The Saccharomyces cerevisiae MYO2 Gene Encodes an Essential Myosin for Vectorial Transport of Vesicles

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Abstract. After the initiation of bud formation, cells of the yeast Saccharomyces cerevisiae direct new growth to the developing bud. We show here that this vectorial growth is facilitated by activity of the MYO2 gene. The wild-type MYO2 gene encodes an essential form of myosin composed of an NH2-terminal domain typical of the globular, actin-binding domain of other myosins. This NH2-terminal domain is linked by what appears to be a short α-helical domain to a novel COOH-terminal region. At the restrictive temperature the myo2-66 mutation does not impair DNA, RNA, or protein biosynthetic activity, but produces unbudded, enlarged cells. This phenotype suggests a defect in localization of cell growth. Measurements of cell size demonstrated that the continued development of initiated buds, as well as bud initiation itself, is inhibited. Bulk secretion continues in mutant cells, although secretory vesicles accumulate. The MYO2 myosin thus may function as the molecular motor to transport secretory vesicles along actin cables to the site of bud development.

Cells of the yeast Saccharomyces cerevisiae display an asymmetric pattern of growth: a progeny cell is produced as a bud on the surface of a mother cell. Initiation of bud formation to produce a daughter cell involves localized cell surface growth. Continued growth and maturation of the bud requires the directed delivery of new cellular constituents to the site of bud development, so that the bud becomes the major recipient of new cell mass. Mother cells thus need not only to specify the site for bud formation, but must subsequently direct the allocation of new cellular constituents to the growing bud itself. This pattern of localized growth at the bud site is accomplished by vectorial transport of secretory vesicles to the cell surface. The continued development of initiated buds, as well as bud initiation itself, is inhibited. Bulk secretion continues in mutant cells, although secretory vesicles accumulate. The MYO2 myosin thus may function as the molecular motor to transport secretory vesicles along actin cables to the site of bud development.

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MY02 gene that encodes an essential myosin involved in localized cell surface growth. The NH₂-terminal region of the MYO2 protein closely resembles a typical myosin-head domain that imparts force-generating activity, and is coupled to a unique COOH-terminal domain. Between these two domains is a region of potential coiled-coil structure suggesting that the MYO2 protein may undergo some self-assembly, perhaps to form a dimer. We show here that the novel MYO2 protein plays a determinant role in the spatial regulation of bud formation. The MYO2 myosin may be the molecular motor that moves secretory vesicles along actin cables for the process of bud formation.

Materials and Methods

Strains and Culture Conditions

The wild-type strain GR2 (Mata ura3 his3) has been described (Johnston and Singer, 1978). Strains GR663-7 (MATH myo2-66 his3 ura3) and GR663-13 (MATH myo2-66 his5 ura3) were generated by repeated backcrosses of an initial myo2 mutant strain (Prendergast et al., 1990) with strain GR2 or the isogenic strain GR6X-7A (MATH ade2 ura3). Diploid strains GRD7 and GRD66 were the results of matings between strains GR2 and GR6X-7A and strains GR663-7 and GR663-13, respectively. Strain JPTA (MATH myo2-66 ade1 his3 leu2-2,112 ura3-52) is a transformable strain produced by successive crosses with strain 21R (Johnston and Hopper, 1982). Strains JY383 (Mata sst-3) and XB88-2C (MATH sst-2,4), kindly provided by J. P. McGrath (Massachusetts Institute of Technology, Cambridge, MA) were tester strains for pheromone secretion assays. Cells were grown in YMI complex medium (Hartwell, 1967) or YNB defined medium (Johnston et al., 1977) supplemented to satisfy auxotrophic requirements. Liquid cultures were maintained in gyratory shaking water baths (New Brunswick Scientific, New Brunswick, NJ). Escherichia coli cells of strain RR1, obtained from J. E. Hopper (M. S. Hershey Medical Center, Hershey, PA) were grown in YT medium (Miller, 1972) and used for plasmid isolation and maintenance.

Assessment of Cellular Parameters

Cell concentrations and morphologies were determined as described (Hartwell, 1970). Before assessment, cells were fixed with formalin and sonicated briefly. DNA content was quantified by a modified diphenylamine procedure (Storms et al., 1984). To determine rates of RNA and protein synthesis, cells were transferred to medium containing radiolabeled precursors of RNA or protein (New England Nuclear, Boston, MA) and incubated for 10 min, after which acid-precipitable radioactivity was quantified (Hanice-Joyce et al., 1987). To visualize chitin, formalin-fixed cells were stained with calcofluor (fluorescent brightener 28; Sigma Chemical Co., St. Louis, MO; Hayashibe and Konoeda, 1973; Johnston et al., 1979). Nuclei were visualized using the DNA stain 4',6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co.). Determinations of individual cell volumes were performed as described (Murray et al., 1987).

Immunofluorescence Procedures

Cells were fixed for 2 h by addition of formalin directly to the culture medium, and then stained with fluorescein isothiocyanate (FITC)-conjugated antibodies to visualize actin and microtubules as described (Adams et al., 1989). Cells were grown to a density of 1-3 × 10⁷ cells per ml and then transferred to low-glucose medium, incubated at 23°C for 15 min and then incubated further at the restrictive temperature of 36°C to assess invertase secretion. External invertase levels were measured by the method of Goldstein and Lampen (1979). Secretion of the mating pheromones α-factor and β-factor was assayed by plate assays described by McLaughlin and Vagelopoulous (1989). Haploid wild-type and mutant cells of each mating type were harvested and washed by centrifugation to remove residual mating pheromones secreted during proliferation at 23°C, and were then transferred to fresh medium and incubated for 3 h at either 23 or 36°C. The cells were then removed by centrifugation and dilutions of the culture supernatants were applied to lawns of tester yeast cells. Manipulations involving transfer to 36°C were carried out in an environmental room.

Genetic Analysis

Standard yeast genetic techniques were used (Mortimer and Schild, 1981).

Cloning and Characterization of the MY02 Gene

The MY02 gene was cloned by complementation (Rothstein, 1986) of the myo2-66 temperature sensitivity of strain JPTA with genomic DNA from a YCP50-based recombinant yeast DNA library, kindly provided by D. Botstein (Genentech, San Francisco, CA). One complementing plasmid, pPI0-2B, was used for further study. Restriction analysis was performed as described (Maniatis et al., 1982). Probes for Southern analysis were prepared by random-primer extension (Boehringer, Mannheim, FRG) of a MYO2 restriction fragment. An integration plasmid was constructed by substituting the smaller EcoRI-Sall fragment of plasmid YIp5 with the EcoRI-Sall fragment from the genomic insert of plasmid pPI0-2B (Fig. 3 A). The resultant plasmid, pLA, contained the wild-type UR43 gene but did not contain yeast sequences that allow autonomous replication, so that only the integrated plasmid could be transmitted to progeny cells. Integration of plasmid pLA at the homologous chromosomal site was directed (Rothstein, 1986) by transformation with pLA DNA that had been linearized within the genomic insert by digestion with HindIII (Figure 3 A). The diploid strain JPD7, heterozygous at the MYO2 locus but homozygous for ura3-52, was transformed to ura3 prototrophy by the linearized pLA plasmid. Integration at the homologous chromosomal locus was confirmed by Southern analysis (data not shown). Subsequent genetic analysis showed that homologous integration of pLA caused the integrated UR43 vector gene to be tightly linked to the myo2 locus (data not shown). Therefore the genomic insert in pPI0-2B harbors the MYO2 gene. Integration of pLA into JPD7 diploid cells also caused disruption of one copy of the MYO2 gene with the result that, upon sporulation, only two spores in each tetrad gave rise to viable cells. Incubation of sporulated diploid cells for varying lengths of time (2-6 d) or germination of spores at different temperatures (14, 23, 36°C) did not improve spore viability.

For DNA sequencing, sets of 3' and 5' MY02 deletion plasmids were generated from plasmids containing subcloned regions from the genomic insert of pPI0-2B (Dale and Arrow, 1987). The deleted single-stranded DNA templates in M13 were sequenced by the dideoxy method (Sanger et al., 1977). In this way both strands of the pPI0-2B insert were sequenced. Nucleotide sequence analysis was performed using the University of Wisconsin Genetics Computer Group (UWCGC) software package (Devereux et al., 1984).

Results

Isolation of the myo2 Mutant

A recently described centrifugation procedure (Prendergast et al., 1990) has been shown to enrich for abnormally large cells. Use of this procedure to enrich for enlarged cells at the restrictive temperature of 36°C enabled us to identify temperature-sensitive mutant cells defective in cell prolifera-
tion but unimpaired in overall mass accumulation. All of the mutant cells identified in this way displayed significant residual biosynthetic activity at the restrictive temperature, but many were unable to perform aspects of the mitotic cell cycle.

One temperature-sensitive mutation, now termed myo2-66 but originally designated cdc66-1 (Prendergast et al., 1990), impaired bud formation. Transfer of proliferating myo2-66 mutant cells to the restrictive temperature of 36°C led to a concerted arrest of proliferation, with arrested cells displaying a uniform unbudded cell morphology (Fig. 1). Despite the cessation of proliferation, myo2-66 mutant cells at 36°C remained biosynthetically active and mass accumulation continued, as evidenced by continued synthesis of protein and RNA (Fig. 2 B), and the continued increase in size of arrested cells. At the restrictive temperature myo2-66 mutant cells also continued to replicate DNA and accumulated DNA to a level twice that for proliferating cells (data not shown). Nuclear staining revealed that a significant proportion of arrested mutant cells (168 out of 360, or 47%) displayed more than one nucleus (Fig. 2 A), whereas each wild-type cell treated in the same way displayed only one nucleus (data not shown). These observations showed that the myo2-66 mutation does not block the nuclear cycle, but makes mutant cells defective in proper morphological development.

The MYO2 Gene

The MYO2 gene was isolated by complementation of the myo2-66 temperature-sensitive defect. Approximately 30,000 transformants were selected by uracil prototrophy and assessed for growth at the restrictive temperature of 36°C. The plasmids from three temperature-resistant transformants that showed concomitant loss of both uracil prototrophy and temperature resistance under nonselective conditions were found to contain overlapping genomic inserts (data not shown). The integration by homologous recombination (Rothstein, 1986) of a plasmid harboring the EcoRI–SalI fragment from plasmid pJP10-2B (Fig. 3 A) into the myo2-66 chromosomal site showed that pJP10-2B contained the wild-type MYO2 gene.

The genomic insert in plasmid pJP10-2B was shown to contain the wild-type MYO2 gene by integration of a plasmid harboring part of that insert into the myo2-66 chromosomal site by homologous recombination (Rothstein, 1986).

The MYO2 Gene Product Is Essential

Sequence analysis (below) showed that the EcoRI–SalI fragment is completely internal to the MYO2 open reading frame, so that integration of this fragment at the MYO2 locus disrupts that chromosomal MYO2 allele. Therefore integration of pL4 into JPD7 diploid cells heterozygous for the myo2-66 allele resulted in disruption of either the wild-type MYO2 allele or the mutant myo2-66 allele. Upon sporulation of one integrant diploid strain, only 2 spores in each of 43 tetrads were viable, in marked contrast to the high spore viability for the untransformed diploid. Each viable spore product from that transformed diploid was a temperature-resistant uracil auxotroph, indicating that viable spores contained neither the integrated plasmid nor the myo2-66 allele. The nonviable spore products must therefore have contained the plasmid integrated into the myo2-66 allele, disrupting the myo2-66 locus. Microscopic examination revealed that all of these nonviable spores germinated and formed a bud, but most (72%) remained as a single budded cell. This finding demonstrates that MYO2 is an essential gene.

myo2-66 Supplies Only Partial Function

The transformed diploid described above was temperature-resistant and grew well with only a single intact copy of the wild-type MYO2 gene. Thus the MYO2 gene product is normally present in excess. However, other Ura+ transformants of the same diploid strain remained temperature-sensitive, and therefore must have been disrupted for the wild-type MYO2 allele. These diploid integrants with only the myo2-66 mutant gene still intact were severely impaired in growth even at 23°C, and were unable to sporulate (data not shown). This growth defect demonstrates that the mutant myo2-66 gene product is only partially functional even at the permissi-
The MY02 gene. (A) Restriction map of plasmid pJP10-2B. The thin line represents the genomic insert containing the MY02 gene, and the thick lines flank the insert vector sequences. The nucleotide and amino acid sequences are numbered on the left and right, respectively. The A nucleotide of the presumed ATG initiator codon is designated +1. The potential p34 phosphorylation site is boxed.

**Figure 3.** The MY02 gene. (A) Restriction map of plasmid pJP10-2B. The thin line represents the genomic insert containing the MY02 gene, and the thick lines flank the insert vector sequences. The nucleotide and amino acid sequences are numbered on the left and right, respectively. The A nucleotide of the presumed ATG initiator codon is designated +1. The potential p34 phosphorylation site is boxed. The ATP-binding and active thiol regions are underlined with a single or double line, respectively. The potential splice donor and acceptor sequences are available from EMBL/GenBank/DDBJ under accession number M35532.
MYO2 Encodes a Myosin I Protein

The nucleotide sequence of a 5,675-bp complementing insert from pJP10-2B revealed an extensive open reading frame that could encode a protein of 1,574 amino acids, with a predicted molecular weight of 180,567 (Fig. 3 B). We assume that the ATG codon at position +1 serves as the translation initiation codon: the sequences flanking this putative initiator codon are identical to the proposed consensus sequence for translation initiation in yeast (Cigan and Donahue, 1987).

The NH₂-terminal half of the deduced amino acid sequence of the MYO2 gene shows striking similarity to the globular head domain of myosin heavy chain genes. Fig. 4 A shows a representative dot-matrix comparison between the MYO2 protein and a myosin heavy chain (a myosin II) from Dictyostelium (Warrick et al., 1986). Like other myosins, the MYO2 protein contains within the NH₂-terminal domain a putative ATP-binding pocket, centered at residue 167, with the characteristic glycine-rich GXXGXGKT motif (Walker et al., 1982). This potential ATP-binding region (Fig. 4 B) displays significant sequence similarity to analogous regions in the myosins from Dictyostelium (Warrick et al., 1986), nematode (Karn et al., 1983), and Acanthamoeba (Hammer et al., 1987). In addition, the putative ATP-binding region of the MYO2 gene shows 62% amino acid identity with a region of the yeast MYO1 gene product (Watts et al., 1987; data not shown). A further region of sequence similarity is evident between residues 443 (phenylalanine) and 523 (glutamic acid). This 81-amino acid stretch has 65% identity to the proposed actin-binding region in nematode myosin and 62% identity to the analogous region in the yeast MYO1 gene product (data not shown).

Reactive cysteine residues are considered important for the actin-activated ATPase activity of myosins (Harrington and Rodgers, 1984). Unlike the situation for the yeast MYO1 gene product, in which the active-thiol region contains only a single cysteine (Watts et al., 1987), the MYO2 protein contains the typical two cysteine residues at positions 692 and 702 (Fig. 4 C). Over a 24-residue stretch encompassing these two cysteine residues, the MYO2 protein displays significant amino acid identity to the analogous regions from Dictyostelium, nematode and Acanthamoeba (Fig. 4 C).

Analysis of the remaining COOH-terminal domain of the MYO2 protein failed to indicate any significant sequence similarity to previously reported myosin proteins (see Fig. 4 C). These structural considerations point to a provisional assignment of the MYO2 protein to the myosin I class of myosins.

Chitin Deposition Delocalized But Septation Normal

At the time of bud emergence, a localized deposition of chitin occurs to form a ring structure, through which the cell surface evacinates to form the bud. This localized deposition of chitin marks the site of bud emergence and remains on the surface of the mother cell after cell separation as a characteristic bud scar (Ballou, 1982; Fig. 5 A). Chitin staining of myo2-66 mutant cells under restrictive conditions revealed that chitin deposition was no longer localized but had become distributed over the entire cell surface (Fig. 5 B). This behavior is similar to the delocalization of chitin distribution seen in actin mutants (Novick and Botstein, 1985) and certain cdc mutant cells (Slot et al., 1981; Roberts et al., 1983; Adams et al., 1990). After 6 h at the restrictive temperature, mutant cells were stained uniformly for chitin. By this time many cells also had become deformed and wrinkled in appearance, consistent with the loss of viability observed after several hours at the restrictive temperature (data not shown).

Chitin deposition also is required to form the final septum during cell separation (Silverman et al., 1988; Cabib et al., 1989). Success of this chitin deposition is indicated indirectly by the phenotype of myo2-66 mutant cells: as shown in Fig. 1, these cells arrest proliferation promptly upon transfer to the restrictive temperature and accumulate as unbudded cells, suggesting that buds that were present at the time of transfer to the restrictive temperature are released normally. Thus, the MYO2 gene, unlike the MYO1 gene (Watts et al., 1987), is not involved in nuclear migration or cytokinesis. Furthermore, prompt completion of the budding process even at the restrictive temperature indicates that the myo2-66 mutation does not compromise the chitin deposition involved in septation.
**Figure 5.** The myo2-66 mutation disrupts actin cables. Proliferating homozygous myo2-66 (B, D, F) or wild-type (A, C, E) diploid cells were incubated at 36°C for 3 h (C-F) or 6 h (A and B) and then stained for chitin (A and B), or subjected to immunofluorescence staining to reveal microtubules (C and D) or actin (E and F). Bars, 10 μm.

**MYO2-encoded Myosin Directs New Growth to the Bud**

The budding process produces a daughter cell that is nearly the same size as its mother cell. This asymmetric pattern of cell enlargement reflects the transport of vesicular material to a localized area of the cell surface (Thaczyk and Lampen, 1972, 1973; Novick and Schekman, 1979; Field and Schekman, 1980; Tschopp et al., 1984). To determine the role of the MYO2 protein in the vectorial transport of vesicles necessary for this asymmetric pattern of growth, we measured the sizes of the new daughter cells produced by myo2-66 mutant cells after the imposition of restrictive conditions. This analysis was facilitated because at the restrictive temperature myo2-66 mutant cells complete ongoing cycles of budding, cytokinesis and cell separation before arrest of proliferation as unbudded cells (Fig. 1). A defect in bud growth at the restrictive temperature should result in new daughter cells that are smaller than normal.

Mutant and wild-type cells were transferred to the restrictive temperature, and incubated for 3 h, at which time cell samples were fixed and cell volumes were determined. As shown in Fig. 6 A, the population of arrested myo2-66 mutant cells generated a disperse cell volume distribution compared to that of the wild-type population. Although most mutant cells were significantly larger than the largest wild-type cell, the population of arrested mutant cells also contained a significant fraction of extremely small cells. To determine the volume of individual wild-type daughter cells, fixed cells were first stained for chitin, and for wild-type cells only newly produced cells, easily identified by the absence of a chitin-rich bud scar, were measured (Fig. 6 B). For cells in the arrested mutant population the classification of cells according to bud scars was compromised by the generalized delocalization of chitin (see above), and for this reason cell volumes of all mutant cells were determined without regard for bud-scar status. As shown in Fig. 6 B, the arrested mutant population contained cells that were significantly smaller than the smallest daughter cells in the wild-type population. Although the arrested cells continued to increase in mass (see Fig. 2), this growth was no longer restricted to the developing bud. This finding indicates that the MYO2 myosin protein is required to direct new growth into the bud, as well as to localize secretion for bud initiation.
An Aberrant Actin Cytoskeleton

Intact Microtubules in Arrested Mutant Cells

Examination of myo2-66 mutant cells using a yeast antitubulin antibody revealed normal spindle structures that persisted after transfer of cells to the restrictive temperature and the eventual arrest of proliferation. As shown in Fig. 5 D, arrested mutant cells maintained well-defined cytoplasmic microtubule structures. These findings are consistent with results showing that the array of extranuclear microtubules in yeast cells is not involved in continued bud growth (Huffman et al., 1988; Jacobs et al., 1988), although this array may affect bud site selection (Byers, 1981; but see Jacobs et al., 1988).

An Aberrant Actin Cytoskeleton

Another structural component involved in localized surface growth is actin (Adams and Pringle, 1984; Novick and Bostein, 1985; Drubin et al., 1988; Haarer et al., 1990). Actin is found in cortical patches that concentrate on the surface of the bud (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Drubin et al., 1988), and in an intracellular network that forms the actin cytoskeleton within the cytoplasm (Adams and Pringle, 1984; Drubin et al., 1988). As assessed by anti-actin antibody staining, proliferating wild-type and mutant cells displayed normal actin cytoskeletons, with pronounced actin cables coursing through the interior of each cell (Fig. 5 E and data not shown). Cortical actin patches were concentrated as expected in the growing bud. At the restrictive temperature the arrested mutant cells remained biosynthetically active (Fig. 2 B) and became enlarged (Fig. 2 C), but now all cells in the population displayed delocalized cortical actin patches (Fig. 5 F), suggesting that cell surface growth was no longer restricted to specific sites. Moreover, the actin cables underwent a striking alteration in mutant cells to produce thick bars of actin within the cell (Fig. 5 F). The actin bars were randomly oriented with respect to the long axis of the arrested, unbudded mutant cells. After 6 h at the restrictive temperature, ~66% of the mutant cells (132 out of 200) contained these actin bars. In contrast, under permissive conditions only 23 out of 200 mutant cells (11%) contained actin bars, and at no time did we observe these structures in wild-type cells regardless of growth temperature (data not shown).

The aberrant actin cytoskeleton in mutant cells suggests that the myo2-66-encoded myosin interacts with the network of actin filaments. Furthermore, the aberrant actin cytoskeletons in some cells even at the permissive temperature reinforces the conclusion derived above from studies of gene dosage that the myo2-66 gene product is partially dysfunctional under permissive conditions.

Impaired Vesicle Movement But Continued Secretion

A defect in bud development could indicate an impairment in general secretory processes, or in the localization of secretion. Many genes have been shown to be involved in the secretion activity of cells (Novick et al., 1980), and mutations that affect steps late in the secretory pathway block secretion and result in the accumulation of vesicles within the cytoplasm (Novick et al., 1981). Therefore in myo2-66 mutant cells, the status of the secretory pathway was assessed. Mutant cells were examined by thin section EM and by freeze fracturing to determine if vesicle transport was affected, and we also measured the export of two well-studied polypeptides, the periplasmic enzyme invertase (Carl-
Figure 8. EM of mutant and wild-type cells. (A–D) Wild-type (A and C) or myo2-66 mutant (B and D) cells were visualized by thin-section EM after growth at 23°C (A and B) or after 3 h at 36°C (C and D). (E and F) myo2-66 mutant cells after growth at 23°C (E) or after 3 h at 36°C (F) were examined by freeze fracturing. Bar, 1 μm.
Invertase is synthesized in two forms: an intracellular, nonsecreted form produced constitutively, and a secreted, extracellular form that is only synthesized under conditions of low glucose concentration. The invertase that is destined for export is processed through the ER and Golgi system to become packaged into vesicles and transported to the growing bud (Esmon et al., 1981). To measure invertase export, proliferating wild-type and \( \text{myo2}-66 \) mutant cells were transferred to medium containing a low glucose concentration (0.1%). A portion of the population then was incubated at the restrictive temperature and invertase secretion was assessed. As shown in Fig. 7, \( \text{myo2}-66 \) mutant cells retained significant capacity to secrete invertase even at the restrictive temperature.

The \( \text{myo2}-66 \) mutant cells also secreted \( \alpha \)-factor at the restrictive temperature. The yeast mating pheromones, \( \alpha \)-factor and \( \alpha \)-factor, are secreted by distinct mechanisms (Julius et al., 1984; McGrath and Varshavsky, 1989). Although \( \alpha \)-factor export is mediated by a yeast homologue of the mammalian P-glycoprotein (McGrath and Varshavsky, 1989), \( \alpha \)-factor is secreted by the same pathway that secretes invertase (Julius et al., 1984). To assess the activity of each of these pathways, we followed the procedure of McGrath and Varshavsky (1989). These assays showed that the secretion of \( \alpha \)-factor by \( \text{MATa} \) mutant cells via the classical secretion pathway, and of \( \alpha \)-factor by \( \text{MATa} \) mutant cells via the P-glycoprotein-mediated pathway, was in each case unimpaired. Indeed, a \( \alpha \)-factor fusion protein is capable of binding calmodulin only when at least one of the \( \alpha \)-factor repeating units is present. The distribution of amino acid sequences within the COOH-terminal region contain features that may reflect \( \text{MYO2} \)-protein function.

Immediately downstream from the highly conserved globular head domain is a region of net basic charge (+33) that encompasses amino acid residues 750–925. This region contains a motif (Fig. 9D) that resembles the calmodulin (CAM)-binding site in neuromodulin, a neural-specific CAM-binding protein (Alexander et al., 1988). The putative \( \text{MYO2} \) CAM-binding sites also resemble a repeated region within the p190 myosin-like protein from vertebrate brain (Espenocek, E., R. Cheney, F. Spindola, M. Coelho, D. Pitta, M. Moosker, and R. Larson. 1990. J. Cell Biol. 111:167a; Cheney, R., and E. Espenocek, personal communication). Indeed, a p190 fusion protein is capable of binding calmodulin only when at least one of the p190 repeating units is present. The distribution of amino acid sequences within the COOH-terminal region contains features that may reflect \( \text{MYO2} \)-protein function.

Downstream from the putative CAM-binding sites in the \( \text{MYO2} \) protein is a region of potential \( \alpha \)-helical structure. The distribution of amino acid residues along these predicted \( \alpha \)-helices (Fig. 9A–C) suggests by analogy that these potential \( \alpha \)-helical domains in \( \text{MYO2} \) could interact with similar domains to form coiled-coil configurations. This \( \alpha \)-helical coiled-coil structure results from the periodicity of amino acids in a seven–amino acid ("heptad") repeat unit; in this heptad repeat (abcdefg) positions \( a \) and \( d \) are occupied by hydrophobic residues (Cohen and Perry, 1986).

Discussion

Myosins belong to a class of proteins that provide mechanical forces along many cytoskeletal structures (Kiehart, 1990; Vale and Goldstein, 1990; Pollard et al., 1991). These force-generating molecules facilitate a wide variety of cellular movements. The \( \text{MYO2} \) gene described here encodes a novel myosin protein that possesses the globular head domain characteristic of all myosin proteins (Fig. 3, B and C), but a unique COOH-terminal tail unlike those found for myosin II molecules (Fig. 4A). Despite the apparent novelty of the COOH-terminal domain within the \( \text{MYO2} \) protein, the predicted amino acid sequences within the COOH-terminal region contain features that may reflect \( \text{MYO2} \)-protein function.

The unbudded phenotype and continued secretion shown by mutant cells suggests that it is the vectorial transport of secretory vesicles, necessary to localize secretion at the site of bud initiation, that is impaired. Indeed, thin sectioning and freeze fracturing showed that incubation of \( \text{myo2}-66 \) mutant cells at the restrictive temperature resulted in the marked accumulation of vesicles within the cytoplasm (Fig. 8, D and F). At the restrictive temperature the delivery of vesicles to the cell surface thus is abnormal. However, the significant bulk secretion shows that secretory vesicles eventually arrive at the cell surface. These findings suggest that vesicles make their way to the plasma membrane of mutant cells, but slowly. The resultant longer transit time consequently leads to the accumulation of transport vesicles.
The predicted amino acid sequence of the COOH-terminal region of the MYO2 protein also displays identity (28%) to the COOH-terminal region of the recently described p190 myosin-like protein from vertebrate brain (Espreafico, E., R. Cheney, F. Spindola, M. Coelho, D. Pitta, M. Mooseker, and R. Larson. J. Cell Biol. 111:167a. 1990; Cheney, R., and E. Espreafico, personal communication). This region of the MYO2 protein also contains nucleotide sequences that could indicate mRNA splicing. The sequence GTATGT suggests that a potential 5'-splice junction (Langford et al., 1984) could be located within codon 1286 (Fig. 3). In addition, the downstream sequence TATTAACC, at nucleotide 4772, closely resembles the TA(C/T)TAACA consensus recognition signal for splicing in yeast (Parker et al., 1987). Use of the 3'-proximal PyAG at nucleotide 4792 as the 3'-splice boundary would generate a truncated polypeptide extended only 11 amino acids beyond the splice junction, whereas use of the next PyAG site at position 4886 would generate an extension of only eight amino acids. However, the similarity of the predicted amino acid sequences of the MYO2 and p190 proteins makes it unlikely that uniform splicing of the mRNA takes place. The novel COOH-terminal domain is undoubtedly involved in functions specific to this MYO2 myosin isoform.

**MYO2 Affects the Actin Cytoskeleton**

The MYO2 protein probably interacts directly with actin. The MYO2 protein exhibits a typical actin-binding region in the NH$_2$-terminal domain and impaired MYO2 function, brought about by incubation of myo2-66 mutant cells at a restrictive temperature, brings about marked disorganization of the actin cytoskeleton and delocalized distribution of actin cortical patches (Fig. 5 F). The rapid loss of actin cables and the presence of actin bars, as well as the random distribution of cortical actin patches, are the same effects caused by mutations in the actin gene itself (Novick and Botstein, 1985), and mutations in other genes encoding various actin-binding proteins (Drubin et al., 1988; Liu and Bretscher, 1989; Haarer et al., 1990).

**MYO2 Is Necessary for Vectorial Secretion**

Proper morphogenesis for the budding yeast requires directed and localized secretion to the area of the cell surface undergoing rapid growth, specifically the bud. Despite the dependence of morphogenesis on an intact secretory pathway, mutations that affect morphogenesis do not necessarily block secretion. For example, the myo2-66 mutation described here does not prevent bulk secretion of either invertase, α-factor, or general cell surface components. Actin mutants have a similar phenotype (Novick and Botstein, 1985).

We presume that the accumulation of intracellular vesicles in myo2-66 mutant cells (and in actin mutant cells; Novick and Botstein, 1985) reflects a decreased efficiency of vesicle transport. In wild-type cells with an active secretory pathway secreting vesicles are rarely seen (Novick et al., 1980), which is thought to reflect the rapidity with which these vesicles are transported to the cell surface (Novick et al., 1981). A mutation that slows this transport could therefore lead to an accumulation of these vesicles, as vesicles are produced at normal rates but are then slower at reaching the cell surface. A defect in efficient vectorial transport of secretory vesicles along actin cables may then liberate these vesicles to embark on a slower course of undirected diffusion to the cell surface.

The enrichment scheme that allowed the isolation of the original myo2-66 mutant depends on the ability of mutant cells to enlarge (Prendergast et al., 1990). We show that the myo2-66 mutation does not compromise cell growth, but does impair the selective partitioning of newly produced cell material to the developing bud, resulting in un budded mother cells that are abnormally large and new daughter cells that are abnormally small. This enrichment scheme also allowed the isolation of many new mutant alleles of the CDC24 gene (Prendergast et al., 1990), another gene implicated in polarized secretion and localized cell surface growth (Sloat et al., 1981). Indeed, an enlarged and un budded cell morphology and random distribution of actin that characterizes the myo2-66 mutation are also features of cdc24 mutations (Sloat et al., 1981). Thus the further use of this enrichment scheme may be a profitable way to identify additional genes involved in morphogenesis.

Figure 9. Amino acid distributions in the MYO2 COOH-terminal region. (A-C) The positions of hydrophobic (A), acidic (B) and basic (C) amino acids in positions a-g are shown for residues 925-981 (solid bars) and 1010-1086 (open bars). (D) MYO2 residues identical or similar to amino acids in the neuromodulin CAM-binding site are boxed. The amino acid position is indicated to the left of each MYO2 segment.
Temporal Regulation of Bud Formation

In addition to the spatial regulation of cell growth that involves the localized delivery of new cell constituents to the site of bud development, the timing of bud formation also is regulated in the mitotic cell cycle. This temporal regulation of bud formation is a function of the central cell cycle regulatory event START (Hartwell, 1974; Hartwell et al., 1974; Singer et al., 1984). One component of this regulatory activity is a highly conserved protein kinase, p34cdc2, that for the budding yeast is encoded by the CDC28 gene (Beach et al., 1982; Draetta et al., 1987). For the budding yeast, activation of p34cdc2 protein kinase instigates processes that form a bud (Wittenberg and Reed, 1988).

The MYO2 protein described here contains a putative p34cdc2 phosphorylation site (Peter et al., 1990a), TPLK, at residue 1097 (Fig. 3). This putative phosphorylation site is within the unique COOH-terminal domain of the MYO2 protein, 10 amino acid residues from the potential coiled-coil domain. Interestingly, two conserved p34cdc2 phosphorylation sites that are modified in lamin also are found next to an α-helical domain; phosphorylation at these sites disrupts lamin protein interactions (Heald and McKeon, 1990; Peter et al., 1990b; Ward and Kirschner, 1990), one of the early changes associated with the onset of mitosis (see Murray, 1989; Moreno and Nurse, 1990; Nurse, 1990). By a similar mechanism, alteration by phosphorylation of the interactions between the MYO2 “motor” protein and its cellular cargo, the vesicles destined for the site of bud formation, may initiate and coordinate bud development.

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