Inp1p is a peroxisomal membrane protein required for peroxisome inheritance in Saccharomyces cerevisiae

Monica Fagarasanu,1 Andrei Fagarasanu,1 Yuen Yi C. Tam,1 John D. Aitchison,1,2 and Richard A. Rachubinski1

1Department of Cell Biology, University of Alberta, Edmonton, Alberta T6G 2H7, Canada
2Institute for Systems Biology, Seattle, WA 98103

Cells have evolved molecular mechanisms for the efficient transmission of organelles during cell division. Little is known about how peroxisomes are inherited. Inp1p is a peripheral membrane protein of peroxisomes of Saccharomyces cerevisiae that affects both the morphology of peroxisomes and their partitioning during cell division. In vivo 4-dimensional video microscopy showed an inability of mother cells to retain a subset of peroxisomes in dividing cells lacking the INP1 gene, whereas cells overexpressing INP1 exhibited immobilized peroxisomes that failed to be partitioned to the bud. Overproduced Inp1p localized to both peroxisomes and the cell cortex, supporting an interaction of Inp1p with specific structures lining the cell periphery. The levels of Inp1p vary with the cell cycle. Inp1p binds Pex25p, Pex30p, and Vps1p, which have been implicated in controlling peroxisome division. Our findings are consistent with Inp1p acting as a factor that retains peroxisomes in cells and controls peroxisome division. Inp1p is the first peroxisomal protein directly implicated in peroxisome inheritance.

Introduction

Compartmentalization of biochemical functions in membrane-bound organelles provides the eukaryotic cell a level of control unavailable to the prokaryotic cell. Because organelles must form from preexisting membranes (Nunnari and Walter, 1996; Warren and Wickner, 1996), maintenance of the advantages afforded by compartmentalization requires the accurate segregation of organelles from mother cell to daughter cell at cell division. This segregation could occur either randomly or by an ordered process requiring cellular machinery. Evidence suggests the second way is more common (Warren and Wickner, 1996; Yaffe, 1999; Catlett and Weisman, 2000), and eukaryotic cells have evolved molecular mechanisms to ensure the faithful inheritance of different organelles.

Unicellular eukaryotes, including notably the budding yeast Saccharomyces cerevisiae, have been used extensively and effectively to dissect the molecular pathways involved in organelle inheritance. Division in S. cerevisiae is asymmetrical, with the formation of a bud that is initially much smaller than its mother. A highly polarized actin cytoskeleton is needed for bud formation and for the faithful delivery of organelles to the emerging bud (Yaffe, 1991). Organelles are duplicated or fragmented within the S. cerevisiae mother cell and then transported along actin tracks by molecular motors to their proper location within the bud.

The class V myosins form a family of actin-associated motors that are necessary for the polarized distribution of organelles. These highly conserved myosins contain an amino-terminal motor domain that binds to actin filaments and a carboxyl-terminal tail domain that binds one or more cargos. The class V myosin, Myo2p, plays a critical role in the bud-directed transport of different organelles, including the vacuole (Hill et al., 1996; Catlett and Weisman, 1998, 2000), secretory vesicles (Govindan et al., 1995; Schott et al., 1999), late Golgi elements (Rossanese et al., 2001), mitochondria (Boldogh et al., 2004; Itoh et al., 2004), and peroxisomes (Hoepfner et al., 2001). Myo2p’s involvement in the transport of many different organelles has been explained by the presence of distinct domains in the globular tail of Myo2p that bind to organelle-specific receptors in a temporal and spatial pattern characteristic for the transport of a particular organelle to the yeast bud (Catlett and Weisman, 1998, 2000; Schott et al., 1999; Itoh et al., 2004). Another class V myosin, Myo4p, has been shown to be involved in the inheritance of the cortical ER that lines the periphery of S. cerevisiae cells (Estrada et al., 2003).

Correspondence to Richard A. Rachubinski: rick.rachubinski@ualberta.ca

Abbreviations used in this paper: 20KgP, 20,000 g pellet; 20KgS, 20,000 g supernatant; 4D, 4-dimensional; G6PDH, glucose-6-phosphate dehydrogenase; Lat A, latrunculin A; mRFP, monomeric RFP; pA, protein A; PTS, peroxisome targeting signal; SM, synthetic minimal.

The online version of this article contains supplemental material.
Although the molecular mechanisms of inheritance of the vacuole, Golgi, ER, and mitochondria become ever more clearly defined, little is known about the inheritance of peroxisomes. Peroxisomes undergo an ordered migration during the cell cycle (Hoepfner et al., 2001). A subset of peroxisomes localizes to the presumptive bud site and is then transported to the nascent bud. Although peroxisomes in the mother cell retain fixed cortical positions, the dynamics of newly inherited peroxisomes correlate with the polarity of the actin cytoskeleton in the bud. Thus, peroxisomes cluster at the bud tip during apical growth and are distributed over the entire bud cortex during the isotropic phase. At cytokinesis, peroxisomes localize to the mother-bud junction, consistent with a reorientation of the actin cytoskeleton for septum assembly at this stage of the cell cycle. The dynamics of peroxisomes during the cell cycle appear to be dependent on Myo2p, because cells of a temperature-sensitive mutant strain of \textit{S. cerevisiae} display a delay in, but not a halt to, the insertion of peroxisomes into the bud at the restrictive temperature (Hoepfner et al., 2001). However, to date, no protein, and in particular no peroxisomal protein, has been shown to have a direct role in peroxisome inheritance.

Systems biology approaches, including transcriptome profiling, organelar proteomics, and comparative gene analysis, have recently led to the identification of a number of novel proteins required for peroxisome assembly (peroxins) in \textit{S. cerevisiae} (Smith et al., 2002; Rottensteiner et al., 2003; Tam et al., 2003; Vizeacoumar et al., 2003, 2004). The construction of a collection of strains expressing full-length, chromosomally tagged GFP fusions covering almost 70% of the genes of the \textit{S. cerevisiae} genome (Huht et al., 2003) has provided another powerful systems biology tool for the identification of novel peroxisomal proteins and potentially novel peroxins. This global analysis of protein localization has led to the tentative identification of a protein of unknown function, Ymr204p, as being peroxisomal. Here, we present evidence that Ymr204p is a peripheral membrane protein of peroxisomes controlling peroxisome size and number and, notably, is required for the inheritance of peroxisomes. Ymr204p, renamed Inp1p, is the first peroxisomal protein directly implicated in peroxisome inheritance.

**Results**

Inp1p is a peripheral membrane protein of peroxisomes

A global analysis of protein localization in \textit{S. cerevisiae} identified Inp1p (Ymr204p), a protein of unknown function, as a...
heretofore unknown peroxisomal protein (Huh et al., 2003). However, the demonstration of a peroxisomal localization for Inp1p remained tentative, because protein localization was done in strains grown in glucose medium, and peroxisomes are dispensable for growth of yeast in glucose medium. We therefore determined the localization of Inp1p in cells incubated in oleic acid medium, the metabolism of which requires peroxisomes and leads to increased numbers of peroxisomes per cell.

A genomically encoded fluorescent chimera of Inp1p and GFP (Inp1p-GFP) was localized in oleic acid-incubated cells by confocal microscopy. Inp1p-GFP colocalized with a fluorescent chimera (mRFP-PTS1) of monomeric RFP (mRFP) and the peroxisome targeting signal (PTS) Ser-Lys-Leu to punctate structures characteristic of peroxisomes (Fig. 1 A).

Subcellular fractionation also showed Inp1p to be peroxisomal. A genomically encoded protein A (pA) chimera of Inp1p, Inp1p-pA, like the peroxisomal matrix protein thiolase, localized preferentially to the 20,000 g per pellet (20KgP) fraction enriched for peroxisomes and mitochondria (Fig. 1 B). Isopycnic density gradient centrifugation of the 20KgP fraction showed that Inp1p cofractionated with thiolase but not with the mitochondrial protein, Sdh2p (Fig. 1 C).

Organelle extraction was used to determine the intraperoxisomal location of Inp1p. Peroxisomes were subjected to hypotonic lysis in dilute alkali Tris buffer, followed by ultracentrifugation to yield a supernatant (T18S) fraction enriched for matrix proteins and a pellet (T18P) fraction enriched for membrane proteins (Fig. 1 D). Inp1p-pA cofractionated with a pA chimera of the integral membrane protein Pex3p to the T18P fraction. The soluble peroxisomal matrix protein thiolase was found almost exclusively in the T18S fraction. The T18P fraction was then extracted with alkali Na$_2$CO$_3$ and subjected to ultracentrifugation. Inp1p-pA fractionated to the supernatant (CO$_3$S) enriched for peripheral membrane proteins and did not cofractionate with Pex3p-pA to the pellet (CO$_3$P) enriched for integral membrane proteins. These results are consistent with Inp1p being a peripheral membrane protein of peroxisomes.

The synthesis of many peroxisomal proteins is induced by incubating yeast cells in oleic acid medium. The expression level of genomically encoded Inp1-pA remained essentially unchanged during incubation in oleic acid medium (Fig. 1 E), as has been observed with some peroxisomal peroxins (Tam et al., 2003; Vizeacoumar et al., 2003, 2004). Under the same conditions, the level of the peroxisomal matrix enzyme thiolase increased considerably, whereas the level of the cytosolic enzyme glucose-6-phosphate dehydrogenase (G6PDH) remained constant and acted as a control for protein loading.
Yeast strains compromised in peroxisome biogenesis often exhibit a growth defect in medium containing oleic acid as the sole carbon source, the metabolism of which requires functional peroxisomes. Cells deleted for \textit{INP1} were compromised in their growth on oleic acid–containing YPBO agar plates, but not to the same degree as the peroxisome assembly mutant \textit{pex3/H9004} (Fig. 1 F), consistent with a defect in some aspect of peroxisome biogenesis and/or function in \textit{inp1Δ} cells.

\textbf{Cells deleted for or overexpressing \textit{INP1} exhibit abnormal peroxisomes}

Wild-type and \textit{inp1Δ} cells expressing the genomically integrated chimeric gene \textit{POT1-GFP} encoding peroxisomal thiolase tagged at its carboxyl terminus with GFP (Pot1p-GFP) were incubated in YPBO medium and observed at different times of incubation by direct fluorescence confocal microscopy (Fig. 2 A). Peroxisomes increased dramatically in size during time of incubation in YPBO medium and were noticeably larger than peroxisomes of wild-type cells, particularly at longer times of incubation. There was also a dramatic decrease in peroxisome number in \textit{inp1Δ} cells compared with wild-type cells with time of incubation. However, there was heterogeneity in the peroxisome phenotype in \textit{inp1Δ} cells, with some cells exhibiting decreased numbers of enlarged peroxisomes and others exhibiting peroxisomes similar in size and number to peroxisomes of wild-type cells (Fig. 2 A, 2 h image). EM (Fig. 2 B) and morphometric analysis (Fig. S1 and Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200503083/DC1) confirmed an overall increase in the size and decrease in the number of peroxisomes in \textit{inp1Δ} cells with time of incubation in oleic acid medium.

The multicopy plasmid YEpl3 containing the \textit{INP1} gene was introduced into wild-type cells synthesizing Pot1p-GFP to determine the effects of \textit{INP1} overexpression on the peroxisome phenotype. Overexpression of \textit{INP1} in cells incubated in oleic acid medium led to the preferential localization of peroxisomes to the cortical regions of cells (Fig. 2 C), as shown by the analysis of individual optical sections in a z-stack. In addition, overexpression of \textit{INP1} led to an apparent irregularity in the distribution of peroxisomes between mother cells and buds, with a significant number of buds not containing any readily evident fluorescent peroxisomes.

\textbf{Deletion of \textit{INP1} leads to increased numbers of mother cells without peroxisomes}

The uneven distribution of peroxisomes in cells in which Inp1p was either absent or overproduced suggested an involvement of Inp1p in partitioning peroxisomes between mother cell and bud during cell division. To investigate this possibility, wild-type and \textit{inp1Δ} cells synthesizing Pot1p-GFP to fluorescently label peroxisomes were incubated in SCIM-containing glucose and oleic acid to allow for cell division and proliferation of peroxisomes. Fluorescent images of budded cells were acquired by confocal microscopy. Mother cells were scored for the presence or absence of fluorescent peroxisomes. Buds were sized according to four categories relative to the volume of the mother cell, expressed as a percentage of the mother cell volume (category I, 0–12%; category II, 12–24%; category III, 24–36%; category IV, 36–48%; see Materials and methods). Quantification was performed on at least 20 budded cells from each category. (B) Wild-type and \textit{INP1}-overexpressing cells synthesizing Pot1p-GFP to label peroxisomes were incubated in SCIM and examined by confocal microscopy as described in A. Buds were scored for the presence or absence of fluorescent peroxisomes, sized and categorized, and quantification was performed, as defined in A. Bars, 1 μm.

**Figure 3.** Deletion or overexpression of \textit{INP1} leads to defects in partitioning peroxisomes between mother cell and bud. (A) Wild-type and \textit{inp1Δ} cells expressing \textit{POT1-GFP} to fluorescently label peroxisomes were incubated for 16 h in SCIM-containing glucose and oleic acid to allow for cell division and proliferation of peroxisomes. Fluorescent images of budded cells were acquired by confocal microscopy. Mother cells were scored for the presence or absence of fluorescent peroxisomes. Buds were sized according to four categories relative to the volume of the mother cell, expressed as a percentage of the mother cell volume (category I, 0–12%; category II, 12–24%; category III, 24–36%; category IV, 36–48%; see Materials and methods). Quantification was performed on at least 20 budded cells from each category. (B) Wild-type and \textit{INP1}-overexpressing cells synthesizing Pot1p-GFP to label peroxisomes were incubated in SCIM and examined by confocal microscopy as described in A. Buds were scored for the presence or absence of fluorescent peroxisomes, sized and categorized, and quantification was performed, as defined in A. Bars, 1 μm.
Peroxisomes retain fixed cortical positions in mother cells. One peroxisome reaches the bud, keeps its mobility for a defined period of time (until bud emergence (0°) gather at the presumptive bud site (30°). Subsequently, all peroxisomes are transported into the growing bud (30°–170°). Inside the bud, peroxisomes localize to sites of active growth, being initially clustered at the bud tip and then relocated to the bud neck region before cytokinesis (Video 2). (C) Peroxisomes present in the mother cell (3°) move into the bud (31°). One peroxisome then returns to the mother cell from the bud (72°; Video 3). (D) Initially, peroxisomes perform saltatory movements (10°–30°) and are then inserted into the growing bud (57°–107°; Video 4). (E) Peroxisomes present in the mother cells before bud emergence move into the buds (72°; Video 5). In the topmost cell, a peroxisome passes with difficulty into the bud due to its large size (0°–3°). In the cell at bottom, left, peroxisomes gather at the bud site (0°–3°) and eventually enter the forming bud. At 92°, one peroxisome returns to the mother cell. Some peroxisomes remain in the mother cell and display chaotic movements. In the cell at bottom, right, peroxisomes display chaotic movements (0°–18°) and then gather at the new bud site. Eventually, all peroxisomes move into the bud (184°; Video 5). (F and G) Wild-type BY4742 cells overexpressing INP1. (F) Peroxisomes appear immobile (0°–145°). Analysis of individual optical sections from the 4D data showed the peroxisomes to be localized at the cell cortex. Both first and second generation buds lack peroxisomes (Video 6). (G) 

Overexpression of INP1 leads to increased numbers of buds without peroxisomes

Overexpression of INP1 in cells led to increased numbers of buds without peroxisomes as compared with wild-type cells (Fig. 3 B). Depending on the size of the bud, from 40% to 55% of buds of INP1-overexpressing cells lacked peroxisomes. In contrast, 10% of only the smallest buds of wild-type cells lacked peroxisomes (Fig. 3 B). These data are consistent with an overproduction of Inp1p resulting in greater retention of peroxisomes in the mother cell, which in its turn leads to compromised peroxisome inheritance.

Impaired peroxisome inheritance in cells lacking or overexpressing INP1

The movement of peroxisomes between mother cell and bud was visualized by 4-dimensional (4D) in vivo video microscopy of wild-type, inf1Δ, and INP1-overexpressing cells containing genomically integrated POT1-GFP to fluorescently la-
bel peroxisomes. In wild-type cells, peroxisomes moved in a directed manner from mother cell to bud (Fig. 4 A and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200503083/DC1). A subset of peroxisomes was delivered to the growing bud, whereas there was concomitant maintenance of the peroxisome population within the mother cell. In \textit{inp1}/H9004 cells, the inheritance of peroxisomes was compromised, resulting in an unbalanced distribution of peroxisomes between mother cell and bud. Frequently, all peroxisomes present in the mother cell before bud emergence were transported to the bud, resulting in a mother cell devoid of detectable fluorescent peroxisomes (Fig. 4, B, D, and E; Videos 2, 4, and 5, available at http://www.jcb.org/cgi/content/full/jcb.200503083/DC1). Once in the bud, peroxisomes accumulated at the sites of polarized growth, being initially localized to the growing tip and, before cytokinesis, relocated to the mother-bud neck. The preference of newly inherited peroxisomes for sites of active growth in the bud is therefore apparently not dependent on Inp1p (Fig. 4 B and Video 2). Interestingly, before being transported to the bud, some peroxisomes in \textit{inp1} cells performed uncharacteristic chaotic movements (Videos 4 and 5). Peroxisomes were also observed that returned from the bud to the center of the mother cell far beyond the region of the bud neck (Fig. 4 C and Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200503083/DC1). Due to the larger size of many peroxisomes in \textit{inp1} cells, a delay was often observed in the passage of a peroxisome into the bud, indirectly affecting peroxisome inheritance (Fig. 4 E and Video 5). In \textit{INP1}-overexpressing cells, peroxisomes appeared immobilized at cortical locations with respect to the other cells and did not pass into the bud (Fig. 4 F and Video 6, available at http://www.jcb.org/cgi/content/full/jcb.200503083/DC1). Occasionally, peroxisomes managed to pass into the bud and initially perform movements similar to those of peroxisomes of wild-type cells (Fig. 4 G and Video 7, available at http://www.jcb.org/cgi/content/full/jcb.200503083/DC1). This mobile behavior would end abruptly, and the peroxisomes would take fixed cortical positions. These observations implicate Inp1p as a factor acting in the retention of peroxisomes within cells. A possible role for actin in the retention of peroxisomes was also investigated, because actin has been shown to have a role in retaining mitochondria in cells (Yang et al., 1999). Treatment of \textit{INP1}-overexpressing cells with the actin-disrupting toxin latrunculin A (Lat A; Rossanese et al., 2001) did not affect the localization of peroxisomes (Fig. 4 H), suggesting that actin is not involved in the retention of peroxisomes at the cell cortex.

The movement of peroxisomes in wild-type, \textit{inp1}/, and \textit{INP1}-overexpressing cells was also analyzed using 3-dimen-

![Figure 5. Quantification of peroxisome mobility.](http://www.jcb.org/cgi/content/full/jcb.200503083/DC1)
Figure 6. Peroxisomes are actively retained in the mother cell. Wild-type BY4742/POT1-GFP cells grown to mid-log phase in YPD medium were arrested in S phase by the addition of 200 mM hydroxyurea for 6 h. Fluorescent images of arrested cells were captured as a z-stack (bottom, middle, top) by confocal microscopy. The bottom cell is the mother cell, and the top cell is the hyperelongated bud. Bar, 1 μm.

Figure 7. Overproduced Inp1p is localized to peroxisomes and the cell cortex. The strain BY4742/POT1-RFP transformed with a multicopy YEp13 plasmid construct overexpressing INP1-GFP were grown to mid-log phase in glucose-containing SM medium and examined by confocal microscopy. Overproduced Inp1p-GFP is localized to both peroxisomes and the cell cortex. Bar, 1 μm.

Discussion

Eukaryotic cells partition their organelle populations during cell division. Here, we report that Inp1p, a protein of unknown function encoded by the S. cerevisiae genome, is required for bud neck. This approach has been used to demonstrate an active retention mechanism for mitochondria in cells (Yang et al., 1999). After treating cells with hydroxyurea, peroxisomes remained equally distributed between the mother cell and the now hyperelongated bud (Fig. 6). In addition, the peroxisomes in the mother cell were cortically localized. These results show that peroxisomes are actively retained in the mother cell.

Overproduced Inp1p localizes to peroxisomes and to the cell cortex

If Inp1p acts to secure peroxisomes to the cell cortex during cell division, overproduced Inp1p should also associate with the cell periphery in glucose-grown cells that have few peroxisomes. To test this, Inp1p-GFP was overproduced in wild-type BY4742/POT1-RFP cells grown in glucose-containing medium. Inp1p-GFP showed both peroxisomal and cortical localizations, supportive of Inp1p being the link between peroxisomes and an anchoring cortical structure (Fig. 7).

The levels of Inp1p vary with the cell cycle

The accurate partitioning of peroxisomes between mother cell and bud is an ordered process that progresses in distinct steps through the cell cycle. Accordingly, it might be expected that Inp1p would be subject to some form of cell cycle–dependent regulation. To test this, cells were subjected to and released from α factor–induced G1-arrest. The levels of Inp1p varied with the cell cycle (Fig. 8), peaking 60 min after α factor release.

Inp1p binds Pex25p, Pex30p, and Vps1p

In vitro binding assays were performed to begin identifying interacting partners of Inp1p. Bacterially produced GST fused to Inp1p (GST-Inp1p) and GST alone were immobilized on glutathione resin and incubated with yeast lysates expressing TAP-tagged Pex11p, Pex17p, Pex19p, Pex25p, Pex30p, and Vps1p. Inp1p was observed to interact with Pex25p, Pex30p, and Vps1p (Fig. 9), all of which have been implicated in controlling peroxisome size and number.

Peroxisomes are actively retained in the mother cell

Our data show a role for Inp1p in retaining peroxisomes in cells. Conceptually, the distribution of peroxisomes between mother cell and bud could be a time-dependent process controlled indirectly by cytokinesis or a process in which peroxisomes are actively retained in the mother cell independently of cell cycle duration. To choose between these views, wild-type BY4742/POT1-GFP cells were treated with hydroxyurea to arrest cells in S phase, which leads to a protracted opening of the mother cell and bud neck. This approach has been used to demonstrate an active retention mechanism for mitochondria in cells (Yang et al., 1999). After treating cells with hydroxyurea, peroxisomes remained equally distributed between the mother cell and the now hyperelongated bud (Fig. 6). In addition, the peroxisomes in the mother cell were cortically localized. These results show that peroxisomes are actively retained in the mother cell.

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Discussion

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peroxisome inheritance. Inp1p is the first peroxisomal protein directly implicated in the inheritance of peroxisomes. Inp1p is not required for peroxisome assembly per se, because cells harboring a deletion of INP1 contain readily identifiable peroxisomes by microscopic analysis and are able to import proteins targeted by either PTS1 or PTS2 (unpublished data).

Cells deleted for INP1 incubated in oleic acid medium showed a progressive decrease in the average number and increase in the average size of peroxisomes with time. However, there was heterogeneity in the peroxisome population, with some cells containing a few enlarged peroxisomes and other cells containing peroxisomes similar in size and number to peroxisomes of wild-type cells. This heterogeneity was suggestive of a defect in peroxisome partitioning. When inp1Δ cells were cultured in medium permitting peroxisome proliferation and rapid cell division, an imbalance in the partitioning of peroxisomes became readily apparent as mother cells without peroxisomes were observed. The overall proportion of mother cells without peroxisomes increased with increasing bud size. These observations, combined with the fact that overexpression of INP1 led conversely to large numbers of buds without peroxisomes and relocation of peroxisomes to the cortical regions of cells, strongly suggested a role for Inp1p in peroxisome inheritance.

The inheritance of organelles in budding yeast consists of two complimentary processes: the retention of a subset population of an organelle in the mother cell and the ordered movement of the remaining portion of the organelle population to the forming bud. The close control of both processes is crucial to the successful distribution of the organelle from mother cell to bud. A retention mechanism within the mother cell has been described for mitochondria (Yang et al., 1999). Retained mitochondria accumulate at the tip of the mother cell distal to the site of bud emergence (the so-called "retention zone"), a process that likely involves the actin cytoskeleton. Retention mechanisms also operate in the bud. In this study we showed that, similar to mitochondria, peroxisomes are actively retained in the mother cell. Both organelles and molecules have been shown to remain anchored to the bud cell cortex at discrete locations, as demonstrated for mitochondria (Simon et al., 1997), ASH1 mRNA (Long et al., 1997; Takizawa et al., 1997), and the protein chitin synthase 3 (DeMarini et al., 1997). Recently, the Rab-like protein Ypt11p was shown to be required for the retention of newly inherited mitochondria within buds of S. cerevisiae (Boldogh et al., 2004).

4D in vivo video microscopy showed that in wild-type cells, a subset of peroxisomes partitioned to the emerging bud, whereas the peroxisomes that remained in the mother cell retained fixed cortical positions. The newly inherited peroxisomes tend to concentrate at the sites of active growth inside the bud. Before cytokinesis, subsets of peroxisomes from both the mother cell and the bud redistribute to the neck region, whereas the remaining peroxisomes remain anchored to the cortices of the mother cell and bud.

Peroxisomes of inp1Δ cells displayed increased mobility relative to peroxisomes of wild-type cells and were never observed to be static. Moreover, in inp1Δ cells, there was no delay as compared with wild-type cells in the passage of peroxisomes to the emerging bud, except in those cells containing greatly enlarged peroxisomes. Therefore, Inp1p is not directly involved in the movement of peroxisomes between mother cell and bud, presumably along actin tracks. How then might Inp1p function in peroxisome inheritance? An interesting feature of the dynamics of peroxisomes in cells lacking Inp1p is that the entire peroxisome population in the mother cell first clusters at the presumptive bud site and then enters the bud, thereby deleting the mother cell of peroxisomes. At times, peroxisomes were observed that failed to be delivered to the growing bud, but they also appeared to be unattached to the mother cell cortex, performing chaotic movements within the mother cell. On occasion, peroxisomes, after having passed to the bud, returned deep into the interior of the mother cell, a phenomenon never observed in wild-type cells. Actin as a whole is apparently normal in inp1Δ cells (unpublished data), and thus a major reorganization of the actin cytoskeleton cannot explain why inp1Δ cells exhibit defects in peroxisome inheritance. In inp1Δ cells, peroxisomes fail to be actively retained in either the mother cell or the bud, which results in the disruption of the ordered vectorial process of peroxisome segregation during cell division. The movements of peroxisomes from buds to mother cells could be explained by proposing that peroxisomes delivered to the bud in inp1Δ cells have a decreased affinity for a structure that retains peroxisomes within the bud, with the possibility
that some peroxisomes actually elude the anchoring mechanism completely. Because the return of newly inherited peroxisomes usually occurred after their performance of the characteristic movements of peroxisomes in the bud observed in wild-type cells, including the initial clustering of peroxisomes at the bud tip, we would predict that other factors must also play a role in maintaining newly inherited peroxisomes in the bud, at least in the early stages. The overproduction of Inp1p results in the retention of peroxisomes in the mother cell at fixed cortical positions and prevents the distribution of a subset of peroxisomes to the growing bud. Occasionally, one peroxisome would be delivered to the bud and, after performing the usual movements in the bud, would gain a fixed position at the bud cortex. The fact that when overproduced Inp1p assumes a cortical distribution in glucose-grown cells containing few peroxisomes strengthened our conclusion that Inp1p acts to tether peroxisomes to anchoring structures localized to the periphery of cells. All in all, our data reveal a major role for Inp1p in tethering peroxisomes to anchoring structures in both mother cell and bud during cell division.

Evidence for Inp1p being regulated during the cell cycle suggests that peroxisome inheritance is tightly controlled by the cell. Increased amounts of Inp1p at certain stages of the cell cycle might be required to ensure the retention of peroxisomes in both mother cell and bud. Inp1p might increase in amount only on a subset of peroxisomes that become prone to anchoring at the cell cortex. Alternatively, Inp1p might be fairly equally distributed on all peroxisomes, and other regional regulatory mechanisms and molecules could themselves act through Inp1p to modulate the anchoring of peroxisomes to the cell cortex. The oscillation of Inp1p levels during the cell cycle correlates with the oscillation of \textit{INP1} mRNA levels during the cell cycle (Spellman et al., 1998), suggesting that the \textit{INP1} gene is subject to cell cycle regulatory control. It is noteworthy that Inp1p is predicted to contain a PEST sequence (a potential signal for rapid protein degradation) between amino acids 279 and 362 (Rechteiner and Rogers, 1996). Whether this PEST sequence functions in the degradation of Inp1p during the cell cycle awaits future experimentation.

A model for Inp1p function in partitioning peroxisomes between mother cell and bud is presented in Fig. 10. A subset of peroxisomes is transported to the bud by a process dependent on Myo2p (Hoepfner et al., 2001), whereas the remaining peroxisomes are retained within the mother cell on a cortical anchor. The peroxisomal peripheral membrane protein Inp1p would link the peroxisome to the cortical anchor. It is noteworthy that overproduction of Inp1p led to a distinctly enhanced cortical distribution of peroxisomes in cells. Whether a given peroxisome will be delivered to the bud or retained in the mother may depend on a tug-of-war between Inp1p and Myo2p. Accordingly, both under- and overproduction of Inp1p would lead to impairment of normal peroxisome inheritance. Once peroxisomes are delivered to the bud, they are prevented from returning to the mother cell. Inp1p also appears to play a role also in retaining peroxisomes within the bud, probably by attaching peroxisomes to cortical anchoring structures present in the bud. Actin structures do not appear to play a role in the Inp1p-dependent anchorage of peroxisomes to the cell cortex, because the treatment of cells overproducing Inp1p with Lat A did not lead to the detachment of immobilized peroxisomes. Moreover, we did not observe a colocalization between the Sac6p-containing actin patches and peroxisomes (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200503083/DC1).

The phenotype of reduced numbers of enlarged peroxisomes seen for \textit{inp1}Δ cells could conceptually arise only as a consequence of unbalanced partitioning of peroxisomes during cell division. However, the interactions of Inp1p with Pex25p, Pex30p, and Vps1p, which have all been shown previously to influence peroxisome division, support a role for Inp1p in peroxisome division. Thus, Inp1p seems to have a dual role in the division and the inheritance of peroxisomes in \textit{S. cerevisiae}. How might these two functions be related? Other proteins are known to influence both the morphology of organelles and their distribution. Mdm10p (Sogo and Yaffe, 1994), Mdm12p (Berger et al., 1997), and Mm11p (Burgess et al., 1994) are mitochondrial outer membrane proteins that affect mitochondrial shape and segregation. Mutation of any one of these proteins results in the presence of giant, spherical mitochondria that exhibit defects in partitioning at cell division. Recent studies (Boldogh et al., 2003) have indicated that these proteins form a complex that connects the minimum heritable unit of mitochondria (mtDNA and mitochondrial membranes) to actin, therefore functioning as a mitochondrial counterpart to the kintochore or the “mitochore.” These proteins affect the retention of mitochondria within the mother cell (Yang et al., 1999) and also Myo2p-independent mitochondrial movement (Boldogh et al., 2001).

In closing, we have presented evidence demonstrating that the peroxisomal peripheral membrane protein, Inp1p, is directly implicated in the inheritance of peroxisomes in \textit{S. cerevisiae}. Inp1p acts as a peroxisome-retention factor, tethering peroxisomes to putative anchoring structures within the mother cell and bud. Inp1p is the first peroxisomal protein shown to be involved in the inheritance of peroxisomes.

Figure 10. A model for Inp1p function in peroxisome retention. Peroxisomes move along polarized actin cables in a Myo2p-dependent manner from mother cell to bud. Concomitantly, a subset of peroxisomes is retained within the mother cell. Inp1p acts to link peroxisomes to a cortical anchor and retain peroxisomes in the mother cell and bud.
Materials and methods

Strains and culture conditions
The *S. cerevisiae* strains used in this study are listed in Table S2, available at http://www.jcb.org/cgi/content/full/jcb.200503083/DC1. All strains were cultured at 30°C, unless otherwise indicated. Strains containing plasmids were cultured in synthetic minimal (SM) medium. Media components were as follows: YPD, 1% yeast extract, 2% peptone, 2% glucose; YPBO, 0.5% yeast extract, 0.5% peptone, 0.5% KH₂PO₄, 0.5% KH₂PO₃, 3.3% Brij 35, 1% oleic acid; SM, 0.67% yeast nitrogen base without amino acids, 2% glucose, 1× complete supplement mixture (Bio 101) without uracil or leucine; SCIM, 0.67% yeast nitrogen base without amino acids, 0.5% yeast extract, 0.5% peptone, 3.3% Brij 35, 0.1% glucose, 0.1% oleic acid, 1× complete supplement mixture; YNO, 0.67% yeast nitrogen base without amino acids, 1× complete supplement mixture without leucine, 3.3% Brij 35, 1% oleic acid.

pA and GFP tagging of genes
Genes were genomically tagged with the sequence encoding Staphylococcus aureus pA or an improved version of GFP (GFP+) from Aequorea victoria (Scholz et al., 2000) by homologous recombination with a PCR-based integrative transformation of parental BY4742 haploid cells (Dillworth et al., 2001). The functionality of fusion proteins was confirmed by the lack of a mutant phenotype in transformed strains.

Plasmids
pmRFP-SKL was constructed by replacing the gene for RFP from Discosoma species in the plasmid pDsRed-PTS1 (Smith et al., 2002) by the gene encoding mRFP (Campbell et al., 2002). Genes to be overexpressed were amplified by PCR and cloned into the plasmid YEp13. For overexpression, the INP1 gene included 523 bp of upstream and 328 bp of downstream sequence.

Microscopy
Strains synthesizing GFP and/or mRFP chimeras were grown to mid-log phase in SM medium and then incubated in YPBO medium for 8 h or SCIM for 16 h. Images were captured on a LSM510 META (Carl Zeiss MicroImaging, Inc.) equipped with a digital fluorescence camera (Spot Diagnostic Instruments). EM of whole yeast cells (Eitzon et al., 1997) and morphometric analysis of EM images (Smith et al., 2000) were performed as described previously.

Quantification of rates of peroxisome inheritance
The rates of peroxisome inheritance were quantified essentially as described previously (Smith et al., 2000) were performed as described previously (Rossanese et al., 2001). The functionality of fusion proteins was confirmed by the lack of a mutant phenotype in transformed strains.

Hydroxyurea arrest
BY4742 cells grown to mid-log in YPD medium were treated with 200 mM hydroxyurea for 6 h at 30°C to arrest cells before cell division as described previously (Yang et al., 1999).

In vitro binding assay
GST and the GST-Inp1p were bound to glutathione Sepharose and incubated with yeast cell lysates containing TAP-tagged proteins as previously described (Marelli et al., 2004). TAP-tagged proteins were detected by immunoblotting.

Antibodies
Antibodies to thiolase and Sdh2p have been described previously (Tam et al., 2003). Rabbit antibodies to *S. cerevisiae* GapDH1 were purchased from Sigma-Aldrich. Rabbit antibodies to the TAP tag were purchased from Open Biosystems. HRP-conjugated donkey anti-rabbit IgG and HRP-conjugated goat anti-guinea pig IgG secondary antibodies were used to detect primary antibodies in immunoblot analysis. Antibigen–antibody complexes in immunoblots were detected by ECL (Amersham Biosciences).

Online supplemental material
Fig. S1 presents a morphometric analysis of peroxisome size in wild-type BY4742 and inp1Δ cells. Fig. S2 shows that peroxisomes do not colocalize with actin patches in cells overproducing Inp1p. Video 1 shows the movement of peroxisomes in wild-type BY4742 cells during cell division. Videos 2–5 show the movement of peroxisomes in inp1Δ cells during cell division. Videos 6 and 7 show the relatively static nature of peroxisomes in cells overexpressing the INP1 gene. Videos 8–10 present animations of 3D kymographs of peroxisome movement in wild-type and inp1Δ cells and in cells overexpressing INP1. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200503083/DC1.

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