissive temperature. In this experiment, the cells were first preincubated 10 min at the restrictive temperature, ^{35}S α-factor was then added and the incubation at 37°C was continued for an additional 10 min. The cells were then shifted either to 24°C in YPUAD medium or to 24°C in YPUAD medium containing cycloheximide and samples were withdrawn and treated as previously described to determine the amount of radioactive pheromone internalized. As a control, a culture was kept throughout all steps of the experiment at 37°C. *end3* Δ*309–327* cells, preincubated at 37°C,

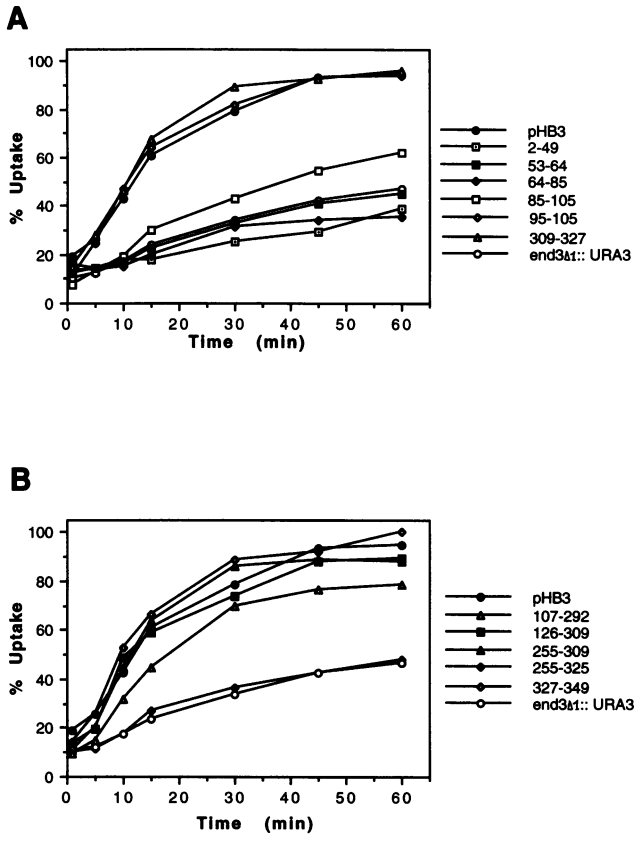


Figure 4. Internalization of α-factor by the different *end3* deletion mutants at 24°C. Cells expressing mutant forms of End3p were incubated with ^{35}S-labeled α-factor and samples were withdrawn in duplicate at the times indicated. The percentage of internalized α-factor was then determined as described in MATERIALS AND METHODS. The values represent averages of at least two independent experiments. The correspondance between the symbols and the mutants is indicated on the side. Panels A and B represent analysis of different mutant *end3* alleles.

internalize α-factor at the same rate and with the same efficiency as wild-type cells when shifted to the permissive temperature in either the presence or absence of cycloheximide (Figure 5B). Therefore, the defect in endocytosis at 37°C can be rapidly reversed at 24°C without synthesis of new protein, supporting the idea that the inactivation of End3p Δ309–327 is fully reversible at 24°C.

Creation of Point Mutations in the Putative Ca^{2+}-binding Site

Deletion of the loop corresponding to the putative EF-hand Ca^{2+}-binding site rendered the protein unstable, thus providing no information on the importance of this region for End3p function (Figure 6B). In an attempt to overcome this problem, we made point mutants in the putative Ca^{2+}-binding loop. The mutants were designed based on the results obtained by Geiser *et al.* (1991) on the Ca^{2+}-binding loops of yeast calmodulin. They observed that changing the aspartate residue at position 1 to an alanine or the glutamate residue at position 12 to a valine as well as the combination of the two mutations greatly reduced the affinity of calmodulin for Ca^{2+}.

Mutation of D53 (residue 1 in the putative Ca^{2+}-binding loop) to alanine did not affect the function of the protein in vivo as shown in Figure 6A. However, cells expressing the single mutant E64V (where the glutamate in position 12 of the putative Ca^{2+}-binding loop is changed to a valine) and the double mutant D53A-E64V could not internalize α-factor at 24°C (Figure 6B) nor grow at 37°C. Furthermore, the level of expression of these two mutant proteins was greatly reduced as compared with the D53A mutant or the wild-type pro-

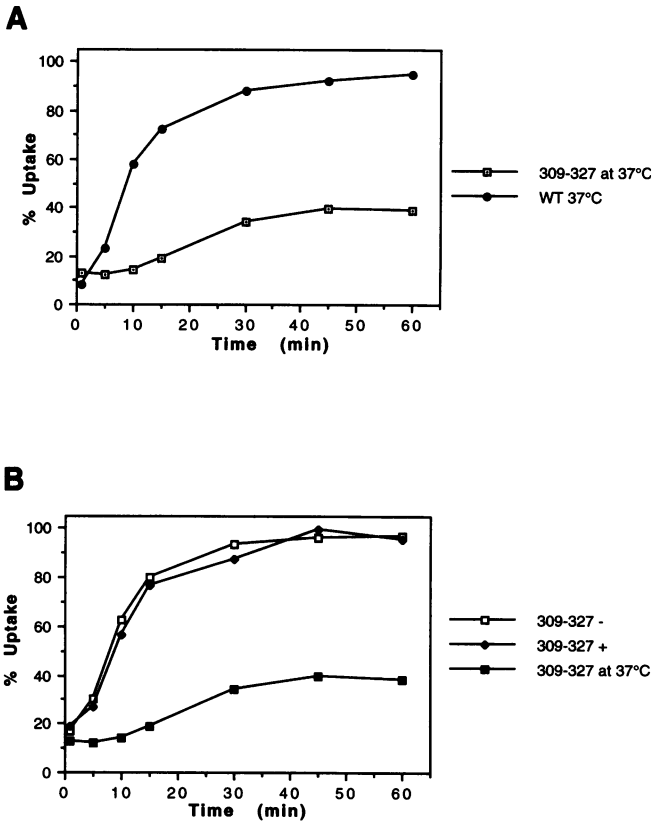


Figure 5. Characterization of the *end3* thermosensitive allele (*Δ309–327*). (A) Internalization of α-factor by wild-type cells and cells expressing End3p Δ309–327 at 37°C. In this experiment, cells were preincubated 10 min at 37°C before the addition of the labeled pheromone as described in MATERIALS AND METHODS. The correspondence between the symbols and the strains is indicated on the side. (B) Reversibility of the Δ309–327 mutation. Cells were preincubated 10 min at 37°C before the addition of α-factor. The incubation was continued for 10 min and the cells were pelleted and resuspended in either 24°C YPUAD (−), 24°C YPUAD containing cycloheximide (1.4×10^{-4} M) (+), or 37°C YPUAD. At the time points indicated, aliquots were withdrawn in duplicate and processed as described in MATERIALS AND METHODS.

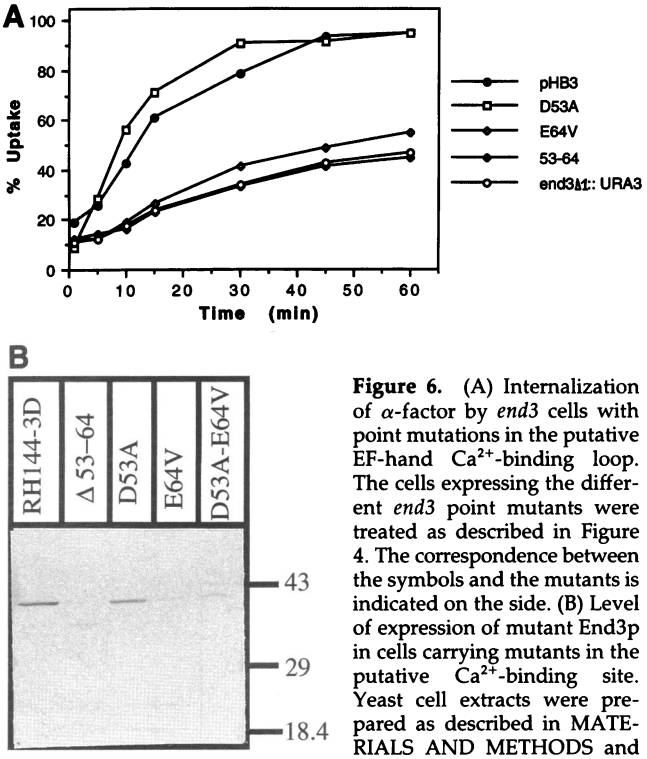


Figure 6. (A) Internalization of α-factor by *end3* cells with point mutations in the putative EF-hand Ca^{2+}-binding loop. The cells expressing the different *end3* point mutants were treated as described in Figure 4. The correspondence between the symbols and the mutants is indicated on the side. (B) Level of expression of mutant End3p in cells carrying mutants in the putative Ca^{2+}-binding site. Yeast cell extracts were prepared as described in MATERIALS AND METHODS and analyzed by immunoblot with the anti-End3p antibody. The same amount of cell extract was loaded in each case. Molecular weight markers are indicated on the side.

teins (Figure 6B). As in the case of the complete deletion of the loop, the results obtained with the E64V and D53A-E64V mutants did not allow any firm conclusions to be made because the protein stability was affected. The phenotype of the D53A mutant, however, supports the idea that the putative binding of Ca^{2+} ions might not be important for End3p function.

End3p Function Is Specifically Involved in the Internalization Step of Endocytosis and Not in the Delivery of α-Factor to the Vacuole

After binding to its receptor, Ste2p, α-factor is internalized in a time-, temperature-, and energy-dependent way and is delivered to the vacuole where it is degraded (Chvatchko *et al.*, 1986; Singer and Riezman, 1990). Upon internalization, α-factor passes through at least two separable membrane-bounded compartments termed early and late endosomes before reaching the vacuole (Singer and Riezman, 1990; Singer-Krüger *et al.*, 1993). Delivery to the vacuole is reduced at 15°C, and the pheromone is mainly recovered in these intermediate compartments upon incubation for 10–20 min at this temperature (Singer-Krüger *et al.*, 1993). Taking advantage of the *end3* thermosensitive allele and the possibility of delaying the endocytic transport of α-factor, we tested at which step of endocytosis End3p is involved. ^{35}S α-factor was bound to *end3* Δ*309–327* and wild-type cells on ice, and the temperature was then shifted to 15°C for 20 min to allow transport of α-factor into endosomes. The temperature was then shifted to 37°C to induce the *end3* defect and aliquots were taken at different times. The internalized radioactive pheromone was extracted from these samples and analyzed using a thin-layer chromatography system (Chvatchko *et al.*, 1986) to determine if it was degraded (and therefore transported to the vacuole) or still intact (and therefore blocked before the vacuole). Results presented in Figure 7B show that internalization of ^{35}S α-factor is arrested upon shift to 37°C in the *end3* Δ*309–327* mutant

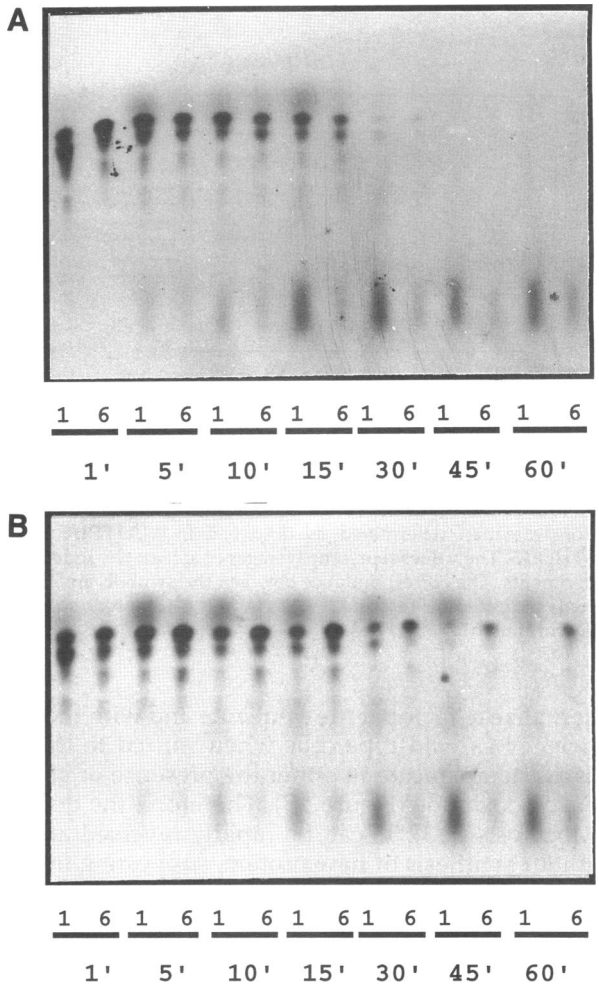


Figure 7. End3p is not required for subsequent transport of internalized α-factor to the vacuole. Wild-type cells (RH144-3D) (A) and cells expressing the *end3* thermosensitive allele (Δ*309–327*) (B) were allowed to bind ^{35}S α-factor 1 h on ice. After eliminating the unbound α-factor, the cells were incubated 20 min at 15°C, then pelleted again, and resuspended in YPUAD prewarmed at 37°C. At the times indicated after the 37°C shift (1′, 5′, 10′, 15′, 30′, and 60′), samples were taken and diluted in pH 1.1 or pH 6 buffer (as indicated above the times) and the radioactivity extracted from the cells and analyzed by TLC as described in MATERIALS AND METHODS.

(persistance of intact α-factor in pH 6 lanes) but continues in wild-type cells. The ^{35}S α-factor internalized during the 20 min at 15°C in the *end3 Δ309–327* mutant (pH 1.1 resistant) and which had accumulated in early and late endosomes was degraded with normal kinetics upon the 37°C shift. This result demonstrates that End3p does not play a role in the transport of α-factor from the endosomes to the vacuole and that it can only be involved in the first step(s) of endocytosis: internalization and perhaps transport to the endosomes.

Actin Distribution Is Modified in end3 Mutant Cells

Recently, Kübler and Riezman (1993) demonstrated that actin and the actin-binding protein Sac6p, the yeast fimbrin homologue, play a direct role in the internalization of α-factor. A functional actin cytoskeleton is thus required for the internalization step of endocytosis in yeast. We wanted to know if the inability of *end3* mutants to internalize α-factor could be related to perturbations in the actin cytoskeleton. Therefore, we compared the actin cytoskeleton in *end3Δ1::URA3* and wild-type cells. After fixation with formaldehyde, the cells were processed as described by Adams and Pringle (1984) and actin was visualized by use of a fluorescent derivative (rhodamine) of the f-actin binding toxin phalloidin.

In wild-type cells, actin distribution is in the form of cables and cortical patches that are arranged in a polarized asymmetric pattern (Adams and Pringle, 1984; Kilmartin and Adams, 1984). The growing portion of the cell, the bud, contains most of the cortical actin patches that have been recently described to be associated with the cell surface via an invagination of the plasma membrane (Mulholland *et al.*, 1994). The nongrowing portion of the cell, the mother cell, contains the cables generally aligned along the axis of bud growth. The actin pattern we detected for wild-type cells was consistent with this description even though the cables were in some cases difficult to detect (Figure 8A). The actin distribution was completely different in *end3Δ1::URA3* cells, which were larger than wild-type cells (Figure 8B). Fewer cortical patches could be detected in the bud, and it seemed that the actin had aggregated in brightly staining patches mostly in the mother cells or at the junction between the mother cell and the bud. The actin visualization was also performed in the RH1995 strain expressing the different truncated End3 proteins. Cells expressing deleted End3 proteins, which restored the defect in endocytosis, exhibited the same actin distribution as wild-type cells. The others showed the same abnormal actin distribution as the RH1995 strain.

In the case of cells expressing the thermosensitive End3p Δ309–327, the distribution of actin was normal for most of the cells at 24°C, but 10–15% of them exhibited the abnormal *end3* mutant typical distribution. After an hour incubation and 2 h fixation of the cells at 37°C, the proportions were reversed. Because the cells have to be fixed for 2 h and we do not know how long it takes for the fixative to take effect, we did not attempt to determine if the immediate defect in endocytosis upon shifting the cells to the nonpermissive temperature correlates with an immediate change in the actin distribution.

The end3 Mutant Has an Aberant Budding Pattern in Diploid Cells and Delocalized Chitin Deposition in Both Haploid and Diploid Cells

Bud emergence in dividing yeast cells is preceded by the appearance of a ring of actin dots and a ring of chitin in the cell wall of the mother cells at the future bud site. After cell division, this chitin ring persists on the mother cell as a bud scar. Haploid and diploid cells each have a distinct budding pattern. In haploid cells, the site of bud emergence is adjacent to the previous one (axial pattern), whereas in diploid cells, the bud can emerge from one of two opposite poles in the cell (bipolar pattern). Chitin rings, which can be visualized using the dye Calcofluor, provide a record of the site of earlier budding events.

Calcofluor-stained wild-type and *end3* disrupted haploid and diploid cells are shown in Figure 9. *end3* disrupted haploid cells exhibit a normal axial budding pattern, but cytokinesis seems to be inhibited and uncoupled from subsequent bud emergence because several mother cells exhibit two adjacent buds. Furthermore, the mother cells are so brightly stained that their bud scars are not easily detectable. This observation indicates that chitin distribution is delocalized.

In *end3* null mutant diploid cells (RH2386), the chitin delocalization is even more pronounced, and the bigger the cells are, the brighter they are stained. In addition, the bud site was not always selected in a bipolar fashion, as in wild-type diploid cells, but at random. As in mutant haploid cells, cytokinesis seems to be slowed down because daughter cells emerge from cells that are not yet separated from their mother cells. This gives rise to multibranched cells and aggregates.

Subcellular Localization of End3p

To obtain information concerning End3p localization, fractionation experiments were performed, as described by Singer and Riezman (1990). After conversion of cells to spheroplasts and gentle lysis with DEAE-dextran, the lysate was subjected to three successive centrifugation steps to roughly separate the organelles. The bulk of plasma membranes, vacuoles, nuclei, cytoskeleton, and unlysed cells were pelleted after 5 min centrifugation at 3500 × *g* (pellet P1). A second round of centrifugation for 10 min at 10 000 × *g* carried out on the resulting supernatant (S1) gives rise to the P2 pellet containing mainly mitochondria. In the P3 pellet, obtained after

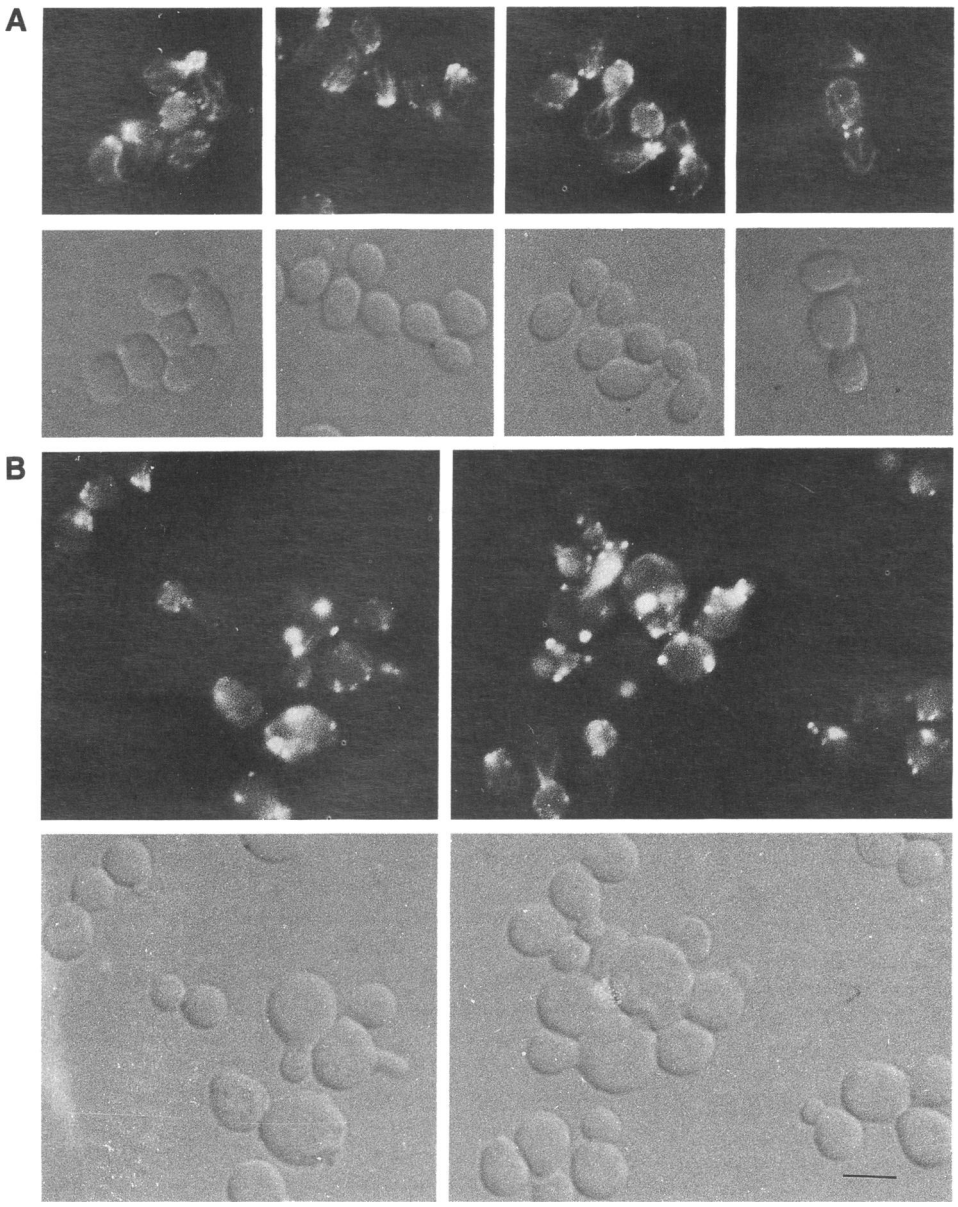


Figure 8. Actin distribution in wild-type and *end3* mutant cells. Wild-type (RH144-3D) (A) and RH1995 (B) cells were stained with rhodamine-conjugated phalloidin as described in MATERIALS AND METHODS. The lower pictures correspond to the same cells viewed by Nomarski optics. Bar, 7.5 μm.

centrifugation of the previous supernatant (S2) at 100 000 × *g* for 1 h, are found ER, Golgi, endosomes, and smaller fragments of the plasma membrane. The final supernatant (S3) contains the cytoplasmic material. These different fractions have been analyzed by Western blotting with the anti-End3p antibody. Several fractionation experiments were performed and after correction for the extent of cell lysis (by using the cytoplasmic marker hexokinase) each indicated the same proportion of End3p in the different fractions: 30% in P1, 5–8% in P3, and the remainder in S3 (Figure 10). Therefore, a fraction of End3p seems to be associated with the low-speed pelleting material. When such a fractionation experiment was performed in carbonate buffer (pH 11) the End3p was entirely recovered in S3, indicating that it is not an integral membrane protein (Figure 10). Upon fractionation in 1% Triton X-100, the amount of End3p in the different fractions did not change (Figure 10). Taken together, these data indicate that End3p pelleting in P1 is associated with a detergent insoluble structure. Electrostatic interactions seem to be involved in this association, because it is broken at high pH. However, we cannot rule out that it is a nonspecific aggregate that is found in P1. Unfortunately, immunofluorescence localization experiments, using the anti-End3p antibody or the monoclonal anti-cmyc antibody after tagging of End3p with the cmyc epitope at its C-terminal extremity, have been unsuccessful thus far.

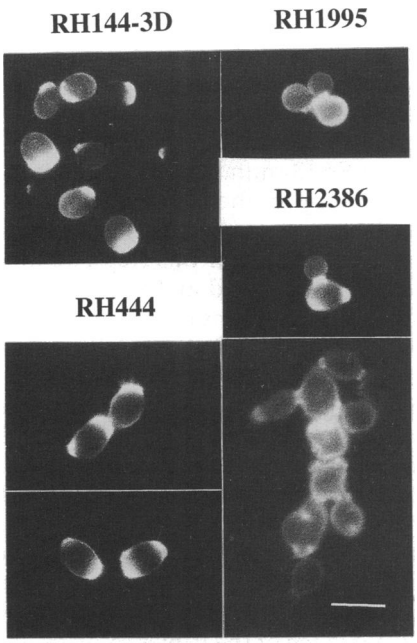


Figure 9. Chitin localization. Cells were grown to log phase and stained for chitin with calcofluor. The strains are indicated above the pictures. Bar, 7.5 μm.

DISCUSSION

In this study, we report the cloning and sequencing of the *S. cerevisiae END3* gene and the characterization of its product, which has been shown to be involved in the internalization step of receptor-mediated endocytosis (Raths *et al.*, 1993). The original *end3* mutant cells were unable to internalize α-factor at any temperature and were unable to grow at 37°C (Raths *et al.*, 1993). They expressed no protein recognized by the anti-End3p antibodies generated against a peptide corresponding to the last 13 aa of the End3 protein. Nevertheless, to be certain of the effect of End3p function on the cells, we constructed a null mutant (*end3Δ1::URA3*). The null mutant cells exhibit the same phenotype as the original *end3* mutant cells, thereby demonstrating a requirement for End3p in receptor-mediated endocytosis. The inability of the *end3* mutant cells to grow at 37°C is a phenotype shared by all the endocytic mutants blocked at the internalization stage that have been isolated thus far (Munn and Riezman, unpublished observations). This observation suggests that a defect in this step of endocytosis is lethal at 37°C.

Searches for functional motifs in the protein databank revealed the existence of a possible EF-hand helix-loop-helix Ca^{2+}-binding site and a putative PIP_2-binding site in the End3p sequence. Usually, Ca^{2+}-binding proteins possess more than one of these consensus sites, but we cannot exclude the possibility that one of these sites is enough for End3p to bind Ca^{2+} and that this putative binding has an influence on End3p function. To test the importance of such a region for End3p function, we deleted the Ca^{2+}-binding loop. Unfortunately, this mutation rendered the protein unstable and we could not draw any conclusion from the experiment. Therefore, we introduced point mutations into the EF-hand structure. The substitutions we made were predicted to reduce the ability of the loop to bind Ca^{2+} (Geiser *et al.*, 1991). One of these mutations had the same destabilizing effect on End3p as the deletion, whereas the second did not modify the protein stability or its function. This result suggests that the putative Ca^{2+}-binding site is important for End3p stability but that binding of Ca^{2+}, if it occurs, may not be required for the function of the protein in endocytosis. This would be similar to calmodulin, which is essential for cell viability (Davis *et al.*, 1986) but can perform its essential function without binding Ca^{2+} (Geiser *et al.*, 1991). Ca^{2+} binding could be required for some other function of End3p or could play a role in regulation of its activity for which we have no assay.

The presence of the putative PIP_2-binding site is interesting because it could have a regulatory function and has been described before for different actin-binding proteins (Yu *et al.*, 1992 and references therein). However, deletion of this region did not modify at all the protein function. As for Ca^{2+} binding, this region could have a regulatory role for End3p function.

Two regions in the N-terminal domain of the protein, on both sides of the EF-hand motif (residues 2–49 and

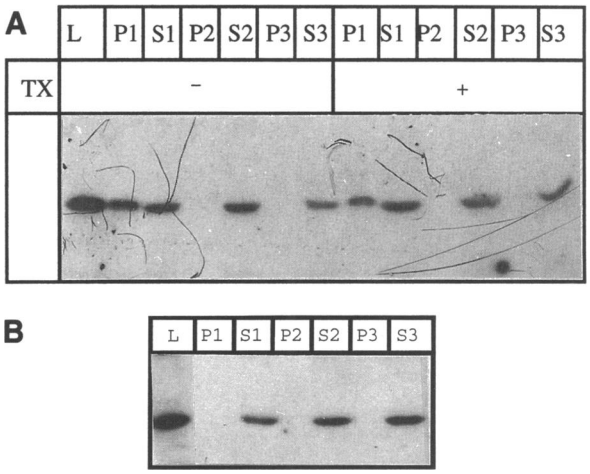


Figure 10. End3p localization after fractionation of yeast cell extracts. Extracts from wild-type cells were prepared and submitted to a series of centrifugations as described in MATERIALS AND METHODS. L, cells lysate before the centrifugations. P1, P2, and P3, three successive pellets obtained. S1, S2, and S3, the corresponding supernatants. These different fractions were separated by SDS-PAGE and probed with antibodies specific for End3p. (A) Fractionation performed in sorbitol medium (0.6 M sorbitol, 5 mM 2-amino-2methyl-1,3-propanediole-Pipes, pH 6.8) with (+) or without (−) 1% Triton X-100. Another exposure of this blot allowed the quantitation of End3p in P3. (B) Fractionation performed in sorbitol medium containing 100 mM Na_2CO_3, pH 11.

85–95), seem to be required for the protein function. A database search identified significant similarity (62% similarity, 41% identity) between the entire N-terminal domain of End3p (residues 1–96) and the three imperfectly repeated regions (of 95–97 aa) constituting domain I of eps15, a substrate of the epidermal growth factor receptor tyrosine kinase (Fazioli *et al.*, 1993) (Figure 11). The tyrosine residue present in the three eps15 repeats is conserved in End3p, but it lacks the flanking motif for a putative tyrosine phosphorylation site (Hunter, 1982). End3p also shares the consensus sequence for an EF-hand Ca^{2+}-binding site with the second and third repeats of eps15 domain I. It has been hypothesized that eps15 domain I would be a regulatory domain, whereas its effector domain would be domain III, which shares homologies with proteins displaying methyltransferase activity (Fazioli *et al.*, 1993). Indeed, End3p and eps15 seem not to be functionally related, because End3p is not implicated in either the transmission or the regulation of the signal induced by binding of α-factor to its receptor (Raths *et al.*, 1993), whereas eps15 is involved in the regulation of the mitogenic signals induced after binding of the epidermal growth factor to its receptor (Fazioli *et al.*, 1993).

Two regions in the C-terminus of End3p (aa 269–302 and 310–342) exhibit 61% homology (Figure 11). When both of these regions are deleted, the protein loses its function; if only one region is deleted, the function is conserved or the protein becomes thermosensitive. Hence, these regions may play a role in protein function. These regions display some similarities with α-actinins, actin-binding proteins that cross-link actin filaments. However, no role has been assigned to this region of the α-actinins. We do not know whether the N-terminal (residues 2–49 and 85–95) and C-terminal (residues 269–302 and 310–342) regions are functional domains or whether they are necessary to allow a proper folding of the protein.

The generation of a thermosensitive allele of *END3* allowed us to investigate the directness and the specificity of End3p function on α-factor internalization and degradation. The block in α-factor uptake is immediate upon shift of *end3* ^{ts} cells to 37°C. Furthermore, this block is immediately reversed without synthesis of new protein upon shifting the temperature back to 24°C. These data imply that a reversible change of conformation of the End3 mutant protein occurs at 37°C. In addition, they support the idea that End3p has a direct effect on α-factor internalization. The action of End3p is probably also specific for this step of endocytosis because α-factor internalized at 15°C was efficiently degraded when the *ts* mutant cells were shifted to 37°C.

Recently, Kübler and Riezman (1993) demonstrated that actin and the actin-binding protein Sac6p (the yeast fimbrin homologue) play a role in the internalization step of α-factor endocytosis. We examined the actin distribution in the *end3* mutants to investigate if End3p is important for the maintenance of the integrity of the actin network. The actin distribution was affected in all the *end3* mutants deficient in endocytosis and in a way quite different from the perturbations described by others, which involve a delocalization of the cortical patches and a disappearance of the cables (Novick and Botstein, 1985; Liu and Bretsher, 1989; Drubin *et al.*, 1990; Dunn and Shortle, 1990; Haarer *et al.*, 1990; Adams *et al.*, 1991; Amatruda *et al.*, 1992). The perturbations in the actin network in *end3* mutants are quite similar to those observed for the null mutant in the *SLA1* gene (Holtzman *et al.*, 1993). The patches seem to be less numerous and much larger than in wild-type cells. Therefore, End3p function, as suggested for Sla1p, could be important for controlling the size of the cortical patches (Holtzman *et al.*, 1993). *SLA1* interacts genetically with *ABP1*, a gene that encodes a cortical actin-binding protein. *ABP1* is not required for α-factor internalization (Kübler and Riezman, 1993), and we therefore tested whether synthetic-lethal interactions could be observed between *end3* and mutations affecting known actin-binding proteins. No synthetic lethality of *end3* with *sac6* (fimbrin), *abp1* (Actin Binding Protein 1), or *cap1*, *cap2* (actin capping protein) mutants could be detected. Therefore, although the defects in Sla1p and End3p

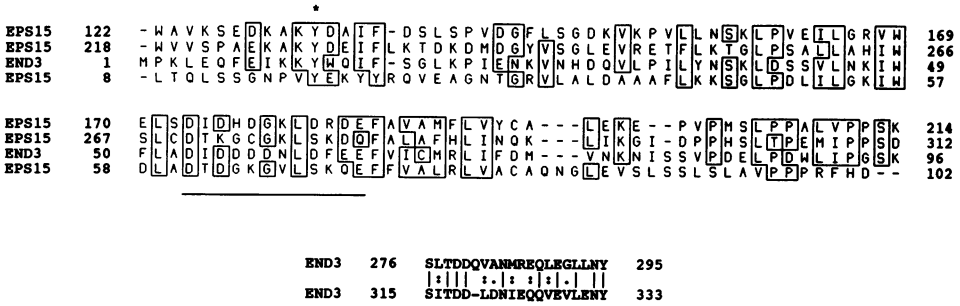


Figure 11. Comparison of the amino acid sequences in the essential domains of End3p. In the upper part, the N-terminus of End3p (third line) is compared with the three repeated regions constituting eps15 domain I using the program Prettyplot (with a plurality of 2.5). Amino acid numbers are indicated on both sides. Dashes represent gaps introduced to optimize the alignment. Boxes enclose positions at which at least two sequences are identical and one is similar. The conserved tyrosine is indicated by an asterisk and the putative EF-hand Ca^{2+}-binding site is underlined. In the bottom part, the homologous regions near the C-terminus of End3p are aligned using the program Bestfit. A line indicates identities and two dots similar residues according to the following groupings: D, E; S, T; I, L, V, M; A, G; F, W, Y; N, Q; H, R, K; C; and P.

seem to have similar effects on the actin cytoskeleton, the two proteins do not share synthetic lethality with the same proteins. Thus, Sla1p and End3p, as judged by their lack of similarity at the level of primary structure, seem to act by different mechanisms on actin cytoskeleton organization. At the present time, we do not know if the disorganization of the actin cytoskeleton is the origin or the consequence of the defect in endocytosis seen in *end3* mutants. If the disorganization of the actin cytoskeleton is the reason why *end3* cells do not internalize α-factor, then the role of End3p would be indirect on endocytosis. An indirect role of End3p would be difficult to reconcile with the behavior of cells carrying the *end3*^{ts} allele, that is, the immediate effect on internalization and rapid reversibility. If the effect of *end3* is via actin, it must be controlling some rapidly changing parameter, such as polymerization, bundling, or nucleation. An alternative explanation would be that endocytosis is required for proper control of the localization and/or size of the cortical actin patches. The *sla1*Δ mutant is also partially defective in α-factor internalization (Wesp, personal communication), and *end4* is allelic to *sla2* (Holtzman *et al.*, 1993; Wesp and Hicke, personal communication), which also shows aberrant actin localization.

As previously observed for conditional actin mutants (Novick and Botstein, 1985) and some null or conditional mutants of actin-binding proteins (Cleves *et al.*, 1989; Novick *et al.*, 1989; Haarer *et al.*, 1990; Amatruda *et al.*, 1992; Liu and Bretscher, 1989), chitin appears to be delocalized in *end3* mutant haploid and diploid cells because it is not only detected at the bud scars but evenly over the mother cells. Cytokinesis is slowed down both in haploid and diploid cells, but chitin delocalization is more pronounced in diploid cells. Because *end3* mutant cells grow slower than wild-type cells and because their division seems to be delayed, the increased chitin content of the cells may simply reflect the prolonged cell cycle time of these cells as already suggested by Shaw *et al.* (1991). However, in *end3* diploid cells the bud site selection is not as tightly controlled as for wild-type diploid cells. Interestingly, *end4* and *end6* (allelic to *RVS161;* Munn personal communication), which are also affected in the internalization step of endocytosis and exhibit an abnormal actin distribution, also have a random budding pattern in diploid cells (data not shown; Bauer *et al.*, 1993). These data support the notion that endocytosis might be involved in bud site selection perhaps by controlling the presence of critical proteins at the cell surface.

Fractionation experiments indicate that ~30% of End3p is associated with a detergent-insoluble structure that pellets after a low-speed centrifugation and from which it dissociates at pH 11. This structure could be the actin cytoskeleton, but further studies will be required to examine this question more closely.

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