Smy1p, a Kinesin-related Protein That Does Not Require Microtubules

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Abstract. We have previously reported that a defect in Myo2p, a myosin in budding yeast (*Saccharomyces cerevisiae*), can be partially corrected by overexpression of Smy1p, which is by sequence a kinesin-related protein (Lillie, S.H., and S.S. Brown. 1992. *Nature*. 356:358–361). Such a functional link between putative actin- and microtubule-based motors is surprising, so here we have tested the prediction that Smy1p indeed acts as a microtubule-based motor. Unexpectedly, we found that abolition of microtubules by nocodazole does not interfere with the ability of Smy1p to correct the mutant Myo2p defect, nor does it interfere with the ability of Smy1p to localize properly. In addition, other perturbations of microtubules, such as treatment with benomyl or introduction of tubulin mutations, do not exacerbate the Myo2p defect. Furthermore, a mutation in *SMY1* strongly predicted to destroy motor activity does not destroy Smy1p function. We have also observed a genetic interaction between *SMY1* and two of the late *SEC* mutations, *sec2* and *sec4.* This indicates that Smy1p can play a role even when Myo2p is wild type, and that Smy1p acts at a specific step of the late secretory pathway. We conclude that Smy1p does not act as a microtubule-based motor to localize properly or to compensate for defective Myo2p, but that it must instead act in some novel way.

In *Saccharomyces cerevisiae*, virtually all growth occurs in the bud rather than in the mother cell. *MYO2* encodes an unconventional class V myosin that has been implicated in this polarized growth by studies of the temperature-sensitive *myo2-66* mutant. This mutant fails to target growth to the bud at restrictive temperature, resulting in abnormally large mother cells (see Fig. 1 *a*; Johnston et al., 1991). Because vesicles were observed to accumulate in the mutant, Johnston et al. (1991) proposed that Myo2p targeted growth by delivering secretory vesicles to the bud. The recent finding that in chick brain a class V myosin is associated with synaptic vesicles (Prekeris and Terrian, 1997) would seem to suggest that other class V myosins might have similar functions. The evidence that Myo2p is a secretory vesicle motor, however, is far from conclusive (Liu and Bretscher, 1992; Govindan et al., 1995), and there are other ways that Myo2p might target growth. For example, it might deliver elements of the ER or Golgi apparatus, which are found in proximity to the bud at a very early stage of bud growth (Preuss et al., 1992). Another possibility is that Myo2p might reside at the bud tip as part of a complex that anchors the actin cytoskeleton or cell wall–synthesizing machinery.

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Smy1p, when overexpressed, can partially compensate for defects in the *myo2* mutant, overcoming lethality and restoring polarized growth at restrictive temperature (Lillie and Brown, 1992, 1994). Furthermore, a *myo2 smy1Δ* (SMY1 deletion) double mutant is dead at permissive temperature, despite the fact that each single mutant appears wild type under the same conditions (Lillie and Brown, 1992). This demonstrates that Smy1p is not simply providing some "spillover function" when overexpressed, but also that it is essential in a *myo2* mutant background. An even stronger indication of the functional significance of the interaction is that Myo2p and Smy1p colocalize, and that their localizations respond identically to several cell perturbations (Lillie and Brown, 1994). Furthermore, overexpression of Smy1p not only restores Myo2p mutant localization, but it also enhances the localization of wild-type Myo2p.

What is particularly surprising about the close relationship between Myo2p and Smy1p is that although both are putative motor proteins, they are predicted to interact with different cytoskeletal filaments. Myo2p is expected to interact with actin filaments, which have also been implicated in polarized growth by their changing localization during the cell cycle (Adams and Pringle, 1984; Kilmartin and Adams, 1984) and by studies of actin mutants (Novick and Botstein, 1985). Smy1p, on the other hand, is by sequence a kinesin-related protein (Lillie and Brown, 1992) and is thus expected to interact with microtubules. Some

time ago, it was believed that cytoplasmic microtubules would be required for bud growth, given their location; they emanate from the spindle pole body at the nuclear membrane and extend into the growing bud. Genetic and nocodazole studies have clearly shown, however, that microtubules are not required for bud growth, but instead, that they are required for nuclear migration and mitosis (Jacobs et al., 1988; Huffaker et al., 1988). Although microtubules together with Smy1p might act as a backup system that is needed for bud growth only when Myo2p is defective, such functional overlap would nonetheless be surprising.

Another consideration is the relative divergence of Smy1p compared to other members of the kinesin superfamily (Lillie and Brown, 1992; Goldstein, 1993), which raises the question of whether it is truly a microtubule-based motor. The three-dimensional structures of the motor domains of both myosin and kinesin have been determined (Rayment et al., 1993; Kull et al., 1996). Whereas these two families of motors show a remarkable similarity in their core structures, the insertions that confer microtubule vs. myosin binding are quite different (Kull et al., 1996; Woehlke et al., 1997). Smy1p has some sequence divergence in regions corresponding to the microtubule-binding regions, but not enough to judge whether it is likely to bind microtubules. For example, in the loop determined by mutagenesis studies to be the most critical for microtubule binding (Woehlke et al., 1997), Smy1p retains an important arginine (corresponding to R278) but has changes in other conserved residues. On the other hand, costal2, another divergent kinesin-related protein, does not retain this arginine but does bind microtubules (Sisson et al., 1997). Smy1p also has changes that could affect other aspects of motor function. It is divergent at two otherwise extremely conserved positions (corresponding to E250 and E311 in Woehlke et al., 1997) where mutations affect the ability of kinesin to be activated by microtubule binding. Other sites of divergence might affect interactions with nucleotide (N-1 through N-4 in Sablin et al., 1996); costal2 shows even more divergence in these regions (Sisson et al., 1997). In summary, sequence comparison does not allow us to predict whether Smy1p can bind microtubules, but it does raise the possibility that Smy1p, like costal2, may be a poor or even nonfunctional motor.

In this report, we have looked for functional evidence to determine whether Smy1p interacts with microtubules. Our results show that Smy1p does not require microtubules to localize properly or to compensate for mutant Myo2p defects.

Materials and Methods

Yeast Strains and Media

All yeast strains used are listed in Table I. The liquid media used were rich medium YM-P (yeast medium-Pringle; Lillie and Pringle, 1980) or yeast extract/peptone/dextroxe medium (YPD)[1] (Sherman et al., 1986) and synthetic complete medium lacking the appropriate supplement to select for plasmid (Sherman et al., 1986). Solid media were made by adding 1.5% agar to YPD or synthetic complete medium. For all media, glucose was autoclaved separately and added to 2%, except that in preparation for sporulation, cultures were often grown in 0.5% glucose instead. Cultures were grown at room temperature (20–23°C) unless otherwise specified.

DNA Manipulations and Genetic Techniques

Standard procedures were used for DNA manipulations and *Escherichia coli* transformation (Sambrook et al., 1989) and for yeast transformation and genetic manipulations (Sherman et al., 1986).

To alter amino acid 119 of Smy1p from Gly to Glu, a missense mutation (*smy1-3*) was introduced by site-directed mutagenesis (using the double-primer method; Sambrook et al., 1989) of a single-stranded template, using the following mutagenic primer: GTTTCAGCGAAAAGTCCTA. The mutation was confirmed by sequencing and then shuttled as a KpnI-XbaI fragment to KpnI-XbaI-cut pBR-SMY1 [pBR322 (Sambrook et al., 1989) with a ~4.5-kb EcoRV *SMY1*-containing insert]. The ~3.3-kb HpaI-EcoRV *smy1-3*–containing fragment from this plasmid was inserted into SmaI-digested YEp352 (Hill et al., 1986) to give YEpsmy1-52. YEpSMY1-52 was constructed by shuttling the corresponding wild-type *SMY1* segment from pBR-SMY1 to YEp352.

Nocodazole Treatment and Immunofluorescence

Nocodazole (Sigma Chemical Co., St. Louis, MO) was added to exponentially growing cultures (1–2 × 10^6 cells/ml) as described by Jacobs et al. (1988), except that stocks were stored in aliquots at −80°C, and these were warmed briefly to 50°C immediately before use to ensure complete solubilization. At each time point, samples were fixed, and an aliquot was processed for Smy1p, Myo2p, or microtubule staining by indirect immunofluorescence, as described by Lillie and Brown (1994). (Formaldehyde was added directly to shaking cultures to minimize loss of labile structures.) Samples were examined using a range of antitubulin concentrations to optimize conditions for detection of microtubules.

Cells eventually escape the effects of nocodazole (Jacobs et al., 1988), and this occurs more rapidly when a greater than optimum concentration is used (see Fig. 2, compare *a* and *d*). Therefore, for each strain and set of experimental conditions, we carried out pilot experiments using a series of nocodazole concentrations to find the narrow window where the drug had its greatest effect. The length of the nocodazole block is also dependent on a number of other factors. Haploids are more resistant to the drug than diploids (our observations and Stearns, T., personal communication), and nocodazole is more effective in rich medium than in selective medium. Furthermore, escape is enhanced at a higher temperature. We took advantage of these findings to maximize the length of the nocodazole block. Diploids were used in nocodazole experiments, cells were shifted to rich medium if they had been grown in selective medium, and the experiment in Fig. 2 was carried out without elevating the temperature. In addition, for the experiment in Fig. 1, we used diploids heterozygous for the *tub1-1* allele. This α-tubulin mutation increases sensitivity to nocodazole, even when heterozygous (our observations and Stearns, T., personal communication), which is useful, because the homozygous but not the heterozygous *tub1* mutant shows an elevated number of large-budded cells even at permissive temperature in the absence of nocodazole. We confirmed that *tub1* does not qualitatively alter the results in experiments using other *myo2* strains that were wild type for *TUB1* (not shown).

Results

Smy1p Does Not Require Microtubules to Compensate for Myo2p Defects

By sequence, Smy1p is clearly a kinesin-related protein (Lillie and Brown, 1992), but its divergence (see Introduction) raised the question of whether it functioned as expected. To ask whether microtubules were required for Smy1p function, we tested the effects of the microtubule-depolymerizing drug nocodazole on the best characterized function of Smy1p, its ability to compensate for *myo2* mutant defects. Nocodazole causes arrest of wild-type cells at the large-budded stage of the cell cycle (Fig. 1 *a*) because mitosis, but not bud growth, is blocked (Jacobs et al., 1988).

1. *Abbreviations used in this paper*: YM-P, yeast medium-Pringle; YPD, yeast extract/peptone/dextrose medium.

Table I. Yeast Strains Used in This Study

Strain	Relevant genotype*	Source‡
ABY167	*MATα, tpm1Δ::LEU2, ura3, leu2, his3, ade2*	A. Bretscher (Cornell University, Ithaca, NY)
APY4ΔD6	*MATa, smy1Δ-1::URA3, ura3, leu2, trp1, ade2, his6*	
BDY4	*MATα, tpm2Δ::HIS3, ura3, leu2, his3, trp1, ade2*	A. Bretscher
DBY1993	*MATa, act1-2, ura3*	D. Botstein (Stanford University, Stanford, CA)
DBY1999	*MATa, "act1-3"* (=*act1-1*), *ura3*	D. Botstein
CUY51	*MATa, tub2-104, ura3, his4*	T. Huffaker (Cornell University)
CUY72	*MATa, tub2-402, ura3, his4, lys2*	T. Huffaker
CUY75	*MATa, tub2-403, ura3, his4, lys2*	T. Huffaker
CUY80	*MATa, tub2-404, ura3, his4, lys2*	T. Huffaker
CUY83	*MATa, tub2-405, ura3, his4, lys2*	T. Huffaker
DPY3	*MATα, tub2-406, ura3, his4*	T. Huffaker
MAY545	*MATα, tub1-1, ura3, leu2 his3, lys2*	M.A. Hoyt (Johns Hopkins University, Baltimore, MD)
MAY755	*MATa, tub3::TRP1, ura3, leu2, trp1, his3*	M.A. Hoyt
MAY2065	*MATα, cin8::URA3, ura3, leu2, his3, lys2, ade2*	M.A. Hoyt
MS524	*MATa, kar3-101::LEU2, ura3, leu2, ade2*	M. Rose (Princeton University, Princeton, NJ)
MS2305	*MATα, kip1-2::URA3, ura3, leu2, trp1*	M. Rose
MS2309	*MATa, kip2Δ1::URA3, ura3, leu2, his3, ade2*	M. Rose
myo3Δ-4Ca§	*MATa, myo3Δ::HIS3, ura3, leu2, trp1, his3*	
NY3	*MATa, sec1-1, ura3*	P. Novick (Yale University, New Haven, CT)
NY17	*MATa, sec6-4, ura3*	P. Novick
NY57	*MATa, sec9-4, ura3*	P. Novick
NY61	*MATa, sec10-2, ura3*	P. Novick
NY64	*MATa, sec15-1, ura3*	P. Novick
NY130	*MATa, sec2-41, ura3*	P. Novick
NY402	*MATa, sec5-24, ura3*	P. Novick
NY405	*MATa, sec4-8, ura3*	P. Novick
NY410	*MATa, sec8-9, ura3*	P. Novick
NY425	*MATα, sec22-3, ura3*	S. Ferro-Novick (Yale University)
NY427	*MATα, sec12-4, ura3, leu2, trp1, his4*	S. Ferro-Novick
NY432	*MATα, sec18-1, ura3*	P. Novick
NY760	*MATα, sec7-1, ura3*	P. Novick
RH268-1C	*MATa, end4-1, ura3, leu2, his4, bar1*	H. Riezman (University of Basel, Basel, Switzerland)
RH299-1C	*MATa, end4Δ::LEU2, ura3, leu2, lys2, his4, bar1*	H. Riezman
RH1995	*MATa, end3Δ::URA3, ura3, leu2, his4, bar1*	H. Riezman
SBY3	*MATα, myo1Δ::LEU2, ura3, leu2, his4*	
SBY4	*MATα, myo1Δ::LEU2, ura3, leu2, his4, trp1*	
SLY34	*MATα, myo2-66, ura3, leu2*	(Lillie and Brown, 1994)
SLY55	*MATa, smy1Δ-2::LEU2, ura3, leu2, his4*	
SLY57	*MATα, smy1Δ:-2:LEU2, ura3, leu2, trp1, his4*	
SLY63	*MATα, myo2-66, ura3, leu2, trp1, his6*	
SLY81	*MATα, myo2-66, leu2*	
SLY82	*MATα, myo2-66, leu2, his3*	
SLY83	*MATa, myo2-66, leu2, his3*	
SLY100	*MATα, smy1Δ-1::URA3, ura3, leu2, trp1, his4*	
SLY102	*MATa, smy1Δ-1::URA3, ura3, leu2, trp1*	
SLY103	*MATa/α, myo2-66/MYO2 SMY1/smy1Δ-2::LEU2 ura3/ura3 leu2/leu2 his4/HIS4*	
SLY109	*MATα, smy1Δ-2::LEU2, ura3, leu2, his3, ade2, ade3*	
SLY111	*MATa, smy1Δ-2::LEU2, ura3, leu2, his3, ade2, ade3*	
SLY163‖	*MATα, cmd1-3, ura3, leu2, his3*	
SLY164‖	*MATa, cmd1-3, leu2, his3*	
SLY165‖	*MATα, cmd1-1, ura3, leu2, his3*	
SLY166‖	*MATa, cmd1-1, leu2, his3*	
SLY191¶	*MATa, myo2-66, smy1Δ-2::LEU2, ura3, leu2, his4,* [YEpsmy1-52]	
SLY192¶	*MATα, myo2-66, smy1Δ-2::LEU2, ura3, leu2,* [YEpsmy1-52]	
SLY194¶	*MATa, myo2-66, smy1Δ-2::LEU2, ura3, leu2,* [YEpSMY1-52]	
SLY195¶	*MATα, myo2-66, smy1Δ-2::LEU2, ura3, leu2, his4,* [YEpSMY1-52]	
SLY248	*MATa/α, ura3/ura3*	
SLY250	*MATa/α, myo2-66/myo2-66, ura3/ura3*	
SLY251	*MATa/α, myo2-66/myo2-66, ura3/ura3*	
SLY254	*MATa, myo2-66, ura3*	
SLY334**	*MATa/α, myo2-66/myo2-66, tub1-1/TUB1, ura3/ura3 leu2/LEU2, his3/HIS3*	
314D5	*MATa/α, cdc4-1/cdc4-1, ura1/ura1, tyr1/tyr1, ade1/ade1, ade2/ade2*	L. Hartwell (University of Washington, Seattle, WA)

*Some strains may carry additional mutations; most or all are S288C derivatives and therefore gal2.
‡This study, except as noted.
§Made by crossing HA1C (H. Goodson and J. Spudich) into our strain background.
‖Made by crossing JGY41 or JGY44-2A (T. Davis) into our strain background.
¶Segregants obtained from SLY103 carrying the indicated plasmids.
**_tub1-1_ allele originally from M.A. Hoyt.

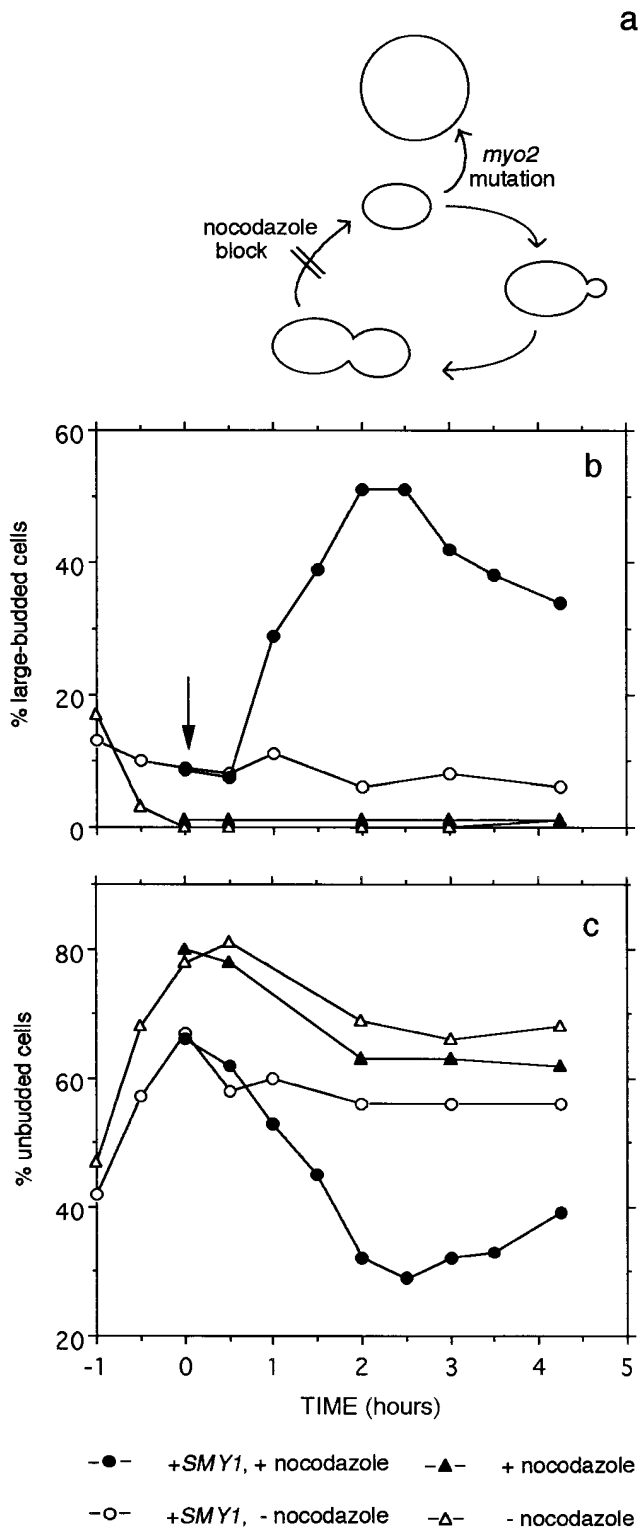

Figure 1. *SMY1*-dependent bud growth in the *myo2* mutant at restrictive temperature does not require microtubules. (*a*) Nocodazole causes arrest at the large-budded stage of the cell cycle because mitosis but not bud growth is blocked (Jacobs et al., 1988), whereas the *myo2* mutation interferes with bud growth giving rise to abnormally large mother cells (Johnston et al., 1991). Counts were made of large-budded (*b*) and unbudded (*c*) cells in cultures of the *myo2* mutant (strain SLY334) carrying multicopy *SMY1* (YEpSMY1-52; *circles*) or control vector (YEp352; *triangles*). Cells growing in selective medium at 25°C were shifted to restrictive temperature (31°C) by a fivefold dilution into prewarmed rich medium (YM-P) (refer to Materials and Methods). 5 μg/ml nocodazole (*closed symbols*) or carrier (DMSO) alone (*open symbols*) was added 1 h later (at the time indicated by the arrow), to allow time for the *myo2* mutation to be "expressed" and for recovery from the transient effects of the temperature shift (Lillie and Brown, 1994). At each time point, samples were fixed and an aliquot was counted after sonication to disperse clumps of cells. For each sample, at least 200 cells were scored as unbudded, small budded, or large budded (cells whose buds were more than three quarters the size of the mother cell). A second aliquot was processed for microtubule staining. In nocodazole-treated cells, all detectable cytoplasmic microtubules had disappeared by 30 min after nocodazole addition although putative spindle pole bodies persisted in many cells and short spindles persisted in an occasional cell. At 2½ h, cytoplasmic microtubules began to reappear in some cells, concomitant with an increase in spindle pole body staining. In mock-treated (DMSO carrier alone) cultures, cytoplasmic microtubules (and spindle pole bodies) were detectable in virtually all cells throughout the experiment, while spindles were present in a fraction of cells.

The *myo2* mutation, on the other hand, interferes with bud growth so that mutant cells at restrictive temperature are not able to reach the large-budded stage. Because this defective bud growth can be overcome by overexpression of Smy1p, we asked whether *myo2*-mutant cells carrying multicopy *SMY1* could reach large-budded arrest when treated with nocodazole, as a test of whether Smy1p could restore bud growth in the absence of microtubules. *myo2* mutant cells containing multicopy *SMY1* or vector alone were shifted to restrictive temperature, and nocodazole (or the DMSO carrier only) was added 1 h later (indicated by the arrow in Fig. 1 *b*). After about a half-hour lag, cells with multicopy *SMY1* rapidly accumulated at the nocodazole block, which was detected as an increase in the percentage of large-budded cells (Fig. 1 *b*, *closed circles*), whereas mock-treated cells (*open circles*) continued to pass through the cell cycle and maintained an approximately constant fraction of large-budded cells. In the control culture (*myo2* mutant cells with vector alone), there was very little bud growth regardless of whether nocodazole was present (consistent with previous observations; Johnston et al., 1991; Lillie and Brown, 1992, 1994). Approximately 15% of these control *myo2* mutant cells produced visible buds at restrictive temperature (inferred from the reduction in the unbudded population; Fig. 1 *c*, *triangles*), but none of those buds grew enough to reach the large-budded stage (Fig. 1 *b*, *triangles*). Cytoplasmic microtubules were no longer detectable 30 min after nocodazole addition, as assessed by immunofluorescence microscopy. We conclude from this experiment that Smy1p can restore bud growth in the absence of microtubules; a model in which Smy1p compensates by carrying the Myo2p cargo along microtubules is ruled out. But what if Smy1p needed microtubules only to reach its location early in the cell cycle, and once localized, could compensate without further microtubule involvement? Such a possibility is not ruled out by the experiment above, but is ruled out by the localization experiments described below.

The experiment above has a couple of complications, which, however, do not abrogate our conclusion. The first is that the shift to restrictive temperature causes a tran-

sient increase in the fraction of unbudded cells, peaking at ~1 h (i.e., at 0 h in Fig. 1 *c* for all four cultures). This has been observed previously with wild-type cells (Johnston and Singer, 1980; Plesset et al., 1987), and it is temporally correlated with transient disorganization of the actin cytoskeleton (Lillie and Brown, 1994). The cultures did not return to the initial percentage of unbudded cells seen at room temperature, but they instead reached a new, higher plateau after temperature shift (compare open symbols at −1 h and >2 h in Fig. 1 *c*). The second complication is that yeast eventually escapes the effects of nocodazole (Jacobs et al., 1988). We used a number of strategies to maximize the length of the nocodazole block (refer to Materials and Methods), but were only able to prolong it long enough to allow about half the cells to reach the large-budded arrest point. Microtubule staining began to reappear at 2 1/2 h of nocodazole treatment in a small fraction of the cells. Concomitant with the reappearance of microtubules, the proportion of large-budded cells began to decline (Fig. 1 *b*, *closed circles*) as the proportion of unbudded cells began to increase (Fig. 1 *c*, *closed circles*), an indication that cells were indeed resuming mitosis.

The most effective way we found to prolong the nocodazole block was to lower the temperature. Therefore, to confirm and extend the results obtained at restrictive temperature, we performed an experiment at room temperature. A change in strains and protocol from that of Fig. 1 was necessitated by the fact that the *myo2* mutant does not require multicopy SMY1 at room [permissive] temperature. Instead, we compared the effects of nocodazole on closely matched homozygous *myo2* and wild-type diploids (made by mating sister segregants obtained after several backcrosses). Since the *myo2 smy1Δ* double mutant is inviable (Lillie and Brown, 1992), we infer that nocodazole should have an effect on the rate of bud growth if microtubules were required for the compensating effect of Smy1p in the *myo2* mutant at nominally permissive temperature. In this experiment, cytoplasmic microtubules were no longer detectable by 30 min after nocodazole addition and started to reappear only at 6¾ h. Most (see below) *myo2*-mutant cells and virtually all wild-type cells were able to reach large-budded arrest by 2½ h with approximately the same kinetics (Fig. 2 *a*). No additional increase in the fraction of large-budded cells was seen in the next 4 h. Furthermore, when either a lower (not shown) or higher (Fig. 2 *d*) dose of nocodazole was used, both of which imposed a shorter block, the *myo2*-mutant strains once again displayed similar rates of bud growth and escape from nocodazole as the wild-type strains did.

We conclude that the room temperature experiment does indeed confirm the results obtained in the experiment performed at restrictive temperature in that a large fraction of mutant cells behave like wild-type cells in response to nocodazole. We were, however, puzzled by the

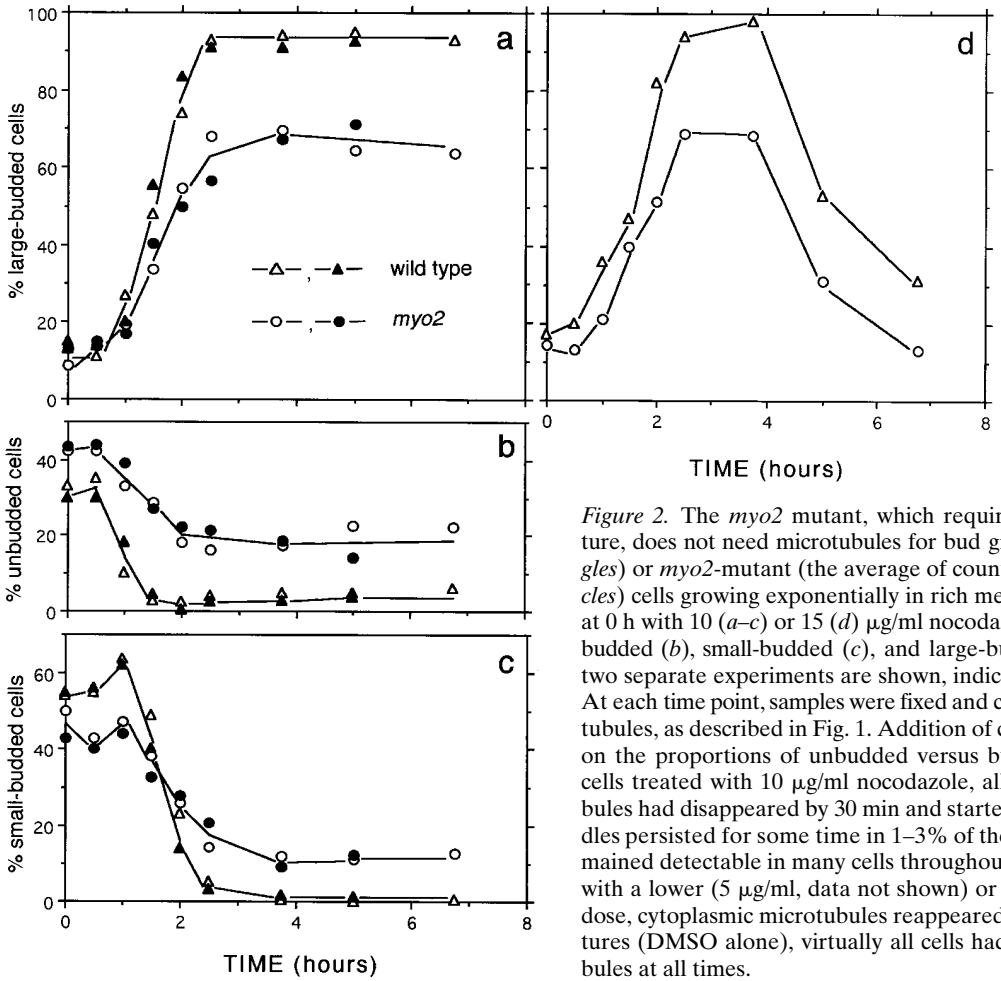

Figure 2. The *myo2* mutant, which requires Smy1p at permissive temperature, does not need microtubules for bud growth. Wild-type (SLY248; *triangles*) or *myo2*-mutant (the average of counts using SLY250 and SLY251; *circles*) cells growing exponentially in rich medium (YM-P) at 22° were treated at 0 h with 10 (*a–c*) or 15 (*d*) μg/ml nocodazole, and counts were made of unbudded (*b*), small-budded (*c*), and large-budded (*a* and *d*) cells. Results of two separate experiments are shown, indicated by open and closed symbols. At each time point, samples were fixed and counted or were stained for microtubules, as described in Fig. 1. Addition of carrier DMSO alone had no effect on the proportions of unbudded versus budded cells (data not shown). In cells treated with 10 μg/ml nocodazole, all detectable cytoplasmic microtubules had disappeared by 30 min and started to reappear only at 6¾ h. (Spindles persisted for some time in 1–3% of the cells, and spindle pole bodies remained detectable in many cells throughout the experiment.) In cells treated with a lower (5 μg/ml, data not shown) or higher (15 μg/ml [*d*]) nocodazole dose, cytoplasmic microtubules reappeared at ~3 3/4 h. In mock-treated cultures (DMSO alone), virtually all cells had detectable cytoplasmic microtubules at all times.

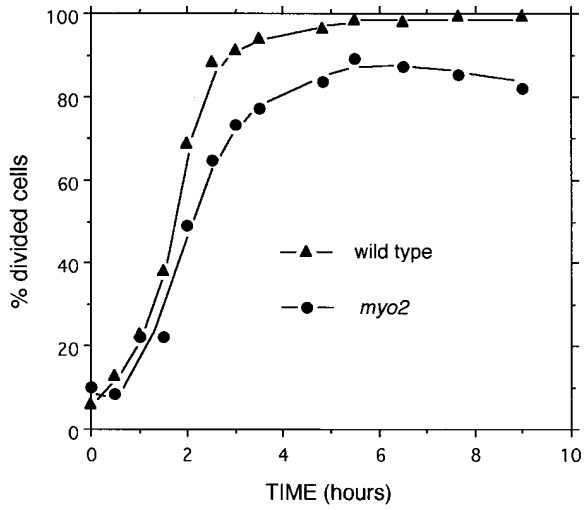

Figure 3. Some *myo2*-mutant cells fail to multiply at permissive temperature. Logarithmically growing *myo2* mutant (SLY251; *circles*) or wild-type (SLY248; *triangles*) cells (in YM-P) were briefly sonicated to disperse clumps and plated as single cells on YPD at room temperature. At each timepoint, the plates were examined microscopically to determine what fraction of the cells had undergone division. Most cells underwent several cell divisions during the time course of the experiment, giving rise to a microcolony. 200 cells and/or microcolonies per strain were examined at each time point.

difference in plateau values for wild-type vs. mutant cells. The mutant cells that failed to reach the large-budded stage did not appear to arrest at a specific stage because both unbudded and small-budded cells remained (Fig. 2, *b* and *c*). It seemed unlikely that mutant cells were more susceptible to some poisoning effect of nocodazole because the same fraction of *myo2* cells failed to reach the large-budded stage using a range of nocodazole concentrations (from 5 to 15 μg/ml; compare Fig. 2, *a* and *d*). We therefore examined cells that had not been treated with nocodazole for their ability to grow. The same strains that we used in Fig. 2 were plated as single cells at room temperature (in the absence of nocodazole) and examined microscopically at various times thereafter. Essentially all of the wild-type control cells and most of the mutant cells were able to grow buds and divide several times during the timecourse of the experiment (Fig. 3). However, 10–20% of the *myo2*-mutant cells never divided; instead, they remained as single cells that were either unbudded or had buds of various sizes. These nondividing *myo2* cells were not dead by the criterion of dye exclusion (not shown). Although we cannot explain this behavior, it could account for the difference in plateau values between wild-type and *myo2*-mutant cells in Fig. 2.

In addition to nocodazole treatment, we tried other approaches predicted to perturb microtubules. We did not observe any genetic interactions between *myo2* and any of several α- or β-tubulin mutations (a total of eight alleles from the three *tub* genes were tested; Table II) despite the fact that *myo2* is synthetically lethal with *smy1Δ* (Lillie and Brown, 1992). Furthermore, we found that *myo2*-mutant strains were not more sensitive than related wild-type strains to benomyl, another drug that acts on microtubules (Stearns and Botstein, 1988). Tetrads of four well-backcrossed *myo2*/*MYO2* diploids were dissected, and segregants were replica-pronged onto YPD plates containing 0, 10, 15, 20, and 25 μg/ml benomyl (Thomas et al., 1985) at 22°C. For one of the crosses, cells were spread on sectors of the same plates to better assess relative growth. No consistent differences between *myo2* and wild-type segregants were seen. Both grew well at 10 μg/ml of benomyl, less well at 15 μg/ml, and very little growth was seen at 20 or 25 μg/ml. These results are consistent with the hypothesis that Smy1p does not require microtubules to compensate for the Myo2p defect.

*Table II. Synthetic Lethal Relationships**

	myo2-66	*smy1Δ*
Microtubules		
α tubulin (*tub1-1, tub3Δ*)	+	ND
β tubulin (*tub2*)‡	+	+
Kinesin-related proteins§	ND	+
Actin (*act1-1, act1-2*)	ND	+
Tropomyosins		
tpm1Δ	−‖	+
tpm2Δ	+‖	+
Myosins		
myo1Δ	±	±
myo2-66	NA	−¶
myo3Δ	+	+
myo4Δ	+**	+**
Calmodulin		
cmd1-1	−‡‡	+
cmd1-3	+‡‡	+
Late secretory		
sec2-41, 4-8	−§§	−
sec5-24, 8-9, 9-4, 10-2, 15-1	−§§	+
sec 1-1, 6-4	+§§	+
Early secretory‖‖	+§§	+
Endocytic (*end3Δ, end4Δ*)	+	+¶¶

*+, double mutants were alive; −, double mutants were dead; ±, live mutants were obtained, but under some conditions, they grew slowly (or not at all) compared to either single mutant. ND, not done; NA, not applicable.
For crosses performed in our laboratory, a temperature permissive for the two parent single mutants was used throughout. For each cross, 9–35 tetrads were dissected on YPD. The segregants were replica pronged to selective plates at permissive temperature and/or YPD plates at appropriate temperatures to infer genotypes, and, in crosses with live double mutants, to look for synthetic growth defects.
‡*tub2-104*, *-402*, *-403*, *-404*, *-405*, *-406*.
§*kar3Δ*, *cin8Δ*, *kip1Δ*, *kip2Δ*.
‖Performed by A. Bretscher laboratory.
¶Lillie and Brown (1992).
**Haarer et al (1994).
‡‡Confirmation of Brockerhoff et al. (1994) in our strain background.
§§Performed by Govindan et al. (1995).
‖‖*sec7-1*, *12-4*, *18-1*, *22-3*.
¶¶*end4-1* instead of *end4Δ* was used.

Smy1p Does Not Require Microtubules for Localization

It seemed likely that Smy1p must localize properly to compensate for the Myo2p defect, given the apparent colocalization of these proteins, the loss of localization of both proteins in the *myo2* mutant at restrictive temperature, and the restoration of localization of both when Smy1p is overexpressed (Lillie and Brown, 1994). We therefore tested whether Smy1p localization was microtubule dependent. Smy1p and Myo2p colocalize as a "cap" at growing bud tips (Lillie and Brown, 1994), and this localization can indeed be seen in nocodazole-treated wild-type cells

and *myo2* mutant cells with multicopy *SMY1* (not shown). These cells, however, were not optimal for quantitation of nocodazole effects. This is because caps are detectable only in (some) unbudded and small-budded cells (Lillie and Brown, 1994), classes that are lost from the population as cells accumulate at the nocodazole block. We circumvented this problem by using *cdc4* mutant cells at restrictive temperature because these cells continue to produce buds even in the presence of nocodazole (Jacobs et al., 1988). Strikingly, treating *cdc4* cells with nocodazole (as described in Fig. 4) had absolutely no effect on the frequency of cells with Smy1p (or Myo2p) caps (illustrative cells shown in Fig. 4, *c* and *d*). Counts made 60, 95, and 120 min after nocodazole addition revealed that 59–64% of the nocodazole-treated cells had Smy1p caps, as compared to 58–66% of the control cells. The frequencies of Myo2p caps in control (84–88%) and nocodazole-treated (85–90%) cells were also indistinguishable. (The frequencies of Myo2p caps were higher than those of Smy1p caps, presumably because Smy1p caps are more difficult to stain; Lillie and Brown, 1994). We confirmed that microtubules were efficiently abolished in these cells (0% of nocodazole-treated cells had detectable cytoplasmic microtubules, as compared to 94–97% of control cells; illustrated in Fig. 4, *a* and *b*). Thus, Smy1p (and Myo2p) clearly remain localized properly without microtubules. We infer that new Smy1p caps can also form without microtubules because the cells continue to make new buds. Furthermore, small buds, which almost certainly formed in the absence of microtubules (see Fig. 6 in Jacobs et al., 1988), displayed prominent Smy1p caps (see arrow in Fig. 4 *d*).

To address this point more directly, we used osmotic shock to eliminate Smy1 caps (see Lillie and Brown, 1994) in *cdc4* cells after microtubules had been abolished by nocodazole (Fig. 5). We then asked whether caps could reform in the absence of microtubules. Control cells that had not been nocodazole treated lost their caps within 5–10 min after the addition of 0.4 M NaCl, and they recovered the caps after about a half hour, as expected (Lillie and Brown, 1994). Nocodazole-treated cells behaved identically despite the lack of cytoplasmic microtubules throughout the experiment (compare *closed* and *open circles* in Fig. 5). Thus, it is clear that microtubules are not required for Smy1p cap formation. This conclusion is relevant to the bud growth experiments of Figs. 1 and 2 for the following reason. Smy1p localization is almost surely necessary for its ability to compensate for the *myo2* mutant defect in bud growth, and we imagine that both formation and maintenance of caps are involved in this process. If only formation were critical, however, microtubule dependence at this step would not have shown up in the bud growth experiments, which, for the most part, looked at cells that would have already formed caps before nocodazole addition. The fact that Smy1p caps can form in the absence of microtubules removes this reservation about the bud growth experiments, allowing us to state unequivocally that Smy1p can function without microtubules.

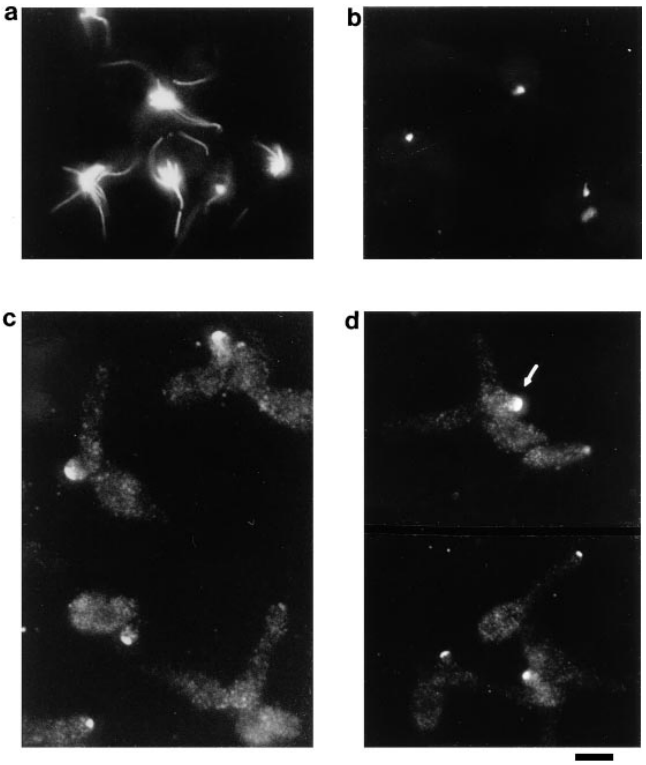

Figure 4. Smy1p caps are present in cells lacking microtubules. Immunolocalization of microtubules (*a* and *b*) and Smy1p (*c* and *d*) in the multibudded *cdc4* mutant (strain 314D5) in the absence (*a* and *c*) or presence (*b* and *d*) of 15 μg/ml nocodazole. *cdc4*/*cdc4* diploid cells growing exponentially in YM-P at 24°C were shifted to restrictive temperature (36°C) and incubated for 165 min. By this time, most cells had produced one or two abnormally elongated buds. Nocodazole or carrier DMSO alone was added, and samples were processed for indirect immunofluorescence microscopy at 60, 95, and 120 min. 200 cells/time point were scored for the presence of Smy1p caps, Myo2p caps, and microtubules; results were indistinguishable at these three time points (see text for the ranges). As expected (Jacobs et al., 1988), virtually every bud contained a prominent bundle of microtubules in the absence (*a*) but not the presence (*b*) of nocodazole. The small dots seen in *b* are caused by residual staining of the spindle pole bodies. A subset of the buds contained Smy1p caps (and Myo2p caps; data not shown) whether (*d*) or not (*c*) nocodazole was present. The arrow in *d* indicates a (putatively) newly forming bud with a bright Smy1p cap. Bar, 10 μm.

Smy1p May Not Require Motor Activity to Localize or Compensate for Myo2p Defects

Our finding that microtubules are not required for Smy1p function raises the question of whether Smy1p is even a motor protein. If it is, alteration of the P loop (which binds to phosphates of ATP) should interfere with Smy1p function. Meluh and Rose (1990) have found that an alteration (*kar3-1*) in the P loop of Kar3p, another yeast kinesin-related protein, severely compromises its function. Therefore, we made the same change (*smy1-3*) in Smy1p, substituting a Glu for Gly 119 (the second G in the P loop consensus sequence GX_4GKS/T). In contrast to the Kar3p results, however, this alteration did not interfere with either the localization or function of Smy1p. Localization of mutated Smy1p was carried out in a *smy1Δ* background to avoid staining of wild-type protein, and was indistinguishable from that of wild-type Smy1p (data not shown). Furthermore, the mutation did not interfere with the ability of

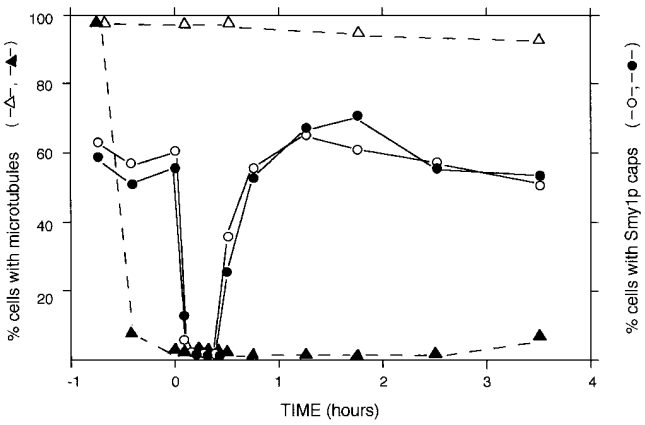

Figure 5. Smy1p caps can form in cells lacking microtubules. *cdc4*/*cdc4* diploid cells (strain 314D5) growing exponentially in YM-P at 24°C were shifted to restrictive temperature (36°C) and incubated for 120 min. By this time, most cells had produced one or two abnormally elongated buds. 15 μg/ml nocodazole (*closed symbols*) or carrier DMSO alone (*open symbols*) was added, and after an additional 45 min of incubation, cells were osmotically shocked (at 0 h) by addition of 0.4 M NaCl (using a 5-M stock). At each time point, samples were processed for indirect immunofluorescence microscopy, and at least 200 cells were scored for the presence of cytoplasmic microtubules (*triangles*) or Smy1p caps (*circles*). As observed previously, nocodazole treatment abolished most cytoplasmic microtubules in <30 min, while residual staining of spindle pole bodies remained (Fig. 4 *b*). We also noted that the NaCl treatment appeared to retard the loss of nuclear microtubules and/or to enhance the formation of an abnormal bar of tubulin in the nucleus (not shown). No change in microtubule structures was observed upon osmotic shock of control cells treated with carrier DMSO alone.

overexpressed Smy1p to overcome the defects of the *myo2* mutant. A *myo2* mutant strain (SLY34) carrying multicopy mutated *smy1-3* (YEpsmy1-52) grew as well as the same strain with wild-type *SMY1* (YEpSMY1-52) when incubated for 4 d on a YPD plate at restrictive temperature (31°C). Subtle differences were sometimes seen; these may be caused by a slight loss of stability of the mutant protein at elevated temperature, given the lack of such ephemeral differences in the experiment below. In contrast, no growth was seen with the control plasmid (Yep352), and all strains grew well on a YPD plate at permissive temperature. A reservation is that the *myo2* mutant has an endogenous wild-type *SMY1* gene in addition to the overexpressed *smy1-3* gene. This could complicate interpretation if wild-type Smy1p formed functional heterodimers with mutant Smy1p (or if there were an unexpectedly high rate of gene conversion). Therefore, we used a second functional test that avoided these complications, asking whether *smy1-3* prevented lethality (Lillie and Brown, 1992) in a *myo2 smy1Δ* double mutant. A heterozygous diploid strain (SLY103) carrying each of the same plasmids as described above was subjected to tetrad analysis at room temperature. We found that in the vast majority of tetrads (those that had retained plasmid) all segregants were viable, except that as expected (Lillie and Brown, 1992), no live double-mutant segregants were recovered from SLY103-carrying control vector. Thus, *smy1-3* was able to prevent lethality in the double mutant. Furthermore, the growth rates of double-mutant segregants carrying wild-type versus mutant SMY1 (strains SLY191, SLY192, SLY194, and SLY195) incubated at 25°C in YPD were indistinguishable when followed by OD over a period of several days (with dilutions to maintain cells in log phase). Therefore, *smy1-3* not only prevented lethality, but it also allowed cells to grow as well as they did with *SMY1.* We conclude from the above experiments that Smy1p does not need motor activity to localize properly and to compensate for Myo2p defects.

The above-mentioned *kar3-1* mutation causes Kar3p localization to microtubules to become detectable (Meluh and Rose, 1990). An attractive interpretation is that the mutant Kar3p cannot bind ATP well and is therefore blocked at a step of the ATPase cycle when it is tightly bound to microtubules (see Hackney, 1994). Therefore, we looked carefully for mutant Smy1p localization to microtubules but were unable to detect any. *kar3-1* has dominant-negative effects, perhaps because tightly bound mutant protein competes with other microtubule interactions. We looked for dominant effects with *smy1-3* but found no evidence of this. These findings are consistent with the evidence presented above that Smy1p is not a microtubule-based motor.

A deletion of amino acids 20–137 (roughly the first third of the Smy1p "motor" domain including the P loop) abolishes localization and the ability to correct *myo2*-mutant defects without reducing the amount of Smy1p present (as judged by Western analysis; data not shown). This is not conclusive proof that the motor domain of Smy1p is functionally important because the deletion might cause folding problems that interfere with other domains. However, a similar deletion in kinesin did not abolish microtubule binding (Yang et al., 1989), suggesting that our deletion may not have such global effects. Assuming that the motor domain is indeed important, it seems likely that this domain is involved in a novel function that Smy1p has acquired.

Synthetic Lethal Relationships

Although they should not be overinterpreted, synthetic interactions (i.e., a more than additive phenotype in a double mutant) can provide valuable clues about function. We have used this approach to ask several questions about Smy1p. A summary of crosses performed with *smy1Δ* (and *myo2*) mutants is provided in Table II. First, we looked for synthetic interactions between *smy1Δ* and deletions of other kinesin-related genes. We reasoned that Smy1p might indeed be a microtubule-based motor, even though this activity is not needed for localization or to compensate for the *myo2* mutation. If so, the lack of a phenotype upon *SMY1* deletion might indicate redundancy with another kinesin-related protein. However, we found no indication of a synthetic interaction between *smy1Δ* and deletions in *KAR3*, *KIP1*, *KIP2*, or *CIN8.* The completion of the yeast genome project has revealed a sixth kinesin-related gene in yeast, *KIP3*, which also does not have a synthetic interaction with *smy1Δ* (DeZwaan et al., 1997; Hoyt, M.A., personal communication). The only other microtubule-based motor in yeast is dynein, and there is no synthetic interaction between *smy1Δ* and deletion of

DYN1 (Cottingham and Hoyt, 1997). Synthetic interactions have implicated all of these microtubule-based motor proteins in partially overlapping aspects of nuclear migration and mitosis (Cottingham and Hoyt, 1997; DeZwaan et al., 1997). *SMY1* stands alone in its failure to show any synthetic interactions.

We have also used this approach to look for further clues as to the nature of Smy1p's relationship with Myo2p and/or other components of the actin cytoskeleton (Table II). No genetic interactions were seen, except for a subtle interaction between *smy1Δ* and deletion of the gene encoding the putative conventional myosin (*MYO1*). We also detected a subtle interaction between the *MYO1* deletion and *myo2*, and we are investigating these interactions further.

The most informative results we obtained were with mutants defective in the secretory pathway (*SEC* mutants). Govindan et al. (1995) have shown that *myo2* is synthetically lethal with mutations in most of the late (but not the early) *SEC* genes, suggesting that Myo2p plays a role at a late stage of secretion. When we tested *smy1Δ* against the same alleles, we found synthetic lethality with two of these late *SEC* mutations (*sec2* and *sec4*), but not with the others nor with the early *SEC* mutations we tested. These results are interesting for two reasons. First, they show that Smy1p plays a role in exocytosis, even when Myo2p is wild type. In other words, they rule out the possibility that the only function of Smy1p is to stabilize a mutant Myo2p. Second, the fact that Smy1p interacts (genetically) with only a subset of the proteins that interact with Myo2p may well be a significant clue to the role it plays in secretion.

Discussion

Our working hypothesis at the beginning of this study was that mutant Myo2p was defective in carrying a cargo along actin filaments, and that Smy1p might compensate by carrying the cargo along microtubules instead. This hypothesis was plausible, given the orientation of both actin filaments and microtubules toward the bud, and given findings in other systems (Fath et al., 1994; Langford, 1995; Morris and Hollenbeck, 1995) that a vesicle or organelle can have both microtubule- and actin-based motors. Our present results, however, have ruled out this model for Smy1p function. It is clear from the nocodazole experiments that microtubules are not required for Smy1p to compensate for the Myo2p defect, and we have ruled out the possibility that Smy1p must travel down microtubules to reach the bud tip. What models remain? We cannot completely rule out the heretical possibility that Smy1p might travel down actin filaments, but this notion is not consistent with evidence (discussed below) that Smy1p does not have motor activity. A second possibility is that Smy1p might interact with Myo2p to stabilize or otherwise enhance its function. In this case, Smy1p might be carried along actin filaments by Myo2p to reach the bud tip. Alternatively, as mentioned in the Introduction, Myo2p may not carry a cargo to the bud tip, but it may instead reside there together with Smy1p to carry out some function. We have recently obtained evidence that Smy1p and Myo2p do, in fact, interact physically (Beningo, K.A., and S.S. Brown, manuscript in preparation). This extends our conclusion that there is a surprising, novel interaction between these proteins, that does not involve microtubules.

Our conclusion that microtubules are not required for Smy1p localization and function depends on knowing that nocodazole has truly abolished all microtubules. Although we have been careful to stipulate that our results were obtained in the absence of "detectable" microtubules, we are confident that cytoplasmic microtubules were completely abolished for several reasons. First, there are not many cytoplasmic microtubules in yeast (an average of three per cell), and cytoplasmic microtubule staining corresponds to individual microtubules (Carminati and Stearns, 1997). Second, Jacobs et al. (1988) went to great lengths to demonstrate that nocodazole completely abolishes microtubules in *S. cerevisiae.* Third, microtubules were functionally absent; cells were unable to undergo mitosis. Furthermore, we did not begin to see escape from mitotic arrest until microtubules began to reappear. Thus, it is thoroughly unlikely that microtubules persisted that we failed to detect.

The fact that microtubules are not required for Smy1p to localize or to function led to the prediction that motor activity would not be required either. Studies of the *smy1-3* mutation support this prediction. This mutation has no apparent effects on Smy1p, in contrast to the dramatic effects of the same mutation (*kar3-1*; Meluh and Rose, 1990) on another yeast kinesin-related protein, Kar3p. The loss of Kar3p function must be caused by a loss of motor activity because the mutation is in the P loop, a motif that interacts with the phosphate portion of nucleotide in many NTPases. The mutation alters a glycine that is absolutely conserved (Saraste et al., 1990) and whose alteration interferes with nucleotide binding and/or inactivates other NTPases (Clanton et al., 1987; Liu and Summers, 1988; Logan and Knight, 1993; Shen et al., 1994). Of particular relevance, given the structural similarity between kinesin and myosin (Kull et al., 1996), is the effect of this mutation on *Dictyostelium* myosin II (Gly184 to Glu); the mutant myosin no longer binds ATP, and actin binding becomes ATP insensitive (Ruppel, K., personal communication). This glycine is critical because of a structural constraint; any other amino acid in this position destroys the conformation of the P loop (Pai et al., 1990), which is essentially identical in all NTPases whose three-dimensional structures have been determined (Kull et al., 1996; Smith and Rayment, 1996). Thus, this mutation should have affected the ability of Smy1p to localize or to correct Myo2p defects if motor activity were required. (Note that this would be the prediction even if Smy1p were to travel along some novel substrate such as actin.) Our results do not rule out the possibility that Smy1p could be capable of motor activity, which is used for some unknown function that has not been revealed by phenotypic studies. However, our efforts to observe in vitro motility, ATP binding or hydrolysis, and cosedimentation with microtubules have all been negative. Furthermore, the divergence of Smy1p at many otherwise highly conserved positions (see Introduction) also suggests that it may not be a motor protein.

The synthetic lethal relationships that we have observed are consistent with earlier evidence that Smy1p is functionally linked with the actin rather than the microtubule cytoskeleton (Lillie and Brown, 1994). Of particular interest is the synthetic lethality observed between *SMY1* dele-

tion and mutations in two of the late *SEC* genes. The products of the late *SEC* genes function in the delivery or fusion of secretory vesicles with the cell membrane, a process in which actin (Novick and Botstein, 1985) and Myo2p (Govindan et al., 1995) have also been implicated. This synthetic lethality provides new clues about Smy1p function. The first is that Smy1p must have a function beyond that of stabilizing mutant Myo2p, since the synthetic lethality takes place in cells where *MYO2* is wild type. Smy1p may nonetheless act via Myo2p; we have previously noted that *SMY1* overexpression enhances the localization of even wild-type Myo2p (Lillie and Brown, 1994). The second clue is that *smy1Δ* is synthetically lethal with mutations in only two of the late *SEC* genes, whereas Govindan et al (1995) have shown that *myo2* is synthetically lethal with most late *SEC* mutations. The two mutations with which *smy1Δ* is synthetically lethal, *sec2* and *sec4*, are not simply the most deleterious of the late *SEC* mutations tested (Nair et al., 1990), thus ruling out the most trivial explanation of these results. Instead, recent studies indicate that the functions of Sec2p and Sec4p are intimately associated. Sec4p is a small GTPase (Salminen and Novick, 1987) that has been postulated to act as a conformational switch regulating either the delivery of vesicles or their subsequent capture at the site of fusion. Interestingly, Sec2p has been found to be an exchange factor for Sec4p (Walch-Solimena et al., 1997). Almost all of the other late *SEC* mutations we tested (*sec3* [not shown], *5*, *6*, *8*, *10*, and *15*), which are not synthetically lethal with *smy1Δ*, are components of the "exocyst," a large complex of copurifying proteins (TerBush and Novick, 1995; TerBush et al., 1996). Sec4p continues to localize at the bud tip in exocyst mutants, indicating that the exocyst is not involved in vesicle localization, but rather, in some subsequent event (Walch-Solimena et al., 1997). Sec4p, however, does not localize in *sec2* or *myo2-66* mutants (Walch-Solimena et al., 1997). Taken together, these data indicate that Smy1p, Myo2p, Sec2p, and Sec4p are involved in event(s) that affect vesicle delivery and/or capture. Since the *myo2* mutation is also synthetically lethal with most of the exocyst mutations, it may be sufficiently detrimental that it also affects subsequent events. We should point out that although the most obvious way for Myo2p to play a role is to be a secretory vesicle motor, there are a number of other possibilities (refer to Introduction).

In summary, Smy1p does not use microtubule-based motor activity to compensate for the mutant Myo2p defect. Regardless of whether or not it is capable of such an activity, Smy1p must function in some novel manner in its interaction with Myo2p. The family of actin-related proteins (Mullins et al., 1996) provides a precedent for proteins with structural similarity but divergent function; Smy1p may represent an example of such divergence in the kinesin superfamily.

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