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A novel protein complex promoting formation of functional α- and γ-tubulin

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We describe the identification of *GIM1/YKE2*, *GIM2/PAC10*, *GIM3*, *GIM4* and *GIM5* in a screen for mutants that are synthetically lethal with *tub4-1*, encoding a mutated yeast γ-tubulin. The cytoplasmic Gim proteins encoded by these *GIM* genes are present in common complexes as judged by co-immunoprecipitation and gel filtration experiments. The disruption of any of these genes results in similar phenotypes: the *gim* null mutants are synthetically lethal with *tub4-1* and super-sensitive towards the microtubule-depolymerizing drug benomyl. All except Δ*gim4* are cold-sensitive and their microtubules disassemble at 14°C. The Gim proteins have one function related to α-tubulin and another to Tub4p, supported by the finding that the benomyl super-sensitivity is caused by a reduced level of α-tubulin while the synthetic lethality with *tub4-1* is not. In addition, *GIM1/YKE2* genetically interacts with two distinct classes of genes, one of which is involved in tubulin folding and the other in microtubule nucleation. We show that the Gim proteins are important for Tub4p function and bind to overproduced Tub4p. The mammalian homologues of *GIM1/YKE2* and *GIM2/PAC10* rescue the synthetically lethal phenotype with *tub4-1* as well as the cold-sensitivity and benomyl super-sensitivity of the yeast deletion mutants. We suggest that the Gim proteins form a protein complex that promotes formation of functional α- and γ-tubulin.
Keywords: chaperonin/spindle pole body/TRiC/Tub4p/γ-tubulin

Introduction

Microtubules are hollow cylinders with a diameter of 25 nm. They are part of the cytoskeleton of eukaryotic cells and play an important role in numerous cellular processes, including chromosome segregation in mitosis and meiosis, organelle positioning and intracellular transport. The wall of the microtubule cylinder consists of 13 protofilaments which are strings of alternating α- and β-tubulin subunits pointing in the same direction (reviewed by Mandelkow and Mandelkow, 1993). Therefore, microtubules are polar structures with biochemically distinct ends. Microtubule formation is a complex process that includes the post-translational folding of α- and β-tubulin, their assembly into the heterodimer tubulin and finally the formation of microtubules from tubulin subunits (Solomon, 1991). All of these steps are assisted by proteins *in vivo*.

The first post-translational step in the pathway leading to the formation of the tubulin heterodimer is the folding of α- and β-tubulin. Genetic (Ursic and Culbertson, 1991; Chen *et al.*, 1994; Vinh and Drubin, 1994; Archer *et al.*, 1995) and biochemical (Frydman *et al.*, 1992; Yaffe *et al.*, 1992; Melki *et al.*, 1993; Sternlicht *et al.*, 1993; Tian *et al.*, 1996) evidence suggests that tubulins undergo facilitated folding via their interaction with cytoplasmic chaperonins. Conditional lethal mutations in *TCP1*, *BIN2*, *BIN3* and *ANC2*, coding for subunits of cytoplasmic chaperonin (TRiC), affect microtubule as well as actin functions (Ursic and Culbertson, 1991; Chen *et al.*, 1994; Li *et al.*, 1994; Miklos *et al.*, 1994; Vinh and Drubin, 1994). For example, *tcp1-1* cells are cold-sensitive for growth, and accumulate multi-nucleated and anucleated cells with abnormal microtubule structures. This mutant is also sensitive towards the microtubule-depolymerizing drug benomyl (Ursic and Culbertson, 1991). Remarkably, *bin3-1* cells display a normal distribution of unbudded, small-budded and large-budded cells, although the microtubules of *bin3-1* cells disassemble at 14°C (Chen *et al.*, 1994).

While the cytoplasmic chaperonin is sufficient for the production of native actin in an ATP-dependent manner (Gao *et al.*, 1992), folding of α- and β-tubulin requires the participation of additional cofactors. Such proteins, named cofactors A, B, C, D and E, have been identified by biochemical approaches (Tian *et al.*, 1996, 1997). Potential yeast homologues of cofactors A, B, D and E are Rbl2p, Alf1p, Cin1p and Pac2p (Archer *et al.*, 1995; Tian *et al.*, 1996; Geiser *et al.*, 1997). Surprisingly, *ALF1*, *RBL2*, *CIN1* and *PAC2* are not essential for growth of yeast cells (Hoyt *et al.*, 1990; Stearns *et al.*, 1990; Archer *et al.*, 1995). However, their deletion causes sensitivity towards benomyl and they show multiple genetic interactions with genes coding for components of the microtubule system. In addition, Rbl2p interacts directly with yeast β-tubulin (Archer *et al.*, 1995).

In many cell types, microtubule assembly is initiated at centrosomes, basal bodies, spindle pole bodies (SPBs) or nucleus-associated bodies, structures for which Pickett-Heaps (1969) coined the generic term microtubule-organizing centre (MTOC). MTOCs assist the assembly of the first tubulin monomers into oligomers, a step known as microtubule nucleation (Mitchison and Kirschner, 1984). A universal component of MTOCs involved in microtubule nucleation is γ-tubulin (Oakley *et al.*, 1990; Horio *et al.*, 1991; Stearns *et al.*, 1991). γ-Tubulin forms a complex with additional proteins (Stearns and Kirschner, 1994;

Moudjou *et al.*, 1996; Akashi *et al.*, 1997), and purification of such a complex from *Xenopus laevis* eggs identified α-, β- and γ-tubulin and proteins with mol. wts of 195, 133, 109 and 75 kDa (Zheng *et al.*, 1995).

In the yeast *Saccharomyces cerevisiae*, the MTOC is known as the SPB. It is a multi-layered structure which is embedded in the nuclear envelope (Byers and Goetsch, 1975; Byers, 1981). The yeast γ-tubulin is encoded by the essential *TUB4* gene (Sobel and Snyder, 1995; Marschall *et al.*, 1996; Spang *et al.*, 1996). Tub4p forms a 6S complex with the SPB components Spc98p and Spc97p (Geissler *et al.*, 1996; Spang *et al.*, 1996; Knop *et al.*, 1997). Purification of this complex suggests that it contains only one molecule of Spc98p and Spc97p, but two or more molecules of Tub4p (Knop and Schiebel, 1997). Fractionation experiments suggest that the Tub4p complex assembles in the cytoplasm followed by its nuclear import via an essential nuclear localization sequence in Spc98p (G. Pereira *et al.*, 1998). Finally, Spc98p and Spc97p of the Tub4p complex interact in the nucleus with the amino-terminal domain of the SPB component Spc110p (Knop and Schiebel, 1997). The binding site for the Tub4p complex at the outer plaque is still unknown.

To identify genes that are involved in yeast γ-tubulin functions, we performed a genetic screen for mutants that are synthetically lethal with *tub4-1*. We identified *SPC98* and *SPC97* coding for components of the yeast γ-tubulin complex and five additional genes, which we named *GIM1–GIM5*. Their products are not associated with the SPB, instead they form cytoplasmic multi-protein complexes which promote formation of functional α-tubulin and Tub4p. Most interestingly, the Gim proteins are phylogenetically conserved proteins, and mouse and human homologues function in yeast, indicating that the mammalian proteins fulfil a very similar role.

Results

Identification of GIM1/YKE2, GIM2/PAC10, GIM3, GIM4 and GIM5 in a screen for mutants that are synthetically lethal with a mutated yeast γ-tubulin

In the yeast *S.cerevisiae*, the γ-tubulin Tub4p forms a complex with the SPB components Spc98p and Spc97p. Physical interaction between Tub4p, Spc98p and Spc97p is reflected by multiple genetic interactions, including synthetic lethality (Geissler *et al.*, 1996; Knop *et al.*, 1997). The latter is an indication of a functional relationship of two gene products. To identify further components that functionally interact with *TUB4*, we performed a genetic screen for mutants that are synthetically lethal with *tub4-1*, yielding 12 mutants with this phenotype. Subsequent analysis showed that two of these mutants were defective in *SPC98* and two in *SPC97*. The other eight belong to five complementation groups which we named *GIM1–GIM5* (genes involved in microtubule biogenesis). Plasmids containing *GIM1–GIM5* were isolated by transforming the mutants with a yeast genomic library. Subcloning of DNA fragments and complementation analysis showed that *GIM1* is identical to *YKE2* (Shang *et al.*, 1994), coding for a protein of 114 amino acids (Table I). *GIM2* had already been identified as *PAC10*, a gene that becomes essential in the absence of the *CIN8*-encoded kinesin motor (Geiser *et al.*, 1997). *GIM3* corresponds to the open reading frame (ORF) YNL153c, encoding a protein of 129 amino acids. The DNA fragment complementing *gim4* cells contained three overlapping ORFs, one of which was interrupted by an intron. The various coding regions were cloned directly behind the *ADH* promoter of plasmid p415-*ADH*. Only the *ADH* promoter fusion with the intron-containing ORF YEL003w complemented *gim4* cells (data not shown). Gim4p is a protein of 132 amino acids. *GIM5* (ORF YML094w) also contains a small intron and encodes a protein of 163 amino acids.

The Gim proteins are phylogenetically conserved coiled-coil proteins

As reported for Gim2p/Pac10p (Geiser *et al.*, 1997), all Gim proteins have a high probability of forming coiled-coils (Lupas, 1996). Sequence comparisons have shown that Gim1p/Yke2p, Gim2p/Pac10p, Gim3p, Gim4p and Gim5p are related proteins. They also have relatives in mammals, *Caenorhabditis elegans* and *Schizosaccharomyces pombe*, revealing that they are phylogenetically conserved (Figure 1). Remarkably, Gim1p/Yke2p and Gim5p are homologous to two proteins of the archaebacterium *Methanococcus jannaschii*, while no relatives were found in eubacteria. We also noticed that the Gim proteins are more closely related to their homologues from other species than to each other (Figure 1).

Deletion of the GIM genes causes microtubule defects

To understand the function of the *GIM* gene products, we investigated whether they are essential for growth of yeast cells. We disrupted the entire coding regions of the *GIM* genes in the diploid yeast strain YPH501. As shown for Δ*gim5* (Figure 2A), spore analysis revealed that *GIM1/YKE2*, *GIM3*, *GIM4* and *GIM5* are not essential for growth. In agreement with a previous report (Geiser *et al.*, 1997), we observed that Δ*pac10* cells were defective in spore germination. Such a defect was not apparent for the other *GIM* mutants, raising the possibility that *GIM2/PAC10* has an additional specialized function in spore germination. The disruption of any of the *GIM* genes resulted in a slow growth phenotype at 30°C (Figure 2A and B). A more detailed analysis indicated that the doubling time of Δ*gim1/yke2*, Δ*gim2/pac10*, Δ*gim3* and Δ*gim5* cells grown in liquid medium at 30°C was increased by a factor of 1.4 compared with the wild-type, while the doubling time of Δ*gim4* cells was only affected 1.1-fold. It has been reported previously that a deletion mutant of *GIM1/YKE2* shows normal growth over a wide range of temperatures (Shang *et al.*, 1994). This discrepancy with our study is explained either by strain differences or by the fact that Shang *et al.* (1994) did not disrupt the entire coding region of *YKE2*.

Common phenotypes of mutants affecting the microtubule cytoskeleton are a cold-sensitive growth defect and an increased sensitivity towards the microtubule-depolymerizing drug benomyl (Neff *et al.*, 1983; Huffaker *et al.*, 1988). Therefore, we investigated whether the growth defects of the haploid *gim* null strains were intensified at lower temperatures. The *gim* null strains showed an enhanced growth defect at reduced temperatures in comparison with the wild-type (Figure 2B; compare 30°C with 23°C and 14°C plates). An exception was

Table I. Properties of the *GIM* genes and the corresponding deletion mutants

	ORF name	Amino acids in encoded protein	Cold-sensitive[a]	Benomyl-sensitive[b] (2.5 μg/ml)	Relative volume[c]	Suppression of benomyl super-sensitivity[d]
GIM1/YKE2	YLR200w	114	yes	yes	2.4	yes
GIM2/PAC10	YGR078c	199	yes	yes	2.2	yes
GIM3	YNL153c	129	yes	yes	2.4	yes
GIM4	YEL003w	132	no	yes	2.4	yes
GIM5	YML094w	163	yes	yes	2.3	yes

[a]Cold sensitivity was determined as described in the legend to Figure 2B.
[b]Benomyl sensitivity was determined as in Figure 2C.
[c]Determination of cell volumes (compared with wild-type) is described in Materials and methods.
[d]Suppression of benomyl super-sensitivity by overexpression of *TUB1* or *RBL2* was determined as in Figure 5C.

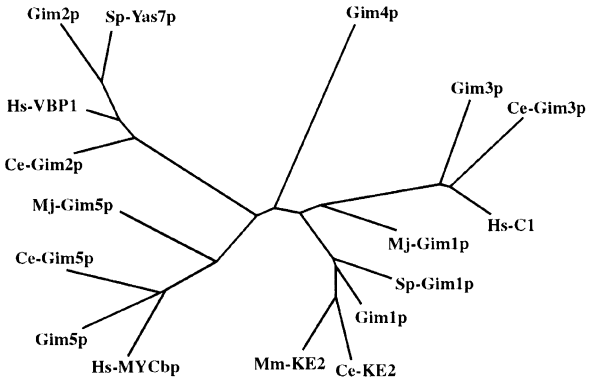

Fig. 1. *GIM1/YKE2*, *GIM2/PAC10*, *GIM3*, *GIM4* and *GIM5* encode phylogenetically conserved proteins. Dendrogram of the Gim proteins and their homologous proteins from *S.pombe* (Sp), *C.elegans* (Ce), mouse (Mm), human (Hs) and *M.jannaschii* (Mj) generated by the PROTDIST and FITCH modules of PHYLIP (Felsenstein, 1996). The dendrogram is based on an alignment by A.Lupas (personal communication) using MACAW (Schuler *et al.*, 1991). Accession Nos: Sp-Gim1p, Z99260; Mm-KE2, I53651; Ce-KE2, P52554; Mj-Gim1p, C64423; Sp-Yas7p, Q10143; Ce-Gim2p, Z81587; Hs-VBP-1, U56833; Ce-Gim3p, Z73102; Hs-C1, U41816; Ce-Gim5p, U00036; Hs-MYCbp, Q99471; Mj-Gim5p, H64418.

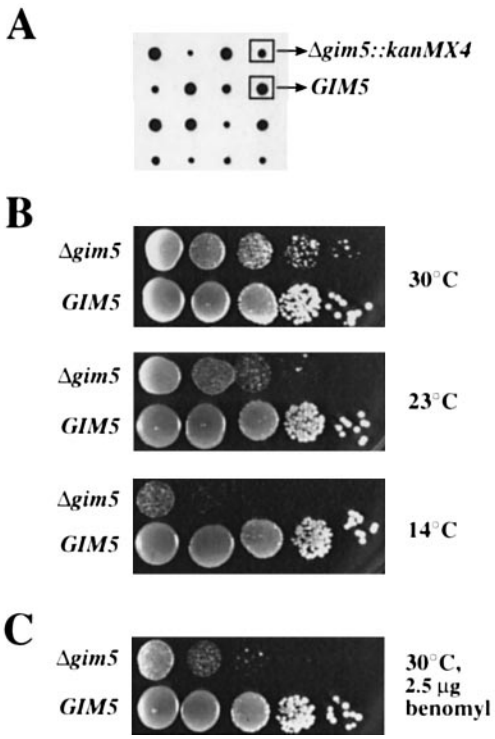

Fig. 2. Δ*gim5* cells are cold- and benomyl-sensitive. (**A**) Tetrads of a diploid *GIM5/Δgim5::kanMX4* strain were analysed for growth at 30°C on YPD plates. All spores from a tetrad germinated and formed colonies. These colonies were tested for growth on YPD plates containing the kanamycin derivative G418. Only cells which carry the *kanMX4* gene grow on these plates. Two of the spores of each tetrad, which were G418 resistant (Δ*gim5::kanMX4*), grew more slowly in comparison with the G418-sensitive *GIM5* cells. (**B**) The growth defect of Δ*gim5::kanMX4* cells on YPD plates is more pronounced at lower temperatures. Serial dilutions of Δ*gim5* and *GIM5* cells were grown on YPD plates. One plate was incubated for 3 days at 30°C. The plates at 23 and 14°C were incubated until the sizes of the *GIM5* colonies were approximately the same as on the 30°C plate. (**C**) Δ*gim5::kanMX4* cells are super-sensitive towards the microtubule-depolymerizing drug benomyl. Serial dilutions of Δ*gim5::kanMX4* and *GIM5* cells were grown on YPD plates containing 2.5 μg/ml benomyl at 30°C.

Δ*gim4* cells, which did not show an enhanced growth defect at lower temperatures (Table I). In contrast to the wild-type, all *gim* null strains were super-sensitive to 2.5 μg/ml benomyl (Figure 2C). Such a strong benomyl sensitivity has only been observed for mutants defective in microtubule biogenesis (Stearns *et al.*, 1990; Chen *et al.*, 1994; Archer *et al.*, 1995; Tian *et al.*, 1997).

We investigated whether the Gim proteins function in parallel pathways. In this case double mutants should have more pronounced defects compared with the single mutants. However, the double deletion mutants of Δ*gim1/yke2* together with any one of Δ*gim2/pac10*, Δ*gim3*, Δ*gim4* or Δ*gim5* were viable and as benomyl-sensitive as the single mutants. We also tested whether overexpression of any of the *GIM* genes rescued the synthetically lethal phenotype of *gim tub4-1* cells. This was the case for *GIM1* which weakly suppressed the defect of *gim4 tub4-1* cells (data not shown).

The strong benomyl super-sensitivity of the haploid *gim* null strains suggests that the gene products are needed for either tubulin formation or microtubule stability. To analyse these possibilities, we investigated, by indirect immunofluorescence, the microtubule arrays of Δ*gim1/yke2*, Δ*gim2/pac10* and Δ*gim3* cells incubated at 14°C for 20 h. The phenotype is shown for Δ*gim1/yke2* cells as a representative, since it is very similar to that of the other mutants. Δ*gim* cells lost most of their cytoplasmic and nuclear microtubules, leaving only a small spot near the nucleus, most likely at the SPB (Figure 3A). This observation suggests that microtubule attachment to the SPB via the Tub4p complex is still taking place in Δ*gim* cells. Instead microtubule stability seems to be impaired. Furthermore, cells with a large bud (Figure 3A, arrow) had

only one 4′,6’-diamidino-2-phenylindole (DAPI) staining region in one of the two cell bodies, a phenotype that is consistent with the observed defect in microtubule organization. Despite these defects, *gim* null cells incubated for 20 h at 14°C were still as viable as wild-type cells when then grown at 30°C (data not shown). Based on the distributions of the DNA content (Figure 3B) and of the cell morphologies (Figure 3A), Δ*gim* cells did not arrest at a defined stage of the cell cycle. This observation was surprising, since many mutants with a defect in spindle formation arrest in mitosis due to a mitotic check point (Hoyt *et al.*, 1990; Li and Murray, 1991). The non-arrest phenotype could point to a role for the Gim proteins in mitotic check point control. However, all *GIM* deletion mutants arrested in the cell cycle like wild-type cells in response to the microtubule-depolymerizing drug nocodazole, while the mitotic check point control mutant *bub2* did not (Hoyt *et al.*, 1990; Li and Murray, 1991; Geiser *et al.*, 1997; data not shown). This led us to conclude that the *GIM* gene products are not part of a mitotic check point. Taken together, the strong benomyl sensitivity and the microtubule defects of the *gim* null mutants at reduced temperature are most consistent with a role for the Gim proteins in tubulin biogenesis.

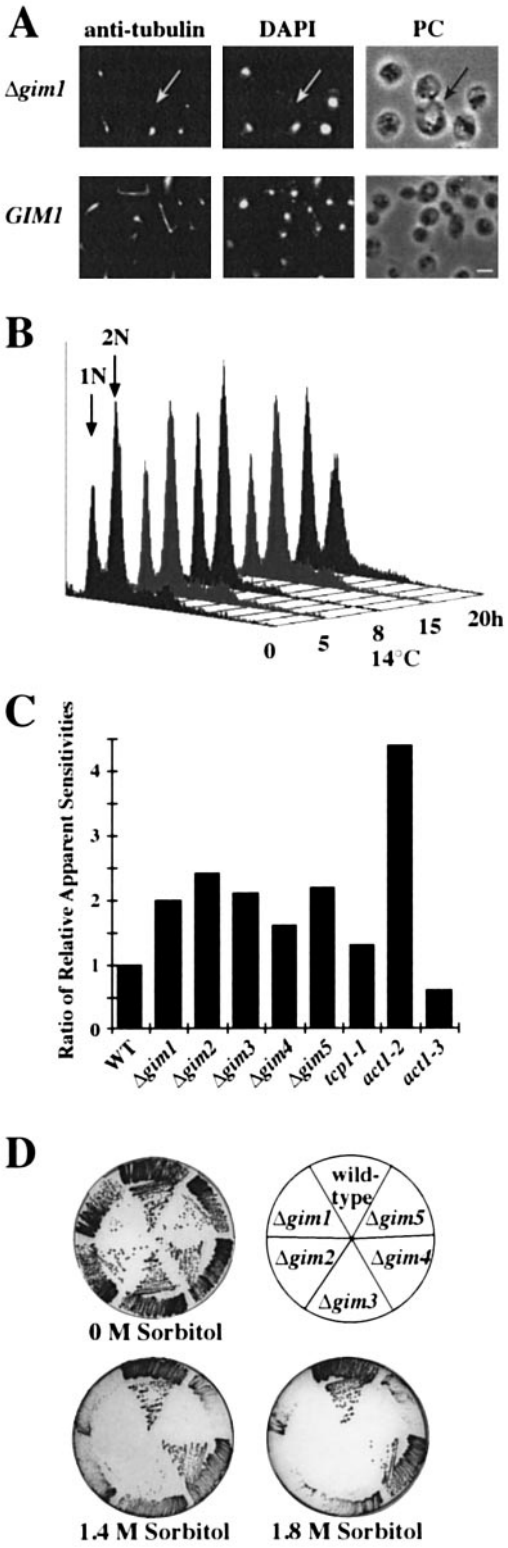

Fig. 3. Microtubule defects of Δ*gim1/yke2* cells. (**A**) Microtubule staining of the *gim1/yke2* null mutant. Cells of Δ*gim1/yke2::kanMX4* and *GIM1/YKE2* were grown in YPD medium at 30°C. The cells were diluted to 5×10^6 cells/ml using pre-cooled YPD medium. The cultures were then incubated at 14°C for two doubling times (20 h). Microtubules were detected by indirect immunofluorescence with anti-tubulin antibodies. DNA was stained with DAPI. Cells were also inspected by phase contrast microscopy (PC). The arrows point to a Δ*gim1/yke2* cell with a large bud that contains no mitotic spindle and only one DAPI-staining region. Bar: 5 μm. (**B**) DNA content of Δ*gim1/yke2* cells. Δ*gim1/yke2* cells were incubated in YPD at 14°C. Samples were taken at the indicated time points. The DNA content of these cells was analysed by flow cytometry. (**C**) Sensitivity of the indicated strains towards latrunculin-A. Relative apparent sensitivity was determined as described (Ayscough *et al.*, 1997). (**D**) Sensitivity of *gim* null strains towards high osmolarity. Wild-type cells, Δ*gim1/yke2*, Δ*gim2/pac10*, Δ*gim3*, Δ*gim4* and Δ*gim5* cells were grown for 3 days at 30°C on YPD plates containing 0, 1.4 or 1.8 M sorbitol.

gim null mutants are sensitive towards the actin inhibitor latrunculin-A and are osmotically sensitive

We noticed that the volume of cells of the *GIM* deletion mutants incubated at 14°C was ~2.2- to 2.4-fold increased compared with the wild-type (Table I). Such a phenotype has been reported, among others, for mutants affecting the actin cytoskeleton (reviewed by Drubin, 1990). Many mutants with actin defects are sensitive towards the actin-binding drug latrunculin-A (Ayscough *et al.*, 1997) and are osmotically sensitive (Drubin, 1990). We found that the *gim* null strains were more sensitive towards latrunculin-A than the wild-type, which indicates an influence of the Gim proteins on the actin cytoskeleton (Figure 3C). This sensitivity was stronger compared with that of the cold-sensitive mutant *tcp1-1*, but was, however, clearly weaker compared with the actin mutant *act1-2*. *TCP1* encodes a component of TRiC which is required for actin folding (Ursic and Culbertson, 1991). Furthermore, similarly to actin mutants, Δ*gim1/yke2*, Δ*gim2/pac10* and Δ*gim5* cells did not grow on plates containing 1.4 M sorbitol. In contrast, Δ*gim3* cells grew slowly, while Δ*gim4* grew as wild-type cells (Figure 3D). At 1.8 M sorbitol, Δ*gim3* cells failed to grow and a weak growth defect of Δ*gim4* cells was observed (Figure 3D). We sought additional evidence for a role for the Gim proteins in actin function. However, deletion of *GIM1/YKE2* was synthetically lethal with neither *act1-2* nor *act1-3*. In addition, Δ*gim1/yke2*, Δ*gim2/pac10*, Δ*gim3* and Δ*gim4* cells did not show an obvious defect in actin organization as judged by indirect

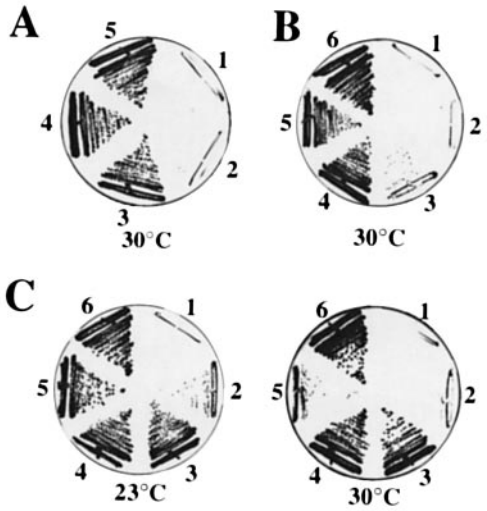

Fig. 4. Genetic interactions of *GIM1/YKE2* with *TUB4*, *SPC98* and *SPC97*. (**A**) *GIM1/YKE2* of strain ESM183 (Δ*tub4::HIS3* pRS316-*TUB4*) was disrupted using the *kanMX4* gene. The resulting strain SGY119 was unable to grow on 5-FOA plates which selects against the *URA3*-based plasmid (sector 1), confirming that *TUB4* is an essential gene (Spang *et al*., 1996). However, strain SGY119 was able to grow on 5-FOA when it contained a *LEU2*-based plasmid carrying *TUB4* (sector 3), but not when it contained *tub4-1* (sector 2), indicating that Δ*gim1/yke2* is synthetically lethal with *tub4-1*. Controls established that *tub4-1* (sector 4) and Δ*gim1/yke2* cells (sector 5) grow on 5-FOA at 30°C. (**B**) In a similar manner as in (A), using strain ESM243 (Δ*spc98::HIS3* pRS316-*SPC98*), we tested for genetic interactions of *GIM1/YKE2* with *SPC98*. Strain SGY120 (ESM243 Δ*gim1/yke2*) was unable to grow on 5-FOA (sector 1), as *SPC98* is essential for growth (Geissler *et al.*, 1996). As expected, growth was observed in the presence of a *LEU2*-based plasmid containing *SPC98* (sector 4). In contrast, *spc98-2* (sector 2) and *spc98-1* (sector 3) did not or hardly support growth, indicating synthetic lethality and synthetic toxicity of Δ*gim1/yke2 spc98-2* (sector 2) and Δ*gim1/yke2 spc98-1* (sector 3). We established that *spc98-2* (sector 5) and *spc98-1* (sector 6) cells grow well on 5-FOA at 30°C. (**C**) *GIM1/YKE2* of strain YMK10 (Δ*spc97::HIS3* pRS316-*SPC97*) was disrupted. The resulting strain SGY121 was unable to grow on 5-FOA (sector 1), unless the plasmid pRS315-*SPC97* was present (sector 4). Since *spc97-20* Δ*gim1/yke2* cells barely grow at 23°C (sector 2), while cells of *spc97-20* (sector 5) grow well, we conclude that Δ*gim1/yke2* is synthetically toxic with *spc97-20*. In contrast, *spc97-14* combined with Δ*gim1/yke2* (sector 3) grow as *spc97-14* cells at 23 or 30°C (sector 6).

immunofluorescence using anti-actin antibodies (data not shown), suggesting that the *gim* null mutants have only subtle actin defects. In conclusion, the *GIM* genes may have multiple functions, since their deletion causes microtubule defects as well as an increased sensitivity towards the actin-specific drug latrunculin-A and towards high osmolarity.

The GIM genes interact genetically with genes involved in microtubule biogenesis

To gain further insight into the role of the *GIM* genes, we investigated their genetic interactions with other genes involved in microtubule function. We first addressed the question of whether total loss of *GIM* gene function is synthetically lethal with mutants of the Tub4p complex. Using the approach described in Figure 4A, we could show that this is indeed the case for *tub4-1*. This result was confirmed by tetrad analysis using a *GIM4/* Δ*gim4::HIS3MX6 TUB4/tub4-1* strain. No viable temperature-sensitive *HIS3* spores were obtained, confirming that Δ*gim4::HIS3MX6* combined with *tub4-1* is lethal. Microscopic inspection of the non-colony-forming spores identified several hundred cells. Therefore, Δ*gim4 tub4-1* cells died only after multiple duplications. A similar result was obtained with *GIM3* (data not shown).

In a similar manner, we tested whether *GIM1/YKE2*, *GIM2/PAC10* and *GIM4* showed interactions with *SPC98* and *SPC97*, coding for components of the yeast γ-tubulin complex. Δ*gim1/yke2* was synthetically lethal with *spc98-2* and synthetically toxic when combined with *spc98-1* (Figure 4B). Allele-specific genetic interactions of *GIM1/YKE2* were also observed with *SPC97*: deletion of *GIM1/YKE2* was synthetically toxic in *spc97-20* cells, while it did not affect growth of the *spc97-14* mutant (Figure 4C). In contrast to Δ*gim1/yke2*, Δ*gim2/pac10* or Δ*gim4* combined with *spc98-1*, *spc98-2* or *spc97-20* hardly affected growth (Table II). It is important to note that the *spc98* and *spc97* alleles have distinct phenotypes and are suppressed differently by *TUB4* (Geissler *et al.*, 1996; Knop *et al.*, 1997). In particular, *spc97-20* has an SPB duplication defect, while *spc97-14* fails to form a mitotic spindle (Knop *et al.*, 1997).

Finally, *GIM1/YKE2*, *GIM2/PAC10* and *GIM4* were in part tested for their genetic interactions with *TUB1*, *TUB2*, *BIN2*, *BIN3* and *RBL2*. *TUB1* and *TUB2* code for α- and β-tubulin in yeast (Neff *et al.*, 1983; Schatz *et al.*, 1986a), while *RBL2* encodes a β-tubulin-binding protein, the homologue of mammalian cofactor A (Archer *et al.*, 1995; Tian *et al.*, 1996). Bin2p and Bin3p are subunits of TRiC (Chen *et al.*, 1994). Δ*gim1* showed synthetic lethality with *tub1-4*, *tub2-403*, *tub2-405*, *bin2-1*, *bin3-1* and Δ*rbl2* (Table II). In contrast, Δ*gim4* was only synthetically lethal in combination with *bin2-1*, *bin3-1* and Δ*rbl2*, but not with *tub2-403* or *tub2-405*. In summary, the *GIM* genes show a broad range of genetic interactions with genes involved in tubulin biogenesis and microtubule nucleation.

Deletion of the GIM genes results in reduced levels of α-tubulin, which explains the benomyl super-sensitivity, but not the synthetic lethality with tub4-1

A defect of the *gim* null mutants in tubulin biogenesis may result in reduced levels of α-, β- or γ-tubulin. The amount of these proteins was determined by immunoblotting using specific antibodies. While the α-tubulin content was about half to a third compared with wild-type, β-tubulin and Tub4p levels were approximately the same (Figure 5A and B). It is noteworthy that deletion of *GIM4* affected α-tubulin less severely than deletion of the other *GIM* genes. We also noticed that the reduction of α-tubulin levels was independent of the incubation temperature of the cultures (data not shown).

An increase of β-tubulin over α-tubulin levels, which is the case in the *gim* null mutants, is toxic for yeast cells (Burke *et al.*, 1989; Katz *et al.*, 1990; Schatz *et al.*, 1986b). Therefore, increasing the α-tubulin content by overexpression of *TUB1* or reducing the pool of free β-tubulin by overproduction of the β-tubulin-binding protein Rbl2p may suppress the tubulin defects. Indeed, the benomyl super-sensitivity of the five Δ*gim* mutants was fully rescued when *TUB1* or *RBL2* were overexpressed using either elevated gene dosage (2μ plasmids) or the strong *ADH* promoter (Table I), while overexpression of

Table II. Genetic interactions of *GIM1/YKE2, GIM2/PAC10, GIM3, GIM4* and *GIM5* with genes involved in microtubule assembly

	tub4-1	*spc98-1/2*	*spc97-20*	*tub1-4*	*tub2-403/-405*	*bin2-1*	*bin3-1*	Δ*rbl2*
gim1/yke2	SL[a]	SL	SL	SL	SL	SL	SL	SL
Δ*gim2/pac10*	SL	+[b]	+	n.d.[c]	n.d.	n.d.	n.d.	SL
Δ*gim3*	SL	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	SL
Δ*gim4*	SL	+	+	n.d.	+	SL[d]	SL	n.d.
Δ*gim5*	SL	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a]SL, synthetic lethality. This was determined by plasmid shuffle unless otherwise indicated. For plasmid shuffle experiments, the mutant strains (see Table III) were transformed with a plasmid-encoded *GIM* gene (*URA* plasmid). The corresponding *GIM* gene on the chromosome was disrupted by use of the *kanMX4* marker as described. The resulting strains were transformed with plasmids containing the *GIM* gene or the wild-type allele of the respective mutated gene and a control vector and were tested for growth on 5-FOA selecting against the *URA3* plasmid. Non-growth of the transformants with the control plasmid on 5-FOA indicates synthetic lethality. However, the transformants with the additional *GIM* gene or the wild-type allele of the mutated gene grew.
[b]+, not synthetically lethal.
[c]n.d., not determined.
[d]Tested for synthetic lethality by tetrad analysis. The *bin2-1* mutant and the *gim4* null mutant were crossed. The resulting strain was sporulated and the spores were analysed.

TUB4 showed no effect (Figure 5C; shown for Δ*gim1/yke2*). We then investigated whether the reduced level of α-tubulin is also the cause for the synthetically lethal phenotype with *tub4-1*. However, overexpression of either *TUB1* or *RBL2* did not suppress the synthetic lethality, while plasmid-encoded *TUB4* and the *GIM* genes complemented the mutants (Figure 5D; shown for Δ*gim1/yke2*). In summary, the benomyl super-sensitivity of the *GIM* mutants is the result of a reduced level of α-tubulin; however, this is not the reason for the synthetically lethal phenotype with *tub4-1*. This suggests that the Gim proteins have at least two functions, one related to α-tubulin and the other to Tub4p.

Gim1p/Yke2p, Gim2p/Pac10p, Gim3p and Gim5p bind to overproduced Tub4p

The failure of overexpressed *TUB1* and *RBL2* to suppress the synthetically lethal phenotype with *tub4-1* and the allele-specific interaction of *GIM1/YKE2* with *SPC98* and *SPC97* point to a more direct role for the Gim proteins in Tub4p complex function. This notion was supported further by the finding that overexpression of *TUB4* was toxic in the Δ*gim1/yke2* null strain (Figure 6A, sector 1), while it hardly affected growth of the wild-type (Figure 6A, sector 3) (Spang *et al.*, 1996; Knop *et al.*, 1997). Δ*gim1/yke2* cells overexpressing *TUB4* frequently were large-budded and showed abnormal bud morphologies (Figure 6A), phenotypes which were observed neither in Δ*gim1/yke2* cells nor in wild-type cells overexpressing *TUB4* (data not shown). Similarly, simultaneous overexpression of chromosomally integrated *TUB4*, *SPC98* and *SPC97* was not toxic for wild-type cells; however, it strongly affected growth of the *gim1/yke2* null mutant (Figure 6B). As expected, this defect was rescued by plasmid-encoded Gim1p.

This result, together with the observed synthetically lethal phenotype with *tub4-1*, suggests that the *GIM* genes become essential either after overexpression of *TUB4* or in the presence of a mutated *TUB4*. This consideration led us to test whether the Gim proteins bind to overproduced Tub4p. A gene fusion consisting of *TUB4* and three repeats of the haemagglutinin (HA) epitope-encoding sequence was used for this experiment. *TUB4-3HA* was overexpressed in wild-type cells (Figure 6C), followed by the immunoprecipitation of Tub4p-3HA using anti-HA antibodies. Interestingly, the precipitate not only contained Tub4p-3HA (lane 3), but also Gim1p/Yke2p (lane 5), Gim2p/Pac10p (lane 7), Gim3p (lane 9) and Gim5p (lane 11). This co-immunoprecipitation was specific as no, or hardly any, Gim protein was detected when the anti-HA precipitation was performed with an extract containing overproduced Tub4p (lanes 6, 8, 10 and 12) instead of Tub4p-3HA. Gim4p was not tested due to the lack of an antibody. In summary, our results show that *GIM1/YKE2* becomes essential after *TUB4* overexpression and that the Gim proteins bind to overproduced Tub4p.

The Gim proteins interact with each other and are present in common complexes

The interactions between the Gim proteins were investigated by immunoprecipitation experiments, using functional, epitope-tagged versions of the Gim proteins (Figure 7A). For example, co-immunoprecipitation of Gim2p/Pac10p-MYC with Gim1p/Yke2p-3HA was observed. Similarly, Gim3p-MYC co-precipitated with Gim2p/Pac10p-3HA and Gim4p-3HA precipitated Gim2p/Pac10p-MYC. The result of the immunoprecipitation was confirmed using a functional gene fusion between *GIM5* and protein A (*GIM5-ProA*). Gim5p-ProA was enriched from cell lysates with IgG–Sepharose. We detected not only Gim5p-ProA in the precipitate, but also Gim1p/Yke2p, Gim2p/Pac10p, Gim3p and Gim4p-3HA (Figure 7B). Similar results were obtained with Gim2p/Pac10p-ProA (data not shown). Multiple interactions between the Gim proteins were also observed using the yeast two-hybrid system (Fields and Song, 1989) (data not shown).

The results of the immunoprecipitations do not allow a decision to be made as to whether the Gim proteins form only one or several complexes. To address this point, yeast extract from wild-type cells was fractionated by gel filtration (Figure 7C). Immunodetection of the Gim proteins revealed that Gim1p/Yke2p, Gim2p/Pac10p, Gim3p and Gim5p co-fractionated over a wide range, suggesting that the complexes were heterogeneous in their composition. However, all four proteins peaked in the same fraction corresponding to an apparent mol. wt of ~170–230 kDa. It is noteworthy that Gim2p/Pac10p was also detected in earlier fractions corresponding to mol.

wts up to 2 MDa. In conclusion, the immunoprecipitation experiments demonstrate that the Gim proteins interact with each other and that they are present in common complexes.

The Gim complexes are localized mainly in the cytoplasm of yeast cells

In order to obtain a better understanding of the Gim complexes, we investigated their cellular localization. Localization studies by indirect or direct immunofluorescence using epitope-tagged or green fluorescent protein (GFP) fusion proteins excluded a localization of the Gim proteins at the SPB. Instead, a cytoplasmic staining was observed (data not shown). This result was confirmed by fractionation experiments (Figure 8). Yeast cell lysates (lane 1) were separated by differential centrifugation into a soluble cytoplasmic fraction (lane 2) and a sediment (lane 3) containing nuclei, mitochondria and other organelles. The distribution of the α- and β-subunits of the cytoplasmic fatty acid synthase (Fas) and the nuclear protein nucleolin (Nop1p) established that the two fractions were hardly contaminated by each other. Gim1p/Yke2p, Gim2p/Pac10p, Gim3p, Gim4p-3HA and Gim5p were detected predominantly in the cytoplasmic fraction (lane 2). Taken together, the localization studies suggest that the Gim complexes are localized predominantly in the cytoplasm of yeast cells.

The mammalian homologues of Gim1p/Yke2p and Gim2p/Pac10p are fully functional in yeast

Gim1p/Yke2p is 47% identical to the mouse *KE2* gene product (Shang *et al.*, 1994) and Gim2p/Pac10p is 43% identical to the human VBP-1 protein (Geiser *et al.*, 1997). We were interested to see whether the *KE2* and *VBP-1* gene products can substitute for Gim1p/Yke2p and Gim2p/Pac10p in yeast. The *KE2* and *VBP-1* genes were amplified by PCR from a mouse and a human cDNA library, respectively. Both genes were cloned behind the yeast *ADH* promoter. Like plasmid-encoded *GIM1/YKE2*, the *ADH–KE2* promoter fusion complemented the synthetically lethal phenotype of Δ*gim1/yke2 tub4-1* (data not shown) as well as the cold- and benomyl-sensitive growth defects of Δ*gim1/yke2* cells (Figure 9A). Similar results were observed with the human *VBP-1* gene using the Δ*gim2/pac10* mutant (Figure 9B). Our results indicate that the Gim proteins are not only structurally conserved, but that they also fulfil very similar functions in mammalian and in yeast cells.

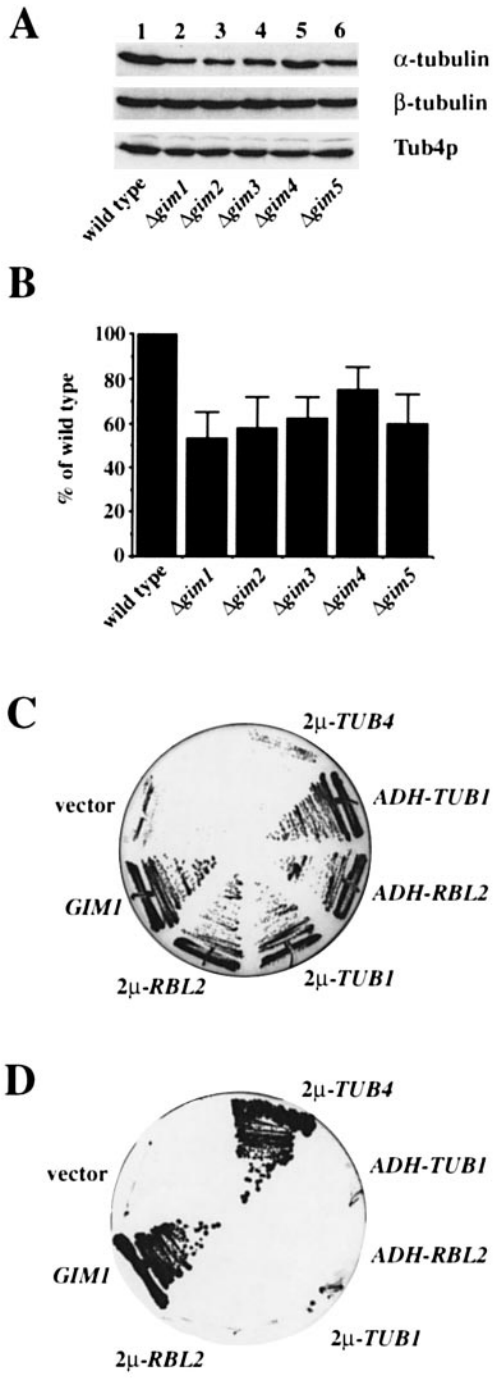

Fig. 5. Haploid *gim* null mutants have reduced α-tubulin levels, resulting in benomyl super-sensitivity. (**A**) Wild-type cells (lane 1) and cells of Δ*gim1/yke2* (lane 2), Δ*gim2/pac10* (lane 3), Δ*gim3* (lane 4), Δ*gim4* (lane 5) and Δ*gim5* (lane 6) were grown in YPD medium at 30°C to mid-log phase. Equal amounts of protein (50 μg) from the six strains were separated by SDS–PAGE, followed by immunodetection of α-tubulin, β-tubulin and Tub4p, using specific antibodies. An identical result was obtained when the cells were incubated at 14°C for 24 h (data not shown). (**B**) The α-tubulin levels of the wild-type and the five *gim* null strains from three independent experiments were quantified by densitometric scanning of the blots using the NIH image program. The bars indicate the variation between the experiments. (**C**) Cells of Δ*gim1/yke2* were transformed with control vector pRS425 (sector: vector), pRS315-*GIM1/YKE2* (*GIM1*), pRS425-*RBL2* (2μ-*RBL2*), pRS425-*TUB1* (2μ-*TUB1*), p415-*ADH-RBL2* (*ADH-RBL2*), p415-*ADH-TUB1* (*ADH-TUB1*) and YEp13-*TUB4* (2μ-*TUB4*). Transformants were tested for growth on YPD plates containing 2.5 μg/ml benomyl at 30°C. (**D**) In a similar manner as in (**C**), cells of strain SGY146 (*tub4-1* Δ*gim1/yke2* pRS316-*TUB4*) were transformed with the indicated plasmids. The transformants were tested for growth on 5-FOA plates at 30°C. Cells of SGY146 with the control vector pRS425 did not grow on 5-FOA, since *tub4-1* is synthetically lethal with Δ*gim1/yke2*. The synthetic lethality was relieved by an additional *TUB4* or *GIM1/YKE2* on a *LEU2*-based plasmid. In contrast to (C), *TUB1* or *RBL2* on pRS425 or under the control of the *ADH* promoter did not suppress the synthetic lethality of Δ*gim1/yke2 tub4-1*, indicated by the non-growth on 5-FOA.

Discussion

Gim1p/Yke2p, Gim2p/Pac10p, Gim3p, Gim4p and Gim5p interact with each other and form complexes

In this study, we performed a genetic screen for mutants that are not viable in combination with a mutated *TUB4*. We expected to find genes coding either for components of the Tub4p complex or for proteins that are involved in the folding of Tub4p or the assembly of the Tub4p complex. Since the Tub4p complex is associated with SPB substructures that are located in the nucleus (inner plaque) and in the cytoplasm (outer plaque) (Rout and Kilmartin, 1990; Spang *et al.*, 1996; Knop *et al.*, 1997), we could also expect to identify factors that play a role in the nuclear import of the Tub4p complex (G.Pereira *et al.*, 1998). Finally, we expected to find either α- or β-tubulin, since it has been proposed that these proteins interact with γ-tubulin (Oakley, 1992).

Our genetic screen identified *SPC98* and *SPC97*, which was not surprising, since previous results indicated that they interact genetically with *TUB4* (Geissler *et al.*, 1996; Knop *et al.*, 1997). In addition, we identified five novel genes that we named *GIM1–GIM5*. Two of the *GIM* genes have been described previously: *YKE2* was analysed due to its homology to the mouse *KE2* gene, located on the mouse MHC locus (Shang *et al.*, 1994), and *PAC10* was found in a screen for genes which become essential in the absence of *CIN8*, encoding a kinesin-related protein (Geiser *et al.*, 1997). The co-immunoprecipitation and the fractionation studies indicate that all five Gim proteins function together and that they are part of either one complex or several complexes containing different Gim proteins and possibly as yet unidentified proteins. In retrospect, our screen was quite selective, as it only identified components of the Tub4p or the Gim complexes.

Gim proteins have multiple functions related to the yeast cytoskeleton

All *GIM* deletion mutants had a reduced level of α-tubulin, while the amounts of β-tubulin and Tub4p were normal. The imbalance of α- and β-tubulin results in an elevated pool of free β-tubulin which is probably the cause of the microtubule defects. Our conclusion is supported by the observation that the deletion of *TUB3*, coding for the minor α-tubulin in yeast, also results in benomyl super-sensitivity (Schatz *et al.*, 1986b) and that overexpression of *TUB2*, coding for β-tubulin, is toxic (Katz *et al.*, 1990). We reasoned, that any manipulation that increases the amount of α-tubulin or decreases the pool of free β-tubulin should suppress the tubulin defects. This was indeed the case, as overexpression of *TUB1* or *RBL2*, both of which reduce the level of free β-tubulin (Archer *et al.*, 1995), cured the benomyl super-sensitivity of the *gim* null strains. We also believe that the synthetically lethal defects of *GIM* deletions with Δ*rbl2* are the result of too little α-tubulin, as loss of Rbl2p will increase the pool of free β-tubulin further, which is then lethal for the cells (Katz

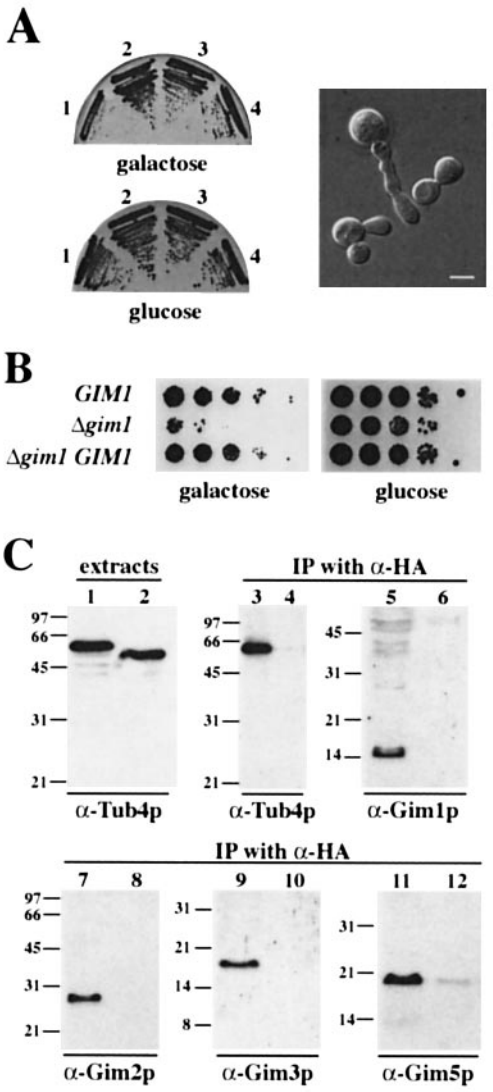

Fig. 6. Genetic interactions of *GIM1/YKE2* with *TUB4* and binding of the Gim proteins to overproduced Tub4p. (**A**) Overexpression of *TUB4* is toxic for Δ*gim1/yke2* cells. Cells of Δ*gim1/yke2* (sectors 1 and 2; strain SGY101) or *GIM1/YKE2* (sectors 3 and 4; strain YPH499) with plasmids pYES2-*TUB4* (*Gal1-TUB4*) (sectors 1 and 3) or pYES2 (sectors 2 and 4) were grown at 30°C on plates containing either raffinose/galactose (induction of the *Gal1* promoter) or glucose (no induction) as carbon sources. Δ*gim1/yke2 Gal1-TUB4* cells grown for 8 h in raffinose/galactose medium at 30°C were inspected by light microscopy. This culture contained a high proportion of cells with a large bud (>70%), and ~5% with a misformed bud. In contrast, 30–40% of the *GIM1* cells overexpressing *TUB4* contained a large bud, and <0.1% of the buds were misformed. Bar: 5 μm. (**B**) Cells co-overexpressing *TUB4*, *SPC98* and *SPC97* require *GIM1/YKE2* for survival. Serial dilutions of cells containing chromosomally integrated *Gal1-TUB4*, *Gal1-SPC98* and *Gal1-SPC97* in either a *GIM1/YKE2* (strain ESM387) or a Δ*gim1/yke2* (strain SGY159) background and cells of SGY159 transformed with plasmid-encoded *GIM1/YKE2* were grown on plates containing either glucose (no induction) or raffinose/galactose (induction) as carbon sources at 30°C. (**C**) Co-immunoprecipitation of Gim1p/Yke2p, Gim2p/Pac10p, Gim3p and Gim5p with overproduced Tub4p-3HA. YPH499 cells carrying plasmid-encoded *Gal1-TUB4-3HA* (pSI57) (lanes 1, 3, 5, 7, 9 and 11) or *Gal1-TUB4* (pSM209) (lanes 2, 4, 6, 8, 10 and 12) were grown in raffinose to a cell density of 5×10^6 cells/ml at 30°C. The *Gal1* promoter was induced for 6 h by the addition of galactose (2%). Cell extracts (lanes 1 and 2) were analysed by immunoblotting with anti-Tub4p antibodies, in order to confirm *TUB4-3HA* and *TUB4* overexpression. Cell lysates from both strains were incubated with anti-HA antibodies covalently coupled to protein A–Sepharose. The immunoprecipitates were analysed for Tub4p (lanes 3 and 4), Gim1p/Yke2p (lanes 5 and 6), Gim2p/Pac10p (lanes 7 and 8), Gim3p (lanes 9 and 10) and Gim5p (lanes 11 and 12) by immunoblotting using affinity-purified antibodies.

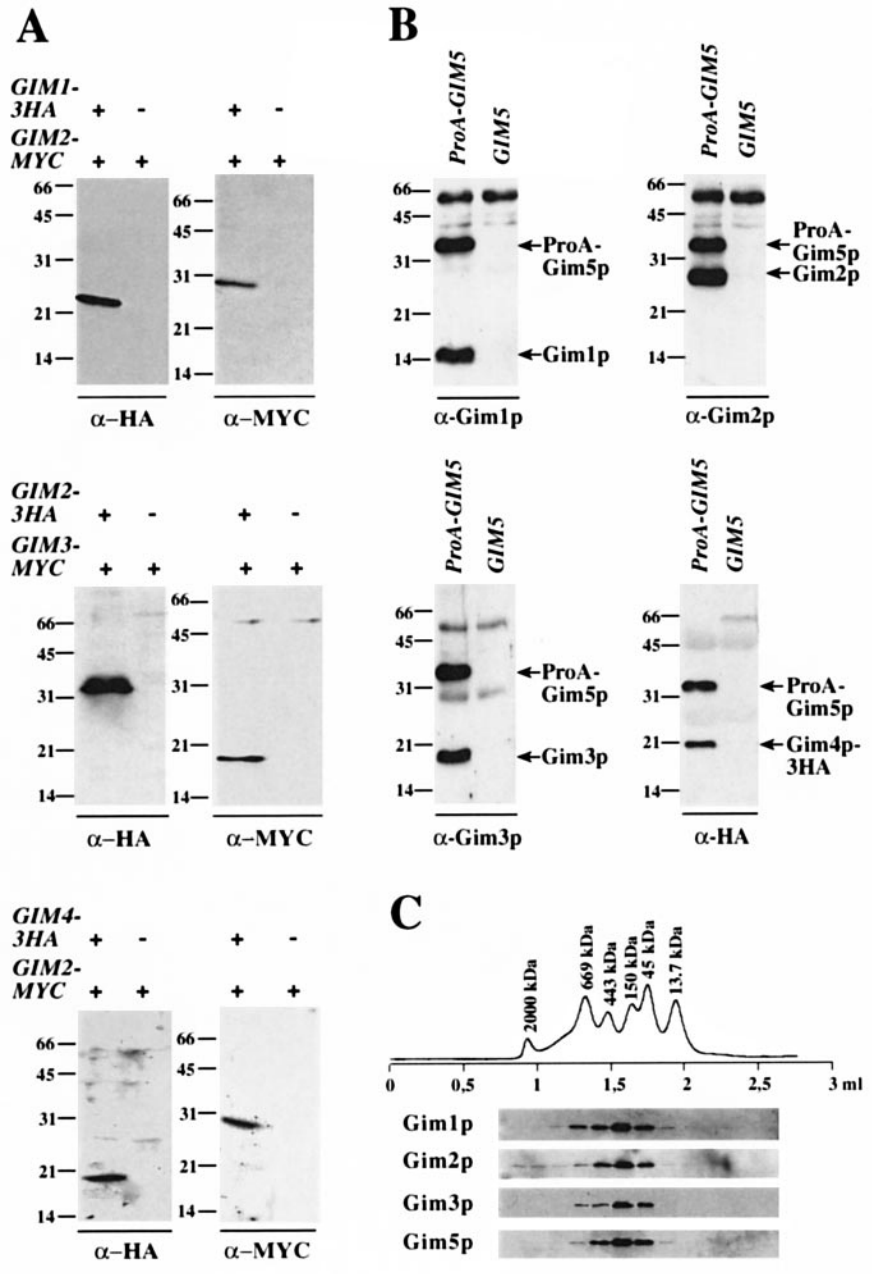

Fig. 7. Co-immunoprecipitation and co-fractionation of the Gim proteins. (**A**) Co-immunoprecipitation of the Gim proteins. Precipitation of the HA-tagged proteins of strains expressing the indicated *HA* and *MYC* gene fusions, or the wild-type genes, was performed with anti-HA antibodies coupled to protein A–Sepharose. Proteins were detected by immunoblotting using anti-HA and anti-MYC antibodies. (**B**) Co-immunoprecipitation of Gim1p/Yke2p, Gim2p/Pac10p, Gim3p and Gim4p-3HA with Gim5p-ProA. A chromosomally integrated gene fusion of *GIM5* with protein A was created. Cell lysates of ProA-*GIM5* or *GIM5* cells were incubated with IgG–Sepharose. The precipitates were analysed for Gim1p/Yke2p, Gim2p/Pac10p, Gim3p and Gim4p-3HA by immunoblotting using polyclonal anti-Gim1p/Yke2p, anti-Gim2p/Pac10p and anti-Gim3p antibodies, or monoclonal anti-HA antibodies. Gim5p-ProA was visualized by the binding of IgG–peroxidase to the ProA region of Gim5p-ProA. (**C**) The Gim proteins co-fractionate in gel filtration experiments. Cell lysates of strain YPH499 were fractionated by gel filtration. Fractions were analysed by immunoblotting using antibodies directed against the indicated proteins. The column was equilibrated using the indicated molecular weight standards.

et al., 1990; Archer *et al.*, 1995). Furthermore, the reduced level of α-tubulin in Δ*pac10* cells may explain why this gene becomes essential in the absence of the microtubule-binding protein Cin8p (Geiser *et al.*, 1997). Alternatively, Pac10p may have additional, motor protein-related functions.

GIM deletion mutants are slightly more sensitive towards the actin inhibitor latrunculin-A, than *tcp1-1* which codes for a mutated subunit of TRiC. Besides having other functions, the latter plays an essential role in actin folding (Ursic and Culbertson, 1991; Chen *et al.*, 1994; Miklos *et al.*, 1994; Vinh and Drubin, 1994). An additional hint of a function for the Gim proteins in actin-related processes comes from the sensitivity of *GIM* deletion mutants towards high osmolarity, which is also a common phenotype of actin mutants (Drubin, 1990). Since we did not observe direct defects of the actin cytoskeleton in *gim* null mutants and since latrunculin-A is highly specific towards actin (Ayscough *et al.*, 1997), the actin defects in the *gim* strains are probably only subtle.

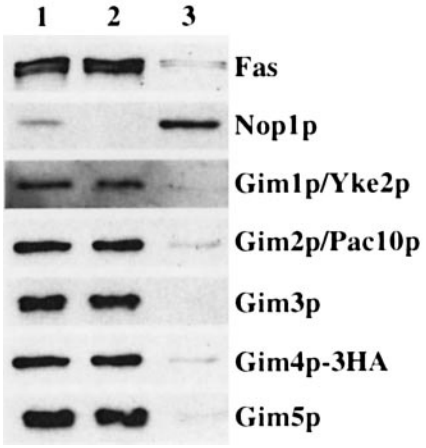

Fig. 8. The Gim complexes are localized mainly in the cytoplasm of yeast cells. Total cell lysates (lane 1) of wild-type (YPH499) and *GIM4-3HA* cells (ESM464) were fractionated by differential centrifugation (Young and Tyk, 1997) into a cytoplasmic 100 000 *g* supernatant (lane 2) and an organelle-containing pellet (lane 3). The sediment was resuspended in the original volume. Equal volumes were analysed by immunoblotting using antibodies directed against the indicated proteins.

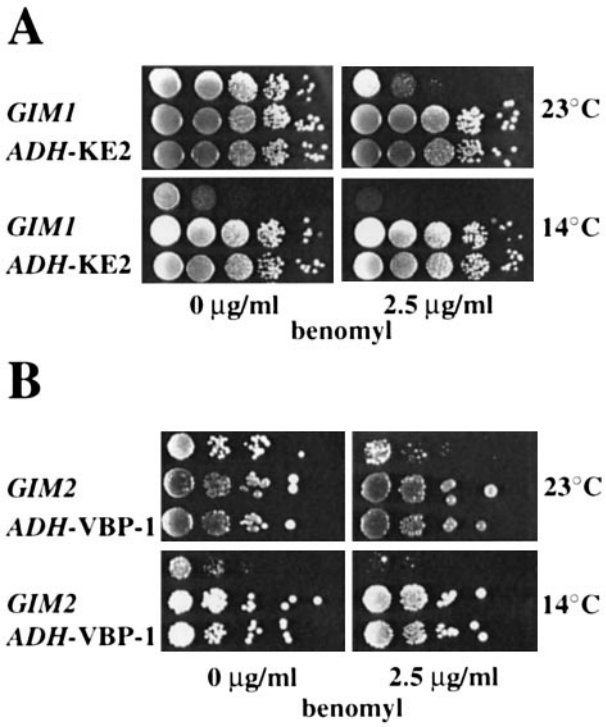

Fig. 9. The mouse *KE2* gene and the human *VBP-1* gene function for *GIM1/YKE2* and *GIM2/PAC10*, respectively, in yeast. (**A**) The cold sensitivity and the benomyl sensitivity of Δ*gim1/yke2* cells were complemented by expression of the *KE2* gene in yeast. Cells of Δ*gim1/yke2* were transformed with a control plasmid (p415-*ADH*, first row), *GIM1/YKE2* on plasmid pRS315 or a plasmid carrying the *KE2* gene under control of the yeast *ADH* promoter (p415-*ADH*-KE2). Serial dilutions of the transformants were tested for growth on YPD plates with or without 2.5 μg/ml benomyl at 23 or 14°C.
(**B**) Expression of VBP-1 rescues the cold-sensitive growth defect and the benomyl super-sensitivity of Δ*gim2/pac10* cells. Cells of Δ*gim2/pac10* were transformed with p415-*ADH* (first row), pRS315-*GIM2/PAC10* or p415-*ADH*-VBP-1. Transformants were tested as described in (A).

A number of results point to specific functions of the Gim proteins with respect to Tub4p. First, all *GIM* genes genetically interact with *TUB4*. Second, *GIM1/YKE2* shows allele-specific genetic interactions with *SPC98* and *SPC97*, coding for components of the yeast γ-tubulin complex. Third, overexpression of *TUB4* or co-overexpression of *TUB4*, *SPC98* and *SPC97* is toxic in a *gim1/yke2* null strain, while it does not affect growth of wild-type cells (Spang *et al.*, 1996; Knop and Schiebel, 1997). This suggests that the *GIM* genes become essential in the presence of a mutated Tub4p protein or after overproduction of Tub4p. Under the latter condition, we could show that the tested Gim1p/Yke2p, Gim2p/Pac10p, Gim3p and Gim5p proteins were associated with Tub4p.

Do all of the Gim proteins have the same function? The very similar phenotypes of the *gim* null strains as well as complex formation suggest that the Gim proteins have at least overlapping functions. However, we also obtained evidence for specialized functions of the Gim proteins. For example, only Δ*pac10* cells but not the other Δ*gim* mutants have a spore germination defect (Geiser *et al.*, 1997). In addition, Δ*gim4* cells have a less severe growth defect and are not as osmotically sensitive as the other *GIM* deletion mutants. Finally, *GIM1/YKE2*, *GIM2/PAC10* and *GIM4* interact differently with *SPC98* and *SPC97*.

The GIM genes encode phylogenetically conserved proteins which may function in folding or assembly processes

How do the Gim proteins function? The cellular localization, the effects of *gim* null mutants on the actin and tubulin systems as well as the phylogenetic conservation of the Gim proteins are reminiscent of the cytoplasmic chaperonin TRiC. Our localization studies suggests that the Gim proteins, like the TRiC proteins, form cytoplasmic multiprotein complexes. While TRiC contains eight subunits (Kubota *et al.*, 1995; Willison and Horwich, 1996; Nitsch *et al.*, 1997), it is still unclear whether additional Gim proteins exist. The tubulin defects of TRiC mutants and *gim* null strains are remarkably similar. TRiC mutants are also frequently super-sensitive towards benomyl, and their microtubules depolymerize at reduced temperatures. In addition, some of the TRiC mutants also do not arrest in the cell cycle, although microtubule structures are impaired (Ursic and Culbertson, 1991; Chen *et al.*, 1994; Miklos *et al.*, 1994). *tcp1-1* was similarly sensitive towards latrunculin-A as the *gim* null strains. Finally, a similar phylogenetic conservation as for the Gim proteins has been observed for the subunits of TRiC (Kubota *et al.*, 1995; Nitsch *et al.*, 1997): like TRiC, the Gim proteins have relatives in human, mouse, *C.elegans* and *S.pombe* (Figure 1). As for the Gim proteins, only two homologues of TRiC subunits were identified in archaebacteria and none in eubacteria. In addition to this, the Gim proteins as well as the subunits of TRiC bear a stronger homology to their relatives from other species than to each other (Kubota *et al.*, 1995; Waldmann *et al.*, 1995; Nitsch *et al.*, 1997).

The Gim complexes may play a role in the biogenesis of α- and γ-tubulin and probably actin, possibly as a molecular chaperone. A number of our results are consistent with this notion. First, the *GIM* genes show

Table III. Yeast strains and plasmids

Name	Genotype/construction	Source or reference
Yeast strain		
YPH499	*MAT**a** ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1*	Sikorski and Hieter (1989)
YPH500	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1*	Sikorski and Hieter (1989)
YPH501	*MAT**a**/α ura3-52/ura3-52 lys2-801/ lys2-801 ade2-101/ade2-101trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1*	Sikorski and Hieter (1989)
239.2C	*MAT**a** ade2-101 lys2-801 ura3-52 tub1-4*	T.Stearns
DBY1991	*MATα his4-619 act1-2*	D.Botstein
DBY1995	*MATα his4-619 act1-3*	D.Botstein
DBY2305	*MAT**a** ura3-52 lys2-801 his4-539 tub2-403*	Schatz *et al*. (1988)
DBY2309	*MAT**a** ura3-52 lys2-801 his4-539 tub2-405*	Schatz *et al*. (1988)
DDY805	*MAT**a** ura3-52 his4-619 bin2-1*	Chen *et al*. (1994)
DDY806	*MAT**a** his3Δ200 lys2-801 ura3-52 bin3-1*	Chen *et al*. (1994)
DUY559	*MATα leu2-3, -112 ura3-52 trp1-7 Δtcp1::LEU2 YCpMS38* (*tcp1-1-TRP1*)	Ursic and Culbertson (1991)
ESM97	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δbub2::HIS3*	Spang *et al*. (1996)
ESM183	*MAT**a** ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δtub4::HIS3* pSM223	Spang *et al*. (1996)
ESM210	*MAT**a**/α ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 TUB4/tub4-1*	Spang *et al*. (1996)
ESM243	*MAT**a** ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1* pSM296	Geissler *et al*. (1996)
ESM387	*MAT**a** ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 leu2Δ1::Gal1-TUB4-LEU2 trp1Δ63::Gal1-SPC97-3HA-TRP1 ura3-52::Gal1-SPC98-URA3*	Knop and Schiebel (1997)
ESM447	*MAT**a** ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 GIM5-ProA-kanMX4*	this study
ESM463	*MAT**a** ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 GIM4-3HA-kanMX4*	this study
K2346	*MAT**a** ade2-1 ade3 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3 Gal$^+$ psi$^+$*	K.Nasmyth
K2348	*MATα ade2-1 ade3 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3 Gal$^+$ psi$^+$*	K.Nasmyth
KSY2	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim2/pac10::kanMX4*	this study
SGY24	*MAT**a** ade2-1 ade3 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3 Gal$^+$ psi$^+$ Δtub4::HIS3* pSG24	this study
SGY33	*MATα ade2-1 ade3 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3 Gal$^+$ psi$^+$ Δtub4::HIS3* pSG24 pSG23	this study
SGY88	*MAT**a** ade2-1 ade3 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3 Gal$^+$ psi$^+$ SPC98::LEU2-SPC98 Δtub4::HIS3* pSG24	this study
SGY101	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim1/yke2::kanMX4*	this study
SGY110	*MAT**a**/α ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 TUB4/tub4-1 Δgim4::HIS3MX6*	this study
SGY115	*MAT**a** ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim4::HIS3MX6*	this study
SGY116	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim1/yke2::kanMX4 Δgim4::HIS3MX6*	this study
SGY117	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim3::kanMX4*	this study
SGY119	*MAT**a** ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δtub4::HIS3 Δgim1/yke2::kanMX4* pSM223	this study
SGY120	*MAT**a** ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc98::HIS3 Δgim1/yke2::kanMX4* pSM296	this study
SGY121	*MAT**a** ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc97::HIS3 Δgim1/yke2::kanMX4* pMK8	this study
SGY123	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim1/yke2::kanMX4 Δgim3::HIS3MX6*	this study
SGY124	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim1/yke2::kanMX4 Δgim2/pac10::HIS3MX6*	this study
SGY140	*MAT**a** ura3-52 lys2-801 his4-539 tub2-403 Δgim1/yke2::kanMX4* pSG62	this study
SGY141	*MAT**a** ura3-52 lys2-801 his4-539 tub2-405 Δgim1/yke2::kanMX4* pSG62	this study
SGY143	*MAT**a** bin3-1 his3Δ200 lys2-801 ura3-52 Δgim1/yke2::kanMX4* pSG62	this study
SGY144	*MAT**a** ade2-101 lys2-801 ura3-52 tub1-4 Δgim1/yke2::kanMX4* pSG62	this study
SGY146	*MATα lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 tub4-1 Δgim1/yke2::kanMX4* pSM223	this study
SGY156	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim5::kanMX4*	this study
SGY158	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim1/yke2::kanMX4 Δrbl2::HIS3MX* pSG62	this study
SGY159	*MAT**a** ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 leu2Δ1::Gal1-TUB4-LEU2 trp1Δ63::Gal1-SPC97–3HA-TRP1 ura3-52::Gal1-SPC98-URA3 Δgim1/yke2::kanMX4*	this study
SGY172	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δgim1/yke2::kanMX4 Δgim5::HIS3MX6*	this study
YMK10	*MAT**a** ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc97::HIS3* pMK8	Knop *et al*. (1997)
Plasmids		
p415-ADH	*CEN6, LEU2*-based yeast–*E.coli* shuttle vector carrying the *ADH* promoter	Mumberg *et al*. (1995)
pCH1122	YCp50 with *ADE3* and mutated centromere sequence	Kranz and Holm (1990)
p2–7–1	YEp13 containing *TUB4*	Geissler *et al*. (1996)
pMK8	pRS316 containing *SPC97*	Knop *et al*. (1997)
pMK10	pRS315 containing *SPC97*	M.Knop
pSG23	pRS314 containing *tub4-1*	this study
pSG24	pCH1122 containing *TUB4*	this study
pSG55	pRS315 containing *GIM1/YKE2*	this study
pSG60	pRS413 containing *GIM1/YKE2*	this study
pSG62	pRS316 containing *GIM1/YKE2*	this study
pSG64	pRS315 containing *MYC-GIM1/YKE2*	this study
pSG72	p415-*ADH* containing the mouse *KE2* gene	this study

Table III. *Cont.*

Name	Genotype/construction	Source or reference
pSG89	pGEX-4T-3 containing *GIM1/YKE2*	this study
pSG96	pRS315 containing *3HA-GIM1*	this study
pSG100	p415-ADH containing *GIM4*	this study
pSI2	pRS316 containing *GIM2/PAC10*	this study
pSI11	pRS315 containing *MYC-GIM2/PAC10*	this study
pSI13	pRS316 containing *MYC-GIM3*	this study
pSI14	pRS315 containing *GIM3*	this study
pSI16	pRS316 containing *MYC-GIM2/PAC10*	this study
pSI18	pRS315 containing *GIM2/PAC10*	this study
pSI25	pGEX-5X-1 containing *GIM2/PAC10*	this study
pSI26	pGEX-5X-1 containing *GIM3*	this study
pSI39	pRS315 containing *3HA-GIM2/PAC10*	this study
pSI41	pRS316 containing *MYC-GIM3*	this study
pSI57	pYES2 containing *TUB4-3HA*	this study
p*spc97-14*	pRS315 containing *spc97-14*	Knop *et al*. (1997)
p*spc97-20*	pRS315 containing *spc97-20*	Knop *et al.* (1997)
pSM204	pRS315 containing *tub4-1*	Spang *et al.* (1996)
pSM209	pYES2 containing *TUB4*	Spang *et al.* (1996)
pSM223	pRS316 containing *TUB4*	Spang *et al.* (1996)
pSM291	pRS315 containing *SPC98*	Geissler *et al.* (1996)
pSM296	pRS316 containing *SPC98*	Geissler *et al.* (1996)
pSM473	p415-*ADH* containing the coding region of *GIM5*	this study
pSM480	pRS315 containing *3HA-GIM4*	this study
pSM495	p415-*ADH* containing human *VBP-1*	this study
pSM553	pRS425 containing *TUB1*	this study
pSM554	pRS425 containing *RBL2*	this study
pSM555	p415-*ADH* containing *TUB1*	this study
pSM556	p415-*ADH* containing *RBL2*	this study
p*spc98-1*	pRS315 containing *spc98-1*	Geissler *et al.* (1996)
p*spc98-2*	pRS315 containing *spc98-2*	Geissler *et al.* (1996)
pRS305	*LEU2*-integrating vector	Sikorski and Hieter (1989)
pRS315	*CEN6*, *LEU2*-based yeast–*E.coli* shuttle vector	Sikorski and Hieter (1989)
pRS316	*CEN6*, *URA3*-based yeast–*E.coli* shuttle vector	Sikorski and Hieter (1989)
pRS413	*CEN6*, *HIS3*-based yeast–*E.coli* shuttle vector	Sikorski and Hieter (1989)
pRS425	2 μm, *LEU2*-based yeast–*E.coli* shuttle vector	Christianson *et al.* (1992)
pGEX-4T-3	*E.coli* expression vector containing GST under control of the *lacZ* promotor	Pharmacia
pGEX-5X-1	*E.coli* expression vector containing GST under control of the *lacZ* promotor	Pharmacia
pYES2	2 μm, *URA3*-based yeast–*E.coli* shuttle vector carrying the *Gal1* promoter	Invitrogen

genetic interactions with components of TRiC, which is expected if both complexes function in related processes. Secondly, the Gim proteins bind to overproduced Tub4p. Thirdly, the reduced level of α-tubulin may be the consequence of a folding defect. Fourthly, the phenotypes of TRiC and *GIM* mutants are very similar. The Gim complexes may assist protein folding either before or after TRiC function. They could also bind to folded monomeric Tub4p or actin, thereby reducing potential toxic effects of the monomers. In any case, the Gim proteins only become essential for growth under certain circumstances, such as in the presence of a mutated Tub4p protein or overexpressed Tub4p.

Our results demonstrate that the mammalian homologues of Gim1p/Yke2p and Gim2p/Pac10p, the *KE2* and *VBP-1* gene products, are functional in yeast. Their expression complemented the synthetically lethal defects of Δ*gim1/yke2* or Δ*gim2/pac10* with *tub4-1* as well as the benomyl super-sensitivity and cold sensitivity. Interestingly, *KE2* mRNA is most abundant in brain and testis (Abe *et al.*, 1988), tissues which have a high content of tubulin. It is expected that these tissues have a high level of proteins that are required for tubulin biogenesis, as is the case for TRiC (Silver *et al.*, 1979, 1987). The homologue of Gim2p/Pac10p, the VBP-1 protein, has been identified in a two-hybrid screen using the von Hippel–Lindau tumor suppressor gene as a bait (Tsuchiya *et al.*, 1996). Whether this interaction has any physiological significance remains to be determined. Homologues of Gim3p and Gim5p were also found; however, we have not yet tested whether they function in yeast. The fact that the KE2 and VBP-1 proteins can substitute for Gim1p/Yke2p and Gim2p/Pac10p in yeast raises the possibility that the homologues of the Gim proteins also form complexes that promote the formation of functional α- and γ-tubulin in mammals and other species.

Materials and methods

Media and general methods

Basic yeast methods and growth media were as described (Guthrie and Fink, 1991). For *Gal1*-controlled gene expression, yeast strains were grown in synthetic complete medium (SC) containing raffinose (2%) as carbon source. Glucose (2%) or galactose (2%) was added for the repression or induction of the *Gal1* promoter, respectively. Yeast strains were transformed by the lithium acetate method (Schiestl and Gietz, 1989). *Escherichia coli* strains were transformed by electroporation (Dower *et al.*, 1988). PCR was performed with Vent polymerase (New England Biolabs). Recombinant DNA methodology was performed as reported (Sambrook *et al.*, 1989).

Plasmids and yeast strains

Plasmids and yeast strains used and constructed during the course of this study are listed in Table III. The mouse *KE2* gene and the human

VBP-1 gene were amplified by PCR from a mouse and human cDNA library, respectively and the PCR products obtained were cloned into p415-*ADH*. Epitope-tagged versions of *GIM* genes were generated by recombinant PCR, cloned into pRS315 (Sikorski and Hieter, 1989) and tested for functionality in *gim* mutants. The coding regions of the intron containing the *GIM4* ORF were amplified by PCR from chromosomal yeast DNA and cloned into p415-*ADH* (Mumberg *et al.*, 1995). The intron of *GIM5* was removed by recombinant PCR. The coding region of *GIM5* was cloned into expression vector p415-*ADH*. Double deletion mutants of Δ*gim1/yke2* together with any one of Δ*gim2/pac10*, Δ*gim3*, Δ*gim4* or Δ*gim5* were generated by subsequent deletion of the respective ORFs using either *kanMX4* (Wach *et al.*, 1994) or *HIS3MX6* (Wach *et al.*, 1997) as selectable markers. Chromosomally integrated C-terminal fusions of the *GIM* genes with GFP, protein A and 3HA were generated by homologous recombination with PCR-amplified *GIM-GFP-kanMX4* (Wach *et al.*, 1997), *GIM-ProA-kanMX4* and *GIM-3HA-kanMX4* cassettes (E.Schiebel, unpublished). *TUB1* and *RBL2* were amplified by PCR and cloned into p415-*ADH* and pRS425 (Christianson *et al.*, 1992), respectively.

Isolation of mutants which are synthetically lethal with tub4-1

In order to identify mutants which are synthetically lethal with *tub4-1*, we used the *ade2/ade3* red/white colony sectoring system (Koshland *et al.*, 1985; Huffaker *et al.*, 1987). The haploid strain K2348 (*ade2 ade3*) was transformed with an *ADE3*/*URA3* plasmid containing *TUB4* (pSG24). This plasmid had a mutated centromere rendering it unstable under non-selective growth conditions. The chromosomal *TUB4* gene was disrupted by insertion of the *HIS3* marker using the Δ*tub4::HIS3* cassette of plasmid pSM219 (Spang *et al.*, 1996). This strain was transformed with a *TRP1*-based plasmid carrying the temperature-sensitive *tub4-1* allele (pSG23), yielding strain SGY33. The *tub4-1* allele harbours a single amino acid substitution which causes cells to die at 37°C while they stay alive at 30°C (Spang *et al.*, 1996). Strain SGY33 grows with red colony colour, since it is genotypic *ade2*, resulting in the accumulation of a red intermediate of the adenine biosynthetic pathway. Loss of the *ADE3* plasmid pSG24 under non-selective conditions changes the genotype from *ade2* to *ade2 ade3*, resulting in white or red/white sectoring colonies. Strain SGY33 was mutagenized with methane sulfonic acid ethyl ester (EMS, Sigma) to ~30% survival. Mutations that conferred synthetic lethality with *tub4-1* were identified on plates without selection for the plasmid pSG24 as red, non-sectoring colonies, since these cells depend on *TUB4* of the *ADE3*-containing plasmid. Among 60 000 screened colonies, 12 colonies with this phenotype were obtained. These were unable to grow on plates containing 5-fluoro-orotic acid (5-FOA), a drug that kills cells harbouring a *URA3* gene. They all regained the red/white sectoring phenotype and their ability to grow on 5-FOA when transformed with a *LEU2*-based plasmid containing the *TUB4* gene but not when transformed with a plasmid carrying *tub4-1*. Although all 12 mutants showed a red/white sectoring phenotype when transformed with an *SPC98*-containing plasmid, it was shown by crossings to strain SGY88 (*SPC98-LEU2*) and subsequent linkage analysis (see below) that the mutation causing synthetic lethality resided only in two cases in *SPC98*. In contrast, the red/white sectoring phenotype of the other mutants was due to suppression of the *tub4-1* phenotype by *SPC98* (Geissler *et al.*, 1996). The mutants were back-crossed twice to strain SGY24, which revealed that the phenotypes were caused by a single, recessive mutation. Crosses between the mutants identified seven complementation groups.

Identification and disruption of the GIM genes

Two of the mutants from our screen were complemented by *SPC97*, recently identified as the third component of the γ-tubulin complex in yeast (Knop *et al.*, 1997). The other eight mutants were transformed with DNA from a *LEU2/CEN*-based yeast genomic library (kindly provided by Dr K.Nasmyth), and the transformants were screened for regaining of the red/white sectoring phenotype. The genomic inserts of plasmids allowing cells to lose the *TUB4*-containing *ADE3/URA3* plasmid after re-isolation and re-transformation were sequenced. Subcloning and sequencing identified five genes which we named *GIM1–GIM5*.

The *GIM*, *SPC98* and *SPC97* genes on pRS305 (Sikorski and Hieter, 1989) were targeted to their chromosomal locations in strain SGY24. The resulting strains were then crossed with the corresponding mutants. Random spore analysis of the diploid strains revealed that, for example, *GIM1* was linked to the mutation conferring the synthetically lethal phenotype with *tub4-1*, since all sectoring haploid progenies did not contain the labelled *GIM1-LEU2*. The *GIM* genes were disrupted by insertion of the heterologous markers *kanMX4* (Wach *et al.*, 1994) or *HIS3MX6* (Wach *et al.*, 1997), resulting in the deletion of the entire ORFs. The *kanMX4* and *HIS3MX6* disruption cassettes were generated by PCR and transformed into diploid YPH501 or haploid YPH499 (in the case of *GIM2/PAC10)*. The deletion of the *GIM* genes in the resulting strains was confirmed by PCR.

Anti-Gim1p/Yke2p, anti-Gim2p/Pac10p, anti-Gim3p, anti-Gim5p antibodies, immunoblots, immunoprecipitation experiments and immunofluorescence microscopy

The entire coding regions of *GIM1/YKE2*, *GIM2/PAC10*, *GIM3* and *GIM5* were cloned into pGEX expression plasmids (Pharmacia). The GST fusion proteins were expressed and purified by affinity purification using glutathione–Sepharose according to the manufacturer's recommendations. Antibodies against the purified proteins were raised in rabbits as described (Harlow and Lane, 1988). Antibodies against Gim1p/Yke2p and Gim2p/Pac10p were affinity purified as described (Spang *et al.*, 1995). The specificity of the antibodies was demonstrated by performing immunoblots with extracts from *GIM* and Δ*gim* strains.

For immunoprecipitations of the Gim proteins, cells of strains carrying 3HA- or MYC-tagged derivatives were grown in selective media at 30°C to a density of 2×10^7 cells/ml. Washed cells were resuspended in H-buffer [50 mM HEPES, pH 7.5, 100 mM NaCl, 2.5 mM $MgCl_2$, 1 mM EGTA, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM benzamidine, 10 μM leupeptin, 20 μg/ml chymostatin and 20 μg/ml E64] and lysed by vortexing with glass beads on ice until >95% of the cells were lysed. Cell debris were removed by centrifugation (5 min, 20 000 *g*). Cell lysates were incubated with anti-HA antibodies (12CA5) covalently bound to protein A–Sepharose for 3 h at 4°C. The anti-HA antibodies were cross-linked to protein A–Sepharose as reported (Harlow and Lane, 1988). The precipitates were washed twice with H-buffer, resuspended in HU-buffer (Knop *et al.*, 1996) and heated for 10 min at 65°C prior to SDS–PAGE (Laemmli, 1970). Gim5p-ProA was precipitated identically using IgG–Sepharose. As molecular weight standards for SDS–PAGE, we used myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa).

For the overexpression of *TUB4-3HA* and *TUB4* from the *Gal1* promoter, yeast cells were grown in SC medium with 2% raffinose as sole carbon source at 30°C to a cell density of 5×10^6 cells/ml. The *Gal1* promoter was induced by the addition of 2% galactose for 6 h. Harvested cells were resuspended in L-buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA, 1 mM EGTA, 5% glycerol, 1 mM GTP and protease inhibitors) and lysed with glass beads as described above. Cell lysates were incubated with anti-HA antibodies covalently bound to protein A–Sepharose for 1 h at 4°C.

For the quantifications of α-tubulin, β-tubulin and Tub4p, cells were lysed as described by Knop *et al.* (1996). The primary antibodies used for immunoblots were mouse monoclonal anti-HA (12CA5, Hiss Diagnostics), mouse monoclonal anti-MYC (9E10; Boehringer Ingelheim), rabbit anti-Gim1p/Yke2p, rabbit anti-Gim2p/Pac10p, rabbit anti-Gim3p, rabbit anti-Gim5p, rabbit anti-Tub4p (Geissler *et al.*, 1996), rabbit anti-α-tubulin, rabbit anti-β-tubulin (α- and β-tubulin antibodies were a gift of Dr F.Solomon), rabbit anti-Nop1p (Dr E.Hurt) and rabbit anti-Fas antibodies (Dr R.Egner). As secondary antibodies, rabbit anti-mouse or goat anti-rabbit IgGs coupled to horseradish peroxidase (Jackson Immuno Research Laboratories) were used. The immunoreaction was visualized by an ECL Kit from Amersham.

Immunofluorescence of formaldehyde-fixed yeast cells was performed as described (Knop *et al.*, 1996) with 1 h fixation time. The primary antibodies were mouse monoclonal anti-β-tubulin WA3 (kindly provided by U.Euteneuer-Schliwa) or mouse monoclonal anti-actin (Amersham). Secondary antibodies were goat anti-mouse IgGs coupled to CY3 (Jackson Immuno Research Laboratories). DNA was stained with DAPI (Boehringer Mannheim).

Nocodazole arrest, flow cytometry, determination of cell size and protein determination

For nocodazole arrest, cells were treated with 15 μg/ml nocodazole (Sigma) in liquid medium at 30°C for 3 h. For flow cytometry, cells were prepared as reported (Hutter and Eipel, 1979). The DNA content of 20 000 cells was determined using a flow cytometer (Facscalibur, Becton-Dickinson). Mean cell volumes were determined using a CASY1 Cell Counter and Analyzer System (Schärfe System GmbH). Protein concentrations were determined according to Bradford (1976).

Size exclusion chromatography

Cell lysates of YPH499 were prepared in H-buffer as described above. Proteins were separated according to their apparent size using a Smart System from Pharmacia with a Superose 6 column (PC 3.2/30). The column was equilibrated with H-buffer, and molecular weight determination was carried out according to a calibration curve obtained with standard proteins (thyroglobulin, apoferritin, β-amylase, alcohol dehydrogenase, bovine serum albumin, ovalbumin, carbonic anhydrase, RNase A, blue dextran; Sigma) separated in H-buffer. Separation was performed with a flow rate of 15 μl/min. The eluate was collected in fractions of 50 μl each. Each three consecutive fractions were pooled and analysed by immunoblotting.

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