Abstract. We identified DNM1, a novel dynamin-related gene in *Saccharomyces cerevisiae*. Molecular and genetic mapping showed that DNM1 is the most proximal gene to the right of centromere 12, and is predicted to encode a protein of 85 kD, designated Dnmlp. The protein exhibits 41% overall identity with full-length dynamin I and 55% identity with the most highly conserved 400-amino acid GTPase region. Our findings show that like mammalian dynamin, Dnmlp participates in endocytosis; however, it is unlikely to be a cognate homologue. Cells with a disruption in the DNM1 gene showed mating response defects consistent with a delay in receptor-mediated endocytosis. The half-life of the Ste3p pheromone receptor was increased two- to threefold in the *dnml* mutant, demonstrating that Dnmlp participates in the constitutive turnover of the receptor. To define the step in the endocytic pathway at which Dnmlp acts, we analyzed mutant strains at both early and late steps of the process. Initial internalization of epitope-tagged pheromone receptor or of labeled pheromone proceeded with wild-type kinetics. However, delivery of the internalized receptor to the vacuole was greatly impeded during ligand-induced endocytosis. These data suggest that during receptor-mediated endocytosis, Dnmlp acts after internalization, but before fusion with the vacuole. The *dnml* mutant was not defective for sorting of vacuolar proteins, indicating that Dnmlp is not required for transport from the late endosome to the vacuole. Therefore, we suggest that Dnmlp participates at a novel step before fusion with the late endosome.
Mx subgroups, although Vps1p is most like the former with respect to homology. VPS1/SPO15 (Rothman et al., 1990; Yeh et al., 1991), is required for proper delivery of proteins destined for the yeast lysosome known as the vacuole (Rothman et al., 1990; Vater et al., 1992), and for retention of Golgi membrane proteins (Wilsbach and Payne, 1993). Vps1p is postulated to function in the formation of Golgi-derived vesicles destined for the vacuole (Nothwehr et al., 1995). Mgm1p is needed for maintenance of the mitochondrial genome (Jones and Fangman, 1992; Guan et al., 1993) and bears the least resemblance to any other superfamily member in terms of both homology and function. In this paper, we investigated the cellular role of a third Saccharomyces cerevisiae dynamin-related protein, Dnm1p. Although Dnm1p was most like Vps1p in terms of overall homology, the primary function appeared to be in endocytosis rather than vacuolar protein sorting.

Like animal cells, yeast undergo both receptor-mediated, and fluid-phase endocytosis. Receptor-mediated endocytosis occurs for the yeast peptide mating pheromone receptors, Ste2p and Ste3p (see review of Sprague and Thorer, 1992). Ste2p and Ste3p are seven-transmembrane segment receptors and are internalized constitutively (Jenness and Spatrick, 1986; Davis et al., 1993) and in response to their respective ligands, α-factor (Chvatchko et al., 1986; Jenness and Spatrick, 1986; Schandel and Jenness, 1994) and α-factor (Davis et al., 1993). The internalized receptors and pheromone traverse the cell in a vesicle-mediated fashion (Singer and Riezman, 1990; Singer-Krüger et al., 1993) to the vacuole where they are degraded (Chvatchko et al., 1986). Unlike some mammalian receptors, the Ste2p pheromone receptor is not recycled after ligand-induced endocytosis (Jenness and Spatrick, 1986). In contrast, fluid-phase endocytosis is commonly measured by the ability of yeast cells to internalize a soluble fluorescent dye, Lucifer Yellow (LY)1 (Riezman, 1985; Dulic et al., 1991). Fluid-phase and receptor-mediated endocytosis appear to share some functional components, since mutants with defects in fluid-phase uptake also show receptor-mediated abnormalities (Munn and Riezman, 1994).

The yeast endocytic components to date may be roughly divided into two groups: those that contribute to internalization, and those that effect efficient delivery of endosomes to the vacuole. Mutations in the genes encoding End3p, End4p/Sla2p (Raths et al., 1993; Bénédict et al., 1994), End6p/Rvs161p, End8p, End9p, End10p (Munn and Riezman, 1994), Sac6p/Fimbrin, Act1p/actin (Kübler and Riezman, 1993), and clathrin heavy chain (Payne et al., 1988; Tan et al., 1993) all effect the internalization step of endocytosis.

The second class of endocytosis mutants effect delivery of internalized material to the vacuole. Certain secretory mutants show fluid phase (Riezman, 1985) and phospholipid (Kean et al., 1993) endocytic defects. In addition, other trafficking proteins that are important for proper vacuolar protein sorting appear to function in the endocytic pathway. The dual function of this class of proteins provided evidence that the endocytic and secretory pathways merge. This class includes small GTPases, or Rab-like proteins, Ypt7p (Wichmann et al., 1992; Schimmöller and Riezman, 1993), Ypt51p/Vps21p, Ypt52p, and Ypt53p (Singer-Krüger et al., 1994), that have been implicated in both endocytosis and vacuolar protein sorting. Other proteins involved in both pathways include End1p/Vps11 (Dulic and Riezman, 1989), Ren1p/Vps2p (Davis et al., 1993), End12p/Vps34p and End13p/Vps4p (Munn and Riezman, 1994). In summary, all of the previously identified endocytic proteins that participate in the delivery of endocytosed material to the vacuole have either a secretory, or a vacuolar protein sorting role as well.

We identified a novel dynamin-related gene in yeast, and characterized a strain in which the gene had been disrupted. The primary defect occurred at a late step of endocytosis: after internalization, but before delivery to the vacuole. The phenotypes of the mutant place Dnm1p at a unique position in the yeast endocytic pathway. Dnm1p represents the first protein to act at the endosomal trafficking stage of endocytosis that does not appear to participate in secretion or vacuolar protein sorting.

**Materials and Methods**

**DNA Manipulations**

Cloning and DNA hybridization methods were conducted as described elsewhere (Sambrook et al., 1989). Yeast genomic DNA was purified according to Rose et al. (1980). The DNM1 disruption plasmid, pMR2357, was made by cloning HIS3 into the region of DNM1 encoding the conserved GTPase domain (Fig. 1 A). A 1.7 kb BamHI fragment containing HIS3 from p8866 (provided by J. Broach, Princeton University) was ligated into the EcoRI site of pMR2310 after blunting the ends with the Klenow fragment of DNA polymerase I. pMR2310 contained a 0.9-kb BglII-MluI fragment of DNM1. A complete deletion of DNM1 (Fig 1 A) was made using the γ method of Sikorski and Hieter (1989). The 5-kb Nac1 fragment from pMR2738, a DNM1 deletion clone containing downstream sequences, was ligated to a 700-bp Smal-MscI fragment containing upstream sequences (bp -300 to 400 in Fig. 1 A) creating plasmid pMR2969.

**Strains, Media, and Genetic Methods**

Yeast strains used in this study are listed in Table I. Yeast media and genetic techniques were as described by Rose et al. (1990). For carbon source utilization experiments, isogenic wild type (MS2288), single (MS2961, MS3128, and MS3165), double (MS3129, MS3163, MS3164), and triple deletion (MS3162) strains were streaked on YEP plates with different carbon sources and incubated at 13, 23, 30, and 27°C. Carbon sources tested were glucose, acetate, ethanol, glycerol, lactate, and glycerol plus ethanol. Bacterial strains (XLI-Blue and HB101) were manipulated as described in Sambrook et al. (1989). Yeast strains were transformed using lithium acetate (Ito et al., 1983).

Gene disruptions and deletions were constructed as described by Rothstein (1991). To disrupt DNM1, cells were transformed with a gel purified 2.7-kb Apal to Sac1II fragment from pMR2357. To confirm the disruption, candidate transformants were analyzed by Southern blotting (Rose et al., 1990). To make a complete deletion of DNM1, MS173, containing the dnm1 disruption was transformed with the 4.9-kb PmlI and EcoRI fragment from pMR2969. The desired integrant replaced the dnm1::HIS3 insertions with the deletion marked by URA3. The presence of the deletion in Ura- "His" transformants was confirmed by Southern blot hybridization. VPS1 deletions were constructed using pCKR3A (T. Stevens, University of Oregon, Eugene, OR) cut with XbaI and SacI. Transformants were confirmed to be vps1::LEU2 by Western blotting with rabbit polyclonal α-Vps1p (also from T. Stevens) and PCR amplification of genomic DNA. MGM1 was deleted using a mgm1::URA3 construct obtained from W. Fangman (University of Washington, Seattle, WA). Transformants were confirmed by their failure to grow on media with glycerol as the sole carbon source and by Southern blot hybridization. Double and triple dy-
namin-related deletion strains were constructed by sequential disruptions and confirmed by the methods listed for each gene.

Degenerate primers based on homology with rat brain dynamin (Obar et al., 1990), mouse Mxl (Staeheli et al., 1986), and Vpslp (Rothman et al., 1986) were obtained by the two step gene replacement method (described in Rothstein, 1991) using pDH107 (G. Sprague). The deletions of STE3 were obtained by the same two gene replacement method (described in Rothstein, 1991) using pDH107 (G. Sprague). The ste3 deletion phenotype was confirmed by the failure of the MATa cells to mate.

**Polymerase Chain Reaction**

Degenerate primers based on homology with rat brain dynamin (Obar et al., 1990), mouse Mxl (Stacheli et al., 1986), and Vpslp (Rothman et al., 1990) were used in the following combinations. (a) 514 and 512, (b) 514 and 513, (c) 515 and 512, and (d) 515 and 513. Primer sequences and degeneracy were: 514:GGGAATTCGXXA(G/A)(T/C)XXGTXG, 6,144-fold; 515:GGGAATTCGXXA(G/A)(T/C)XXGTXG, 65,536-fold; 512:GGGGATCCXCXGGX(A/T)(C/G)XGGXAT, 131,072-fold; and 513:GGGGATCCCXGGX(A/T)(C/G)XGGXAT, 6,144-fold.

PCR was conducted in 50 &mu;l under mineral oil (Sigma, St. Louis, MO). Reaction conditions were 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 100 pmol of each primer, 200 &mu;M deoxynucleotides dATP, dCTP, dGTP, and dTTP, (Pharmacia LKB Nuclear, Gaithersburg, MD), 2.5 &mu;g genomic DNA and 1.25 U of Taq Polymerase (Perkin-Elmer Cetus, Norwalk, CT). Yeast strains were SF383-1Da/pCR3 (pvs1Δ) and SF383-1Da (VP51) from T. Stevens. Reactions were conducted as follows: 5 cycles of 95°C 1 min, 37°C 2 min, 72°C 1 min, then 25 cycles of 95°C 1 min, 42°C 2 min, 72°C 1 min. Controls were run without genomic DNA and with each primer individually. Primer 515 amplified spurious sequences when acting as sole primer. Gel purified PCR products amplified from pvs1 deletion strain DNA were digested with BamHI and EcoRI, cloned into pRS306 (Sikorski and Hieter, 1989), and sequenced. PCR reactions were repeated with genomic DNA from an isogenic wild-type strain and were repeated with genomic DNA from an isogenic wild-type strain and were repeated with genomic DNA from an isogenic wild-type strain and were repeated with genomic DNA from an isogenic wild-type strain.

**Cloning, Physical Mapping, and DNA Sequencing of DNM1**

Colony hybridization of a yeast genomic library (Rose et al., 1987) in HB101 using DNM1-specific probes were performed according to Sambrook et al. (1989). Yeast chromosomes were electrophoretically separated, blotted to membranes and hybridized with a DNM1-specific probe as described in Rose et al. (1990). Prime a-cleavage grid filters were obtained from Linda Riles (Washington University School of Medicine, St. Louis, MO) and probed according to the protocol provided. The DNM1 probe hybridized to clone 4355.

Both strands of a 4.0-kb region containing the DNM1 gene and centromere 12 (CEN112) were sequenced using a combination of nested deletions (Ozkaynak and Putney, 1987) and subclones in Sikorski vectors (Sikorski and Hieter, 1989). Templates were sequenced using oligonucleotides (Princeton University), [35S]dATP (Amersham Corp., Arlington Heights, IL), and the Sequenase Kit (US Biochemicals, Cleveland, OH). Stretches of the second strand were sequenced using DNM1-specific oligonucleotides (Princeton University).

**Vacuolar Protein Sorting Assays**

Strains MS3031, MS3107, MS3161, and MS3349 were grown at 30°C to mid-exponential phase, washed and diluted in fresh YEPD media to equivalent concentrations. Cells were allowed to grow an additional 2 h. Proteins in the cell pellet were processed as described by Ohashi et al. (1982). Proteins in the supernatant were precipitated by adding trichloroacetic acid to 10%. Proteins samples were separated by SDS-PAGE, and analyzed by Western blotting with a 1:5,000 dilution of rabbit α-carboxypeptidase (α-CPY) (R. Schekman, University of California at Berkeley). The secondary antibody was conjugated to horseradish peroxidase (Amersham) and bands were visualized using an ECL Western detection kit (Amersham).

For the CPY pulse-chase experiments, cells were grown in synthetic complete media at 30°C until an OD600 of 0.5-0.7 was reached. Cultures were washed and resuspended to 2-3 OD600/ml in synthetic media lacking cysteine and methionine. Cells were starved for methionine for at least 30 min at 30°C. [35S]Translabeled was added to 25 to 100 μCiODs00 (ICN Radiochemicals, Irvine, CA). After 10 or 15 min labeling, the chase cocktail was added to a final concentration of 0.1% Tween, 0.1% methionine, 0.1 M (NH4)2SO4. Aliquots were taken at the indicated times, placed on ice and treated with NaN3 (to 10 μM) and cycloheximide (to 100 μg/ml). Cell lysis, immunoprecipitations, and autoradiography were conducted as described previously (Sclodine et al., 1993). Strains used in the assay were MS2288, MS2961, MS3128, and MS3161 (Fig. 3B). The levels of P1, P2, and mature CPY were quantitated using a PhosphorImager and ImageQuant Software (Molecular Dynamics, Sunnyvale, CA). Cricket Graph Software (Malvern, PA) was used to plot the rates of turnover or accumulation of the forms of CPY.

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**Table I. Yeast Strains Used in This Study**

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<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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Purification of 35S-ct-factor was described previously and showed specific binding to cells expressing the Ste2p receptor. Internalization Assays

The first time point (t = 0) was taken before the chase. Chase time points were pulse labeled and chased as described above. Immunoprecipitations of 35S-ct-factor were conducted with affinity-purified α-ct8p rabbit polyclonal antibody (a generous gift from T. Stevens, University of Oregon) as described previously (Nothwehr et al., 1995). A 6.5% SDS-polyacrylamide gel was used to resolve the bands and the precursor and mature forms were quantitated as described for CPY immunoprecipitations.

Recovery From Pheromone Arrest

MS3172 and MS3173 were grown to 10^7 cells/ml at 30°C. Cultures were split and half were exposed to 0.59 µM α-factor (synthesized at Princeton University). Pheromone treated cells were grown until the culture showed no further increase in optical density. Cells were large with single or multiple shmoo projections. Untreated controls were grown until they reached stationary phase as determined by optical density. Cells were washed several times in fresh YEPD and diluted to 10^7 cells/ml. Recovery from pheromone arrest was assessed by three methods: (a) Optical density was measured by a Klett-Summerson Colorimeter (Klett MFG Co., NY), (b) Bud formation was assessed by microscopy, and (c) Viable cell number was measured by plating dilutions onto YEPD agar. Recovery from stationary phase was assessed by optical density. Recovery after nonpheromone-induced G1 arrest was tested with strains containing the ade2-84 mutation. MY3468 and MY3542 were cultured at 37°C for 4 h at which point the cells were fully arrested (>95% unbudded). The cultures were then incubated at 23°C and analyzed for bud formation every 15 min.

Quantitative Matting After Repression of Ste3p Synthesis

MATa strains, MS3827 and MS3832, containing the ste3 deletion, were transformed with pSL552, a plasmid with STE3 under the GAL1 galactose inducible promoter (G. Sprague). The strains were grown at 30°C in synthetic media lacking uracil, with 2% raffinose as the sugar source (-URA raffinose), to midexponential phase. Galactose was added to 2% and the cultures were grown for an additional 4 h at 30°C after which glucose was added to 4% to repress synthesis of Ste3p. The t = 0 time point represents cells mating without repression. Subsequent time points represent the time in glucose media before mating. The mating partner, MS2298, was grown in YEPD media to a concentration of 1–2 x 10^7 cells/ml. Quantitative filter matings were conducted for 6 hours as described by Rose et al. (1990). Diploids were selected on plates lacking adenine and tryptophan.

Pulse–Chase Analysis to Determine the Turnover Rate of Ste3p

Isogenic MATa strains MS3031, MS3107, MS3385, and MS3386 and the negative control strains SY1372 MATa ste3A (G. Sprague) and MS3666 MATa were pulse labeled for 10 min as described above for CPY sorting. The first time point (t = 0) was taken before the chase. Chase time points were taken at 15, 30, and 60 min. At each time point 500 µl of cells were added to an equal volume of ice-cold stop solution (20 mM Na3, 20 µM KCl). Cells were kept on ice for 15 min and centrifuged. Cell pellets were frozen in liquid nitrogen and stored at -70°C. Proteins were solubilized as described in Davis et al. (1993). Before immunoprecipitation, the labeled extracts were preincubated with PAS (Pharmacia) for 30 min on ice. The extracts were preclarified by centrifugation for 30 s at ~14,000 g and the supernatant was added to PAS that had been preadsorbed with α-Ste3p polyclonal antibody (from G. Sprague). The remaining steps of the immunoprecipitation were conducted as described previously (Davis et al., 1993). Separate experiments confirmed that the turnover of Ste3p was dependent on the vacuolar protease Pep4p. The half-life of Ste3p was quantitated as described for CPY.

Internalization Assays

Strains and protocols for purification of the ^35S-α-factor were generously provided by D. Jenness (University of Massachusetts Medical Center, Worcester, MA). Purification of ^35S-α-factor was described previously (Schandel and Jenness, 1984). The pheromone had a specific activity of 2 Ci/mmol at the time of the binding studies. The quality of the purified pheromone was assessed by thin layer chromatography (Dulic et al., 1991). A minor oxidized product was present in the binding analyses. The ^35S-α-factor showed specific binding to cells expressing the Ste2p receptor (MS3173), but not to those lacking the receptor (MS3107, MATa or D1213-7, 3, MATa ste2Δ). In addition, binding was restored to D1213-7 when the STE2 gene was provided on a plasmid (pJBK008). Finally, specific binding to cells possessing Ste2p was competitively inhibited with unlabeled pure synthetic α-factor. Internalization experiments were conducted as described in detail by Dulic et al. (1991).

Lucifer yellow accumulation was assessed as described in Dulic et al. (1991) except that the washes were conducted as specified by Munn and Riezman (1994). Exponentially growing strains MS3172 and MS3173 were incubated for 0.5, 1.0, and 1.5 h in the presence of Lucifer Yellow CH (Sigma). Photographs were taken with identical exposure times and developing conditions.

Immunofluorescence of Ste3ΔΔ65p After Pheromone Treatment

Immunofluorescence of Ste3ΔΔ65p after pheromone treatment was as described by Davis et al. (1993) with modifications. MS3385 and MS3386, MATa pep4 strains, were transformed with pSL2006, to express c-myc tagged Ste3ΔΔ65p, (Nick Davis, University of Michigan). Strains were grown at 30°C in -URA raffinose synthetic media to midexponential phase. Galactose was then added to 2% and the cultures were allowed to grow for an additional 4 h. For the t = 0 time point, a 10-ml aliquot of the culture was taken for immunofluorescence analysis (Roberts et al., 1991). To repress further receptor synthesis, glucose was added to the remaining cultures to a final concentration of 4%. The experimental cultures were treated with an equal volume of cell-free α-factor culture supernatant, while the control cells were treated with equivalent supernatants from cells overexpressing α-factor (SY70). The α-factor supernatant was from a saturated culture of SM1587, a strain overexpressing α-factor (S. Michaelis, Johns Hopkins University, Baltimore, MD). The cultures were placed back at 30°C and assayed at 0.5, 1.0, and 1.5 h after pheromone treatment.

Preparation of cells for immunofluorescence was as described previously (Roberts et al., 1991) except that spheroplasting was performed with Oxalyticase (Enzogenetics, Corvalis, OR) at 20 µg/ml for 1.5 h (Davis et al., 1993). 9E10 α-c-myc (Princeton Monoclonal Facility) was the primary antibody and FITC-α-mouse IgG (Sigma) was the secondary antibody. Cells were quantitated for the percentages of staining and for the percentages of cells displaying the reported phenotype. Controls included strains carrying the Ycp50 vector instead of pSL2006 and experimental cells incubated with no primary antibody.

Results

Identification of a Novel Dynamin-related Gene, DNM1

PCR was used to identify a new dynamin-related family member in Saccharomyces cerevisiae. We designed degenerate oligonucleotides to highly conserved regions (Fig. 1 B) in three family members: mouse Mxl (Staeheli et al., 1986), Vps1p (Rothman et al., 1990), and rat brain dynamin I (Obar et al., 1990). Genomic DNA from a strain with a deletion in the VP51 gene was used to enrich novel family members. Two of the four possible primer combinations gave specific products. Primer combination 515-512 amplified a region of MGM1, a dynamin-related gene found to be important in maintenance of the mitochondrial genome (Jones and Fangman, 1992; Guan et al., 1993). Primer combination 515-512 amplified a portion of DNM1, a new family member. A fragment of the cloned DNM1 PCR product was used to probe a centromeric based yeast genomic library (Rose et al., 1987). Two full-length clones, and four partial clones were obtained by screenings of the library.

The DNM1 locus was mapped using genetic and molecular techniques. Hybridization of a DNM1 probe to a blot of yeast chromosomes separated by pulsed-field gel electrophoresis (Rose et al., 1990) localized DNM1 to chromosome 12 (data not shown). The placement of DNM1 on
**Figure 1.** (A) Map of the insertion and deletion at the DNM1 locus. Restriction sites shown are as follows: H, HindIII; B, BglII; P, PstI; E, EcoRI; X, XbaI; M, MluI. CEN12 is depicted as a black dot. The numbers below the DNM1 gene correspond to the numbers in the sequence in B. The numbers below the URA3 containing fragment correspond to those published in Sikorski and Hieter (1989). (B) The nucleotide sequence of the DNM1 gene. The open reading frame for Dnmlp is shown below the nucleotide sequence. The numbers in the left hand column are for the nucleotide sequence and the numbers in the right hand column are for the amino acid sequence. The arrows show the location and orientation of the degenerate primers discussed in the Materials and Methods. The CGARRI box marks the end of extensive homology between the dynamins and the DnmlpNpslp sub-group. The consensus GTP binding site is indicated by asterisks. Sequence data are available from EMBL/Genbank/DDBJ under accession number L40588.

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chromosome 12 was further defined using an ordered yeast genomic library. A probe from within the *DNM1* gene hybridized with clone 4355 from the prime λ phage library. A map of the phage clone suggested that *DNM1* might be linked to *CEN12*. Genetic mapping confirmed that *DNM1* is tightly linked to a fully functional centromere (data in Gammie and Rose, in press). Sequence analysis revealed that *DNM1* is the most proximal gene to the right of *CEN12* and is oriented such that transcription would proceed towards the centromere (Fig. 1 A). The chromosomal configuration of *DNM1* is strikingly similar to *VPS1*, another dynamin-related gene, which is immediately adjacent to *CEN11* and also is oriented with the reading frame directed toward the centromere (Yeh et al., 1991).

Nucleotide sequence analysis showed that the 2.3-kb open reading frame of the *DNM1* gene encodes a putative protein of 85 kD, designated Dnmlp (Fig. 1 B). Like other dynamin-related family members, Dnmlp contains a tripartite GTP binding consensus site in the amino-terminus (starred residues, Fig. 1 B). A dendrogram of representative family members (Fig. 2) allowed for the classification of four subgroups, each named for a prototypic member: dynamin, Vpslp, Mx, and Mgm1p. The Mgm1p subgroup at present contains a single member. Dnmlp and Vpslp are placed in a separate subfamily, although the homology of these proteins with dynamin extends quite a bit beyond that which is detected for the Mx proteins and Mgm1p. For example, Dnmlp, Vpslp, and the dynamin/shibire isoforms, but not the other superfamily members, contain a S/CGARRI box (amino acids 397–404 in Dnmlp). This site marks the carboxy-terminal end of the highly homologous region for Dnmlp and Vpslp with the dynamin/shibire subgroup.

In spite of the extended homology, Dnmlp and Vpslp lack structural elements correlated with important properties in the dynamins. Dynamin and shibire isoforms contain a pleckstrin homology (PH) domain (Haslam et al., 1993), corresponding to amino acids 510–633 in dynamin. The PH domain is postulated to be important for protein-protein interaction or for membrane association (Ferguson et al., 1994). The homology of Dnmlp and Vpslp with the dynamins is extremely low in the region corresponding to the PH domain, suggesting that the regulatory signals for Dnmlp and Vpslp are different. Moreover, Dnmlp and Vpslp also lack a proline-rich extension in the carboxy-terminal region, shown to be important for regulating the GTPase activity of dynamin (Herskovits et al., 1993b).

Proline-rich tails are present in all of the dynamin and shibire isoforms isolated thus far. Microtubules (Shpetner and Vallee, 1992), acid phospholipids (Tuma et al., 1993), and certain SH3 containing proteins (Booker et al., 1993; Gout et al., 1993; Herskovits et al., 1993b; Ando et al., 1994; Miki et al., 1994; Scaife et al., 1994; Seedorf et al., 1994) can bind to the carboxy-terminal proline-rich region and stimulate the GTPase. In addition, protein kinase C phosphorylation sites appear to reside primarily in this proline-rich extension (Liu et al., 1994). Phosphorylation by protein kinase C also stimulates the GTPase of dynamin (Robinson et al., 1993). Hence, two regions thought to be important regulatory domains in dynamin, the PH domain and the proline-rich tail, are lacking in the yeast proteins Dnmlp and Vpslp.

Dnmlp and Vpslp share other distinguishing features. In the extended superfamily the conserved GTPase domain is often interrupted by a region with low homology of variable length (from 2–46 amino acids). Dnmlp and Vpslp possess the largest spacer regions, which are of comparable size (44 and 46 amino acids respectively) and charge (estimated pI of 4.7 and 5.7, respectively). The functional significance of these spacers is unknown.

Direct sequence comparisons showed that Dnmlp is most like Vpslp. However, using a program that allowed for significant gaps, Vpslp is as related to dynamin and shibire as it is to Dnmlp with respect to total amino acid sequence identity (Fig. 2). However, both Dnmlp and Vpslp are more like shibire in the conserved amino-terminal portion of the protein than they are to each other, suggesting that what has been conserved from a common progenitor is not the same in the two yeast proteins (Fig. 2). Dnmlp and Vpslp are most like each other in the carboxy-terminal region, with the *Caenorhabditis elegans* dynamin (Dynl) being the next closest relative in that less conserved domain. Finally, both Dnmlp and Vpslp are most distantly related to the third yeast family member,
Mgm1p. Taken together, the sequence comparisons suggest that Dnm1p is not the cognate homologue of dynamin.

A DNM1 Deletion Mutant Is Viable

Both a disruption of DNM1 within the highly conserved GTPase region, and a complete deletion of the gene (Fig. 1 A) showed that the protein is not essential for life under a variety of growth conditions. Isogenic single, double and triple deletion strains were constructed with the three yeast dynamin-related genes, DNMI, VPS1, and MGM1, to determine if any of the known family members are functionally redundant. All of the mutant strains were viable indicating that the family members do not share an essential overlapping function. A nonessential, redundant function might exist, but was not detected in the vacuolar protein sorting or mitochondrial function assays that we performed.

Although viable, each of the yeast dynamin-related deletion strains had a unique profile for growth on different carbon sources. All of the strains were able to grow on glucose, even at elevated temperatures. Consistent with a strong mitochondrial defect, each strain containing a deletion at the MGM1 locus was unable to grow on any of the non-fermentable carbon sources, regardless of the temperature.

In contrast, dnml and vps1 disruption strains showed impaired utilization for certain non-fermentable carbon sources. However, these defects were evidenced primarily at elevated temperatures, e.g., 37°C. Mutant dnml strains showed impaired growth on ethanol and acetate, while vps1 strains did not grow well on glycerol, lactate, and acetate. The effects were superimposable in that the dnml vps1 double mutant was defective for use of all of the non-fermentable carbon sources tested. Ethanol and acetate are two-carbon compounds that require normal peroxisomal and mitochondrial function to be metabolized. In contrast, glycerol and lactate breakdown requires only normal mitochondrial function. Therefore, the dnml defects may be due to impaired peroxisomal function.

One consequence of the impaired utilization of acetate in the dnml mutant may be the observed deficiency in sporulation. As is the case with vps1/spol5 (Yeh et al., 1991), homozygous dnml diploids showed a sporulation defect (47% of wild type); however, the effect was not as severe as vps1, which was unable to sporulate. In summary, since dnml and vps1 deletion strains show only subtle temperature-sensitive growth defects on certain nonfermentable carbon sources, we concluded that these phenotypes were most likely due to indirect effects of their principal roles in the cell.

Dnm1p Is Not Involved in Sorting or Secretion of the Vacular Proteins CPY and Pho8p

Given the high degree of homology between Vpslp and Dnm1p we checked for defects in vacuolar protein sorting in dnml disruption strains. Carboxypeptidase Y (CPY) is a soluble vacuolar protein used to identify vacuolar protein sorting mutants (reviewed in Rothman et al., 1989). In wild type, CPY is found in three forms, the two glycosylated precursors (P1) and (P2), and the mature, proteolytically cleaved form (m-CPY). m-CPY is produced from the P2 form only after successful delivery to the vacuole. The predominance of m-CPY in the cell is indicative of proper vacuolar protein sorting. If CPY is not sorted correctly, the P2 form is secreted into the culture medium.

Western blotting analysis with α-CPY antibody showed that a dnml disruption strain and the isogenic wild-type control do not accumulate appreciable levels of P2 in the culture medium, whereas vps1 deletion strains did secrete the P2 form (Fig. 3 A). The sorting defect of vps1 was unaffected in the dnml vps1 double mutant. The Western blot analysis of the cell pellets also showed that the dnml disruption strain was indistinguishable from the isogenic wild-type control, both of which contain m-CPY, while in the vps1 strains no m-CPY was detectable. The upper band present in all four lanes for the cell pellet fractions is a cross-reacting band with slower mobility than all of the forms of CPY (compare cell pellet lanes with lanes 3 and 4 containing the largest form, P2).

Although the dnml strain did not appear to have a CPY sorting defect by Western analysis, we performed pulse-
chase experiments to examine the extent and rate of CPY sorting. The first experiment compared the four strains described for the Western blot. For the *dnml* disruption strain and the isogenic wild-type control, the ratios of the forms of CPY in the pulse, and the levels of m-CPY in the chase were indistinguishable. In contrast, *vpsl* strains showed strong defects in CPY processing. Again, we found that the *vpsl* defect was unaffected by a disruption of the *DNM1* gene (Fig. 3 B).

To determine whether the rate of sorting was altered in a more subtle fashion, we performed an extensive time-course of sorting for the *dnml* and isogenic wild-type control. Quantitative analyses of the time course of processing of CPY showed that the disappearance of P1 (Fig. 3, C and D) and the concomitant loss of P2 and accumulation of m-CPY (Fig. 3, C and E) were indistinguishable for the *dnml* mutant and the wild-type control. We conclude from these data that Dnmlp was not required for the rate of sorting of the soluble vacuolar protein CPY.

From the CPY experiments we may also conclude that *dnml* strains do not have an obvious secretory defect. Accumulation of P1 found in the endoplasmic reticulum, or of the Golgi-associated P2 form would have indicated a possible early secretory defect. We observed that pre-CPY forms did not accumulate in *dnml* strains when compared to the wild-type strain. Therefore, Dmnlp is unlikely to play a role in early secretion. Strains with a disruption in the *DNM1* gene also do not appear to have a late secretory defect since the CPY levels secreted in *vpsl* is the same as in the *vpsl dnml* double mutant (Fig. 3 A). In addition if late secretion were affected, we would have expected to see P2-CPY accumulate in the double mutant cells during the pulse–chase, but the levels were unchanged when compared with the *vpsl* single mutant (Fig. 3 B).

Membrane and soluble proteins have been reported to have different requirements for proper sorting to the vacuole (reviewed in Nothwehr and Stevens, 1994). For this reason we performed a pulse–chase experiment to follow the fate of a membrane associated vacuolar protein, Pho8p. The rate of loss of the Pho8p precursor and accumulation of the mature form occurred with identical kinetics in the *dnml* and wild-type strains (Fig. 4 A). The half life of the precursor for both strains was ~5 min. We therefore conclude that Dmnlp is also not required for sorting of the membrane associated vacuolar protein Pho8p. These data, combined with the results from the CPY experiments, allowed us to conclude that Dmnlp does not participate in the primary cellular role of its highly homologous family member Vpslp.

**dnml** Mutant Strains Are Not Defective for Lucifer Yellow Accumulation, a Marker for Fluid Phase Endocytosis

For Dmnlp, the next most homologous subfamily of dynamin-related proteins is comprised of the dynamin/shibire isoforms. Given this homology and that dynamin/shibire isoforms are involved in endocytosis, we tested *dnml* disruption strains for endocytic function. The accumulation of Lucifer Yellow within the vacuole is an assay for fluid-phase endocytosis (Dulic et al., 1991). The uptake of LY is time and energy dependent (Riezman 1985), and requires certain proteins important for endocytosis (Munn and Riezman, 1994 and references therein).

Looking at various time points (30, 60, and 90 min), we found that the LY accumulation in *dnml* strains (Fig. 5 D)
was indistinguishable from the wild-type control (Fig. 5 B). These data indicate that there is no obvious fluid-phase defect associated with a disruption of the \textit{DNM1} gene. However, although the extent of accumulation looked identical in the two strains, a minor effect on the rate of fluid-phase endocytosis might not be detected by these methods.

Finally, Nomarski optics showed that the vacuolar morphology of \textit{dnm1} (Fig. 5 C) is identical to the isogenic wild-type control (Fig. 5 A), a condition that is necessary for proper assessment of LY accumulation. From the vacuolar morphology of the Nomarski and LY uptake images, we may also conclude that \textit{dnm1} strains are not class C \textit{vps} mutants, which lack coherent vacuoles (Raymond et al., 1992).

\textbf{Disruption of the DNM1 Gene Results in a Lag in Recovery from Pheromone Arrest}

Our LY data suggested that there was no obvious fluid-phase endocytic defect in the \textit{dnm1} mutant. However, a role for Dnm1p in receptor-mediated endocytosis had not been excluded. It is of interest that dominant-negative mutations in mammalian dynamin affect receptor-mediated, but not bulk fluid-phase, endocytosis (Herskovits et al., 1993a; Damke et al., 1994).

Initial evidence for a role for Dnm1p in receptor-mediated endocytosis came from our analysis of the recovery rate of fluid-phase endocytosis might not be detected by these methods.

The liquid assays were performed with MATa strains lacking Bar1p, a protease that degrades extracellular \(\alpha\)-factor (reviewed in Sprague and Thorner, 1992). The delay was quantitated by several methods: culture optical density, colony forming units, and bud formation. Changes in colony forming units (not shown) and in culture optical density (Fig. 6 A) corresponded to when daughter cells were liberated from the mother cell. These methods showed that the delay was \(~90\) min. Bud formation was more sensitive for detecting when the cells resumed normal growth. The delay measured by this method was \(~60\) min (Fig. 6 C).

Two controls were performed to ensure that the defect was specific to recovery from pheromone arrest rather than cell cycle arrest. There was no difference between disrupted and control \textit{DNM1} strains in the recovery after stationary phase arrest (Fig. 6 B). In addition, the \textit{dnm1} disruption strains resumed growth after \(\alpha\)-factor arrest with wild-type kinetics. We analyzed the rate of bud formation after releasing the cells from \(\alpha\)-factor arrest caused by a temperature sensitive mutation in the \textit{CDC28} gene. After release, there was no difference between the \textit{dnm1} disrupted and \textit{DNM1} control strains (Fig. 6 D). Hence, the \textit{dnm1} strains showed a lag in recovery specifically from pheromone arrest, a finding that is consistent with a receptor-mediated endocytic defect.

\textbf{Disruption of DNM1 Delays the Turnover of the Ste3p Pheromone Receptor}

Davis et al. (1993) developed a screen to obtain mutants with receptor-mediated endocytic defects. The screen relied on the fact that \textit{MATa} cells must express Ste3p receptors to mate (Michaelis and Herskowitz, 1988) and that the receptors are rapidly turned over (Davis et al., 1993). An enhanced ability to mate after turning off Ste3p synthesis is the initial criterion for an endocytic defect in the screen. Quantitative matings were performed to determine whether \textit{dnm1} disruption strains exhibited an increased ability to mate after blocking Ste3p synthesis. The data from these experiments reflect receptor function over the period of mating (6 h). The time points signify the time elapsed be-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Delay in recovery from pheromone arrest. The figure shows the recovery of \textit{dnm1} (black squares) and of the wild-type \textit{DNM1} control (open diamonds) from (A) pheromone arrest, and (B) stationary phase arrest, plotted for OD (Klett Units); and from (C) pheromone arrest, and (D) \textit{cdc28-4} G1 arrest plotted for bud emergence over time in min.}
\end{figure}
Table II. Quantitative Mating of MATα dnmlΔ and Wild Type After Repression of Ste3p Synthesis

<table>
<thead>
<tr>
<th>Strain (MATα)</th>
<th>Diploids Formed</th>
<th>Mating Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
<td>15 min</td>
</tr>
<tr>
<td>dnml</td>
<td>2.7 × 10⁴</td>
<td>2.0 × 10⁴</td>
</tr>
<tr>
<td>Wild type</td>
<td>9.7 × 10⁴</td>
<td>4.7 × 10⁴</td>
</tr>
</tbody>
</table>

*Diploids assayed by complementation of auxotrophic markers after matings on glucose of MATa cells to the MATa cells which received no prior repression (0 min) or repression of Ste3p synthesis with glucose for 15 or 60 min before mating.

tween receptor repression and exposure to the mating partner. These experiments showed that the dnml strain mated threefold more efficiently than did the isogenic wild-type control at all time points (Table II). These data suggested that the Ste3p receptor functioned longer in the dnml disruption strain than in the wild type. This effect was not as strong as that reported for a mutation affecting the late endosome, renl-1 (Davis et al., 1993). However, the extent of the defect is similar to that reported for a pep4 deletion mutant, in which the receptor half-life is increased (Davis et al., 1993). In summary, the data from the quantitative matings are consistent with a defect in the pathway leading to the turnover of the Ste3p receptor.

We determined the half life of the Ste3p pheromone receptor using pulse–chase analyses. These experiments allowed us to assay more directly for an endocytic defect in dnml disruption strains. Routinely, after immunoprecipitation with a-Ste3p antibody, a single major band was detectable upon autoradiography (Fig. 7 B). Based on independent experiments we found that the rate of Ste3p turnover decreased 2.4 ± 0.6-fold in the dnml disruption strain over the isogenic wild-type control (Fig. 7 A), thereby changing the half life from 20 min for wild type to 40–60 min for dnml mutants. These data suggested that Dnmlp is involved in the constitutive turnover of the Ste3p receptor. The pulse–chase experiments assay total cellular protein and do not identify the cellular localization of the receptor. Therefore, we wanted to next determine if the defect was a consequence of impaired internalization, or inefficient delivery of the receptor to the vacuole.

Dnmlp Does Not Act at the Earliest Step in Endocytosis of 35S-α-Factor

The early steps of receptor-mediated endocytosis, binding and internalization of ligand, were examined quantitatively using 35S-α-factor. The rate and extent of internalization in the dnml disruption strain were indistinguishable from the isogenic control strain (Fig. 8). We observed a half time of internalization of 15 min for both strains. A two- to threefold difference in the kinetics of uptake would have given an easily detected difference in the half-time for internalization of 30–45 min for dnml rather than the observed 15 min. Hence, a lag in internalization would have significantly shifted the curve to the right for dnml. From these data, we conclude that the rate and extent of the earliest step of receptor-mediated endocytosis seemed to be unaffected by the dnml disruption. These experiments suggested that the dnml defect must be accounted for in later steps of endocytosis.

A Disruption of DNMI Impedes Delivery of Ste3Δ365p to the Vacuole During Ligand-induced Endocytosis

The steps in the endocytic pathway that are subsequent to internalization involve transit of endosomes to the vacuole.
ole. The fate of the Ste3p pheromone receptor after removal from the plasma membrane can be traced using immunofluorescence (Davis et al., 1993). Normally Ste3p is endocytosed by both the constitutive and ligand-induced pathways (Davis et al., 1993). A truncation of Ste3p, designated Ste3Δ365p, fails to be turned over effectively via the constitutive pathway but will be internalized upon exposure to the α-factor ligand (Davis et al., 1993). Therefore, the Ste3Δ365p deletion construct allowed us to examine the ligand-induced endocytic pathway specifically. An epitope-tagged, Ste3Δ365p was expressed in galactose containing media via a galactose regulated promoter, and receptor synthesis was shut off by the addition of glucose to the cultures. An extract containing α-factor was also added, to trigger ligand-induced endocytosis and time points were taken 0.5, 1.0, and 1.5 hours after exposure. The experiments were conducted in MATα strains with a deletion in PEP4, to prevent intracellular degradation, allowing delivery of the tagged receptor to the vacuole to be assessed by immunofluorescence.

Before exposure to pheromone, the wild-type and dnm1 strains both showed the expected surface staining (Fig. 9, A–D). Receptor synthesis was shut off with glucose, and the controls were exposed to extracts containing α-factor, for which MATα cells have no receptor. Under these control conditions, after 0.5, 1.0, and 1.5 h the cells continued to show prominent surface staining (98% of stained cells for both wild-type and dnm1 strains) (Fig. 9, E–H), indicating that the receptor had been retained on the surface in the absence of true ligand. Some staining was detectable in the vacuole of both strains over time (for example, see F), however the predominant staining was on the surface.

In the experimental condition, further receptor synthesis was blocked with glucose and the cells were exposed to α-factor extracts, the true ligand. These conditions revealed a major difference between the dnm1 and the wild-type DNM1 control strains. Internalization and delivery to the vacuole in the DNM1 strain proceeded rapidly, and the receptor was found predominantly in the vacuole (88% of the stained cells by 1.5 h) (Fig. 9, I–J). As early as 30 min after exposure to ligand, the majority of the staining was in the vacuole for the wild-type strain (data not shown). Staining of the vacuole with a vacuolar specific antibody confirmed that the large organelle visualized with Nomarski optics is indeed the vacuole (data not shown). In addition, under identical conditions, except that Pep4p was functional, the staining with the α-c-myc antibody disappeared rapidly (within 30 min) after α-factor exposure for the wild-type DNM1 strain, showing that the receptor was being efficiently delivered to the vacuole, the organelle containing Pep4p-dependent degradative activity (data not shown).

After exposure to α-factor pheromone, the staining pattern in the dnm1 disruption strain differed significantly from the wild-type control strains. For both strains, the majority of the Ste3Δ365p receptor was cleared from the surface within 30 min after ligand addition (data not shown). In the wild type, vacuolar staining was seen within 30 min to 1 h. However, in 90% of the dnm1-stained cells, the protein was not delivered to the vacuole even after 1.5 h (Fig. 9, K–L). Vacuolar-exclusion was observed to a limited extent in wild type at the earliest time point (30 min, data not shown), suggesting that the dnm1 phenotype is a lag in the normal pathway, and not a condition peculiar to the mutant.

The staining pattern in dnm1 is different from that exhibited by another endocytosis mutant, renl, where the receptor is concentrated in a pre-vacuolar compartment, or endosome (Davis et al., 1993). In addition, the localization of Ste3Δ365p within the dnm1 cells was not affected in a

![Figure 9. Immunofluorescence of Ste3Δ365p after exposure of pheromone. A, C, E, G, I, and K are the Nomarski images of the cells and B, D, F, H, J, and L are the corresponding FITC image of the cells showing Ste3Δ365p localization before pheromone treatment (B for wild-type DNM1 and D for dnm1), 1.5 h after mock pheromone treatment (F for wild-type DNM1 and H for dnm1), and 1.5 h after α-factor treatment (J for wild-type DNM1 and L for dnm1).](image-url)
PEP4 strain, indicating that the dispersed endosome-like structures did not have Pep4p-dependent hydrolytic activity (not shown). Thus, we conclude from these experiments that the dnml mutant is able to internalize the receptor, but is defective for delivery of the receptor to the vacuole during ligand-induced endocytosis.

Discussion

Dnm1p and Dynamin-related Proteins

We have found that Dnm1p, a dynamin-related protein, is important in the yeast receptor-mediated endocytic pathway. In dnml disruption strains, initial binding and internalization of ligand and receptor occur at normal rates, but subsequent delivery of the receptor to the vacuole is hindered. Hence, like dynamin and shibire, Dnm1p plays a role in endocytosis. However, the phenotypes of the dnml disruption suggest that Dnm1p acts at a different step than the one postulated for mammalian dynamin. Unlike Dnm1p, dynamin acts at the level of internalization (van der Bliek et al., 1993; Herskovits et al., 1993a; Damke et al., 1994; Takei et al., 1995; Hinshaw and Schmid, 1995). Dynamin is thought to act at the level of coated vesicle formation at the plasma membrane (Hinshaw and Schmid, 1995; Takei et al., 1995). At least two explanations could account for the differences in phenotypes between dynamin and dnml mutants. The disruption of the DNM1 gene used in this study was a null mutation, while the dynamin proteins used in the endocytosis assays were dominant negatives. The dominant dynamin proteins might perturb endocytosis at an earlier stage of the process than would the absence of wild-type protein.

A more likely alternative is that Dnm1p might not be a cognate homologue of dynamin, implying that different dynamin-related proteins act at a variety of steps in the endocytic pathway. Important regulatory domains found in dynamin are lacking in Dnm1p, leading one to conclude that Dnm1p responds to different cellular cues. This conclusion leaves open the possibility that a true functional dynamin homologue exists in yeast. The degenerate primers that amplified the three known yeast family members were used in a PCR reaction with genomic DNA from a triple deletion strain. No new bands appeared, suggesting that all of the dynamin-related proteins in yeast that are detectable with these primers had been identified. However, in light of mounting sequence data from a variety of dynamin-related proteins, new primers might yield different results. Furthermore, the rapid progress of the yeast genome project will eventually reveal all of the dynamin-related family members.

The Role of Carbon Source Utilization and Endocytosis

Like other yeast genes involved in endocytosis (Raths et al., 1993), DNM1 is not an essential gene. A disruption in DNM1 does result in subtle growth defects at elevated temperatures on certain nonfermentable carbon sources. The metabolic deficiencies could simply reflect a generalized cellular defect such as impaired membrane function caused by endocytic abnormalities. For example, peroxisomal function, might be particularly sensitive to the changes caused by a dnml disruption (see Results). An alternative explanation is that the role in metabolism is more direct, but not essential. One possibility is that Dnm1p-dependent endocytic function is important in a process such as autophagy. Autophagy is characterized, in part, by the rapid turnover of cytosolic glycolytic components under nutrient deficient conditions such as growth on non-fermentable carbon sources (Chiang and Schekman, 1991; Takeshige et al., 1992). At least one endocytosis mutant has been implicated in autophagy (Chiang and Schekman, 1994). Support for this hypothesis would come from direct measurements of the autophagic response in dnml disruption strains.

Dnm1p and Receptor-mediated Endocytosis

Our results suggest that Dnm1p is unlikely to play a role in fluid-phase endocytosis, but is involved in both constitutive and ligand-induced receptor-mediated endocytosis. The half life of turnover during constitutive endocytosis was increased two to three fold, from 20 min for wild type to 40-60 min in a dnml disruption strain. However, the block visualized during ligand-induced endocytosis showed that the majority of the receptor persisted in the cytoplasm up to 90 min. The more dramatic difference seen with the immunofluorescence assay might result from saturation of the endocytosis system with overexpressed receptor protein. Alternatively, the Ste3A365p receptor used in the ligand-induced assay might transit through the endocytic pathway more slowly than the full-length receptor. A final possibility is that Dnm1p may be more important for ligand-induced than for constitutive endocytosis.

Dnm1p and the Yeast Endocytic Pathway

Dnm1p seems to have a unique placement in the yeast endocytic apparatus. As discussed in the introduction, the yeast endocytic components either contribute to internalization, or affect efficient delivery of endosomes to the vacuole. In addition, all of the previously identified endocytic mutants that alter endosomal trafficking to the vacuole have either a secretory defect, or vacuolar protein sorting abnormalities. The dnml mutant is unique in that the intracellular trafficking defect appears to be specific to endocytosis. The kinetics of sorting of vacuolar proteins was not affected by the dnml mutation. From these results we conclude that Dnm1p is not required for vacuolar protein sorting and that the two- to threefold effect on constitutive endocytosis is not likely to be a consequence of generalized slowing of membrane trafficking events.

Previous studies have shown that internalized α-factor is found in two biochemically distinct compartments in the cell, representing early and late endosomes (Singer and Riezman, 1990; Singer-Krüger et al., 1993). In keeping with this model, Dnm1p might act before the late endosome, where the endocytic pathway merges with the post-Golgi secretory branch destined for the vacuole. This placement is consistent with the absence of a vacuolar protein sorting defect in dnml mutants. An alternative explanation is that Dnm1p acts in a parallel endocytic pathway that does not merge with the late endosome used in vacuolar protein sorting.

Since Dnm1p acts at the level of delivery to the vacuole in the endocytic pathway, we must reconcile this with the
delay in recovery from pheromone arrest, and the prolonged mating in Ste3p shut-off experiments seen in the *dnml1* mutant strains. Several explanations are possible, one is that a block in delivery to the vacuole might result in a generalized slowing of the endocytic process, leaving sufficient quantities of signaling receptors on the surface to produce the in vivo effects. Surface staining of the untruncated, fully functional α-factor pheromone receptor even under overexpression conditions was difficult to detect by immunofluorescence (Gammie and Rose, unpublished observations). For this reason, the possibility that some receptors are retained on the surface cannot be ruled out. However, the internalization experiments make the explanation of slowed receptor removal from the surface much less likely.

Alternatively, the intracellular block in the pathway might allow for recycling of the receptor to the surface, an effect that is not seen under normal conditions (Jenness and Spatrick, 1986). Recycling has been postulated for the phenotype of *renl-1*, a mutation that results in defects in the late endosome (Davis et al., 1993). Our experiments suggest that if recycling is the mechanism for prolonged mating in *renl-1* strains after repressing Ste3p synthesis, then by comparison, *dnml1* strains appear to have less efficient recycling (see the quantitative matings of Davis et al., 1993). If this hypothesis is correct, then different compartments within the cell may be more amenable to recycling. For example, recycling may be more efficient from the late endosome than from an early endosome.

A final alternative is that removal of the pheromone and receptor from the surface is not sufficient to block intracellular signaling. Endocytosis is typically thought to down-regulate intracellular signaling by first causing dissociation of the ligand from the receptor and ultimately by targeting the internalized material to a degradative organelle. Binding studies (Dulic et al., 1991) have shown that α-factor binding persists in low pH (a pH of 1.1 is used to strip the ligand from the receptor). Thus, the acidity of the early endosome might be insufficient to dissociate the ligand from the receptor. In addition, the early endosome would not contain proteases in transit from the Golgi to the vacuole. Hence, in the *dnml1* disrupted strain, during delayed transit to the vacuole a pheromone–receptor complex might remain competent for transmitting the signal for the mating response. If this hypothesis is correct, our data suggest that delivery to the vacuole and subsequent degradation of the ligand–receptor complex is an important aspect of attenuation of signal transduction during the mating response.

In conclusion, one of the broader implications of our findings with Dnm1p is that the superfAMILY of dynamin-related proteins can act at multiple steps of vesicular trafficking. It will be of considerable interest to identify specific roles of dynamin-related proteins in other organisms in which the pathways are well characterized.

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