Isolation and characterization of cold-sensitive mutations at the benA, β-tubulin, locus of Aspergillus nidulans

Berl R. Oakley, C. Elizabeth Oakley, Kimberly S. Kniepkamp, and Janet E. Rinehart
Department of Microbiology, The Ohio State University, 484 W. 12th Avenue, Columbus, OH 43210, USA

Summary. We have isolated large numbers of conditionally lethal β-tubulin mutations to provide raw material for analyzing the structure and function of β tubulin and of microtubules. We have isolated such mutations as intragenic suppressors of benA33, a heat-sensitive (hs-) β-tubulin mutation of Aspergillus nidulans. Among over 2,600 revertants isolated, 126 were cold-sensitive (cs-). In 41 of 78 cs revertants analyzed, cold sensitivity and reversion from hs- to hs+ were due to mutations linked to benA33. In three cases reversion was due to mutations closely linked to benA33. Thirty-three of the revertants in which cold sensitivity and reversion were linked to benA33 were sufficiently cold-sensitive to allow us to select for rare recombinants between benA33 and putative suppressors in a revertant × wild-type (wt) cross. We found only one recombinant among 1,000 or more viable progeny from crosses of each of these revertants with a wt strain. Reversion is thus due to a back mutation or very closely linked suppressor in each case. We have analyzed 17 of these 33 revertants with greater precision and have found that, in each case, reversion is due to a suppressor mutation that maps to the right of benA33. The recombination frequencies between benA33 and the suppressors are very low (less than 1.2 × 10^-4) in all cases. Five of these 33 revertants have been examined microscopically and in each of them nuclear division and nuclear migration are inhibited at a restrictive temperature. We conclude that at least some and perhaps all of these revertants carry intragenic suppressors of benA33 that, in combination with benA33, cause cold sensitivity by inhibiting the functioning of microtubules at low temperatures. Of the 17 suppressors mapped, 11 map to two clusters. These clusters are likely to define regions particularly important to the functioning of the β-tubulin molecule.

Introduction

Significant progress has been made in recent years in identifying the components of microtubules, in understanding some of the basic principles underlying microtubule assembly, in determining what cellular phenomena are microtubule-mediated and, in flagella, in understanding how microtubules function in force production. Nevertheless many of the fundamental questions in microtubule research remain unanswered. The regulatory mechanisms for microtubule assembly in vivo are not understood, nor are the precise intermolecular interactions that hold microtubules together, nor are the mechanisms of force production in microtubule-mediated movements such as mitosis and organelle translocation. Recently genetic approaches have been initiated that promise to be of great value in answering some of these questions. Oakley and Morris (1981) have, for example, isolated a β-tubulin mutation of Aspergillus nidulans that appears to block chromosomal movement and nuclear translocation by inhibiting the disassembly of microtubules, thereby providing evidence that microtubule disassembly is necessary for these forms of movement.

Since microtubule functioning is essential for mitosis and thus for cell reproduction, mutations that block the functioning of essential microtubules under all conditions will prevent cell replication. Mutations that disrupt microtubule function can, therefore, be isolated only if they are conditionally lethal (i.e. microtubule function is disrupted under some conditions but not others) or if they affect microtubules that are not essential for the propagation of the organism [e.g. flagellar microtubules (reviewed by Luck 1984; Huang 1984)] or, in multicellular organisms, tissue specific microtubules (reviewed by Raff 1984). Since we are interested in determining the mechanisms of force production in essential processes such as mitosis and organelle movement, we have chosen to isolate conditionally lethal mutations.

Three approaches have been used to isolate conditionally lethal microtubule mutants. The first has been to isolate tubulin mutations as drug resistance mutations and to examine these mutants for conditional blockage of growth. This approach has produced heat-sensitive (hs-) β-tubulin mutations of Aspergillus nidulans (Morris et al. 1979; Oakley and Morris 1981), hs- x- and β-tubulin mutants of CHO cells (Cabral et al. 1982; Abraham et al. 1983), and hs- and cs- β-tubulin mutations of Saccharomyces cerevisiae (Neff et al. 1983). The second approach has been to isolate conditionally lethal mutations that block a microtubule-mediated process such as mitosis and to determine the identity of the mutant genes. Cold-sensitive (cs- x- and β-tubulin mutations of Schizosaccharomyces pombe have been discovered in this way (Toda et al. 1984; Hiraoaka et al. 1984). The third approach has been to isolate conditionally lethal tubulin mutations among revertants of other condi-
tionally lethal tubulin mutations. Morris et al. (1979) isolated an \(\alpha\)-tubulin mutation, \(\mu\)Al, as a revertant of a hs\(^+\) \(\beta\)-tubulin mutation, and \(\mu\)Al was subsequently shown to be super-sensitive to anti-microtubule agents (Oakley and Morris 1981). Thomas et al. (1984) have isolated temperature-sensitive revertants of a cs\(^-\) \(\beta\)-tubulin mutant of \textit{Saccharomyces cerevisiae} and have found one intragenic suppressor and 26 extragenic suppressors in 16 complementation groups.

We have chosen to employ the third approach, to isolate conditionally lethal tubulin mutations among revertants of \(\text{benA}\)33, a heat-sensitive (hs\(^-\)) \(\beta\)-tubulin mutation of \textit{Aspergillus nidulans} (Oakley and Morris 1981). This mutation inhibits nuclear division and nuclear movement and causes nuclei to be blocked in mitosis at high temperatures (Oakley and Morris 1981). Oakley and Morris (1981) and Gambino et al. (1984) have provided evidence that this mutation blocks chromosomal movement and nuclear translocation by blocking the disassembly of microtubules. Revertants of this mutant that are able to grow at the restrictive temperature must carry mutations that reverse the effects of \(\text{benA}\)33. Among such revertants one would expect to find mutations that restore the \(\beta\) tubulin encoded by the \(\text{benA}\) gene to its original, wild-type amino acid sequence (back mutations), mutations at other sites within the \(\text{benA}\) gene that compensate for the alteration of structure caused by \(\text{benA}\)33 (intragenic suppressors), and mutations in other genes, whose products interact with \(\beta\) tubulin, that compensate for the effects of \(\text{benA}\)33 (extragenic suppressors). Further, one would expect that in some instances intragenic and extragenic suppressors might themselves cause conditional lethality. One might, for example, find cold-sensitive (cs\(^-\)) suppressors of \(\text{benA}\)33.

Here we report the isolation of 41 mutations that confer cold sensitivity and are closely linked to \(\text{benA}\)33. We have examined five of them microscopically and have found that nuclear division and movement is inhibited in each of them. We have also mapped 17 of them at high resolution with respect to \(\text{benA}\)33 and have found that all of them map to the right of \(\text{benA}\)33 with recombination frequencies of less than \(1.2 \times 10^{-4}\) and that many of them map to two clusters. These mutations are likely to define some of the regions of the \(\text{benA}\) gene that are essential to microtubule function.

Materials and methods

**Strains.** BRO2 (\(\text{benA}\)33, \(\text{yA}\)2) is a strain constructed by Berl R. Oakley in the lab of Dr. N.R. Morris (UMDNJ, Rutgers Medical School). R153 (\(\text{wA}\)3, \(\text{pyroA}\)4) was obtained from Dr. C.F. Roberts (Leicester University). LO167 was constructed by C.E. Oakley from FGSC475 (\(\text{fpaB}\)37, \(\text{galD}\)5, \(\text{suA}\)1\text{adE}\)20, \(\text{riboA}\)1, \(\text{anA}\)1, \(\text{pabaA}\)1, \(\text{yA}\)2, \(\text{adeE}\)20, \(\text{biA}\)1, \(\text{sD}\)85, \(\text{fwA}\)2) from the Fungal Genetics Stock Center (Humboldt State University) and G81 (\(\text{ornB}\)7, \(\text{fwA}\)1) from Dr. A.J. Clutterbuck (University of Glasgow). LO167 carries \(\text{ornB}\)7 and \(\text{sD}\)85 as well as a \(\text{fwA}\) mutation. Since the two parental strains carry different \(\text{fwA}\) alleles, we do not know which allele is carried in LO167.

**Mutagenesis and isolation of revertants.** A suspension of conidia of BRO2 (10\(^8\)/ml) was irradiated with a 15 Watt General Electric germicidal lamp, model G15T8 at 200 \(\mu\)W/cm\(^2\) for 4 min with gentle agitation. Approximately 75% of the conidia were killed under these conditions. Aliquots of 0.1 ml were spread on YAG plates which were incubated at 42°C for three days. The hs\(^-\) revertants were isolated and tested subsequently for growth at 25°C and 42°C.

**Light microscopy.** Conidia were inoculated into YG medium containing 0.1% agar (the agar inhibits conidial clumping) and incubated under the conditions specified in the text. 0.9 ml samples were taken and fixed with 0.1 ml of 10% glutaraldehyde for 10 minutes. The samples were then washed twice for 10 min in distilled water, once for 15 min in acetone, twice for 10 min in distilled water and resuspended in distilled water containing 4',6-diamidino-2-phenylindole (DAPI) at a final concentration of 0.015 \(\mu\)g/ml. Samples were observed and photographed with a Zeiss standard microscope equipped for epifluorescence.

**Media.** YAG and FYG were used as complete media. YAG is 5 g/l yeast extract, 20 g/l dextrose and 15 g/l agar supplemented with a trace element solution devised by Dr. C.F. Roberts (personal communication). FYG is identical to YAG except that the agar is replaced by 25 g/l Pretested Burtonite 44c (TIC Guns, Inc, New York, NY, USA). For growth of strains carrying the \(\text{ornB}\) mutation, YAG and FYG were supplemented with arginine (100 mg/l). YG (YAG without agar or trace elements) was used as a complete liquid medium. Tests for cold sensitivity and heat sensitivity were carried out on FYG or YAG. Tests for benomyl resistance were carried out on YAG or FYG with 1.2 \(\mu\)g/ml benomyl. Tests for nutritional markers were carried out on minimal medium (Pontecorvo et al. 1953) with appropriate supplements.

**Chemicals.** Benomyl (technical grade, 98% pure) was a generous gift from DuPont (Wilmington, Delaware). DAPI was from Sigma, St. Louis, MO, USA.

**Fine structure mapping.** For these crosses we used progeny of the crosses of the revertants to R153 that carried \(\text{wA}\)3, \(\text{pyroA}\)4 and that had the revertant phenotype. We used these progeny rather than the revertants because \(\text{wA}\)3 is a white conidial color marker that is advantageous for determining whether cleistothecia are twinned and the \(\text{pyroA}\)4 nutritional marker is useful for forcing the crosses to occur. These progeny were crossed to LO167. Cleistothecia were harvested, cleaned and each crushed to form an ascospore suspension. Hybrid cleistothecia were detected by streaking a small aliquot of each suspension onto a FYG plate, incubating the plate and examining the streaks for recombination of color markers. \textit{Aspergillus nidulans} sometimes produces twinned cleistothecia in which a portion of the ascospores are from one strain crossed with itself and the remainder are from the two strains crossed with each other. Such cleistothecia distort recombination frequencies and can be detected by examining the recombination frequencies of the color markers in each cleistothecium. We, consequently, examined at least 100 progeny from each cleistothecium and determined the ratios of the color markers. Cleistothecia in which the color ratios differed by more than 5% from expected values were not used for further study. Serial dilutions were made from each ascospore suspension and the number of viable (colony-forming) ascospores determined at a permissive temperature of 37°C. To determine the relative viabilities of the ascospores having the phenotypes of the revertant and wild-type parents,