The Role of Myo2, a Yeast Class V Myosin, in Vesicular Transport

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Abstract. Previous studies have shown that temperature-sensitive, myo2-66 yeast arrest as large, unbudded cells that accumulate vesicles within their cytoplasm (Johnston, G. C., J. A. Prendergast, and R. A. Singer. 1991. J. Cell Biol. 113:539–551). In this study we show that myo2-66 is synthetically lethal in combination with a subset of the late-acting sec mutations. Thin section electron microscopy shows that the post-Golgi blocked secretory mutants, secl-1 and sec6-4, rapidly accumulate vesicles in the bud, upon brief incubations at the restrictive temperature. In contrast, myo2-66 cells accumulate vesicles predominantly in the mother cell. Double mutant analysis also places Myo2 function in a post-Golgi stage of the secretory pathway. Despite the accumulation of vesicles in myo2-66 cells, pulse-chase studies show that the transit times of several secreted proteins, including invertase and α factor, as well as the vacuolar proteins, carboxypeptidase Y and alkaline phosphatase, are normal. Therefore the vesicles which accumulate in this mutant may function on an exocytic pathway that transports a set of cargo proteins that is distinct from those analyzed. Our observations are consistent with a role for Myo2 in transporting a class of secretory vesicles from the mother cell along actin cables into the bud.

The yeast Saccharomyces cerevisiae exhibits a high degree of cell polarity. The localized incorporation of cell surface constituents causes the daughter cell to approach the size of the mother cell at which time cell division ensues. Bud initiation as well as bud development rely on a wide array of gene products including ACT1, BUD1-5, BEM1, CDC24, CDC42, and CDC43 (for a recent review see Madden et al., 1992). Polarized growth occurs via the fusion of secretory vesicles with the plasma membrane of the bud. Recent work (Preuss et al., 1992) suggests that components of the secretory pathway, such as the ER and Golgi complex, may be transported into the bud to enable polarized growth. A polarized actin cytoskeleton is associated with the localized deposition of cell surface components (Adams and Pringle, 1984; Kilmartin and Adams, 1984), whereas intact microtubules are not necessary for this process (Huffaker et al., 1988). Temperature-sensitive mutations in the essential yeast gene encoding actin, ACT1, result in vesicle accumulation, the predominance of unbudded cells, and the accumulation of the secretory protein invertase within cells at the restrictive temperature (Novick and Botstein, 1985).

A variety of proteins which interact with actin have been identified in yeast (see references within Welch et al., 1994; Brown, 1993; Bretscher et al., 1994). These include yeast capping protein (Capi and Cap2) (Amatruda et al., 1990), actin-binding protein (Abpl), fimbrin (Sac6), profilin (Pfyl), cofilin (Cofil), and tropomyosin (Tpm1). SLA1 and SLA2 encode proteins required for the formation of the cortical actin cytoskeleton (Holtzman et al., 1993). Recently, the requirement for actin and fimbrin in yeast endocytosis has been demonstrated (Kubler and Riezman, 1993). The characterization of the yeast actin cytoskeleton at the ultrastructural level has provided new insight into its possible functions in the cell. Cortical actin patches were found associated with the cell surface via plasma membrane invaginations, giving rise to speculations about the role of this structure in cell wall growth (Mulholland et al., 1994). Since polarized growth in yeast is associated with a polarized actin cytoskeleton, it has been suggested that an actin-based motor fulfills the role of transporting vesicles into the bud.

To date, four myosins have been identified in yeast. First, the MYO1 gene product was identified as the conventional (or class II) myosin in yeast and shown to be required for cytokinesis (Watts et al., 1987). A class I myosin, Myo3, has been identified, but its function is as yet unknown (Goodson, H. V., and J. A. Spudich. 1993. Mol. Biol. Cell. 4:156a). In the past few years, a rapidly growing number of unconventional myosins have been identified from a variety of organisms (Cheney and Mooseker, 1992). The yeast MYO2 and MYO4 gene products are class V myosins (Johnston et al., 1991; Haarer et al., 1994). They contain a myosin-like head domain which is conserved among the members of this class, a series of IQ motifs involved in calmodulin binding, and a relatively short coiled-coil region followed by a globular distal tail domain. The COOH-terminal portion of these myo-
sins are more similar to each other than to any other myosins. Class V myosins have been implicated in the transport of membrane-bounded organelles in a variety of organisms (Titus, 1993). Mutations in the murine Dilute locus, which encodes an essential class V myosin, leads to the loss of coat color in dilute mice (Merce et al., 1991). This phenotype arises from a defect in the transfer of pigment-containing melanosomes into the keratinocytes of the developing hair shaft. Melanosomes in dilute mice exhibit mostly a perinuclear distribution, whereas in wild-type mice the melanosomes are evenly distributed between the cell body and dendrites (Wei, M. C., V. Ipe, and J. Mercer. 1994. Mol. Biol. Cell. 5:381a). More severe alleles of dilute result in death from neurological seizures. An additional member of the class V myosin family is the chick brain myosin V protein (Espindola et al., 1992). Chick brain myosin V has been well-characterized biochemically as an actin-activated ATPase, and it has been localized to the perinuclear region and the tips of cellular processes in neurons (Espindola et al., 1992; Cheney et al., 1993). This localization suggests an association of chick myosin V with vesicles. Temperature-sensitive myo2-66 mutants, defective in a yeast class V myosin, arrest as large, un budded cells with mislocalized actin and chitin (Johnston et al., 1991). Furthermore, myo2-66 cells accumulate vesicles throughout their cytoplasm upon shifts to the restrictive temperature. Nonetheless, the perinuclear enzyme invertase is still secreted in myo2-66 mutants. Based on these findings Johnston et al. (1991) proposed that Myo2 functions as a motor to transport post-Golgi secretory vesicles into the growing bud. It was hypothesized that the vesicle accumulation in this mutant is due to a kinetic lag in their transport, since the vesicle motor is defective.

To investigate the possible role of Myo2 in various vesicular transport pathways, we have further characterized the myo2-66 mutant through analysis of genetic interactions, epistasis tests and through pulse-chase studies of protein transport. Using electron microscopy, we have also examined the phenotype of myo2-66 cells shifted to 37°C for short time periods to determine the primary site of vesicle accumulation. These studies indicate that Myo2 is involved in a post-Golgi stage of the secretory pathway. Our observations are consistent with the model that Myo2 functions to transport vesicles from the mother cell into the bud.

Materials and Methods

Yeast Strains, Media, and Reagents

The Saccharomyces cerevisiae strains used in this study are described in Table 1. Cells were grown in YP medium containing 1% Bacto-yeast extract and 2% Bacto-peptone (Difco Laboratories Inc., Detroit, MI), with either 2% glucose (rich medium, YPD) or 0.1% glucose (low glucose media). To change the growth medium, cells were pelleted and resuspended in prewarmed, fresh medium. For the pulse-chase studies, cells were grown in minimal media containing 0.7% yeast nitrogen base without amino acids (Difco), 2% glucose, and supplemented for auxotrophic requirements as described by Sherman et al. (1974) when necessary. The absorbance of cell suspensions was measured at 599 nm in a model No. 4054 spectrophotometer (Pharmacia LKB Biotechnology, Piscataway, NJ).

Yeast Genetic Techniques

Yeast transformation was performed by the alkali cation treatment method (Ito et al., 1983) and transformants were selected on minimal medium supplemented by the appropriate amino acids at 25°C. Crosses of strains, sporulation of diploids, tetrad dissections, complementation, and mating type determination were performed as described by Sherman et al. (1974). Double mutants were constructed between myo2-66 and various sec or end genes by crossing the appropriate strains, sporulating the diploids at 25°C, and dissecting the resulting tetrads. In cases where 2:2 segregation of temperature-sensitive: temperature-resistant spores was observed, the temperature-sensitive spores were picked and tested for mating type and auxotrophy. MATa spores were selected and complementation analysis confirmed that the strains were defective in both MYO2 and SEC or END genes.

Thin Section Electron Microscopy

Cultures were grown overnight in YPD medium to an A900 of 1.0. For synchronization experiments, 13.2 OD units were harvested and incubated in...
60 ml of YPD, pH 4.0, containing 1 μM α factor for 4.5 h at 25°C. Cells were then washed in YP, resuspended in 60 ml fresh YPD, and incubated at 25°C for 1 h to induce small bud formation. Temperature shifts were then performed by incubating the cells at 37°C for short periods. At time points, cells were harvested by filtration and washed with 10 ml 0.1 M cacodylate, pH 6.8, reharvested and resuspended in 10 ml 0.1 M cacodylate, pH 6.8, containing 1% glucose at 37°C for 1 h. After incubation at 37°C for 1 h, the cells were incubated on ice overnight at 4°C. Fixed cells were then washed twice in 50 mM phosphate buffer, pH 7.0, and incubated in 2 ml 50 mM phosphate buffer, pH 7.0, containing 0.125 mg/ml Zymolyase 100-T for 40 min at 37°C. After being washed twice in ice cold 0.1 M cacodylate buffer, the cells were resuspended in 2 ml 0.1 M cacodylate and incubated for 1 h at 4°C. The cells were washed in sterile H2O three times, resuspended in 2 ml of 2% aqueous uranyl acetate, and incubated at room temperature for 1 h. This was followed by two washes in sterile H2O, formation into 2% agar blocks, and finally dehydration by a series of graded ethanol washes (1 wash each of 50, 70, 90, and 100% EtOH), and then four washes of fresh 100% EtOH. The dehydrated blocks were washed in 100% acetone and embedded in a mixture of SPURR epoxy medium. Thin sections were cut, stained with lead citrate, and poststained with uranyl acetate. Sections were analyzed on a Philips 301 electron microscope at 80 kV.

to count vesicles, budded cells containing a nucleus and/or vacuole and a well-defined neck region were selected at random at low magnification. The neck region was considered as part of the mother cell in determining vesicle number. A vesicle was considered to be in the bud when the bud neck was ~3% wider than the mother cell width, and the membranes could be clearly distinguished were counted. The cytoplasmic area of each cell was determined using an NIH Image program with a Zepha ME-3 magnetic tablet (Carl Zeiss, Inc.) by outlining the surface of each mother cell and bud, and subtracting all internal surfaces including the nucleus, mitochondria, lipid droplets, and vacuoles. The number of vesicles per μm² was determined for both the mother and bud of each cell. At least 25-30 samples from 1-3 independent experiments were analyzed per time point.

**Pulse Chase Experiments**

**myo2-66 and wild-type (NY 1006 and NY 1033) strains were transformed with pRSB81 and 10 μM plasmid containing the** SUC2 **gene which encodes invertase (Carlson and Botstein, 1982). The resulting invertase-overproducing strains were grown overnight in supplemented minimal media with 2% glucose at 25°C to an OD₆₀₀ of 0.25. 25 OD units of cells were harvested by centrifugation and resuspended in medium containing 0.1% glucose to derepress invertase synthesis. After incubation in this medium for 30 min at 25°C, cells were pelleted and resuspended in 25 ml of spheroplast buffer containing 0.1% glucose, 1 M sorbitol, 50 mM KPi (pH 7.5), 50 mM β-mercaptoethanol, and 1 mg/ml Zymolyase-100 T. Spheroplasts were formed by incubation at 25°C for 90 min with gentle agitation, and then pelleted gently and resuspended in 50 ml recovery buffer containing supplemented minimal media, 0.1% glucose, 1 M sorbitol, and 20 mM Pipes (pH 6.8). The spheroplasts were incubated at 37°C for 60 min to allow cell membrane permeabilization. The recovered spheroplasts were then shifted to 37°C for 30 min, pelleted gently and resuspended in 1 ml of recovery buffer. After incubating the recovered spheroplasts for 10 min at 37°C, 35S-trans label was added to 0.3 MCI/ml and the spheroplasts were labeled for 4 min at 37°C. The chase was initiated by the addition of cysteine and methionine (in recovery buffer) to a final concentration of 5 mM, at various time points of chase (at 37°C), cell aliquots were removed to tubes on ice and pelleted in a microfuge to separate cells from supernatants (containing the periplasm). The cell pellets were resuspended in 100 μl of 1% SDS and the supernatants were made 1% in SDS and both samples were boiled for 3 min. Both samples were diluted with 900 μl of IP buffer (2% Triton X-100 in PBS) and microcentrifuged for 10 min at 4°C to remove insoluble material. The supernatants were added to tubes containing 2 μl anti-invertase antibody (generous gift of Dr. Susan Ferro-Novick) and incubated overnight at 4°C. The immunoprecipitates were collected on protein A-Sepharose beads (60 μl of a 5% suspension per sample), washed twice with urea wash buffer containing 2 M urea-200 mM NaCl-100 mM Tris (pH 7.2)-1% Triton X-100, washed twice with 0.5% β-mercaptoethanol and solubilized by boiling in 50 μl 1× SDS sample buffer for 5 min. Aliquots were boiled and treated as described above. The samples were then boiled for 5 min, diluted with 0.9 ml of IP buffer containing 10 mM Tris-CI, pH 8.0, 0.1% Triton-X100, 2 mM EDTA, 1 mM PMSF, and 1:1000 vol of PIC and clarified as described above. The supernatant was removed to a tube containing 3 μl of anti-S73 antibody (generous gift of Dr. George Sprague) plus 70 μl of a 3% suspension of protein A-Sepharose CLAB and incubated for 90 min at 4°C. (The antibody was first preadsorbed to the protein A-Sepharose beads for 4 h at 4°C). An aliquot of the supernatant was also counted by liquid scintillation. Immunoprecipitates were collected as described above and washed four times with IP buffer plus 0.1% SDS. The samples were then boiled for 5 min in SDS sample buffer containing 1% β-mercaptoethanol, counted by liquid scintillation and loaded on 10% SDS gels with prestained high molecular weight standards. After electrophoresis, the gels were treated as described above.

**Secretion of factor was examined by the method described in Graham and Emr (1991).** MATα cells were grown to mid-log phase in supplemented minimal media with 2% glucose overnight at 25°C, and then 10 ODU/ml cells in media containing 1 mg/ml BSA were shifted to 37°C for 30 min.

**Abbreviations used in this paper.** ALP, alkaline phosphatase; CPY, carboxypeptidase Y; DB, digestion buffer; wt, wild type.

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[Image 0x0 to 612x792]
Whole cells were pulse labeled at 37°C for 8 min with 200 µCi/ml 35S-
label (or in vitro labeling mix), and then chased with 10 mM cysteine
and methionine in media. At time points, cell aliquots were removed and
pelleted in a microfuge to separate cells from media. Both samples were
precipitated with 3% TCA to terminate the chase, washed twice in cold ace-
tone, dried, resuspended in 100 µl buffer containing 50 mM Tris, pH 7.5,
6 M urea, 1 mM EDTA, 1% SDS, and lysed by vortexing with glass beads.
After boiling the samples, a MAb lysate of equivalent cell density was
added to reduce background in the immunoprecipitation. 900 µl of dilution
buffer containing 1.25% TX-100, 190 mM NaCl, 6 mM EDTA and 50 mM
Tris, pH 7.5, was added to each sample. Samples were clarified as described
above, and then anti-a factor antibody was added to the supernatant and
incubated overnight at 4°C. The immune complexes were collected as de-
scribed and washed twice in dilution buffer and once in 1% β-mercapto-
ethanol. The washed immunoprecipitates were boiled for 5 min in 20 µl
SDS sample buffer containing β-mercaptoethanol. These samples were
counted by liquid scintillation and loaded onto a 17% SDS-polyacrylamide
gel with prestained low molecular weight standards. After electrophoresis,
the gels were fixed and treated as described elsewhere (Julius et al., 1984a).

Results

Double Mutants of myo2-66 and a Subset of
Late-acting sec Genes Are Inviable at 25°C

Mutant alleles of genes which function on the same pathway or on parallel pathways often display synthetic effects in combina-
tion with each other. Many of the temperature-sensitive sec mutants blocked at the post-Golgi stage of the secretory
pathway are inviable even at the permissive temperature when combined to generate haploid double sec mutants (Salminen
and Novick, 1987). If the MYO2 gene product is directly or indirectly involved in facilitating the transport of post-Golgi
vesicles, one might predict that MYO2 would exhibit interactions with genes required for secretion. Thus we initially ex-
amined double mutants between myo2-66 and alleles of the early- and late-acting sec genes (Novick et al., 1980, 1981).

Crossovers were made between myo2-66 and representative alleles of ER-Golgi (early) sec genes and post-Golgi (late)
sec genes. Diploids were sporulated at 25°C and 12–24 tetrads per cross were dissected and analyzed. In crosses with
seven of the late-acting sec genes (sec2-41, sec4-8, sec5-24, sec8-9, sec9-4, sec10-2, and sec13-1), approximately
one-fourth of the meiotic products were inviable at 25°C. This subset of late-acting sec genes also genetically interacts
with sec4-8 (Salminen and Novick, 1987). Duplication of SEC4 efficiently suppresses the myo2-66 growth defect at 30°C and partially suppresses at 34°C (data not shown). Together, these data demonstrate that myo2-66 genetically interacts with a subset of late-acting SEC genes, and suggests that the Myo2 protein may function at the post-Golgi stage of the secretory pathway or on a parallel pathway that shares many of the same compo-
nents.

myo2-66 Mutants Are Not Defective in the Processing or Transport of Invertase or α Factor

Previous studies have shown that the myo2-66 mutation does not block the secretion of invertase at the restrictive tempera-
ture (Johnston et al., 1991). However, these studies did not test for the accumulation of an intracellular pool of invertase
or for a kinetic lag in export. We examined the transit time of invertase secretion by pulse-chase analysis. In yeast, in-
vertase is found in the internal and external fraction of the Golgi-PM
gel with prestained low molecular weight standards. After electrophoresis,
the gels were fixed and treated as described elsewhere (Julius et al., 1984a).

Table II.* Summary of Viability of myo2-66, sec Double
Mutants at 25°C

| ER-Golgi | myo 2-66 x | sec 4-8 x |
|----------|------------|-----------|
| sec 7-1  | +          | +         |
| sec 12-4 | +          | +         |
| sec 13-1 | +          | +         |
| sec 14-3 | +          | +         |
| sec 16-2 | +          | +         |
| sec 17-1 | +          | +         |
| sec 18-1 | +          | +         |
| sec 19-1 | +          | +         |
| sec 20-1 | +          | +         |
| sec 22-3 | +          | +         |
| Golgi-PM | sec 1-1    | +         |
|          | sec 2-41   | +         |
|          | sec 3-2    | +         |
|          | sec 4-8    | NA        |
|          | sec 5-24   | +         |
|          | sec 6-4    | +         |
|          | sec 8-9    | +         |
|          | sec 9-4    | +         |
|          | sec 10-2   | +         |
|          | sec 15-2   | +         |

* Strains were crossed at 25°C, diploids sporulated, and tetrads dissected and
analyzed on YPD plates at 25°C.
both mutant and wild-type strains. After 5 min of chase, most of the glycosylated invertase was found secreted into the external fraction in both myo2-66 and wild-type samples. By 10 min of chase, all of the glycosylated invertase was found externally in both strains. As a control for cell lysis we have quantitated the cytoplasmic, unglycosylated form of invertase by scanning densitometry and found that less than 15% of it is found in the secreted fraction (data not shown).

Furthermore, no other form of invertase was found within the cells, indicating that there was no internal accumulation of the secretory form of the protein or of any degradation products. This experiment clearly shows that newly synthesized invertase in myo2-66 cells is efficiently transported into the periplasm with wild-type kinetics.

To address whether myo2-66 cells are defective in the processing or export of a protein which is normally secreted into the media, we next examined the transport of the peptide pheromone α factor. The α factor mating pheromone is processed through the secretory pathway (Julius et al., 1984a), and its final modification occurs via the conversion of pro-α factor to mature α factor (a tridecapeptide) by peptidases in a late Golgi compartment (Julius et al., 1984b; Wilcox and Fuller, 1991). The pulse chase experiment in Fig. 2 shows that mature α factor is secreted from both wild-type and mutant cells within 5 min of chase after labeling for 8 min. Furthermore, although α factor is found associated with the cells at the initial zero time point, a significant amount is rapidly chased into the medium by 2 min. In addition, no other form of α factor is found accumulated within the cells (not shown). Thus, the α factor processing compartment is functional in myo2-66 cells, and the secretion of α factor and invertase is unimpaired.

**Transport of Major Glycoproteins Is Normal in myo2-66 Cells**

To examine the fates of additional exported proteins in myo2-66 cells, we studied the transport of several major secreted glycoproteins which bind to Con A. A pulse chase protocol was performed on regenrated wild-type and myo2-66 spheroplasts, followed by Con A precipitation. Five major secreted glycoproteins (30, 35, 45, 116, and 200+ kD) were observed in both strains (Fig. 3). As Fig. 3 illustrates, wild-type (B) or myo2-66 (A) cells show similar kinetics of glycoprotein transport out of the cell. While the 116-kD glycoprotein is secreted even at the 0-min chase time point (after a 5-min pulse), the 200+, 45, and 35 kD glycoproteins appear by 15 min of chase. Finally, the 30-kD glycoprotein is chased into the secreted fraction by 30 min. Recently a gene encoding a high copy suppressor of myo2-66, SMT1, has been identified and shown to have homology to kinesin (Lillie and Brown, 1992). We reasoned that if Smyl functions as a microtubule-based motor to bypass a myo2-66 defect, disrupting microtubules in a myo2-66 mutant may cause an observable block in secretion. To address this possibility, we treated myo2-66 cells with nocodazole during the Con A precipitation experiment. Samples processed in parallel for immunofluorescence showed that essentially all of the tubulin staining had disappeared in the nocodazole-treated cells (data not shown). Fig. 3 shows that myo2-66 and wild-type strains exhibit similar kinetics of glycoprotein secretion whether treated with (+) or without (−) nocodazole. It
treatment was applied to samples in the invertase pulse-chase experiment described in Fig. 1. In this case also, both mutant and wild-type samples. A similar nocodazole in nocodazole did not affect the kinetics of invertase secretion nocodazole-treated cells at the 15-min chase time point in pulse chase experiments on regenerated spheroplasts followed by protease protection and Con A precipitations to examine the pathway of glycoprotein transport to the cell surface in myo2-66 and wild-type cells. From these pulse-chase/protease protection studies, we determined that the same set of four glycoproteins (roughly 30, 50, 80, and 200+ kD in size) reached the cell surface and became protease-sensitive with the same kinetics in both myo2-66 and wild-type cells (data not shown). The pattern of protease-resistant bands was also found to be identical in both strains (not shown). Thus, a number of different membrane-bound and secreted proteins are correctly processed and delivered normally in myo2-66 mutants, even under conditions of microtubule disruption.

Figure 3. Glycoproteins are secreted normally from myo2-66 cells, even after nocodazole treatment. Regenerated spheroplasts treated (+) with 15 μg nocodazole and 1% DMSO or mock-treated (−) with 1% DMSO were pulse labeled with 35S-trans label for 5 min, and then chased for the times indicated. The cellular and media fractions were separated by microcentrifugation, and the media fraction was denatured by boiling in 1% SDS, and then precipitated with Con A Sepharose beads. The precipitates were washed, boiled in sample buffer, and loaded onto a 10% SDS polyacrylamide gel. The arrows indicate the appearance of the major secreted glycoproteins. All of these glycoproteins are transported out of the cell in both myo2-66 (A) and wild-type (B) cells shifted to 37°C. Their approximate molecular masses are (from top to bottom): 200+, 116, 45, 35, and 30 kD.

seems that the 35-kD glycoprotein is more evident in the nocodazole-treated cells at the 15-min chase time point in both mutant and wild-type samples. A similar nocodazole treatment was applied to samples in the invertase pulse-chase experiment described in Fig. 1. In this case also, nocodazole did not affect the kinetics of invertase secretion in myo2-66 cells (not shown).

Eleven major proteins are found on the yeast cell surface, and most of these bind to Con A (Novick and Schekman, 1983). It has previously been shown that secretory mutants are defective in the transport of all of these major cell surface glycoproteins (Novick and Schekman, 1983). We conducted pulse chase experiments on regenerated spheroplasts followed by protease protection and Con A precipitations to examine the pathway of glycoprotein transport to the cell surface in myo2-66 and wild-type cells. From these pulse-chase/protease protection studies, we determined that the same set of four glycoproteins (roughly 30, 50, 80, and 200+ kD in size) reached the cell surface and became protease-sensitive with the same kinetics in both myo2-66 and wild-type cells (data not shown). The pattern of protease-resistant bands was also found to be identical in both strains (not shown). Thus, a number of different membrane-bound and secreted proteins are correctly processed and delivered normally in myo2-66 mutants, even under conditions of microtubule disruption.

Since the plasma membrane ATPase accumulates in secretory vesicles found in late-acting sec mutants (Walworth and Novick, 1987), we sought to determine if this protein was associated with vesicles in myo2-66 cells. Through immunofluorescence staining, pulse chase experiments, and column fractionation, we found that the plasma membrane ATPase of myo2-66 cells is correctly distributed on the yeast cell surface, is stable, and is not found on vesicles (data not shown).

Vacuolar Transport Is Not Defective in myo2-66 Mutants

Since transport to the cell surface and out of the cell seemed to occur normally in myo2-66 cells, we next examined whether transport from the Golgi to the vacuole is impaired in the myo2-66 mutant. It has recently been shown (Raymond et al., 1992; Vida et al., 1993) that the transport of vacuolar proteins is directed from the Golgi complex via a prevacuolar compartment (or endosome) to the vacuole. Since the delivery of soluble and membrane-bound vacuolar proteins is thought to occur by somewhat different mechanisms (Klionsky and Emr, 1989), we chose to study the transport of a representative protein from each class.

The soluble vacuolar protease carboxypeptidase Y (CPY) undergoes modifications in the ER and Golgi before arriving in the vacuole where it is cleaved by the PEP4 gene product to its mature 61-kD active form. The ER (67 kD) form or Golgi (69 kD) form accumulate in mutants which are blocked at these stages of the secretory pathway (Stevens et al., 1982; Franzusoff and Schekman, 1989). Pulse chase studies on whole cell lysates indicated that the transit time for CPY processing and transport was similar to wild type in myo2-66 mutants (Fig. 4). By 2 min of chase, the ER (pl) form of CPY was partially chased into the Golgi (p2) and the mature (m) form in both the mutant and in wild-type cells. By 5 min of chase, CPY was completely in its mature vacuolar form in both strains. Furthermore, by immunoblot analysis, CPY was not inappropriately secreted from myo2-66 cells (data not shown).

Alkaline phosphatase (ALP) is an integral membrane glycoprotein of the yeast vacuole (Klionsky and Emr, 1989; Kaneko et al., 1987). ALP is processed from an inactive

Figure 4. The soluble vacuolar protease carboxypeptidase Y (CPY) is processed and transported normally in myo2-66 mutants. Cells (NY 1006, myo2-66, and NY 1033, wt) were shifted to 37°C for 30 min, and then were pulse labeled for 2 min with 35S-trans label and chased with 10 mM cysteine and methionine for the times indicated. At each time point, cells were washed into 10 mM NaN₃ and lysed with glass beads. The samples were immunoprecipitated with anti-CPY antibody as described in Materials and Methods. The final washed, solubilized precipitates were loaded onto 8% SDS gels and processed for autoradiography. The ER precursor form of CPY (pl = 67 kD) is found in both myo2-66 (A) and wild-type (B) samples at the zero time point, and then it is partially chased into the Golgi form (p2 = 69 kD) and mature form (m = 61 kD) by 2 min in both strains. All of the CPY is in its mature, vacuolar form by 5 min in both strains.
precursor of 76 kD to a mature enzyme of 72 kD upon reaching the vacuole. Klionsky and Emr (1989) proposed that ALP may differ somewhat from CPY in its final targeting to the vacuole. To examine this transport pathway, whole cells were pulse labeled and immunoprecipitated with anti-ALP antibody. In both mutant and wild-type cells, the precursor form of ALP was converted to the mature 72-kD enzyme within 5 min of chase after a 5-min pulse (Fig. 5). Therefore, both a membrane-bound and a soluble vacuolar protein are processed and transported efficiently in both wild-type (B) and myo2-66 (A) cells.

Figure 5. Alkaline phosphatase (ALP) is processed and delivered to the vacuole normally in myo2-66 and wild-type cells. Cells (NY 1005, myo2-66, and NY 180, wt) were pulse labeled with 35S-trans label for 5 min and chased with 10 mM cysteine and methionine for 0, 5, and 20 min as described in Fig. 3. The washed, solubilized precipitates were loaded onto 10% gels and processed for autoradiography. Within 5 min of chase, the precursor form (76 kD) of ALP is converted to the mature 72-kD vacuolar form in both wild-type and myo2-66 cells.

myo2-66 Cells Are Not Defective in the Transport or Internalization of the a-Factor Receptor

Since we found that neither the secretory pathway nor the vacuolar transport pathway are slowed or disrupted in myo2-66 cells, we sought to determine if endocytosis or transport of a membrane receptor is affected in this mutant. It has recently been shown that myo2-66 mutants are not defective in the uptake of labeled α factor (Kubler et al., 1994), indicating that they do not exhibit a defect in receptor-mediated endocytosis. Therefore we examined the transport and constitutive endocytosis of the a-factor receptor. This receptor is normally found on the surface of cells where it functions to bind the a-factor mating pheromone and induce the mating response. The a-factor receptor undergoes internalization after a transient association with the cell surface, and is delivered to the vacuole via endosome-like structures (Davis et al., 1993). Once it reaches the vacuole, the receptor is degraded by the PEP4 gene product. To follow the a-factor receptor as it is brought to the cell surface, and then retrieved back into the cell, we employed the protocol described by Davis et al. (1993). PEP4 deficient myo2-66 strains were pulse labeled and chased as described (see Materials and Methods) and before spheroplasting, the cells were treated with and without protease to degrade any a-factor receptor at the cell surface. We found that the transport of newly synthesized a-factor receptor to the cell surface in myo2-66 mutants occurred at rates similar to wild-type cells, as a portion of the labeled receptor became accessible to exogenous protease within 10 min of chase after a 10-min labeling period (Fig. 6). However, from the amount of full-length receptor observed at the 10-min chase time (Fig. 6), it appears that the amount of receptor that becomes protease sensitive in the mutant is somewhat reduced compared to wild type. Protease sensitivity of the receptor was also confirmed by the appearance of a 30-kD digestion product at the 10-min chase time point (Fig. 6). The a-factor receptor became largely inaccessible to protease by 30 min of chase in the myo2-66 mutant, as indicated by the absence of the 30-kD digestion product and the increase in the abundance of the full-length protein by 30 min. In contrast, the full-length protein was still reduced in abundance at 30 min of chase in wild-type cells and the 30-kD proteolytic fragments persisted through 90 min of chase. These results demonstrate that a large fraction of a-factor receptor does reach the cell surface by 10 min in both the mutant and wild-type strains. However, a pool of receptor may either fail to reach the surface in the myo2-66 cells or it may reach the surface and become rapidly internalized by 10 min of chase. A block in receptor export should result in an increased vesicular pool of a-factor receptor in myo2-66 cells. However, no such vesicular pool of a-factor receptor was found in fractionation.

Figure 6. Surface accessibility of the a-factor receptor in myo2-66,pep4::URA3 cells. Mat α, PEP4 deficient, wild-type (NY 579) or myo2-66 (NY 1125) cells were shifted to 30°C for 30 min, and then pulse labeled for 10 min with 35S in vitro labeling mix, chased with 10 mM cysteine and methionine for 0, 10, 30, 60, 90, and 150 min, washed into 10 mM KF and NaN3 on ice and digested with 1 mg/ml Pronase E for 1 h at 37°C before spheroplasting and lysis. Both mutant and wild-type strains show the receptor protected from protease at zero min, but then at 10 min of chase the receptor band diminishes, while a 30-kD proteolytic fragment appears (arrow), indicating that the receptor has reached the cell surface. The receptor then becomes internalized into a protease-protected compartment by 30 min in the mutant strain as evidenced by the absence of the degradation product, while in wild-type cells a portion of the 30-kD fragment persists even at 90 min of chase.
experiments of myo2-66 lysates (not shown). In addition, we have observed that the a-factor receptor appears to be internalized more completely in the myo2-66 mutant (Fig. 6).

**myo2-66 Mutants Accumulate Vesicles Predominantly in the Mother Cell upon Short Incubations at 37°C**

Wild-type yeast cells (Fig. 7 a) contain very few secretory vesicles within their cytoplasm, as vesicle turnover is rapid. Johnston et al. (1991) have shown that in myo2-66 mutants, 80-100 nm vesicles accumulate throughout the cell after a 3-h shift to the nonpermissive temperature. To understand the origin and destination of these vesicles, we characterized the vesicle-accumulation phenotype of myo2-66 cells as a function of time and cell polarity through an electron microscopic study performed on cells synchronized at the small budded stage. Mating type "a" cultures were arrested with α-factor at the unbudded stage of the cell cycle, and then small

![Figure 7. Electron microscopy of wild-type or sec6-4 cells shifted to 37°C for 10 min. Cells were prepared for TEM as described in Materials and Methods. a is a representative wild-type cell (NY 13) which does not accumulate secretory vesicles, and b is a typical sec6-4 (NY 17) cell showing vesicle accumulation mainly in the bud. Bar, 1 μm.](image)
buds were allowed to form in fresh medium at the permissive temperature. Cells were then shifted to 37°C for 0, 10, or 20 min, and subsequently fixed and sectioned for TEM (see Materials and Methods).

In two post-Golgi sec mutants, sec1-1 and sec6-4, upon a short incubation at the nonpermissive temperature, secretory vesicles accumulated within the bud of the cell (Fig. 7b). These cells showed a quantitative increase in both vesicle number and vesicle density in the bud up until 15 min at 37°C (Fig. 10A). After this time point, the vesicle density in the mother cell (mainly the neck region) began to increase as the bud was saturated (Fig. 10B). This reflects the inability of these vesicles to fuse with the plasma membrane of the bud. In contrast, myo2-66 cells shifted to 37°C for 10 min accumulated vesicles almost exclusively in the mother cell (Fig. 8b). Even at the permissive temperature (0 min) myo2-66 cells showed some vesicle accumulation within the cytoplasm of the mother cell and few vesicles in the bud (Fig. 8a). After 20 min (Fig. 8c) at the restrictive temperature, myo2-66 mutant cells continued to show increased vesicle accumulation predominantly in the mother cell while few vesicles were found in the bud. Quantitation of vesicle accumulation in myo2-66 cells confirmed that the number and density of vesicles in the mother cell increased linearly with time (Fig. 10B), while staying constant, and low in number, in the bud (Fig. 10A). This phenotype of myo2-66 cells indicates a defect in the transport and/or fusion of vesicles with their correct target membrane and may reflect a block in vesicle transport into the bud.

**EM of Double Mutants**

Phenotypic analysis of double mutants can establish epistatic relationships (Huffaker et al., 1988; Kaiser and Schekman, 1990; Novick et al., 1981). We first sought to identify the relationship of MYO2 function with respect to the function of three different classes of SEC genes: those involved in ER to Golgi, intra-Golgi, or post-Golgi transport. Since the EM phenotype of the various sec mutants is clearly distinct from the myo2-66 phenotype, we were able to establish the relationships between several of these gene functions by performing EM analysis on various double mutants.

If MYO2 functions in a SEC-dependent pathway, a double mutant should exhibit a phenotype reflecting the earlier block. A myo2-66, sec6-4 double mutant was initially examined. sec6-4 mutants accumulated post-Golgi secretory vesicles in the bud when shifted to the restrictive temperature (Fig. 7b). In contrast, the myo2-66, sec6-4 double mutant accumulated vesicles in the mother cell (Fig. 9a), as did the myo2-66 single mutant (Fig. 9b). The quantitation of vesicle density in the mother and bud of the myo2-66, sec6-4 mutant was similar to the myo2-66 single mutant in that vesicle accumulation in the mother cell increased with time but remained constant in the bud (Fig. 10A and B and Table III). Thus, by this criteria alone, MYO2 appears to function before SEC6. However, unlike the myo2-66 mutant, the myo2-66, sec6-4 double mutant displays the same tight block in invertase secretion as the sec6-4 mutant alone (data not shown). Thus myo2-66 does not bypass a SEC6-requiring step in the secretory pathway. Taken together, the EM phenotype combined with the assay for internal invertase in the double mutant shows that this double mutant exhibits some phenotypic characteristics of each single mutant.  

![Figure 8](image_url)  
*Figure 8. Time course of myo2-66 cells shifted to 37°C for short periods. Cells (NY 829) were synchronized with a factor, released from G1 arrest, and allowed to form small buds at 25°C, and then incubated at 37°C for the following times (a) 0 min, (b) 10 min, and (c) 20 min. At the various time points, cells were fixed for TEM. Vesicle accumulation occurs predominantly in the mother cell with time, while few vesicles appear in the bud. Bar, 1 μm.*

To define the myo2-66 block with respect to other landmarks on the secretory pathway, double mutants in which myo2-66 was combined with ER to Golgi blocked sec alleles were examined. SEC13 facilitates ER to Golgi transport by catalyzing the budding of carrier vesicles (Salama et al.,...
1993), and secl3-1 mutant cells accumulate only ER membranes at the restrictive temperature. Double mutant, secl3-1, myo2-66, cells displayed the same morphological phenotype as the secl3-1 single mutant (Fig. 9 b). SEC18 is also required for transport between the ER and Golgi complex, catalyzing the fusion of carrier vesicles (Rexach and Schekman, 1991; Graham and Emr, 1991). A sec18-1 mutant builds up ER membranes and small (50 nm) ER-derived transport vesicles at the restrictive temperature (Novick et al., 1981). Double mutant, myo2-66, sec18-1, cells also accumulated ER membranes and some small vesicles at the non-permissive temperature (Fig. 9 d). Thus, Sec13 and Sec18

Figure 9. Electron microscopy of double mutants between myo2-66 and representative sec mutants after 10 min at 37°C. Double mutants were constructed and prepared for TEM as described in Materials and Methods. (a) myo2-66,sec6-4 (NY 895) accumulates vesicles predominantly in the mother cell, as seen in the myo2-66 single mutant. (b) myo2-66,sec13-1 (NY 922) accumulates only ER membrane as observed in the sec13-1 single mutant. (c) myo2-66,sec7-1 (NY 919) accumulates Golgi membranes as does the sec7-1 single mutant. (d) myo2-66,sec18-1 (NY 896) accumulates both ER-derived vesicles and ER membrane as does the sec18-1 single mutant. Bar, 1 μm.
A double mutant of myo2-66 must be active in order to generate the vesicles found in myo2-66 cells.

Next, a sec7-1, myo2-66 double mutant was examined. SEC7 is involved in intra-Golgi transport and possibly ER-Golgi transport (Franzusoff and Schekman, 1989; Franzusoff et al., 1991). At the nonpermissive temperature sec7-1 mutant cells accumulate Golgi membranes which are not readily observed in wild-type yeast cells by usual fixation methods. sec7-1, myo2-66 double mutant cells shifted to 37°C also exhibited the characteristic Golgi stacks of the sec7-1 mutant (Fig. 9 c). Therefore, myo2-66 appears to block after sec7-1, and Sec7 function is required to form myo2-66 vesicles.

Finally, to determine whether the vesicles in the myo2-66 mutant could be derived from an endocytic or recycling pathway, we constructed double mutants of myo2-66 with representatives from two stages of the endocytic pathway.

### Table III. Vesicle Accumulation in myo2-66 and sec6-4 Mutants at 37°C

| Strain          | Time at 37°C | Bud Density (vesicles/μm²) | Mother Density (vesicles/μm²) | Average number of vesicles |
|-----------------|--------------|---------------------------|-----------------------------|---------------------------|
|                 | min          | Bud                       | Mother                      |                           |
| sec7-1          | 0            | 6.6 ± 5.0                 | 0.5 ± 0.2                   | 12.2 ± 5.6                |
|                 | 10           | 17.2 ± 5.5                | 1.0 ± 0.3                   | 46.0 ± 12.9               |
|                 | 20           | 27.2 ± 8.0                | 2.7 ± 0.9                   | 46.0 ± 14.7               |
| sec6-4          | 0            | 18.6 ± 8.4                | 1.0 ± 0.4                   | 21.1 ± 10.4               |
|                 | 5            | 27.2 ± 8.6                | 1.7 ± 1.0                   | 48.1 ± 20.6               |
|                 | 10           | 33.4 ± 10.4               | 2.5 ± 1.1                   | 59.3 ± 32.3               |
|                 | 15           | 37.8 ± 9.8                | 5.2 ± 1.4                   | 59.0 ± 60.1               |
| myo2-66         | 0            | 7.5 ± 2.2                 | 5.0 ± 2.1                   | 17.0 ± 67.0               |
|                 | 10           | 6.9 ± 2.3                 | 7.3 ± 1.9                   | 16.0 ± 104                |
|                 | 20           | 7.5 ± 2.3                 | 9.5 ± 2.2                   | 16.0 ± 137                |
| myo2, sec6      | 0            | 3.2 ± 2.4                 | 3.1 ± 2.7                   | 9.0 ± 47.0                |
|                 | 10           | 3.3 ± 2.3                 | 5.5 ± 2.2                   | 8.3 ± 80.0                |
|                 | 20           | 3.5 ± 2.3                 | 7.9 ± 2.7                   | 9.8 ± 114                 |

*The density and number of vesicles located in both the bud and mother cell of sec7-1, sec6-4, myo2-66, and {myo2-66,sec6-4} cells after brief incubations at 37°C. Vesicle density was determined by dividing the number of vesicles within a particular region of the cell (bud or mother) by the corresponding area. Each value is the average of measurements made on 25-30 individual cells from 1-3 independent experiments ± SD.

**Discussion**

We have presented further characterization of the myo2-66 mutant, and investigated the possible role of Myo2 in various processes related to vesicular transport.
vesicular transport pathways. First, we examined the genetic interactions of this mutation with known sec mutations, and found that myo2-66 displays synthetic lethality with a subset of post-Golgi blocked sec mutations, but not with any mutations which act earlier in the secretory pathway. These interactions suggest that Myo2 functions on a pathway that also requires the late-acting SEC gene products. We can therefore speculate that Myo2 facilitates a transport pathway that involves fusion of vesicles with the plasma membrane, and not in transport from the ER to the Golgi complex. Liu and Bretscher (1992) have reported that myo2-66 is synthetically lethal with a disruption of the yeast tropomyosin gene, TPM1, and since the mutant phenotype of TPM1 disrupted cells is very similar to myo2-66 mutants (Liu and Bretscher, 1992), the two genes may be involved in concerted functions.

The myo2-66 mutant was previously shown to accumulate vesicles within its cytoplasm upon a 3-h shift to the restrictive temperature. We have observed that upon short shifts to 37°C, vesicles accumulate predominantly in the mother cell, and not in the bud of myo2-66 cells. This is in contrast to the pattern we observed in late-acting sec mutants, such as sec6-4 and sec1-1, in which vesicles are found predominantly in the bud after brief incubations at the restrictive temperature. In the case of sec6-4 and sec1-1, post-Golgi secretory vesicles reach the bud, but are unable to fuse with its plasma membrane. In the myo2-66 mutant, however, vesicle accumulation in the mother cell may reflect a targeting or delivery defect, and this is consistent with a model in which Myo2 acts as a motor to move vesicles from the mother cell into the bud. Alternatively, it may reflect a defect in segregating components of the secretory apparatus, such as the Golgi complex, into the bud. Analogous phenotypes have been observed in dilute mice and unc-104 mutants of C. elegans. In the case of dilute, loss of function of this class V myosin leads to a defect in the transport of pigment granules in melanocytes (Mercer et al., 1991). In the case of unc-104, the defect in this kinesin-related gene product causes the accumulation of synaptic vesicles specifically in the neuronal cell bodies due to a failure in anterograde axonal transport (Hall and Hedgecock, 1991).

Even though myo2-66 cells rapidly accumulate vesicles, previous studies demonstrated that bulk secretion still continues in these cells, although perhaps in a nonpolarized fashion. The appearance of vesicles without a concomitant block in protein export led to the proposal that secretion continues because secretory vesicles make their way to the cell surface more slowly in the myo2-66 mutant (Johnston et al., 1991). This hypothesis would predict a kinetic lag in the transport of secretory proteins. We have shown, however, that there is no detectable lag in the transport of a number of secreted, cell surface or vacuolar proteins in myo2-66 cells. It is possible that secretory proteins accumulate in myo2-66 vesicles, but get degraded. However, in our pulse-chase experiments, we have not observed any degradation products or internal intermediates. It should be noted that after a shift to 37°C, wild-type cells mislocalize actin (by immunofluorescence) for up to 60 min. It is possible that Myo2 might normally act to speed vesicular transport, but the loss of actin polarity resulting from the temperature shift masks this effect. However, a temperature shift does not, by itself, slow secretion or result in the accumulation of vesicles. The efficient vesicular transport seen in wild-type cells after a temperature shift presumably reflects the presence of resid-
ual actin structures that are not detected by immunofluorescence, since temperature sensitive actin mutants do exhibit a partial block in secretion as well as vesicle accumulation.

The absence of a secretory block in myo2-66 cells might indicate that vesicles can use an alternate cytoskeletal track and motor to move into the bud. This is an appealing proposal since a kinesin-like suppressor of myo2-66 has been identified (Lillie and Brown, 1992). In squid axoplasm, crosstalk between microtubule-based and actin-based transport mechanisms has been observed; vesicles were found to switch from moving along microtubules to actin filaments sequentially (Kuznetsov et al., 1992). In addition, Golgi-derived vesicles in the brush border possess both dynein and myosin-I, suggesting that a vesicle can use more than one cytoskeletal track (Path et al., 1994). However, we found that disrupting microtubules with nocodazole in myo2-66 cells does not lead to a tighter block in invertase or glycoprotein secretion. This suggests that Myo2 does not operate in parallel with a microtubule-based transport system.

One could postulate that the myo2-66 vesicles contain a specific subset of secretory proteins that are as yet unidentified, and in a recent review, Bretscher et al. put forth a similar proposal to resolve the surprising observation of vesicle accumulation without concomitant secretory defects in both myo2-66 and tpml mutants (Bretscher et al., 1994). It is possible that Myo2 and Tpml both function on a pathway that requires the late SEC gene products, but which facilitates the transport of a specific class of proteins. Since myo2-66 was originally identified as a cell cycle mutant, cdc66-1 (Johnston, 1991), it may be that the vesicles which accumulate in this mutant contain cargo needed for a particular stage of the cell cycle. The cargo of the myo2-66 vesicles could be components necessary for cell wall assembly, such as chitin synthetases, chitinase, or endoglucanases. Alternatively, they could ferry proteins needed for mating (e.g., Fus1). Once a marker for purifying these vesicles is found, analysis of the composition and cargo of the vesicles should be enlightening.

Recently, two classes of post-Golgi secretory vesicles have been identified in a late-acting sec mutant (Harsay, E., and A. Bretscher. 1994. Mol. Biol. Cell. 5:443a). If Myo2 were required for transport of only one set of vesicles, then the other set should still accumulate in the bud of the myo2-66, sec6-4 double mutant. However, our EM analysis of the double mutant indicates that there is no large class of vesicles transported to the bud in a Myo2 independent fashion. Perhaps the invertase-containing vesicles represent only a minor pool of the total post-Golgi vesicles. Another possible model is that Myo2 is required to move both types of vesicles into the bud. The subset of vesicles which accumulate in myo2-66 cells must be targeted to the bud in order to fuse, while the invertase-containing vesicles can fuse equally well with the plasma membrane of the mother cell. This model is consistent with the very large mother cells seen in myo2-66 cultures.

The Myo2 protein is found predominantly at the tip of the bud in wild-type yeast (Lillie and Brown, 1994) and along with calmodulin and actin, it changes its distribution throughout the cell cycle to regions of active growth (Brockerhoff et al., 1994). This localization may reflect the destination of a Myo2-dependent transport pathway involved in establishing and maintaining polarized growth. However, we must also consider other, less direct, roles for Myo2 in vesicular transport. An alternate function of Myo2 may be to organize polarized growth at the cell surface by recruiting components required for bud assembly and growth. Myo2 may facilitate the polarized distribution of these bud site proteins by organizing the actin cytoskeleton. Finally, Myo2 may also act to support the dynamic changes of the actin-plasma membrane interface in facilitating cell wall growth (see Mulholland et al., 1994). To sort out these possible avenues of Myo2 function, it will be important to determine if Myo2 functions on the surface of the vesicles and/or at the plasma membrane.

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