Myosin-driven peroxisome partitioning in
S. cerevisiae

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In Saccharomyces cerevisiae, the class V myosin motor Myo2p propels the movement of most organelles. We recently identified Inp2p as the peroxisome-specific receptor for Myo2p. In this study, we delineate the region of Myo2p devoted to binding peroxisomes. Using mutants of Myo2p specifically impaired in peroxisome binding, we dissect cell cycle–dependent and peroxisome partitioning–dependent mechanisms of Inp2p regulation. We find that although total Inp2p levels oscillate with the cell cycle, Inp2p levels on individual peroxisomes are controlled by peroxisome inheritance, as Inp2p aberrantly accumulates and decorates all peroxisomes in mother cells when peroxisome partitioning is abolished. We also find that Inp2p is a phosphoprotein whose level of phosphorylation is coupled to the cell cycle irrespective of peroxisome positioning in the cell. Our findings demonstrate that both organelle positioning and cell cycle progression control the levels of organelle-specific receptors for molecular motors to ultimately achieve an equidistribution of compartments between mother and daughter cells.

Introduction

Membrane-bound organelles organize the eukaryotic cell into distinct compartments that are each specialized for specific cellular functions. Compartmentalization allows a range of different processes to occur simultaneously within optimal microenvironments to increase the overall metabolic efficiency of cells. To maintain the advantages of compartmentalization, eukaryotic cells have evolved molecular mechanisms to ensure the faithful inheritance of their organelles during cell division.

The yeast Saccharomyces cerevisiae divides asymmetrically by budding. The accurate inheritance of its organelles is achieved by delivery of a portion of its organelles to the bud concomitant with retention of the remaining organelles in the mother cell (Fagarasanu and Rachubinski, 2007). This feature makes detection and isolation of mutants defective in organelle partitioning easier in budding yeast than in cells that divide by median fission, thus facilitating investigation of the spatial and temporal control of organelle motility.

The bud-directed movement of organelles is mediated by class V myosins, which are motors specialized in carrying cargo along actin filaments. The amino termini of all class V myosins contain a conserved motor domain that generates actin-based movements, whereas their divergent carboxyl termini form a globular domain called the tail that is specialized in capturing various organelles. Myo2p and Myo4p are the S. cerevisiae class V myosins (for reviews see Reck-Peterson et al., 2000; Bretscher, 2003; Pruyne et al., 2004). Myo4p is involved in the movement of cortical ER (Estrada et al., 2003), whereas Myo2p powers the bud-directed movement of most other membrane-bound organelles, including Golgi elements (Rossanese et al., 2001), the vacuole (Ishikawa et al., 2003; Tang et al., 2003), peroxisomes (Hoeppner et al., 2001; Fagarasanu et al., 2006a), and mitochondria (Itoh et al., 2002, 2004; Boldogh et al., 2004; Altman et al., 2008). Myo2p also drives the polarized transport of secretory vesicles, which is essential for cell growth (Govindan et al., 1995; Schott et al., 1999), and carries the plus ends of cytoplasmic microtubules into the bud for orientation of the nucleus before mitosis (Yin et al., 2000).

Ensuring the efficient transport of the different types of organelles carried by Myo2p requires tight control and coordination of Myo2p’s attachment to and detachment from different...
organelles. Importantly, distinct Myo2p functions are genetically dissectible within the Myo2p tail. For example, mutations in the Myo2p cargo-binding domain were found that specifically disrupt either vacuole inheritance or polarized secretion (Schott et al., 1999; Catlett et al., 2000). Therefore, it was proposed that each organelle has its own Myo2p-specific receptor/adaptor that binds to a specific region in the Myo2p tail. Receptor proteins that physically connect Myo2p to its organelle cargoes have been shown to be indeed different and specific for each type of organelle (Beach et al., 2000; Ishikawa et al., 2003; Itoh et al., 2004; Fagarasanu et al., 2006a; Arai et al., 2008; Lipatova et al., 2008). Interestingly, although most yeast organelles are carried by the same motor, Myo2p, they move to distinct locations at different times in the cell cycle (Fagarasanu et al., 2006b; Pashkova et al., 2006). For example, at cytokinesis, both late Golgi elements and peroxisomes relocate to the mother–bud neck where Myo2p accumulates. In contrast, vacuoles do not display Myo2p-dependent movements at this stage of the cell cycle, and no vacuolar structures are found at the mother–bud neck. Also, late compartments of the Golgi follow Myo2p to the shmoo tips in G1-arrested cells, which is in contrast to peroxisomes and vacuoles (Rossanes et al., 2001; Tang et al., 2003; Fagarasanu et al., 2006a). Thus, Myo2p associates with each type of organelle at a different and specific time in the cell cycle. The position of Myo2p receptors as mediators between the various organelles and the molecular engine driving their movement makes them ideally suited as regulatory targets for the organelle-specific patterns of movement occurring during the cell cycle.

We previously identified Inp2p as the peroxisome-specific receptor for Myo2p (Fagarasanu et al., 2006a). The levels of Inp2p fluctuate during the cell cycle in a pattern that correlates with the dynamics of peroxisome inheritance observed in wild-type cells (Fagarasanu et al., 2006a, 2007). Inp2p levels are low during early budding when peroxisomes are first observed to perform vectorial movements toward the bud and peak in medium-sized budded cells when most peroxisomes are inserted into daughter cells. Later in the cell cycle, when about half of the peroxisomes have been delivered to the bud, Inp2p levels start to decrease and return to basal values before cytokinesis (Fagarasanu et al., 2006a,b). Inp2p does not associate uniformly with all peroxisomes but accumulates preferentially on a subset of peroxisomes (Fagarasanu and Rachubinski, 2007). A correlation exists between the levels of Inp2p on different peroxisomes and their segregation fates, as only peroxisomes containing detectable amounts of Inp2p are selectively carried to the daughter cell (Fagarasanu et al., 2006a). These findings show that the availability of Inp2p on the peroxisomal membrane is an important determinant for the timing of Myo2p’s attachment to peroxisomes. How Inp2p–Myo2p interactions are regulated is currently unknown.

In this study, we identify the surface region of the Myo2p tail devoted to binding Inp2p and show that this region partially overlaps the region that binds secretory vesicles. By introducing point mutations in the peroxisome-binding region of Myo2p, we artificially uncoupled peroxisome inheritance from cell cycle progression to study the regulation of Inp2p by cell cycle-dependent and organelle positioning–dependent cues. Inp2p accumulates aberrantly on most peroxisomes in the mother cell if peroxisome inheritance is abolished. Our findings suggest that the inheritance of peroxisomes involves feedback mechanisms that regulate the levels of Inp2p relative to the positioning of peroxisomes in the dividing cell. We also show that Inp2p is a phosphoprotein whose level of phosphorylation is coupled to the cell cycle. Given that common principles govern the partitioning of all organelles (Weisman 2003, 2006; Fagarasanu and Rachubinski, 2007; Li and Nebenführ, 2008; Peng and Weisman, 2008; for review see Pryne et al., 2004), our findings point to the existence of regulatory events affecting the activity of organelle-specific receptors for molecular motors in response to organelle positioning and cell cycle progression that help to achieve an equidistribution of each organelle type between mother cell and daughter upon cell division.

Results

Isolation of mutants of the Myo2p globular tail defective in peroxisome inheritance

The globular tail of Myo2p is composed of subdomains I (residues 1,131–1,309 and 1,528–1,574) and II (residues 1,310–1,527; Fig. 1; Pashkova et al., 2005, 2006). Analysis of the surface residues of the Myo2p tail to identify regions of high sequence conservation among the class V myosins from phylogenetically distant organisms led to the discovery of several patches of conserved residues in both subdomains I and II (Pashkova et al., 2006). Conserved surface residues generally indicate sites of protein–protein interaction. Therefore, the conserved patches on the surface of the Myo2p cargo-binding domain are likely to be regions that attach to specific cargoes. Indeed, the vacuole-binding site on Myo2p occupies a small region in subdomain I, whereas the residues required for Myo2p attachment to secretory vesicles reside within subdomain II (Fig. 1; Catlett and Weisman, 1998; Schott et al., 1999; Catlett et al., 2000; Pashkova et al., 2005, 2006).

Following this reasoning, we set out to identify the peroxisome-binding domain on the surface of Myo2p by first screening cells harboring single point mutations in the conserved exposed regions of the Myo2p tail for defects in peroxisome inheritance (Fig. 1). To do this, we systematically introduced the various myo2 alleles previously constructed by Pashkova et al. (2006; Table S2) as sole copies of the MYO2 gene in cells expressing Pot1p-GFP to label peroxisomes (see Materials and methods). Cells were scored using a stringent all or none criterion for the presence of peroxisomes within buds of the mother cell and daughter upon cell division. Interestingly, Y1415 resides in the region of the Myo2p tail specialized in binding secretory vesicles (Fig. 1; Pashkova et al., 2006). Quantification showed that when the bud volume reached 0–24% of the mother cell volume (small-budded cells), only 21% and 39% of the buds of the myo2-Y1415E and myo2-W1407F mutants, respectively, contained at least one peroxisome. In contrast, 84% of
Mutants of Myo2p defective in peroxisome inheritance display decreased affinity for Inp2p

If Y1415, W1407, Y1483, and E1484, whose mutation affects peroxisome transport to the bud, form a region on the surface of Myo2p specialized in binding peroxisomes, we expect mutations in these amino acids to decrease the affinity of Myo2p for its peroxisome-specific receptor, Inp2p. We have previously shown direct interaction between GST–Myo2p and maltose-binding protein (MBP)–Inp2p fusion proteins made in Escherichia coli (Fagarasanu et al., 2006a). We used a similar approach to quantify the interaction between Inp2p and the various Myo2p point mutants initially tested by microscopy for defects in peroxisome inheritance (Fig. 3 A). To delineate the peroxisome-binding domain on Myo2p, we systematically replaced surface residues in the region neighboring Y1415 and W1407 with alanine (see Materials and methods) and assessed the ability of the resultant mutants to sustain peroxisome transport to buds (Fig. 2 and Table S2). Two additional substitutions, Y1483A and E1484A, were observed to disrupt the transport of peroxisomes to daughter cells (Fig. 2 B). Quantification of rates of peroxisome inheritance showed that only 33% and 47% of small-budded cells and 61% and 70% of large-budded cells of the myo2-Y1483A and myo2-E1484A strains, respectively, displayed peroxisomal fluorescence within buds. Another mutation, myo2-K1408A, displayed a slight defect in peroxisome distribution, with 64% of small buds and 88% of large buds containing peroxisomes. This result may suggest that K1408 is part of the peroxisome-binding region of Myo2p; however, care should be taken in making such a conclusion because the ε-ammonium of the K1408 side chain is predicted to form a hydrogen bond with the backbone carbonyl oxygen of F1334 (Fig. 2 A). Therefore, the effect of the K1408A substitution on peroxisome partitioning could be caused by a destabilization of the local architecture in subdomain II of the Myo2p tail.
and Myo2p-E1484A displayed less affinity for Inp2p than did wild-type Myo2p (Fig. 3 B). Interestingly, the L1411R mutation in the secretory vesicle–binding region of Myo2p also disrupted in vivo. Consistent with both microscopy-based quantification of peroxisome inheritance (Fig. 2 B) and in vitro binding (Fig. 3 A), Myo2p-Y1415E, Myo2p-W1407F, Myo2p-Y1483A, and Myo2p-E1484A displayed less affinity for Inp2p than did wild-type Myo2p (Fig. 3 B). Interestingly, the L1411R mutation in the secretory vesicle–binding region of Myo2p also disrupted...
Figure 3. Mutants of Myo2p defective in peroxisome inheritance display decreased affinity for Inp2p. [A] Glutathione Sepharose beads containing either GST fused to the cargo-binding tail of wild-type or mutant Myo2 proteins or GST alone were incubated with extracts of *E. coli*–synthesized MBP–Inp2p. (top) Bound MBP–Inp2p was analyzed by immunoblotting with anti-MBP antibodies. (middle) Total GST–Myo2p or GST protein levels were visualized by immunoblotting with anti-GST antibodies. Numbers at right denote the approximate sizes of proteins in kilodaltons. (bottom) Quantification of bound MBP–Inp2p, normalized to wild type and expressed as percent bound, was performed by densitometry. Graphic results show the means ± SEM of three independent experiments. (B) Myo2p point mutations specifically disrupt the ability of Myo2p to interact with Inp2p (peroxisomes) but not with Vac17p (vacuoles) in a yeast two-hybrid assay. Total growth of strains (top) and growth arising specifically from protein interaction (bottom) are shown. The pattern presented is representative of seven independent experiments. AD, activation domain; BD, binding domain. (C) Crystal structure of the Myo2p globular tail with the positions of point mutations that showed either weak or no interaction with Inp2p highlighted in red. The asterisk at L1411 indicates that the L1411R mutation was able to disrupt the Myo2p–Inp2p interaction in the yeast two-hybrid assay. The secretory vesicle–binding region is outlined in black. (D) Semitransparent surface view of the Myo2p structure overlaid on a ribbon diagram displaying the side chains of residues (red) implicated in binding Inp2p.
interaction between Myo2p and Inp2p (Fig. 3 B); however, this mutation was not tested for defects in peroxisome inheritance because it does not support yeast viability (Pashkova et al., 2006). The inability of L1411R to bind Myo2p in the yeast two-hybrid assay could potentially extend the peroxisome-binding region of the Myo2p tail to the L1411 residue (Fig. 3, C and D). However, the other two mutations at L1411 that were tested, i.e., Myo2p-L1411A and Myo2p-L1411S (Fig. 2 B), preserved their ability to interact with Inp2p in yeast two-hybrid analysis (Fig. 3 B). Moreover, both the L1411S and L1411R mutations have been shown to severely affect yeast cell growth (Pashkova et al., 2006), indicating that the L1411S substitution is sufficient to disrupt the attachment of Myo2p to secretory vesicles. The effect of the leucine to arginine mutation at L1411 in disturbing the Myo2p-Inp2p interaction may therefore reflect a steric occlusion of the Inp2p-binding site in the Myo2p tail resulting from the long arginine side chain’s hindrance of the ability of Inp2p to gain access to the neighboring amino acids, i.e., Y1415 and W1407, rather than a direct involvement of L1411 in attachment to Inp2p. The results of both in vitro binding and in vivo yeast two-hybrid analysis agree well with the findings of our microscopy analysis, wherein myo2-Y1415E, myo2-W1407F, myo2-Y1483A, myo2-E1484A, and, only partially, myo2-K1408A exhibited defects in peroxisome localization to the bud. This strong correlation demonstrates a clear relationship between the strength of interaction between Myo2p and Inp2p and the efficiency of peroxisome inheritance.

Myo2p point mutants defective in peroxisome inheritance are not impaired in vacuolar or mitochondrial inheritance

There is a valid concern that the identified Myo2p point mutants defective in peroxisome distribution not only impair the process of peroxisome inheritance but in fact affect the overall structure of the Myo2p tail. To test the specificity of these Myo2p mutants for peroxisome inheritance, we examined whether they retained the ability to segregate other organelles. The distribution of mitochondria was followed with the mitochondrion-specific fluorescent label MitoTracker red. The distribution of mitochondria in cells harboring one of the four myo2 alleles, myo2-Y1415E, myo2-W1407F, myo2-Y1483A, and myo2-E1484A, identified as being compromised in peroxisome inheritance, was essentially the same as that observed for cells expressing wild-type MYO2 (Fig. 4 A). The segregation of vacuoles, labeled by the vacuole-specific fluorophore FM4-64, was also unchanged in these point mutants (Fig. 4 B). The observation that the myo2-Y1415E, myo2-W1407F, myo2-Y1483A, and myo2-E1484A alleles support both vacuole and mitochondria distribution clearly shows that these mutations do not affect the overall structure of Myo2p. Moreover, the specificity of these mutants in altering peroxisome distribution strongly suggests that the steady-state levels of the Myo2p mutant proteins are likely to be normal.

Interestingly, the myo2-Y1415E mutation confers temperature sensitivity in that it supported growth at 23°C but caused severe growth defects at 30°C (Fig. 4 C). This is consistent with the previously determined requirement of Y1415 for the polarized distribution of secretory vesicles, the only known essential cargo of Myo2p (Pashkova et al., 2006). However, other MYO2 mutations, i.e., myo2-W1407F, myo2-Y1483A, and myo2-E1484A, supported growth at both 23°C and 30°C with the same efficiency as the wild-type MYO2 allele (Fig. 4 C), indicating that these amino acyl residues are outside the secretory vesicle–binding region of Myo2p (Fig. 4 D).

Collectively, our findings demonstrate that residues Y1415, W1407, Y1483, and E1484 define the surface region of the Myo2p tail devoted to binding peroxisomes. This region resides in subdomain II of the Myo2p globular tail and partially overlaps the region that binds secretory vesicles (Fig. 4 D; Pashkova et al., 2006; Lipatova et al., 2008). Because single point mutations in the peroxisome-binding region are sufficient to cause a significant defect in peroxisome inheritance, all four residues must cooperate to anchor Myo2p securely to the peroxisomal membrane during bud-directed movement.

Inp2p aberrantly decorates peroxisomes in mother cells of a myo2 strain mutant for peroxisome inheritance

The newly identified myo2 mutants are the only strains reported so far in which peroxisome delivery to the bud is impaired despite the presence of an intact INP2 gene. Cells harboring such a mutant myo2 allele as the sole copy of the MYO2 gene produce buds that lack peroxisomes but have a normal cell cycle, thus artificially dissociating peroxisome partitioning from cell cycle progression. An analysis of the dynamics of Inp2p in these cells would therefore permit an evaluation of the relative contributions made by cell cycle progression and peroxisome partitioning to the regulation of Inp2p. We used confocal microscopy to analyze the localization of a genomically encoded fluorescent chimera of Inp2p and GFP (Inp2p-GFP) relative to the distribution of a fluorescent peroxisomal marker, monomeric RFP (mRFP) tagged with a peroxisome-targeting signal 1 (mRFP-SKL). We restricted this analysis to strains containing either the wild-type MYO2 allele or the mutant allele myo2-Y1483A, which exhibits the greatest impairment in peroxisome distribution without a concomitant defect in the targeting of secretory vesicles.

Consistent with our previous study (Fagarasanu et al., 2006a), the Inp2p-GFP signal in cells containing the wild-type MYO2 gene was observed to be polarized and its intensity to be cell cycle dependent (Fig. 5 A). The Inp2p-GFP signal in unbudded cells was below the threshold of detection, and only cytoplasmic autofluorescence was seen. Upon bud formation, Inp2p-GFP fluorescence was detected and colocalized with mRFP-SKL. The amounts of Inp2p on individual peroxisomes varied dramatically, with only a subset of peroxisomes displaying detectable Inp2p-GFP fluorescence. Inp2p-labeled peroxisomes were delivered to the bud and congregated at the bud tip. Inp2p was detected on peroxisomes in mother cells only when buds were small. This resulted in a highly polarized Inp2p-GFP fluorescent signal along the mother–bud axis. The Inp2p-GFP signal was weak at cytokinesis and concentrated in those peroxisomes that relocated to the mother–bud neck region.

In contrast, in cells harboring the myo2-Y1483A mutation in which peroxisome partitioning is disrupted (Fig. 5 B), most peroxisomes in mother cells were decorated by detectable amounts
However, irrespective of the outcome of peroxisome inheritance, Inp2p levels decreased significantly at the end of the cell cycle. Regulation of Inp2p levels and posttranslational modification by peroxisome positioning–dependent and cell cycle–dependent cues

The presence of Inp2p-GFP on most peroxisomes in the myo2-Y1483A mutant is suggestive of an overall increase in Inp2p levels during the cell cycle when peroxisome inheritance is impaired. We have previously reported that Inp2p levels vary with the transfer of some peroxisomes to the bud had occurred. However, irrespective of the outcome of peroxisome inheritance, Inp2p levels decreased significantly at the end of the cell cycle.

**Regulation of Inp2p levels and posttranslational modification by peroxisome positioning–dependent and cell cycle–dependent cues**

The presence of Inp2p-GFP on most peroxisomes in the myo2-Y1483A mutant is suggestive of an overall increase in Inp2p levels during the cell cycle when peroxisome inheritance is impaired. We have previously reported that Inp2p levels vary with...
To determine whether this migration pattern was caused by post-translational modification of Inp2p-pA by phosphorylation, we treated cell lysates with alkaline phosphatase. This resulted in a collapse of the multiple Inp2p-pA species into a single, faster migrating species, demonstrating that Inp2p is indeed phosphorylated (Fig. 5 D). Phosphorylation of Inp2p-pA was coupled to the cell cycle and more pronounced at its beginning and end, whereas Inp2p at the peak of its expression 60 min after release from ɑ-factor arrest migrated as a single, fast migrating species in both MYO2 wild-type cells and myo2-Y1483A mutant cells (Fig. 5 C). The observation that the timing of Inp2p phosphorylation was indistinguishable for both the wild-type MYO2 strain and the myo2-Y1483A mutant strain (Fig. 5 C) suggests that there are temporally distinct phosphorylation events of Inp2p that are coordinated by cell cycle–dependent cues.

Our experiments also showed that Inp2p-pA from both the wild-type MYO2 and the mutant myo2-Y1483A strains migrated as multiple species on SDS-PAGE (Fig. 5, C and D).
The increased levels of Inp2p in the myo2-Y1483A mutant strain could be the result of increased Inp2p synthesis, decreased Inp2p degradation, or a combination of the two. Therefore, we assessed the steady-state levels of INP2 transcript in both wild-type cells and myo2-Y1483A mutant cells to test whether Inp2p synthesis is regulated by peroxisome positioning–dependent cues. Northern blot analysis revealed equal levels of INP2 transcript in cells expressing either wild-type MYO2 or the mutant myo2-Y1483A allele (Fig. 5 E). Therefore, the increased levels of Inp2p in the myo2-Y1483A mutant are not the result of increased transcription of the INP2 gene but rather reflect a posttranscriptional change at either the translational or posttranslational level.

The polarized distribution of Inp2p-GFP observed in wild-type cells could suggest that only a select subset of peroxisomes can accumulate Inp2p, which results in their transfer to the daughter cell. However, the observation that Inp2p is distributed over most, if not all, peroxisomes in myo2-Y1483A mutant cells is consistent with a model in which all peroxisomes can accumulate Inp2p, and Inp2p levels in the mother cell are reduced upon delivery of peroxisomes to the daughter cell. To test this model, we followed the dynamics of Inp2p-GFP in cells lacking the dynamin-like protein Vps1p. Cells lacking Vps1p typically contain only one enlarged peroxisome, probably because of inefficient peroxisome fission (Hoepfner et al., 2001). During cell division, the large peroxisome present in vps1Δ cells emits a tubular projection that passes through the mother–bud neck into the daughter cell. This peroxisomal tubule eventually undergoes fission, resulting in two peroxisomes: one located in the mother cell and the other in the bud (Hoepfner et al., 2001). Inp2p-GFP in small-budded cells was present over the entire peroxisomal tubule, but its distribution was asymmetrical, with Inp2p being concentrated on that part of the peroxisomal tubule present in the bud (Fig. 6 A). However, in cells where the elongated peroxisome divided, the Inp2p-GFP signal was not detectable in the mother cell, and Inp2p-GFP decorated only the peroxisome in the bud (Fig. 6 A). In contrast, in the myo2-Y1483A mutant lacking Vps1p, the one or two peroxisomes that were present remained in the mother cell and were decorated by Inp2p-GFP (Fig. 6 A). Interestingly, we never observed elongated peroxisomes in this Myo2p mutant, suggesting that elongation of the enlarged peroxisome observed in vps1Δ cells carrying wild-type Myo2p is the result of pulling forces exerted by Myo2p–Inp2p transport complexes on the peroxisomal membrane (Fagarasanu et al., 2006b).

We have previously shown that overproduction of Inp1p, a protein involved in the attachment of peroxisomes to the cell cortex, retains peroxisomes at fixed cortical positions in the mother cell, thereby preventing their transfer to the daughter cell (Fagarasanu et al., 2005). We analyzed the distribution of Inp2p-GFP in cells overexpressing INP1. Similarly to what was observed in myo2-Y1483A cells, overproduction of Inp1p led to an aberrantly depolarized Inp2p-GFP signal, with Inp2p decoratiing most peroxisomes found in the mother cell (Fig. 6 B). This finding is consistent with our conclusion from observations in myo2-Y1483A cells that Inp2p levels in the cell are regulated by peroxisome-positioning cues.

Discussion

Most intracellular movement of organelles in S. cerevisiae is powered by the class V myosin motor Myo2p. Interestingly, each Myo2p cargo displays Myo2p-dependent motility at a distinct time in the cell cycle. Also, the ultimate destination of Myo2p-driven transport is specific for each type of organelle (Fagarasanu et al., 2006b; Weisman, 2006). Therefore, Myo2p’s attachment to and detachment from organelles are independently regulated for each type of organelle.

It is crucial to understand the structural basis for Myo2p’s association with its cargoes to elucidate the regulatory mechanisms that allow it to move multiple cargoes to distinct places at different times. The regions on the surface of Myo2p required for binding two of its cargoes, namely the vacuole and secretory vesicles, have been identified (Schott et al., 1999; Catlett et al., 2000). These regions were found to be distant from one another and simultaneously exposed on the surface of the globular tail of Myo2p (Pashkova et al., 2006), suggesting that the tail does not have a major role in regulating cargo binding. Rather, the availability of cargo-specific receptors must dictate the timing of organelle attachment to Myo2p (Fagarasanu et al., 2006b; Pashkova et al., 2006; Weisman, 2006). In this study, we defined the surface region of the Myo2p tail devoted to binding peroxisomes and showed that this region is distinct from the region previously identified to bind vacuoles but partially overlaps the region that binds secretory vesicles. Recently, two surface residues that participate in vacuole binding were found to also function in the Myo2p-driven transport of mitochondria (Altmann et al., 2008). Therefore, it is likely that the yet unidentified mitochondrion-binding region of Myo2p overlaps its vacuole-binding region. These findings challenge the currently held view that the spatial segregation of various organelle-binding regions is an important feature of Myo2p, allowing it to function as a scaffold that exposes all its cargo-binding sites at the same time while still avoiding competition for the transport of different cargoes (Weisman, 2006). Overlap in the regions on the Myo2p surface specialized in binding different organelles suggests that different types of organelle could potentially compete with one another for access to Myo2p. This capacity for steric exclusion in myosin–organelle interactions would impose a tight temporal regulation on the activities of organelle-specific Myo2p receptors during the cell cycle and result in different receptors acting at different times in the inheritance of their specific organelles.

In this study, we elucidated the spatial and temporal parameters that contribute to the regulation of Inp2p, the peroxisome-specific receptor for Myo2p. The levels of Inp2p fluctuate during the cell cycle, being maximal when peroxisome inheritance occurs and decreased later in the cell cycle when about half of the peroxisomes have been delivered to the bud (Fagarasanu et al., 2006a). To gain insight into how Inp2p is regulated during the cell cycle, we followed the dynamics of Inp2p when peroxisome transfer to the bud was prevented or delayed using mutants of Myo2p that are defective in binding and transporting peroxisomes. Cells harboring such Myo2p mutants as the sole copy of Myo2p produced buds devoid of peroxisomes but still progressed normally through the cell cycle, thereby effectively dissociating
the two processes of peroxisome segregation and cell cycle progression. We found that under these conditions, Inp2p levels were increased, indicating that the cellular abundance of Inp2p is not intrinsically linked to the cell cycle but rather determined by the distribution of peroxisomes in the dividing cell, i.e., by organelle positioning–specific cues. Moreover, in contrast to wild-type cells in which the Inp2p signal is highly polarized toward the bud, most peroxisomes in the mother cells of myo2 mutants contained increased amounts of Inp2p. Therefore, the misplacement of peroxisomes caused by point mutations in the Myo2p tail influenced both the localization and levels of Inp2p. These data are consistent with a scenario in which the synthesis of Inp2p in the mother cell and its initial accumulation on a subset of peroxisomes precede its Myo2p-driven transport along with peroxisomes to the bud. In the bud, Inp2p–Myo2p transport complexes are eventually disassembled through the regulated degradation of Inp2p, releasing the transferred peroxisomes from Myo2p. Accordingly, when the association of peroxisomes with the translocation machinery is disrupted, as is the case for the myo2 mutants with compromised peroxisome inheritance, Inp2p is protected from the bud-specific proteolytic turnover. A degradation of Inp2p in the bud to terminate peroxisome motility agrees with current models for the inheritance of other organelles, such as yeast vacuoles. Vac17p, the vacuole-specific receptor for Myo2p, was proposed to be degraded in the bud after the transfer of vacuolar membranes into the bud (Tang et al., 2003).
Our data are also consistent with the existence of additional regulatory mechanisms that survey the intracellular distribution of Inp2p (Fig. 7). If the increase in Inp2p levels in the myo2 peroxisome inheritance mutants resulted exclusively from a lack of Inp2p degradation at its destination, i.e., in the bud, one would expect approximately the same number of peroxisomes to contain detectable levels of Inp2p in the wild-type and myo2 mutant strains. However, this is not the case, as the inheritance mutant myo2-Y1483A displays increased amounts of Inp2p on most, if not all, peroxisomes in the mother cell. These findings strongly suggest the existence of a regulatory feedback mechanism from bud to mother cell that causes Inp2p to accumulate aberrantly on peroxisomes in the mother cell upon disruption of peroxisome inheritance (Fig. 7). Because the levels of INP2 mRNA are essentially the same in wild-type cells and cells of the myo2-Y1483A mutant, this proposed feedback mechanism must operate posttranscriptionally, most likely through degradation machinery acting on Inp2p. Cellular surveillance mechanisms that monitor peroxisome partitioning could be envisioned to trigger the degradation of Inp2p in response to efficient peroxisome inheritance (Fig. 7). If the Inp2p degradation machinery is present in both mother cell and bud, its activation would not only cause the release of transferred peroxisomes from Myo2p but would also prevent new recruitments of additional peroxisomes from the mother cell. Thus, proteolytic degradation of the receptor/adaptor protein in the myosin-capturing complex could represent an effective mechanism not only for depositing a moving organelle at its proper destination (Weisman, 2006; Fagarasanu and Rachubinski, 2007; Li and Nebenführ, 2008) but also for terminating organelle inheritance.

The presence of an Inp2p signal on most peroxisomes in the mother cell was also observed when peroxisome inheritance was blocked, i.e., by overproduction of Inp1p. This result clearly showed that cells alter Inp2p distribution and levels in a compensatory manner in an attempt to reestablish correct peroxisome placement irrespective of the cause of the defect in peroxisome segregation. This result also demonstrated that it is not the lack of engagement of Inp2p by the Myo2p motor that leads to the changes in Inp2p distribution observed in the myo2-Y1483A mutant cells.

Support for this model comes from several different observations. First, the highly polarized Inp2p-GFP signal along the cell division axis in wild-type cells not only demonstrates the selectivity of Myo2p in carrying those peroxisomes that have increased amounts of Inp2p to the growing bud (Fagarasanu et al., 2006a,b) but also shows that Inp2p is not degraded as soon as it is exposed to the bud environment. The degradation of Inp2p is probably influenced by the extent of peroxisome transfer and is thus triggered after a sufficient number of peroxisomes have been partitioned to the daughter cell. Second, although the Inp2p-GFP signal in wild-type cells is not uniformly distributed among different peroxisomes, most likely all peroxisomes can acquire Inp2p, but its levels on some peroxisomes may be below the threshold of microscopic detection. Evidence to support this comes from the observation that in cells lacking the peroxisomal anchor protein Inp1p, wherein all peroxisomes have lost their ability to remain attached to the cell periphery, the entire peroxisome population is eventually transferred to daughter cells (Fagarasanu et al., 2005), presumably in an Inp2p- and Myo2p-dependent manner (Fagarasanu and Rachubinski, 2007). This shows that in the myo2 peroxisome inheritance mutants, the distribution of Inp2p on peroxisomes is probably similar to that found in wild-type cells, but the amount of Inp2p is increased. The delay in peroxisome partitioning in these mutants is sufficient for Inp2p levels on all peroxisomes to increase above the detection threshold, allowing for direct monitoring of how peroxisome inheritance feeds back onto Inp2p levels in the mother cell. Third, we observe a decrease in the Inp2p-GFP signal on peroxisomes in the mother cell in an inheritance mutant when peroxisomes are segregated to the bud, which provides further proof that the Inp2p degradation machinery acting on Inp2p is loaded onto all peroxisomes. Those peroxisomes with more Inp2p have a greater probability of being carried by Myo2p into the bud. The presence of peroxisomes in the bud prevents further accumulation of Inp2p on peroxisomes remaining in the mother cell, probably by degradation of Inp2p. At the end of the cell cycle, Inp2p is turned over irrespective of peroxisome location.

Figure 7. A model for Inp2p regulation. At the beginning of the cell cycle, Inp2p is loaded onto all peroxisomes. Those peroxisomes with more Inp2p have a greater probability of being carried by Myo2p into the bud. The presence of peroxisomes in the bud prevents further accumulation of Inp2p on peroxisomes remaining in the mother cell, probably by degradation of Inp2p. At the end of the cell cycle, Inp2p is turned over irrespective of peroxisome location.
Inp2p turnover can occur both in the mother cell and the bud (Fig. 7). The degradation complex might be present in the cytosol or even on the peroxisomal membrane, but it appears that its activity is determined by both peroxisome positioning and cell cycle cues.

The presence of only one greatly enlarged peroxisome in cells lacking Vps1p facilitates the analysis of Inp2p dynamics because it leads to the accumulation of all Inp2p molecules in the membrane of a single peroxisome. Under these circumstances, it was evident that Inp2p-GFP initially sampled the entire peroxisome, with its leading edge containing higher levels of Inp2p. However, later in the cell cycle, only that part of the enlarged peroxisome present in the bud preserved Inp2p, and no Inp2p-GFP could be detected in the mother cell. In contrast, in cells lacking Vps1p and expressing the myo2-YFP1483A mutation, the peroxisomes in mother cells contained detectable amounts of Inp2p. This observation is consistent with our model in which the peroxisomes that initially accumulate Inp2p, or in the case of vps1Δ cells, the region of the tubule that initially accumulates Inp2p, are delivered to the bud first and ultimately trigger the down-regulation of Inp2p in the peroxisomes remaining in the mother cell (Fig. 7).

Feedback regulation of Inp2p could be provided by a post-translational modification, such as phosphorylation, that would make Inp2p susceptible to degradation. In this study, we show that Inp2p is a phosphorylase whose level of phosphorylation correlates with the cell cycle, being more pronounced at the start and end of the cell cycle. Notably, Vac1p was shown to be activated in the mother cell by phosphorylation by Cdk1p (Peng and Weissman, 2008) and in the bud by Cla4p and Ste20p, an event that leads to its turnover (Bartholomew and Hardy, 2009). The existence of two temporally distinct phosphorylation events of Inp2p hints at the possibility of successive steps of checkpoint-dependent activation and inactivation of Inp2p, such as its preparation for binding to Myo2p early in the cell cycle and its preparation for degradation late in the cell cycle. However, because the times at which the phosphorylated forms of Inp2p appear and disappear are the same in wild-type cells and cells of the peroxisome inheritance mutant myo2-YFP1483A, the timing of phosphorylation of Inp2p is probably coupled to the cell cycle and not related to intracellular peroxisome placement.

In closing, we have shown that Inp2p, the peroxisome-specific receptor for Myo2p, is subject to both spatial and temporal regulation. Our findings point to the existence of cell cycle–dependent and organelle positioning–dependent mechanisms that control the activity and levels of organelle-specific receptors for the molecular motors that drive the intracellular motility of different membrane-bound compartments. Given that the different types of organelles compete for access to these motors, cells have developed multiple levels of regulation for their organelle-specific receptors so as to ultimately achieve an equal distribution of compartments between mother and daughter cells.

Materials and methods

Strains and culture conditions

S. cerevisiae strains used in this study are listed in Table S1. Strains were cultured at 30°C with the exception of temperature-sensitive myo2 mutants, which were cultured at 24°C. The media components used were YPD (1% yeast extract, 2% peptone, and 2% glucose), SCIM (0.67% yeast nitrogen base without amino acids, 0.5% yeast extract, 0.5% peptone, 3.3% Brij 35, 0.3% glucose, 0.3% oleic acid, and 1× complete supplement mixture [CSM; Bio 101]), SM (0.67% yeast nitrogen base without amino acids, 2% glucose, and 1× CSM), and nonfluorescent medium (6.61 mM KH2PO4, 1.32 mM K2HPO4, 4.06 mM MgSO4·7H2O, 26.64 mM (NH4)2SO4, 1× CSM, 2% glucose, and 1% agarose).

Construction of myo2 mutant strains

Individual point mutations in MYO2 were made by the site-directed mutagenesis method (QuikChange; Agilent Technologies) using pRS413-MYO2 (HIS3; Pashkova et al., 2006) as a template. Each individual mutation was verified by DNA sequencing. The list of all myo2 mutants used in this study, including ones previously made by Pashkova et al. (2006) using a similar method, is presented in Table S2.

The heterozygous deletion diploid strain MYO2/myo2Δ (Table S1) was transformed with pRS416-MYO2 (URA3). Successful transformants were sporulated, and tetrads were dissected to select for the haploid MATα strain myo2Δ/pRS416-MYO2 (Table S1). Either the POT1 Δ gene encoding the peroxisomal matrix enzyme 3-ketoacyl-CoA thiolase (Pat1p) or the INP2 gene was genomically tagged with sequence encoding an improved version of GFP (GFP+) from Aequorea victoria (Scholz et al., 2000) by homologous recombination with a PCR-based integrative transformation, making the strains myo2Δ/POT1-GFP/pRS416-MYO2 and myo2Δ/INP2-GFP/pRS416-MYO2 (Table S1). Cells of these strains were transformed with either wildtype MYO2 or myo2 harboring a point mutation. Transformants were streaked onto SM lacking histidine and uracil (SM-His-Ura) to select for the presence of both plasmids and then onto SM containing 5-fluoroorotic acid to remove pRS416-MYO2. The resulting strains contained pRS413-MYO2 or pRS413-myo2 as sole copies of the MYO2 gene. To visualize peroxisomes in strains expressing INP2-GFP, cells were transformed with the plasmid pMFp-SKL encoding mRFP from Discosoma species (Campbell et al., 2002) tagged at its carboxyl terminus with the peroxisome-targeting signal 1, Ser-lys-Leu. The strain myo2Δ/vps1Δ/INP2-GFP/pmRFP-SKL/pRS416-myo2Δ (Table S1) was constructed by replacing the VPST1 gene with the LEU2 gene by homologous recombination using a PCR-based integrative transformation.

To construct the strain myo2Δ/POT1-GFP/pRS416-MYO2 (Table S1), INP2 was first genomically tagged with sequence encoding Staphylococcus aureus pA by homologous recombination using a PCR-based integrative transformation of myo2Δ/POT1-GFP/pRS416-MYO2 cells. A gene confering hygromycin B resistance was substituted for the HIS5 gene in the pA cassette using the plasmid pAG26 cherisharos (Eurosarf). The pRS416-MYO2 (URA3) plasmid in the resulting strain was swapped with the pRS413 (HIS5) plasmids containing either wildtype MYO2 or myo2 harboring a point mutation (as described for INP2-GFP).

Microscopy

Cells were grown in YPD and incubated in SCIM for 6 h. Slides were prepared according to Adames et al. (2001) with modifications. Essentially, 200 µl of hot nonfluorescent medium was used to prepare a thin agarose pad on a slide with two 18-mm square wells (Cel-line Brand). 1–2 µl culture was placed onto the slide, covered with a coverslip, and sealed with Vapal (1:1:1 mixture of vaseline, lanolin, and paraffin). Cells were incubated at room temperature for image capture. Images were acquired as described previously (Hammond and Glick, 2000) using a modified confocal microscope equipped with a 63× 1.4 NA Plan Apo objective (LSM 510 META; Carl Zeiss, Inc.). A piezoelectric actuator was used to drive continuous objective movement, allowing for the rapid collection of 200 optical sections spaced 0.125 µm apart were captured. Accumulated images were deconvolved using algorithms provided by Huygens Professional Software (Scientific Volume Imaging BV). For this method, 3D datasets were processed to remove noise and reassign blur through an iterative classical maximum likelihood estimation algorithm and an experimentally derived point-spread function. The transmission image was treated differently. In Huygens, a Gaussian filter was applied to the transmission image, and blue color was applied to the transmission image using Imaris software (version 6.1; Bitplane, Inc.). The level of the transmission image was modified, and the image was processed until only the circumference of the cell was visible. To prevent interference of internal structures captured in the transmission images, the internal structures were removed in Photoshop (Adobe). Imaris was subsequently used to display the deconvolved 3D dataset with the processed transmission image and to prepare the image files before final figure assembly in Photoshop and InDesign (Adobe).
Quantification of rates of peroxisome inheritance

Rates of peroxisome inheritance were quantified essentially as described previously (Fagarasanu et al., 2005, 2006a). Cells synthesizing genomically encoded Pot1p-GFP were grown in YPD, transferred to SCIM, and incubated in SCIM for 16 h to achieve an OD$\_{600}$ of 0.5. Peroxisomes were visualized by direct fluorescence confocal microscopy. For each randomly chosen field, three optical sections of 5-µm thickness were collected at each time point as indicated, and total cell lysates were subjected to autoradiography. Protein pellets were dissolved in 20 µl 1 M Tris base and 100 µl 2× SDS sample buffer (150 mM Tris-HCl, pH 6.8, 15% glycerol, 2% SDS, 4% 2-mercaptoethanol, and bromophenol blue [trace]) and boiled before SDS-PAGE.

Antibodies

Inp2p-pA was detected by immunoblotting with affinity-purified rabbit IgG (MP Biomedicals). Rabbit antibodies to S. cerevisiae glucose-6-phosphate dehydrogenase (G6PDH) were obtained from Sigma-Aldrich. Rabbit antibodies to Cib2p were provided by R. Wozniak (University of Alberta, Edmonton, Alberta, Canada). Horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibodies were used to detect primary antibody complexes in immunoblots. Antigen–antibody complexes in immunoblots were detected by enhanced chemiluminescence (GE Healthcare).

Total RNA was extracted with hot acidic phenol. Contaminating chromosomal DNA was removed by treatment with DNase I, and 30 µg RNA was separated in 1.25% agarose gels containing 6% formaldehyde. RNA was blotted onto nylon membranes (GE Healthcare) in 20× SSC and cross-linked to the membrane by transillumination. Blots were hybridized under stringent conditions in Rapid-hyb buffer (GE Healthcare) with INP2- and ACT1-specific probes, which were 32P radiolabeled using the Prime It II random primer labeling kit (Agilent Technologies). Blots were washed in 50% formamide and subjected to autoradiography.

Additional method

All figures showing the Myo2p protein structure were made using PyMOL software (http://www.pymol.org).

Online supplemental material

Table S1 lists the S. cerevisiae strains used in this study. Table S2 lists the myo2 point mutants used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200904050/DC1.

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Peng and Weisman, 2008. In brief, protein pellets were resuspended in 140 µl 0.3 M sorbitol, 10 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM MgCl$_2$, and 1 mM EDTA, and 60 µl 1 M Tris base, 133 µl 10% SDS, and 7 µl 2-mercaptoethanol were added. 100 µl of these lysates were diluted with 900 µl 50 mM Tris-HCl, pH 8.8. Phosphatase reactions were started by addition of 20 U calf intestine alkaline phosphatase (0.5 U/µl; New England Biolabs, Inc.) and incubated at 30°C for 1 h. Total protein was precipitated for 30 min on ice by addition of 50% TCA to a final concentration of 15% and pelleted by centrifugation. Protein pellets were dissolved in 20 µl 1 M Tris base and 100 µl 2× SDS sample buffer [150 mM Tris-HCl, pH 6.8, 15% glycerol, 2% SDS, 4% 2-mercaptoethanol, and bromophenol blue [trace]] and boiled before SDS-PAGE.
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