Yeast Vacuoles Fragment When Microtubules Are Disrupted

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Abstract. To identify whether microtubules are involved in the maintenance of vacuolar morphology, we treated *Saccharomyces cerevisiae* with nocodazole and methyl benzimidazole-2-yl-carbamate, drugs which inhibit the polymerization of microtubules. Treated cells arrest with a single large bud in the G2/prophase portion of the cell cycle. Labeling the vacuole with either quinacrine or FITC–dextran revealed vacuole fragmentation that was not found in untreated cells or in cells arrested in G2 by unrelated means. A drug-resistant mutant in beta tubulin does not show vacuolar fragmentation when treated with drug. We propose that microtubules are involved in the regulation of vacuole morphology.

SINGLE copy organelles, such as the nucleus or Golgi apparatus, must divide during each cell cycle and partition accurately into daughter cells (Warren, 1985). In higher eukaryotic cells the Golgi apparatus, endoplasmic reticulum, and nuclear membranes fragment at the start of mitosis (Porter and Machado, 1960; Burke et al., 1982; Lipsky and Pagano, 1985; Zeligs and Wollman, 1979; Gerace and Blobel, 1980). The Golgi apparatus reassembles in the centriolar region during telophase as the new nuclear membranes form (Hiller and Weber, 1982; Burke et al., 1982).

There is substantial evidence that organelle division, segregation, and morphology entails interactions with cytoskeletal elements. During interphase, microtubules radiate from the cell center throughout the cytoplasm to the cell periphery. In conjunction with other cytoskeletal elements, microtubules are thought to direct the spatial organization of organelles (Birky, 1983). For example, Golgi interaction with microtubules has been shown in vivo using double immunofluorescence microscopy (Rogalski and Singer, 1984). In vitro, organelle movement can occur on both microtubules and actin filaments (Schroer and Kelly, 1985).

Mitotic cells have very different microtubule dynamics from interphase cells (Saxton et al., 1984). At the start of mitosis, cytoplasmic microtubules depolymerize and the tubulin is used to form the mitotic spindle. Since microtubules interact with organelles during interphase, the loss of cytoplasmic microtubules at mitosis might facilitate organelle division and ensure partitioning of single copy organelles to daughter cells. When the interaction between the Golgi apparatus and microtubules is disrupted by drug-induced depolymerization of microtubules, the Golgi apparatus loses its perinuclear organization and disperses throughout the cytoplasm (Lin and Queally, 1982; Rogalski and Singer, 1984). This suggests that the Golgi fragmentation normally seen at the start of mitosis may be caused by the disassembly of cytoplasmic microtubules. Using colchicine and nocodazole, Swanson et al. (1987) have shown that tubular lysosomal morphology is dependent on the integrity of cytoplasmic microtubules.

Cell division in *Saccharomyces cerevisiae* and mammalian cells differs in several respects. Cells grow asymmetrically, beginning with the formation of a bud early in S phase. Cell surface growth is restricted to the bud (Field and Schekman, 1980). During mitosis, the nuclear envelope does not disassemble, but rather elongates across the division septum and divides (Byers, 1981). Little is known about the structures of yeast Golgi apparatus or endoplasmic reticulum during cell division. Yeast have a cell cycle–dependent rearrangement of tubulin and actin (Kilmartin and Adams, 1984). Actin and beta-tubulin are encoded by single-copy genes that are essential for cell growth (Neff et al., 1983; Shortle et al., 1982). Two genes encoding alpha-tubulin have been identified, as well as a gene encoding myosin heavy chain (Schatz et al., 1986; Watts et al., 1985). Drug-induced microtubule disruption prevents mating-mediated nuclear fusion in *S. cerevisiae*, although cell fusion and cytoplasmic mixing occur normally (Delgado and Conde, 1984).

While mammalian cells have multiple lysosomes, yeast have only one or a few vacuoles throughout the cell cycle (Weisman et al., 1987). This raises the question of how the division and segregation of the vacuole is achieved and coordinated with the cell cycle. In this paper, we describe experiments which suggest an interaction of microtubules with the yeast vacuole. We show that treatment of cells with microtubule-disrupting drugs causes fragmentation of the vacuole into many small vesicles.

Materials and Methods

Materials

Nocodazole was obtained from Aldrich Chemical Co. (Milwaukee, WI), methyl benzimidazole-2-yl-carbamate (MBC)[1] from DuPont Co. (Wil-

1. *Abbreviations used in this paper*: DAPI, diaminophenylindole; MBC, methyl benzimidazole-2-yl-carbamate.

mington, DE) glusulase from Boehringer Mannheim Diagnostics, Inc. (Houston, TX), and FITC-dextran (70 S), quinacrine, polyethylene imine, phenylenediamine, and beta-glucuronidase were from Sigma Chemical Co. (St. Louis, MO).

Drug Treatment

Wild-type *S. cerevisiae* (X2180-1A) was grown in YEPD media (Sherman et al., 1974) at 30°C to early log phase. Cells were shaken with either nocodazole (2.5 μl of a 5-mg/ml stock solution in DMSO per ml of culture) or MBC (5 μl of an 8 mg/ml stock solution in DMSO per ml of culture) for 2.5 h at 30°C. Control cultures were treated with the same concentrations of DMSO alone. Strains 1326 (parental) and 1323 (tub201), obtained from T. Huffaker and D. Botstein (Massachusetts Institute of Technology, Cambridge, MA) (Thomas et al., 1985), were grown and treated with drug at 26°C. Strain X2180-1A-R was selected as an MBC-resistant spontaneous mutant on a YEPD plate with 40 μg/ml of MBC. UKY403 was grown at 30°C in YEPG (Sherman et al., 1974) and shifted to YEPD for growth arrest.

Transfer of the tub2-201 Mutation into the X2180 Background

Exponentially growing X2180 (MAT alpha) and DBY1323 (MAT a) were mated in YEPD at room temperature with gentle agitation. After 5 h, the cells were streaked onto a YEPD plate and zygotes were dissected with the use of a micromanipulator. Colonies were plated onto sporulation media (Sherman et al., 1974) and spores were dissected. Strains bearing the tub2-201 mutation were identified by the ability to grow in the presence of 40 μg/ml MBC. Subsequent matings (four) used the complementary X2180 mating type to the strain bearing the tub2-201 mutation.

FITC-Dextran Labeling

Equal volumes of drug-treated cells and 200 mg/ml FITC-dextran (Makarow, 1985) with the appropriate drug concentration in YCM medium, pH 7.4, (Rogers and Bussey, 1978) were mixed and incubated 30 min at 26°C. Cells were collected by centrifugation (30 s in a microfuge; Brinkman Instruments Co., Westbury, NY.), suspended at room temperature in 1 ml of YCM with the appropriate drug concentration, and collected by centrifugation (30 s in a microfuge; Brinkman Instruments Co.). This procedure was repeated two to four times. Cells were finally suspended in 20 μl of YEPD containing 1% low melting agarose with the appropriate drug concentration and placed on a slide for microscopy. Alternatively, cells were placed on a slide coated with 0.5 mg/ml concanavalin A.

Quinacrine Labeling

A 1-ml aliquot of drug-treated cells was centrifuged in a microfuge for 30 s. The cells were resuspended in 1 ml of YEPD containing 50 mM NaH_2PO_4, pH 7.6, and 10 μM quinacrine. Cells were shaken for 4.5 min at 30°C and then centrifuged in a microfuge for 30 s. The pellet was resuspended in YEPD with 1% low melting agarose and the appropriate drug concentration.

Microscopy

Labeled cells were observed using a Zeiss microscope (Carl Zeiss, Inc., Thornwood, NY) fitted for epifluorescence and differential interference contrast (or Nomarski) optics. Excitation wavelengths were 450–490 nm and the emission was viewed using a 520-nm cut-off filter. A Nikon F3 camera (Nikon Inc., Garden City, NY) with Kodak Tri-X-pan film was used for photography. Photographs were taken using a Planaplo 100×, oil-immersion objective.

Scoring of Cells

Cells with a bud of approximately the same diameter as the mother cell were scored for vacuolar morphology. Vacuoles were scored as single, multiple, or fragmented (see Fig. 1 for morphologies).

Immunofluorescence

Immunofluorescence microscopy of MBC- and DMSO-treated cultures was performed as described by Kilmartin and Adams (1984), with the following minor modifications. Formaldehyde was added to cultures without centrifugation to a final concentration of 5% (vol/vol). The cells were permeabilized using 50 U lyticase per OD_{600} of cells for 10 min at 30°C. Slides were pretreated with 1% polyethyleneimine for 5 min, then rinsed with distilled water before incubation with the cells. Slides were "blocked" by incubation with 5 μl of blocking buffer (1% bovine albumin, 0.05% NP-40 in PBS) per sample chamber for 15 min, then 10 μl of antibody was added. Antitubulin antibody was the generous gift of Dr. Frank Solomon (Massachusetts Institute of Technology, Cambridge, MA). Slides were washed in 0.05% NP-40 in PBS, then in PBS. Blocking buffer (5 μl) was placed in each well. After 10 min, secondary antibody (a 1:200 dilution of FITC-coupled goat anti-rabbit serum, purchased from Boehringer Mannheim Diagnostics, Inc.) was added. Slides were incubated in the dark for 1 h at room temperature, then washed as above.

To stain the cells with diaminophenylindole (DAPI), 10 μl of 1-μg/ml DAPI in PBS was placed in each well. Slides were incubated for 5 min in a light-shielded, moist chamber, then rinsed with PBS. Mounting solution was as in Kilmartin and Adams (1984) but without DAPI. Coverslips were sealed to the slides with clear nailpolish. Photographs were taken with Kodak Tri-X-pan film, ASA 400, with an 8-s exposure through a Zeiss standard fluorescence microscope.

Results

To reveal the effect of microtubule disruption on vacuolar morphology, early log phase cells were treated with either nocodazole or MBC to prevent microtubule polymerization (Quinlan et al., 1980; Pillus and Solomon, 1986; Hasek et al., 1986). These drugs specifically affect beta tubulin; each of 173 mutants obtained as spontaneous benomyl-resistant colonies mapped to the gene encoding beta tubulin (Thomas et al., 1985). MBC and benomyl are closely related; benomyl releases MBC and butyl isocyanate upon hydration (Hammerschlag and Sisler, 1973). After incubation with drug for 2.5 h, the majority of cells arrested with a single large bud. Cells retained their viability during the drug treatment and entered the cell cycle within 1 h after drug removal. Two fluorescent markers were used to label the vacuoles: FITC-dextran, a fluorophore that labels vacuoles by diffusion (Preston et al., 1987), and quinacrine, a weak base that diffuses through membranes and concentrates in the acidic vacuole (Weisman et al., 1987). Using these fluorescent markers, vacuoles were scored as single (Fig. 1, *A* and *B*), multiple (Fig. 1 *C*), fragmented (Fig. 1, *D* and *E*), or unlabeled. Nocodazole-treated cells displayed marked vacuole fragmentation (Table I, lines *1* and *2*). Control cells, which were not treated with these drugs, had a predominant morphology of one or a few major vacuoles and very rarely showed vacuole fragmentation. When stained with FITC-dextran, 70% of the vacuoles of nocodazole-treated cells were fragmented, while no fragmented vacuoles were found in control cultures (Table I, line 2). When labeled with quinacrine, 46% of these nocodazole-treated cells showed fragmentation (Table I, line *1*).

Several control experiments were performed to test whether the fragmentation was mediated by the effect of the drug on microtubules. (*a*) Fragmentation similar to that in nocodazole-treated cells was also seen in MBC-treated cells, whether visualized with FITC-dextran or quinacrine (Table I, lines *1* and *2*). (*b*) We obtained strain DBY 1323, which has a spontaneous point mutation (tub2-201) in the beta-tubulin gene which renders it resistant to benomyl and MBC (Thomas et al., 1985). Unlike the isogenic parent strain (DBY 1326), these cells do not arrest in the G2 phase of the cell cycle when treated with MBC. These cells show fragmented vacuole structure in the bud (Fig. 2). We transferred the tub2-201 mutation into our wild-type, drug-sensitive

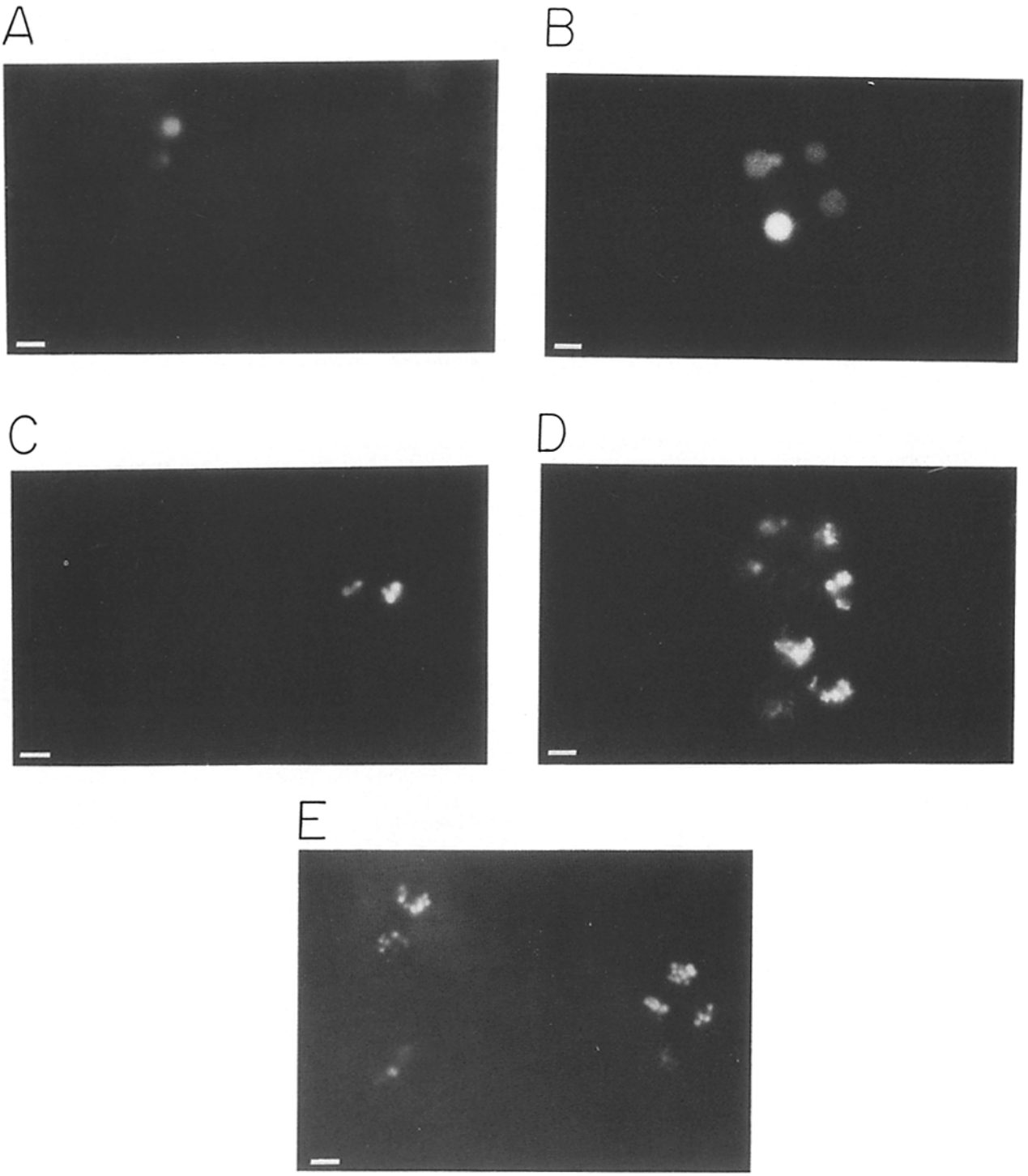

Figure 1. Examples of vacuole morphologies in control and drug-treated *S. cerevisiae.* (*A*) X2180-1A cell in G2, no drug treatment, quinacrine stained. There are single vacuoles on each side of the doublet. (*B*) Strain UKY403, no drug treatment, quinacrine stained. Arrested by histone depletion, it displays single vacuoles. (*C*) Strain X2180-1A, no drug treatment, FITC–dextran stained. This cell (in G2) has multiple vacuoles. (*D*) Strain X2180-1A, nocodazole treatment, FITC–dextran stained. This cell has fragmented vacuoles. (*E*) Strain X2180-1A, MBC treatment, FITC–dextran stained. This cell has fragmented vacuoles. Bars, 1 μm.

strain by successive backcrosses. The resulting strain, BGY 1079X9, still has a high frequency of fragmented vacuoles. The observation of vacuole fragmentation as a result of genetic alteration of beta-tubulin also supports the idea that microtubules affect vacuole integrity. (*c*) Cultures of strains DBY 1323 and BGY 1079X9 were treated with MBC. There was no change in vacuole morphology in these resistant strains upon drug treatment (Table I, lines *4* and *5*). In addition, we isolated an independent, spontaneous MBC-resistant mutant of X2180-1A. This mutant, termed X2180-1A-R,

Table I. Vacuole Morphology

Strain	Fluor	No drug treatment					MBC treatment					Nocodazole treatment				
		Cells scored	Single	Multiple	Fragmented	Unlabeled	Cells scored	Single	Multiple	Fragmented	Unlabeled	Cells scored	Single	Multiple	Fragmented	Unlabeled
		n	%	%	%	%	*n*	%	%	%	%	*n*	%	%	%	%
1. X2180-1A	Quinacrine	55	53	36	0	11	109	6	50	36	7	94	21	32	46	1
2. X2180-1A	FITC	133	24	76	0	0	98	3	34	63	0	184	1	29	70	0
3. 1326	FITC	84	1	95	4	0	96	0	48	52	0					
4. 1323	FITC	112	0	38	62	0	175	1	38	61	0					
5. RGY1079X9	FITC	96	0	64	36	0	102	0	64	36	0					
6. X2180-1A-R	FITC	125	1	82	17	0	171	1	83	16	0					
7. UKY403	Quinacrine	119	95	5	0	0										
8. UKY403	FITC	70	73	27	0	0										

Cells were grown to early log phase and then treated with drug or DMSO as described in Materials and Methods. Vacuoles were labeled with either quinacrine or FITC-dextran, then assayed for vacuole morphology by fluorescence light microscopy. Morphologies are expressed as the percentage of the total scored.

showed some vacuole fragmentation in the absence of drug (Table I, line *6*), as seen with BGY 1079X9. However, there was neither an arrest of cell growth nor increased vacuole fragmentation when these cells were treated with the drug. (*d*) A dose–response curve (Fig. 3) shows that the characteristic cell cycle arrest (*open circles*) and fragmentation (*solid circles*) occur to similar extents at each concentration of drug in the wild-type strain X2180-1A. (*e*) Immunofluorescence of drug-treated cells shows a complete absence of cytoplasmic microtubules (see below).

To determine whether this fragmented morphology was simply due to an arrest in G2, we studied the morphology of vacuoles in the yeast strain UKY403. In this strain a histone H4 gene has been placed under control of the inducible promoter (Kim, U. J., P. Kayne, and M. Grunstein, manuscript in preparation). When these cells are shifted from galactose media to glucose media, their inability to form nucleosomes during replication causes a cell cycle arrest in late G2/prophase. Under these conditions, the majority of vacuoles (73–95%) appear as large single organelles on each side of the doublet (Fig. 1 *B* and Table I, lines *7* and *8*). This experiment shows that the phenotype of vacuole fragmentation is neither a property of G2 nor a result of cell cycle arrest in G2. To confirm that the drugs which cause vacuole fragmentation were in fact causing microtubule disruption in our strain, we examined MBC-treated cells (and cells treated with DMSO, the solvent for the MBC) by fluorescence microscopy, using antitubulin antibody and an FITC fluorophore conjugated to the second antibody (Fig. 4). MBC treatment caused cells to arrest in G2, but disrupted the characteristic pattern of cytoplasmic microtubules seen in normal G2 cells (Fig. 4 *A*). DAPI staining confirms that the arrest in G2 occurs before segregation of the DNA (Fig. 4 *B*). As shown above, examination of the vacuoles of such cells by fluorescence microscopy (Fig. 4 *C*) shows the characteristic MBC-induced fragmentation.

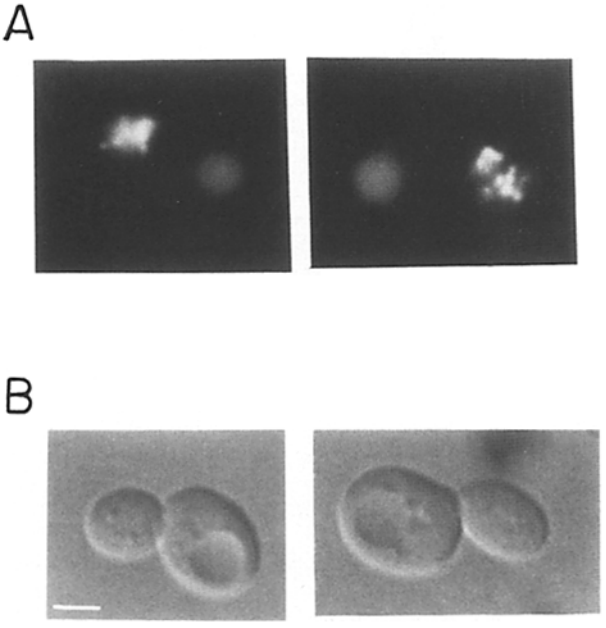

Figure 2. (*A*) Strain DBY1323 (without drug) was stained with FITC-dextran. The vacuole fragmentation is restricted to the bud. (*B*) The corresponding Nomarski optics photomicrograph. Note that the vacuoles in the bud are too small to be readily visualized by Nomarski optics. Bar, 1 μm.

Discussion

To explore the mechanism of organelle partitioning, we have exploited the numerous technical advantages of study-

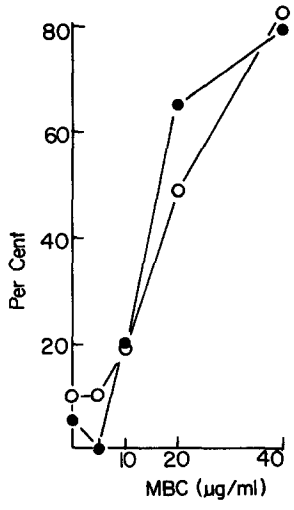

Figure 3. Strain X2180-1A was treated with the indicated concentrations of MBC, as described in Materials and Methods. Each cell in random fields was scored for arrest in G2 (*open circles*) or for vacuole fragmentation (*solid circles*). The tabulated results were expressed as a percentage of the total.

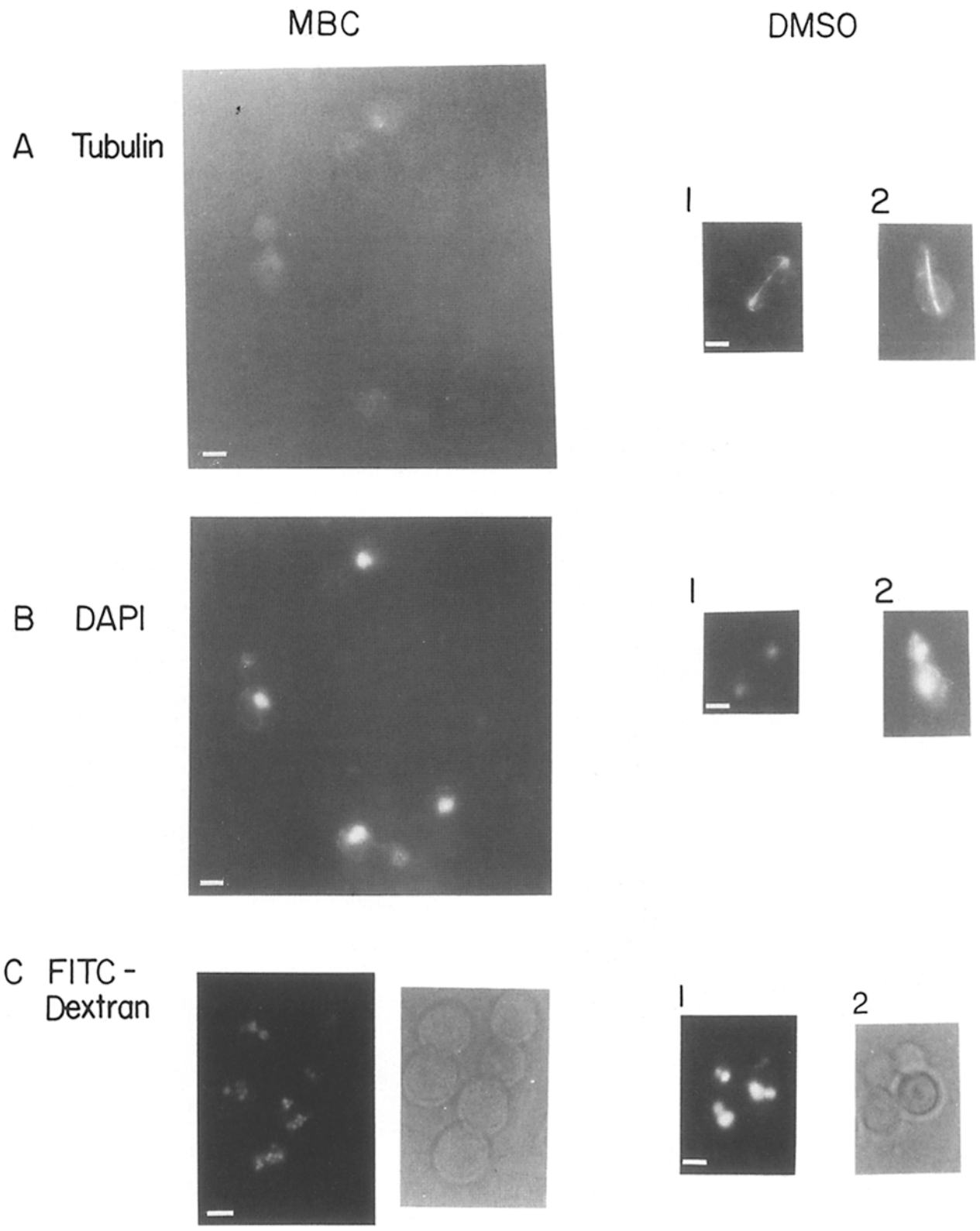

Figure 4. Fluorescence light microscopy of MBC-treated or control cells. Cells were treated with MBC and DMSO, or with DMSO alone, as described in Materials and Methods. (*A*) Immunofluorescence with antibody to tubulin. Note the absence of microtubules in MBC-treated cells, as opposed to control cells (*1* and *2*). (*B*) DAPI staining of DNA. In MBC-treated cells, the DNA fails to segregate into the bud. (*C*) Vacuole morphology as visualized by FITC–dextran labeling. Note the fragmented morphology of vacuoles in drug-treated cells.

ing the yeast vacuole. This organelle is usually present in a single or low copy number, in contrast to the lysosomes of higher eukaryotes. These lysosomes may associate with actin (Mehrabian et al., 1984), and there is evidence for microtubular control of secretion and endocytosis of lysosomal enzymes (von Figura et al., 1978). Lysosomes are present in

multiple copies (Reggio et al., 1984), which may be sufficient to allow their partitioning to daughter cells during mitosis (Warren, 1985). However, an extensive study of yeast vacuole morphology in asynchronous cultures has shown that the most common morphology in either the mother cell or bud throughout the cell cycle is 1–3 vacuoles (Weisman et al., 1987). These facts suggest that a specific mechanism, different from that proposed for lysosomes, must govern vacuolar division and segregation between parental and daughter cells.

Although the morphology of the vacuole seems to remain constant during the cell cycle, the vacuole may, like the Golgi apparatus of mammalian cells, be in a continual state of flux, with fission and fusion of small vacuole-derived vesicles occurring throughout the cell cycle. Segregation of vacuole components into the bud could involve transport of these vesicles into the developing daughter cell. Perhaps the vacuole fragmentation seen upon microtubule disruption implies that microtubules, or a microtubular network, are required for the fusion of vacuolar vesicles. Disruption of this interaction may favor the formation of a vesiculated, or fragmented, vacuole structure. Nuclear fusion in yeast zygotes has also been shown to be inhibited in benomyl-treated cells (Delgado and Conde, 1984).

While we do not know the nature of the fragmented, vacuole-related vesicles seen upon microtubule disruption, at least three possibilities merit mention. (*a*) They may represent breakdown products of vacuoles, and perhaps have no corresponding counterpart in normal cell physiology. (*b*) They may represent, as suggested above, vesicles which normally transit between the vacuoles of the parent cell and the bud, and thus be an intermediate of great interest. (*c*) It is also possible that at least some of them are endocytic vesicles. However, we have, in hundreds of drug-treated and mutant cells, never seen fragmented vacuoles in the same parent cell or bud with a large, intact vacuole. The loss of the large vacuole must lead the vacuole membrane and contents to form smaller derivative vesicles. Further studies may be required to determine the nature of these vesicles.

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